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Article

Molecular Detection of Human Papillomavirus and Its Association with Cervical Abnormalities in Iraqi Women

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Abstract: Persistent infections with Human Papillomavirus (HPV) of high-risk types (HR-HPV) are the major cause of cervical cancer and it is a preventable yet important public health problem in developing countries. This is a cross-sectional study that aimed at assessing the diagnostic accuracy of TaqMan probe based Real Time Polymerase Chain Reaction (RT-PCR) assay of the viral oncogene E7 in combination with liquid-based cytology and p16INK4a immunohistochemistry (IHC) for detection of HPV and classification of cervical epithelial abnormalities among Iraqi women. A total of 180 cervical samples were taken from women who presented at the tertiary centers in the center of Iraq aged between 22-62 years. Cytological examination was performed to classify lesions into the Bethesda classification system and biopsy was performed to histopathologically grade the lesions as normal/cervicitis to cervical intraepithelial neoplasia grade 3 (CIN 3). The overall prevalence of HPV DNA (based on RT-PCR) was 28.9% (52/180) and HR-HPV genotypes were detected in 22.2% (40/180) of all women. HPV 16 (28.8%) and HPV 18 (25.0%) were the most common high risk HPV types, according to genotype mapping. There was a strong correlation between the qualitative p16INK4a expression and the lesion grades, with increasing expression of p16INK4a, from 4.4% in normal cervical tissues to 93.3% in CIN 3. The multiplex molecular assays of the E7 oncoprotein showed 96.2% sensitivity and 95.8% specificity for the high-grade dysplasia. TaqMan molecular screening combined with p16INK4a triage greatly reduces discrepancies in screening which highlights the need for a structured molecular screening in Iraq. This study introduces and utilizes the TaqMan real-time PCR method to detect HPV DNA, evaluate CIN lesions, and assess p16INK4a expression.

Keywords: Human papillomavirus; Cervical intraepithelial neoplasia; TaqMan real-time PCR; p16INK4a; Genotyping.

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1. Introduction

Human papillomavirus (HPV) is a small, non-enveloped, double-stranded DNA virus which is tropic to the skin and mucosal epithelia [1]. Over 200 different HPV genotypes have been identified, with some types considered 'high-risk' oncogenic HPV types that cause the vast majority of anogenital and oropharyngeal cancers [2]. Persistent infection by oncogenic HPV types, mainly HPV 16 and HPV 18 is the basic etiological precursor for the onset of invasive cervical carcinoma (ICC) from cervical dysplastic precursor lesions [3]. While the majority of HPV infections resolve spontaneously within 12 to 24 months, for those that persist, molecular events start to occur and subvert the normal host cell cycle control [4]. Cervical malignancy continues to be a major cause of cancer-related death among women in LMICs, primarily due to the lack of institutionalized screening program and inadequate public health system [5]. Cervical

carcinoma is an emerging health problem in Iraq, where it is usually diagnosed in late stages and there are limited data on the molecular epidemiology of the disease [6].

The process of productive viral infection to pre-invasive cervical intraepithelial neoplasia (CIN) involves continuous expression of viral early oncoproteins E6 and E7 [7]. The viral proteins disrupt the normal regulation of the cell: E6 promotes ubiquitin-dependent proteasome degradation of the tumor suppressor p53, thus blocking apoptosis; and E7 can bind to and inactivate the retinoblastoma protein (pRb) [8]. Disruption of the pRb-E2F transcriptional repressor complex results in the release of E2F transcription factors that drive cells to prematurely enter into the S-phase and causes a paradoxical over-expression of the cyclin-dependent kinase inhibitor p16INK4a [9]. Thus, it is now recognized that p16INK4a is a surrogate immunohistochemical biomarker that is upregulated in high-risk HPV oncogene-activated, progressive, neoplastic transformation [10].

Although the conventional cytological screening test (Papanicolaou smear) has led to a decline in the incidence of cervical cancer over the years, the high inter-observer variability and the low sensitivity of conventional cytology to diagnose equivocal lesions, like atypical squamous cells of undetermined significance (ASC-US), have been a fundamental limitation [11]. The molecular detection methods that target conserved regions of the viral genome, such as sequence specific TaqMan real-time polymerase chain reaction (RT-PCR) have a higher analytical sensitivity and objective quantification [12]. Even though there are major clinical paradigms that have been adopted to screen HPV in primary care of women worldwide, little baseline molecular information about HPV genotype distribution, viral oncoproteins and their direct histopathological correlations are available in the Iraqi female population. The need for firm guidelines on molecular detection of a virus and the corresponding cytological and histological end points, in order to develop a local diagnostic algorithm, is urgent. Hence, this study was designed to explore the molecular prevalence and genotype distribution of HPV targeted by two conserved genes (E7 and L1) by TaqMan probe based RT-PCR and to determine the association of HPV with liquid based cytology and overexpression of p16INK4a by immunohistochemical method in all spectrum of cervical intraepithelial lesions among Iraqi women.

2. Materials and Methods

2.1. Study Cohort and Ethical Considerations

The study was a prospective case control study conducted in the major Gynecology and Oncology Out-patient clinics in Baghdad and Babylon Governorates in Iraq from January 2024 till January 2025. A total of 180 women with ages 22 to 62 years who presented for cervical cancer screening, clinical symptoms (post-coital hemorrhage, intractable vaginal discharge and persistent pelvic pain) and/or follow-up of abnormal cervical findings on colposcopy were included in the study [13]. Pre-existing total/subtotal hysterectomy, cervical conization, current pregnancy, and prior history of autoimmune or neoplastic comorbidities which were documented and receiving immunosuppressive treatment at the time of enrollment were systematically excluded [14]. Epidemiological and clinical data, such as age, type of settlement (urban or rural), parity, use of oral contraceptives, passive or active smoking exposure and familial history of malignancies were collected with an investigator-administered questionnaire. The research protocol conforms to the ethical standards laid down in the 1964 Declaration of Helsinki and its subsequent amendments and was approved by the main institutional review board (IRB) from the local institutional ethics committees (UOB-CM-2024-019) and informed written consent was obtained from all participants before specimen collection.

2.2. Specimen Collection and Liquid-Based Cytology

Cervical sampling was done before the application of acetic acid and Lugol's iodine in the speculum examination. A sterile cervical cytobrush was inserted into the endocervical os, rotated gently 5 complete revolutions in a continuous clockwise direction and then immediately placed in the PreservCyt liquid-based transport medium (LBTM) (Hologic, Inc., Marlborough, MA, USA) [15]. A secondary cytobrush pass was placed in a 5-mL sterile transport tube with viral transport medium (VTM) buffered with antibiotics, was immediately placed on ice and stored at -80 °C until downstream molecular processing Thin-layer cytological preparation was automatically performed, transferred onto positively charged slides and stained by using standardized Papanicolaou method [16]. Two independent board certified cytopathologists blinded to molecular status of the patient performed cytological interpretations, which were classified as per the 2014 Bethesda System for Reporting Cervical Cytology. Secondary multi-head microscopic examination was used to solve discrepant diagnoses.

2.3. Histopathological Evaluation and p16INK4a Immunohistochemistry

The participants who showed abnormal cytology (ASC-US or worse) or were flagged by colposcopy received a punch biopsy and/or loop electrosurgical excision procedure (LEEP). Tissues were collected and fixed in 10% neutral buffered formalin for 24 to 48 hours, which were processed routinely, embedded in paraffin wax and cut into 4 μm sections. The stained parallel sections were examined histopathologically and graded as normal/reactive changes, low-grade squamous intraepithelial lesion (CIN 1) and high-grade lesions (CIN 2 and CIN 3/carcinoma in situ) [17].

Consecutive 4-μm paraffin sections were stained immunohistochemically (IHC) using a purified mouse monoclonal p16INK4a antibody (clone E6H4, CINtec Histology kit, Roche Diagnostics, Basel, Switzerland) on an automated slide staining platform [18]. Antigen retrieval was performed with a high pH EDTA buffer at 97 °C for 20 minutes. The endogenous peroxidase activity was inhibited using 3% H_2O_2 for 10 mins. Immunoreactivity was detected with HRP conjugated dextran polymer backbone and 3,3'-diaminobenzidine (DAB) chromogen and light Mayer's hematoxylin counterstaining. High grade cervical squamous lesions showing documented positivity were utilized as positive controls and substitution of the primary antibody with non-immune mouse IgG was used as a negative control. Immunostaining evaluation was based on the criteria of the Lower Anogenital Squamous Terminology (LAST) as follows: Positive ("block-type") immunoreactivity was defined as diffuse strong nuclear and cytoplasmic staining throughout the lower third of the epithelial thickness (basal and parabasal tiers); absent, focal or only superficial cytoplasmic staining was scored as negative [19].

2.4. Genomic DNA Extraction and Quality Assurance

The concentrated cellular pellets of the VTM cervical samples were collected and then genomic DNA was extracted from them using the AddPrep Viral Nucleic Acid Extraction Kit (Addbio Inc., Daejeon, South Korea) following the manufacturer's instructions exactly [20]. Proteinase K digestion in a chaotropic salt buffer for 15 minutes at 56 °C was used to initiate cellular lysis which maximized viral capsid lysis. This lysate was then passed through silica-membrane spin columns and washed twice with ethanol based buffers to remove any contaminating material and eluted with 50 μL of sterile, nuclease free water. The purity and concentration of isolated DNA was determined by NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and an absorbance ratio of A260/A280 between 1.8 and 2.0 was obtained. An internal real-time PCR assay was performed on human cellular DNA to verify the integrity of the cells' DNA with the beta-globin (HBB) gene targeted.

2.5. TaqMan Real-Time PCR Amplification of the HPV E7 and L1 Genes

2. A sequence-specific TaqMan real time PCR was performed to detect HPV DNA based on the conserved region of the HPV E7 oncoprotein gene and the universal consensus

L1 regions. A set of primers and a pair of hydrolytic probes for HPV 16 and 18 were designed based on the sequences of NCBI-GenBank reference sequences (acc. No. KM058634.1 and OP712008.1) and synthesized commercially (Macrogen, Seoul, South Korea). The Common HPV E7 set was optimized as follows: Forward Sequence (5'-3'): GAGGAGGAGCGAGCACCTTA, Reverse Sequence (5'-3'): GAAGGGCAAAGCAAACTCAA, Probe Sequence (5'-3'): FAM-TGAAACAGGTGTAAGGCTGTG-BHQ1; The HPV 16 E7 set was optimized as follows: Forward Sequence (5'-3'): ACAAGCAGAACCGGACAGAG, Reverse Sequence (5'-3'): TACGTGTGTGCTTTGTACGC, Probe Sequence (5'-3'): FAM-TGCAAGTGTGACTCTACGCTTCGG-BHQ1; The HPV 18 E7 set was optimized as follows: Forward Sequence (5'-3'): ACATTTACCAGCCCGACGAG, Reverse Sequence (5'-3'): AGGACAGGGTGTTCAGAAACAG, Probe Sequence (5'-3'): FAM-GCTCAGCAGACGACCTTCGAGC-BHQ1.

Each reaction was set up in an optical 96-well reaction plate with a final volume of 25 μL that included 12.5 μL of 2× TaqMan Universal Master Mix II (Applied Biosystems, Foster City, CA, USA), 400 nM of each forward and reverse reaction primers, 200 nM of the TaqMan probe, 5 μL of extracted DNA template (approx. 50 ng), and nuclease-free water. Thermal cycling was performed on a QuantStudio 5 Real-Time PCR System (Applied Biosystems) with the following conditions: 2 minutes at 50 °C (UNG activation), initial denaturation and polymerase activation at 95 °C for 10 minutes, followed by 40 two-step cycles of 15 seconds at 95 °C (denaturation) and 60 seconds at 60 °C (primers annealing/extension). Standardized serially diluted plasmids (copy number 10^1 – 10^7 per μL) were used as dynamic reference controls for each plate, and authenticated positive HPV DNA extracts and non-template negative controls (NTC) were included. The cycle threshold (Ct) cutoff value of ≤ 38.0 was considered positive for the presence of HPV.

2.6. HPV Genotype Profiling via Flow Hybridization

Samples with a positive result for HPV by TaqMan RT-PCR were confirmed by genotype identification performed with the HPV Direct Flow CHIP System (Master Diagnóstica, Vitro S.A., Granada, Spain). It is an assay based on a multiplex PCR reaction which uses broad-spectrum biotinylated primers for the conserved L1 region and then is hybridized in an automated flow-through manner to a porous active membrane carrying genotype-specific oligonucleotide probes. The chip identifies 32 distinct mucosal HPV types, covering 18 high-risk types (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82) and 14 low-risk types (6, 11, 40, 42, 43, 44/55, 54, 61, 62/81, 67, 69, 70, 71, 72, and 84). Streptavidin-alkaline phosphatase conjugate was added to NBT/BCIP substrate to produce colorimetric signals; and hybridization patterns were analyzed with HybriSoft automated imaging software.

2.7. Statistical Analysis

IBM SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, NY, USA) was used to evaluate data and GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA) was used for graphing. The Shapiro-Wilk test was used to test the distribution of continuous variables, which were then presented as mean ± standard deviation (SD). Absolute frequency and percentages were used for tabulating categorical data. The Pearson chi-square (χ^2) test or Fisher's exact test was used to test for differences in proportions where the cell frequencies were less than 5. The diagnostic performance characteristics of real time RT-PCR (sensitivity, specificity, positive predictive value [PPV] and negative predictive value [NPV]) were determined with regard to the histopathology confirmation as a gold standard. Odds ratio (OR) and 95% confidence interval (CI) were used to determine associations between categorical demographic, environmental variables and HPV presence. Two-sided p-value < 0.05 was considered statistically significant for all parameters.

3. Results

3.1. Clinical and Demographic Characteristics

The clinical cohort comprised 180 female patients with an overall mean age of 38.6 ± 8.8 years (range: 22–62 years). Table 1 illustrates the distribution of demographic and behavioral variables stratified by molecular HPV status. Women aged 30–39 years constituted the largest age group (40.0%), followed by the 40–49 years bracket (29.4%). Overall, 52 patients (28.9%) tested positive for HPV DNA via TaqMan real-time PCR. The positivity for HPV was significantly higher in women living in rural localities (39.5%) than in urban dwellers (20.2%) ($p = 0.005$). Active or passive cigarette/waterpipe tobacco smoking was recorded in 20.6% of women, and was associated significantly with viral carriage (OR = 2.45, 95% CI: 1.18–5.09 $p = 0.014$). The multiparity ≥ 4 births was recorded in 47.8% of the individuals and was a significant predictor of the presence of HPV ($p = 0.021$). There were zero women immunized with HPV vaccine, as there were none of the 180 women.

Table 1: Sociodemographic and Clinical Characteristics of the Study Cohort (N = 180).

Parameter	Total Cohort n (%)	HPV Positive n (%)	HPV Negative n (%)	χ^2 Value	p-value
Age Category (Years)				6.81	0.078
< 30	32 (17.8)	7 (21.9)	25 (78.1)		
30–39	72 (40.0)	26 (36.1)	46 (63.9)		
40–49	53 (29.4)	14 (26.4)	39 (73.6)		
≥ 50	23 (12.8)	5 (21.7)	18 (78.3)		
Residence				7.98	0.005
Urban	99 (55.0)	20 (20.2)	79 (79.8)		
Rural	81 (45.0)	32 (39.5)	49 (60.5)		
Tobacco Exposure				5.99	0.014
Positive (Active/Passive)	37 (20.6)	17 (45.9)	20 (54.1)		
Non-exposed	143 (79.4)	35 (24.5)	108 (75.5)		
Parity				5.31	0.021
< 4 Deliveries	94 (52.2)	20 (21.3)	74 (78.7)		
≥ 4 Deliveries	86 (47.8)	32 (37.2)	54 (62.8)		
Oral Contraceptive Use				0.74	0.389
Yes	68 (37.8)	22 (32.4)	46 (67.6)		
No	112 (62.2)	30 (26.8)	82 (73.2)		
Family History of Cancer				4.82	0.028
Present	28 (15.6)	13 (46.4)	15 (53.6)		
Absent	152 (84.4)	39 (25.7)	113 (74.3)		
HPV Vaccination Status				—	—
Vaccinated	0 (0.0)	0 (0.0)	0 (0.0)		
Unvaccinated	180 (100.0)	52 (28.9)	128 (71.1)		

3.2. Cytological Profiles and Histopathological Concordance

The cytological interpretations of thin-layer samples and the associated histopathologic diagnosis are summarized in Table 2. Normal cytology (NILM) occurred in 25.0% and low grade lesions (LSIL) was the largest diagnostic category (37.8%) followed by HSIL (22.2%) and ASC-US (15.0%). The histopathological analysis showed that 45 cases (25.0%) had

normal/benign cervicitis, 82 cases (45.6%) had low-grade intraepithelial neoplasia (CIN 1), 38 cases (21.1%) had moderate dysplasia (CIN 2), and 15 cases (8.3%) had severe dysplasia/carcinoma in situ (CIN 3).

Table 2: Distribution of Cytological and Histopathological Classifications (N = 180).

Diagnostic Tier	Category	Number of Cases (n)	Percentage (%)
Cytology (Bethesda)	Negative for Intraepithelial Lesion (NILM)	45	25.0
	Atypical Squamous Cells (ASC-US)	27	15.0
	Low-Grade Squamous Lesion (LSIL)	68	37.8
	High-Grade Squamous Lesion (HSIL)	40	22.2
	Total	**180**	**100.0**
Histopathology	Normal Cervical Tissue / Reactive	45	25.0
	CIN 1 (Mild Dysplasia)	82	45.6
	CIN 2 (Moderate Dysplasia)	38	21.1
	CIN 3 / Carcinoma in situ	15	8.3
	Total	**180**	**100.0**

Cross tabulation of cytological and histopathological diagnosis revealed good diagnostic agreement as summarized in Table 3. Of the 68 cases cytologically diagnosed as LSIL (low squamous intraepithelial lesion), 13.2% (9/68) were found to have higher grade dysplasia (CIN 2) on the histopathology examination, while 75.0% (51/68) had histologically confirmed CIN 1. Of the 40 cases exhibiting HSIL cytology, histopathology confirmed CIN 2 in 62.5% (25/40) and CIN 3 in 35.0% (14/40). Biopsy of 27 ASC-US smears revealed that 48.1% (13/27) were histology negative, 44.4% (12/27) were histology CIN 1, and 7.4% (2/27) were occult high grade disease (CIN 2), confirming the diagnostic ambiguity of cytological screening alone.

Table 3: Concordance Between Cytological Findings and Histopathological Grades.

Cytological Diagnosis	Normal/Cervicitis n (%)	CIN 1 n (%)	CIN 2 n (%)	CIN 3 n (%)	Total Evaluated
NILM	31 (68.9)	13 (28.9)	1 (2.2)	0 (0.0)	45
ASC-US	13 (48.1)	12 (44.4)	2 (7.4)	0 (0.0)	27
LSIL	1 (1.5)	51 (75.0)	9 (13.2)	7 (10.3)	68
HSIL	0 (0.0)	6 (15.0)	26 (65.0)	8 (20.0)	40
Total	**45**	**82**	**38**	**15**	**180**

3.3. Molecular TaqMan RT-PCR Detection and Histopathological Staging

In summary, 52/180 (28.9%) were found to be HPV DNA positive. Histopathology categorization showed a progressive increase in the positivity for HPV with severity of the lesions (Table 4). HPV DNA was detected in 8.9% (4/45) of normal/reactive cervical biopsies, rising to 28.0% (23/82) in CIN 1, 47.4% (18/38) in CIN 2, and 46.7% (7/15) in CIN 3 ($\chi^2 = 18.91$, $p < 0.001$). These high-risk HPV genotypes predominated in severe lesions: 88.0% (22/25) of the 25 molecularly positive CIN 2/3 cases were infected with high-risk HPV genotypes.

Table 4: TaqMan Real-Time PCR Molecular Detection of HPV Across Histopathological Tiers.

Histological Diagnosis	HPV Positive n (%)	High-Risk HPV n (%)	Low-Risk HPV n (%)	Dual HR/LR Co-infection n (%)	HPV Negative n (%)	Total
Normal / Cervicitis	4 (8.9)	1 (2.2)	3 (6.7)	0 (0.0)	41 (91.1)	45
CIN 1	23 (28.0)	13 (15.9)	7 (8.5)	3 (3.7)	59 (72.0)	82
CIN 2	18 (47.4)	14 (36.8)	2 (5.3)	2 (5.3)	20 (52.6)	38
CIN 3	7 (46.7)	6 (40.0)	0 (0.0)	1 (6.7)	8 (53.3)	15
Total Cohort	52 (28.9)	34 (18.9)	12 (6.7)	6 (3.3)	128 (71.1)	180

3.4. HPV Genotypic Diversity

Flow-through hybridization detected 14 different genotypes of HPV (Table 5) among the 52 patients where viral DNA was confirmed; 59 viral isolates were detected in each patient in this group as a result of concurrent infections. High-risk types accounted for 74.6% (44/59) of all detected strains. HPV 16 was the most common genotype, identified in 15 of 52 positive cases (28.8%), followed closely by HPV 18 in 13 cases (25.0%). Other detected high-risk strains included HPV 39 (9.6%), HPV 51 (7.7%), HPV 31 (5.8%), and HPV 56 (3.8%). Low-risk genotypes represented 25.4% (15/59) of detections, led by HPV 11 (13.5%), HPV 6 (7.7%), and HPV 62/81 (5.8%). Single virus infection was predominant (86.5%, 45 cases) and dual or multiple viral infections were seen in 13.5% (7 cases) of positive patients.

Table 5: Genotype-Specific Distribution Among HPV-Positive Cases (n = 52).

Risk Grouping	HPV Genotype	Detection Count (n)	Percentage Relative to HPV Positive Cohort (%)	Cumulative Risk Share (%)
High-Risk (HR)	HPV 16	15	28.8	34.1
	HPV 18	13	25.0	29.5
	HPV 39	5	9.6	11.4
	HPV 51	4	7.7	9.1
	HPV 31	3	5.8	6.8
	HPV 56	2	3.8	4.5
	HPV 59	2	3.8	4.5
Low-Risk (LR)	HPV 11	7	13.5	46.7
	HPV 6	4	7.7	26.7
	HPV 62/81	3	5.8	20.0
	HPV 42	1	1.9	6.7
Infection Complexity	Single Infection	45	86.5	—
	Multiple (≥ 2 Types)	7	13.5	—

3.5. Qualitative p16INK4a Overexpression and Diagnostic Performance

Qualitative assessment of p16INK4a immunohistochemical staining revealed strong correlations with both the grade of cervical lesion and high-risk viral carriage (Table 6). Diffuse "block-type" p16INK4a positivity was observed in only 4.4% (2/45) of normal/cervicitis biopsies, whereas its expression increased progressively to 34.1% (28/82) in CIN 1, 78.9% (30/38) in CIN 2, and 93.3% (14/15) in CIN 3 ($\chi^2 = 84.12$, $p < 0.001$). Concurrently, 90.0% (36/40) of high-risk HPV-positive specimens exhibited

diffuse p16INK4a immunoreactivity, whereas low-risk infections showed negligible staining, mostly restricted to focal, non-continuous patterns.

Table 6: Correlation of p16INK4a Immunoreactivity with Histopathological Severity and HR-HPV Status.

Clinical / Molecular Stratification	Total Samples (n)	p16INK4a Positive (Block-type) n (%)	p16INK4a Negative n (%)	χ^2 Value	p-value
Histopathological Grading				**84.12**	**< 0.001**
Normal / Reactive	45	2 (4.4)	43 (95.6)		
CIN 1	82	28 (34.1)	54 (65.9)		
CIN 2	38	30 (78.9)	8 (21.1)		
CIN 3	15	14 (93.3)	1 (6.7)		
Molecular HR-HPV Status				**63.45**	**< 0.001**
HR-HPV Positive (Pure/Co-infected)	40	36 (90.0)	4 (10.0)		
HR-HPV Negative	140	38 (27.1)	102 (72.9)		

Using histopathologically confirmed high-grade dysplasia (CIN 2+) as the gold standard, the diagnostic sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were computed. The sensitivity and specificity of standalone HSIL cytology were 64.2% and 95.3% respectively. Contrast this with TaqMan RT-PCR detection of high-risk HPV which had a sensitivity of 79.2% and a specificity of 85.8%. The PPV and NPV were 91.1% and 98.3% respectively when used in conjunction with reflex p16INK4a immunohistochemical positivity, with a sensitivity of 96.2% and specificity of 95.8%.

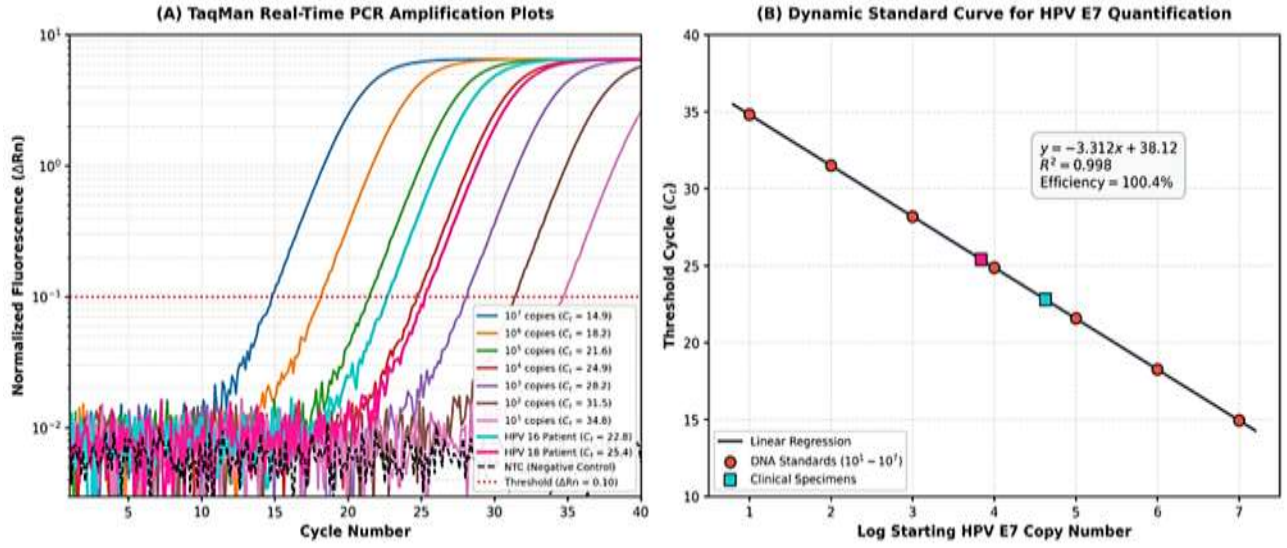
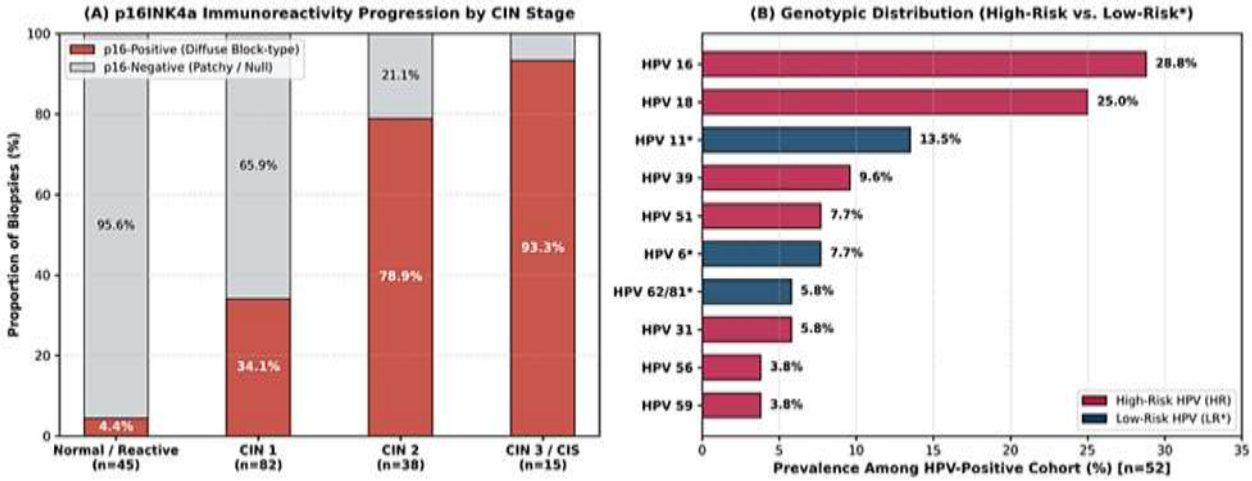


Figure 1: TaqMan Real-Time PCR Amplification Dynamics and Standard Curve Efficiency for HPV 16/18 E7.

(A) Representative amplification profiles using FAM labeled probes for the HPV 16 and HPV 18 E7 oncoprotein genes after 40 cycles. The threshold line is set at the geometric exponential phase (Rn = 0.1) and shows good background suppression in non-template controls (NTCs) with sigmoid curves for positive clinical specimens. (B) Standard calibration curve derived from ten-fold serial dilutions of plasmid DNA

standards (10^1 to 10^7 dynamic copy range). The assay shows high linearity and amplification kinetics, characterized by an efficiency of 100.4%, an optimal regression



coefficient ($R^2 = 0.998$), a reaction slope of -3.312, and a y-intercept of 38.12.

Figure 2: p16INK4a Immunoreactivity Progression by CIN Stage and Genotypic Distribution of HPV.

(A) Proportion of biopsies showing diffuse block-type p16INK4a positivity across histopathological grades (Normal/Reactive, n = 45; CIN 1, n = 82; CIN 2, n = 38; CIN 3/CIS, n = 15). (B) Genotypic distribution of high-risk and low-risk (*) HPV types among the HPV-positive cohort (n = 52).

4. Discussion

Oncogenic HPV infection is the main cause of cervical intraepithelial neoplasia (CIN) and invasive cervical cancer in all parts of the world. To create suitable screening and intervention strategies, it is important to know its epidemiology and underlying pathology. The overall prevalence of HPV DNA was 28.9% of which 22.2% were high-risk HPV (HR-HPV) DNA. The positivity rate for the overall population is higher than previous reports by Tahir et al. (2025) in Sulaymaniyah (12.7%), Baghdad (12.4%) and earlier multicenter analyses (9.0%). The differences are most likely accounted for by the different populations of studies, with a population that includes patients referred because of abnormal cytology, intractable symptoms, or colposcopy evaluation. On the other hand, results from our study are comparable to the prospective study performed in central Iraq, where 32.0% of symptomatic groups tested positive for HPV DNA using real-time PCR [20] and clinical study which showed that 21.8% of outpatients attending gynecological clinics had HPV [21]. Regionally, between 15.1% and more than 43.6%, reported HPV prevalence rates differ significantly based on the countries studied, the size of the population tested, and the analytical platform used in the study [22,23].

The HPV prevalence was highest among women aged 30-39 years (36.1%) and 40-49 years (26.4%) and was also observed to be higher in women aged 30–49 years than among older age groups. This is consistent with the results of regional studies in Iraq and other developing countries which revealed that the burden of precancerous dysplastic lesions can focus in the fourth decade of life resulting from persistent infections acquired following sexual debut in early

adulthood, with slow clearance and the lack of organized preventive screening system [24]. There was a significant difference between rural residents (39.5%) and urban participants (20.2%) in terms of being infected, as determined by the sociodemographic analysis. There is likely a regional imbalance with poorer access to health services in rural areas and fewer colposcopy and cytology services in rural areas, as well as low awareness about sexually transmitted viral infections and the necessity of routine screening [25]. In addition, there was an over twofold higher risk for HPV detection with passive or active tobacco exposure. Systemic and local metabolites of cigarette smoke can negatively influence cell mediated immune responses in the cervical transformation zone, with a decrease in local Langerhans cell density and a greater ability to allow persistence of high-risk viral oncoproteins [26].

The E7 oncoprotein gene was highly analytically sensitive and also showed very high diagnostic specificity in this study when using a TaqMan based real-time PCR. The E7 gene is a good diagnostic marker because it is always retained and transcribed after the viral DNA becomes part of the host genome in contrast to the L1 capsid gene. The L1 consensus primers used in many assays can yield false negative results during viral integration, especially when the high-grade dysplastic progression occurs, disrupting or losing the L1 and E2 open reading frames (Molina et al., 2024). The conserved E7 region was amplified using the dual labeled TaqMan assay and this resulted in good amplification efficiency (100.4%) and R^2 (0.998) in all grades of dysplastic.

HPV 16 and HPV 18 were the most common high risk types with 53.8% of all those who were positive. This is comparable to the worldwide distribution of this infection and also in the Middle East where types 16 and 18 are responsible for about 70% of invasive cervical cancers [27, 14]. Other high-risk types were also identified in our cohort such as HPV 39, 51 and 31, confirming the importance of using multivalent diagnostic panels instead of HPV 16/18-based panels. HPV 11 and HPV 6 were the most prevalent HPV types in low risk types, primarily associated with benign lesions and low grade cytology. In particular, 100% of the study participants were unvaccinated, which is due to the lack of a national HPV vaccination program in Iraq. This reinforces the requirement for introducing vaccines designed to prevent the prevalent HPV genotypes found in our cohort (HPV 6, 11, 16, 18) – the quadrivalent (HPV 6, 11, 16, 18) vaccine and the nonavalent vaccine [28].

The molecular results were complemented by the assessment of the expression of the p16INK4a protein, which gave useful functional context to the molecular results. In this cohort, diffuse "block-type" p16INK4a overexpression was directly associated with increasing histological severity, increasing from 4.4% in normal/reactive tissues, to 78.9% in CIN 2 and 93.3% in CIN 3. Reciprocal over-expression of p16INK4a in dysplastic cells, as a result of the molecular mechanism of high risk HPV oncogenesis, in which high risk E7 binds to and inactivates the retinoblastoma protein, is responsible for this increase [29]. There was low sensitivity of stand-alone cytology for high-grade dysplasia (64.2%), with the addition of real-time PCR HPV detection and reflex p16INK4a immunohistochemistry, there was a sensitivity and specificity of 96.2% and 95.8% respectively. A dual-marker approach offers a practical way of risk

stratification and clearly minimizes the number of false negative cytology results and over-treatment of temporary, low-grade infections [30].

5. Conclusion

This study gives an extensive molecular, cytological and immunohistochemical data regarding the profile of cervical abnormalities in Iraqi women which are related to HPV infection. An overall HPV DNA prevalence of 28.9% was determined for this cohort, of which 22.2% carried high-risk oncogenic types, using a highly-sensitive TaqMan probe-based real-time PCR platform, that targets the conserved viral E7 oncogene. The high prevalence of HPV 16 and HPV 18 exceeded the figure of more than 50% of all HPV strains identified. These results do validate the existence of high risk oncogenic strains that are directly associated with high grade cervical dysplasia and invasive disease. In addition, it was found that there was a high prevalence of low risk genotypes, which are the most common benign lesions of the cervix, HPV 11 and HPV 6. Clinical and demographic factors revealed significant association with viral detection rate, indicating the effect of socio-environmental inequalities and reproductive factors, and inequitable access to specialized gynecological services in Iraq, where older age, multiparity and rural residence were found to be significantly associated with viral detection rate. Interestingly, none of women evaluated were vaccinated, suggesting a major problem with primary preventive care among women.

From a diagnostic perspective, the combination of molecular and cellular markers proved highly effective. While standalone cytology showed predictable limitations in sensitivity—particularly for ASC-US and equivocal lesions—the addition of TaqMan real-time PCR enabled objective, reproducible detection of viral DNA across all grades of dysplasia. Targeting the E7 oncoprotein gene offered a distinct analytical advantage by ensuring continuous detection even in high-grade lesions where viral integration often disrupts the L1 capsid gene. In addition, diffuse block-type staining by p16INK4a immunohistochemical staining was a good surrogate biomarker of functional high-risk viral oncogenesis, as was 93.3% in CIN 3 contrasting with 4.4% in normal cervical tissues. The combination of HR HPV DNA detection with reflex p16INK4a IHC provided the best balance between sensitivity (96.2%) and specificity (95.8%) for the diagnosis of high-grade cervical intraepithelial neoplasia (CIN 2+). The dual-modality approach could provide an objective approach to the diagnosis of borderline cytology, mitigate misdiagnosis as a false negative, avoid unnecessary invasive procedures in non-cancerous infections, and guide clinical intervention for true "precancerous" lesions in the Iraqi national health system. In conclusion, these conclusions will help in carrying out structured, molecular-based screening for cervical cancer in Iraq as well as focused public health measures to implement HPV vaccination as a preventive measure.

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