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The Correlation Between HPV-16 Viral Load and Cytological Grades In Anbar

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Abstract

Background: Human papilloma virus 16 (HPV-16) is the most oncogenic type of HPV and it is in the centre of oncogenesis of cervical cancer. The HPV-16 viral load measured in real-time by the cycle threshold (Ct) values of PCR has been suggested as a potential disease severity biomarker.

Material and Methods: A cross-sectional study was conducted 121 females who attended gynecology clinics. The research was conducted in the obstetrics and gynecology units of Fallujah teaching hospitals and the outpatient clinics in Fallujah city. The research was done in the period between September 2025 and January 2026. Assessment of cervical cytology was based on the Bethesda system 2014. The viral load of the virus and HPV-16 were detected using the multiplex real-time PCR of E6/E7 oncogenes. The correlation was tested using GraphPad Prism software.

Results: HPV-16 was found to be most frequently detected with 15.7 (19/121) in general. The statistical significance between HPV-16 positivity and the severity of cytological grade was found to be statistical ($p < 0.001$). Prevalence of HPV-16 slowly rises to 4.5% in Negative intraepithelial Lesion or Malignancy (NILM) to 67.9% in High-Grade Squamous Intraepithelial Lesions (HSIL). The quantitative analysis demonstrated that such negative correlation of lesion grade and viral load was significant; mean of Ct of HSIL and Atypical Glandular Cells (AGC) were significantly lower than that of NILM (28.42 and 28.16, respectively). High grade lesions could be differentiated well with a Ct cut off of 30 or below.

Conclusion: HPV-16 viral load is a good discriminant against the degree of lesions in the cervix. A large viral load is an excellent indicator of high-grade pathology, which means that quantitative PCR can be used to supplement cytology in the risk stratification.

Keywords: Bethesda System, Cycle Threshold (Ct), Cervical Cytology, HPV-16, Real-Time PCR, Viral Load.

الملخص

يُعد فيروس الورم الحليمي البشري النمط 16 **(HPV-16)** الأكثر ارتباطًا بإحداث الأورام، ويحتل دورًا محوريًا في تطور سرطان عنق الرحم. وقد طُرح قياس الحمل الفيروسي لهذا النمط باستخدام قيمة العتبة الدورية **(Ct)** في تفاعل البوليميراز المتسلسل اللحظي **(Real-Time PCR)** كمؤشر محتمل لشدة المرض.

أُجريت دراسة مقطعية شملت 121 امرأة راجعن عيادات أمراض النسائية. نُفذت الدراسة في وحدات النسائية والتوليد في مستشفيات الفلوجة التعليمية والعيادات الخارجية في مدينة الفلوجة، خلال الفترة من أيلول 2025 إلى كانون الثاني 2026. تم تقييم لطاخة عنق الرحم وفق نظام بيثيسدا 2014. كما تم الكشف عن الحمل الفيروسي لفيروس **HPV-16** باستخدام تقنية **PCR** اللحظي المتعدد **(Multiplex Real-Time PCR)** باستهداف جيني**E6/E7** أُجري تحليل الارتباط الإحصائي باستخدام برنامج**GraphPad Prism.** اظهرت

اظهرت النتائج انه تم الكشف عن **HPV-16** بنسبة 15.7% (19/121) من الحالات. وُجد ارتباط ذو دلالة إحصائية عالية بين إيجابية **HPV-16** وشدة التغيرات الخلوية **(p < 0.001).** ارتفعت نسبة انتشار الفيروس تدريجيًا من 4.5% في الحالات السلبية للآفات داخل الظهارية أو الخبيثة **(NILM)** إلى 67.9% في الآفات الحرشفية عالية الدرجة **(HSIL).** أظهر التحليل الكمي وجود علاقة عكسية ذات دلالة إحصائية بين درجة الآفة وقيم**Ct** ؛ حيث كانت القيم المتوسطة لـ **Ct** في حالات **HSIL**وخلايا الغدد الشاذة **(AGC)** أقل بشكل ملحوظ مقارنةً بحالات **NILM (28.42** و28.16 على التوالي). كما تبين أن حد القطع لقيمة **Ct ≤ 30** يُعد مناسبًا للتمييز بين الآفات عالية الدرجة.

استنتجت الدراسه انه يُعد الحمل الفيروسي لفيروس **HPV-16** مؤشرًا فعالًا للتمييز بين درجات آفات عنق الرحم. إن ارتفاع الحمل الفيروسي يرتبط بوجود آفات عالية الدرجة، مما يشير إلى إمكانية استخدام **PCR** الكمي كأداة مساندة للفحص الخلوي في تصنيف الخطورة المرضية.

الكلمات المفتاحية :نظام بيثيسدا، قيمة العتبة الدورية(Ct) ، **الخلايا العنقية، فيروس الورم الحليمي البشري-16، تفاعل البوليميراز المتسلسل اللحظي، الحمل الفيروسي.**

Introduction

Cervical cancer is a major social health problem, and one of the leading causes of cancer related death among women across the entire world, particularly in the low and middle income countries[1] which70% are caused by HPV-16 and HPV-18 genotype infection. [2, 3]. The cause of cervical cancer has been

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recognized as a necessary requirement of perpetual infection with the high-risk human papillomavirus (HR-HPV), and HPV-16 is the most frequent and oncogenic genotype [4, 5]. The incidence of HPV-16 in cervical cancer is estimated to be 50–60 percent and it has a strong association with high-grade cervical intraepithelial neoplasia and invasive carcinoma[4, 6-8] . Oncogenicity mediated by the HPV-16 is carried out by the viral E6 and E7 oncoprotein which interfere with the p53 pathway and tumor suppressor pathway of retinoblastoma (Rb) and cause genomic instability and malignant transformation[4, 9, 10]. Unlike the popular HPV, DNA screening in the cervix, qualitative detection is not sufficient to differentiate between the transient infections and those with a higher likelihood of developing the disease [11, 12]. Consequently, clinical utility of HPV viral load as a prognostic biomarker is in increased interest [13, 14]. Using real time PCR, semi-quantitative analysis of the viral load can be performed by utilizing Ct values where low Ct values indicate high viral replication and transcriptional activity[15-17]. Various studies have found that a comparison exists between high HPV-16 viral load and severity of cervical cytology abnormalities, particularly high-grade lesions[18-20]. However, population based data in most regions including Iraq are still few[21, 22]. The study was aimed at measuring the correlation between HPV-16 viral load and cervical cytological grades of Iraqi women on a multiplex real-time PCR. It has been shown that the level of viral DNA, the so-called viral load, can be considered as a predictive biomarker. Ct value applied in Real-Time PCR (qPCR) is inversely related to viral load; lower Ct values indicate higher viral copy numbers, whereas higher Ct values reflect lower viral loads.

2. Materials and Methods

2.1 Study Design and Setting

The research was a cross-sectional study conducted in the . The research was done in the period between September 2025 and January 2026, which included the time of patient recruitment, specimen, lab work, and interpretation of results. which was in line with the ethical guidelines in the Declaration of Helsinki.

2.2 Informed Consent and Ethical Approval.

Each participant received written informed consent before enrolment. The permission form addressed the purpose, methods, risks, and advantages of the study and the fact that no information of the patients would be disclosed and that the participation was voluntary only. The subjects were assured of the freedom to discontinue with the study whenever they wanted without compromising their medical care. Ethical approval for this study was obtained from the Research Ethics Committee, College of Applied Science, University of Fallujah (Approval No. AS-EC/0007).

2.3 Research Group

2.3.1 Criteria for Inclusion.

These included 20 years old and above women that visited the gynecological clinics to have a routine checkup or those who complained of having such symptoms which were suggestive of cervical pathology. All subjects were required to agree to the pelvic exam and vaginal swab specimen, active sex life, and not pregnant.

2.3.2 Criteria for Exclusion

To eliminate any contamination or interference of the molecular analysis, women who had undergone total hysterectomy, were pregnant, were menstruating at the time of specimen collection, or had taken vaginal medicines or douches within 48 hours before specimen collection were not included in the study.

2.4 Calculating specimen Size

The specimen size of 115 individuals was determined as the required specimen size with a goal prevalence of approximately 15% based on regional data that is estimated to be 15, a 95 confidence margin, and a 5 margin of error. A target specimen of 125 was established to

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ensure that there was consideration of the invalid or dropout specimen. Finally, the subjects that had complete data were 121.

2.5.1 Information and Clinical Assessment gathering.

Each participant was provided with a systematic questionnaire that contained clinical (symptoms, obstetric history, use of contraceptives) and demographic (age, marital status) information. The clinical symptoms that were documented included pelvic pain, vaginal discharge that is atypical (watery, pink, or purulent), post-coital bleeding, abnormal vaginal bleeding and post-menopausal hemorrhage. A doctor conducted a gynecological test to identify the presence of apparent lesions to the cervix.

2.6 Collection and Management of specimen .

2.7 Cytological Evaluation

Sterile cytobrushes were used to take vaginal swabs. A brush was put in the endocervical canal to rotate it 360 degrees so that it ensures sufficient exfoliated epithelial cells were collected. The swabs were frozen on ice after 4 hours then placed in sterile tubes with the nucleic acid preservation buffer and dispatched The Papanicolaou method was applied to stain the specimen when made as Pap smears and fixed in 95% ethanol. Two independent cytopathologists who had no information about the PCR results were used to do the cytological evaluation. The slides were categorized into the following, in descending order, by the Bethesda System 2014: Atypical Squamous Cells-cannot exclude HSIL (ASC-H). Inflammatory changes were also present to the molecular biology lab.

2.8 HPV-16 Molecular Detection

2.8.1 Extraction of DNA

Genomic DNA was prepared out of the vaginal swab specimen based on a commercial DNA extraction kit (e.g. QIAamp DNA Mini Kit, Qiagen, Germany). The concentration of the extracted DNA and the purity of the DNA were determined using the Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). A260/A280 of 1.8 to 2.0 was to be regarded as clean PCR.

2.8.2 Multiplex Real Time PCR

Primer design

In this study, primers were designed to target the HPV-16 E6 and E7 oncogenes. For the E6 gene, the forward primer sequence was 5′-CAAAAGAGAACTGCAATGTTTCAGGAC-3′ and the reverse primer 5′-CACCGACCCCTTATATTATGGAATCTTTG-3′, yielding a 420 bp amplicon. The melting temperatures (Tm) were 59.8 °C and 62.5 °C, and GC contents 40.0% and 42.9% for the forward and reverse primers, respectively. For the E7 gene, the forward primer was 5′-TGTTAGATTTGCAACCAGAGACAAC-3′ and the reverse primer 5′-AGTGTGCCCATTAACAGGTCT-3′, producing a 226 bp amplicon. The Tm values were 60.0 °C and 59.3 °C, and GC contents 40.0% and 47.6% for the forward and reverse primers, respectively. A multiplex real-time PCR assay based on SYBR Green chemistry was used for HPV-16 detection, with β-globin primers included as an internal control to verify DNA quality and the absence of reaction inhibitors. Each 25 µL reaction mixture contained 12.5 µL of 2× SYBR Green Master Mix, 1 µL of HPV-16 primer mix, 1 µL of β-globin primer mix, 5.5 µL of nuclease-free water, and 5 µL of template DNA. Positive and negative controls were included in every run. Amplification was carried out on a real-time PCR thermal cycler using the following cycling conditions: initial activation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 1 min. Threshold cycle (Ct) values were recorded for each sample. A sample was considered positive for HPV-16 when the target gene Ct value was ≤ 38 and the internal control (β-globin) Ct value was detectable. No gel

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electrophoresis of amplification products was required
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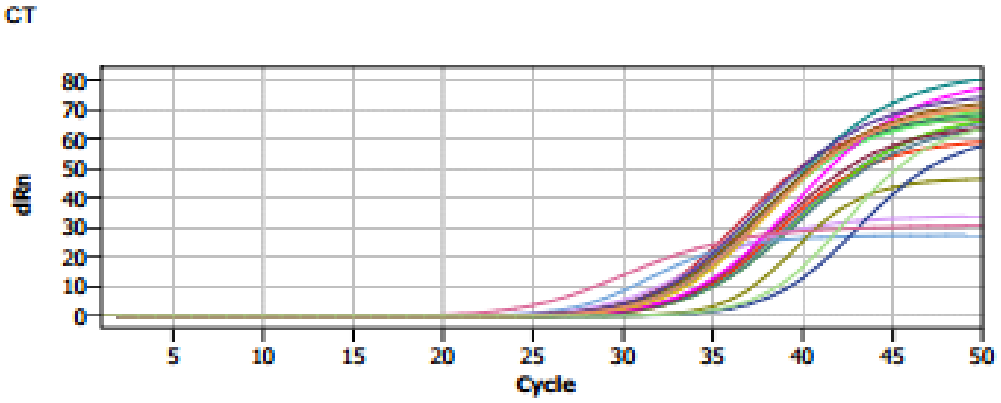

Figure 1: Multiplex real-time PCR for detection of HPV-16.

2.8.3 Results interpretation

In case the cycle threshold (Ct) was below 40, specimen were considered positive with HPV-16. A Ct value greater than 40 was considered negative. The viral load was semi-quantitatively assessed based on the Ct value: Ct less than 30 (high viral load), Ct between 30 and 35 (moderate), and Ct more than 35 (low viral load).

2.8.4 Statistical Analysis

All data are expressed as (Mean ± Standard Deviation) of mean (SD). Statistical analysis was performed using GraphPad Prism software (version 9.5.1, LLA, CA, USA) used for statistical analysis. The parametric analysis was performed using Chi-square (and Fisher's exact) test to find the significant difference between different groups using; NS: Non significant difference: $p > 0.05$, *: significant: $p \leq 0.05$, **: Very significant: $p \leq 0.01$, ***: Highly significant: $p \leq 0.001$ and ****: Excellent significant: $p \leq 0.0001$ were considered statistical significant.

3.1 Results

The cytological profile and prevalence were provided in Table 1. The final analysis comprised 121 women (100%). As per the classification given by the Bethesda System, most of the participants (78.5%- n=95) showed normal cytology (NILM). The presence of abnormalities in epithelial cells was detected in 14.1 percent of the study population, and the most frequent abnormality was LSIL (5.0 percent). Table 3 provides in details cytological distribution.

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Table (1): Prevalence and Distribution of Cervical Cytological

Cytological Diagnosis	*n*(%)	*p*-value
Negative for Intraepithelial Lesion or Malignancy (NILM)	95(78.5%)	<0.0001 ****
Atypical Squamous Cells of Undetermined Significance (ASCUS)	2(1.7%)	
Low-Grade Squamous Intraepithelial Lesion (LSIL)	6(5.0%)	
Atypical Squamous Cells – cannot exclude HSIL (ASC-H)	5(4.1%)	
High-Grade Squamous Intraepithelial Lesion (HSIL)	3(2.5%)	
Atypical Glandular Cells (AGC)	1(0.8%)	
Inadequate / Missing	9(7.4%)	
NS: Non significant difference: $p > 0.05$, *: significant: $p \leq 0.05$, **: Very significant: $p \leq 0.01$, ***: Highly significant: $p \leq 0.001$ and ****: Highly significant: $p \leq 0.0001$.		

3.2 HPV-16 Detection Rate

Molecular diagnosis has revealed HPV-16 DNA in 19 of 121 specimen, which result in the total prevalence of 15.7. The rest 102 specimen (84.3 percent) were negative to HPV-16, as shown in Figure 1. There was a highly significant statistical association between HPV-16 positivity and the severity of cytological findings (Chi-square, $p < 0.0001$). The infection rate was significantly higher in women with epithelial cell abnormalities compared to those with NILM.

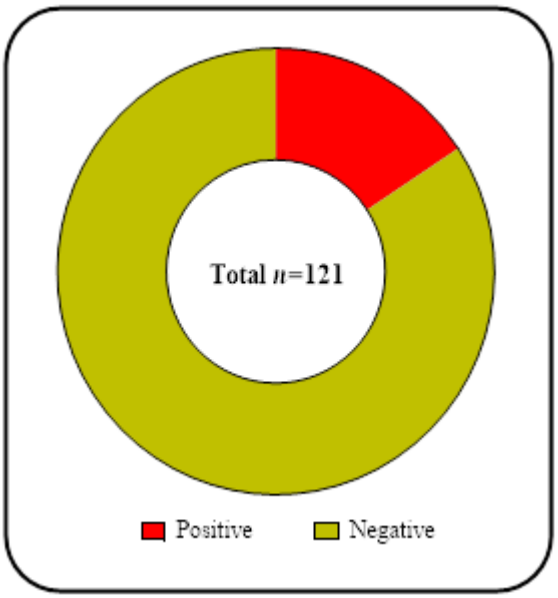

Figure 2: Percentage distribution of HPV-16 positive and negative cases among the study specimen.

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Compares the mean Ct values across diagnostic groups. High-grade lesions (HSIL) and glandular abnormalities (AGC) demonstrated the lowest mean Ct values (28.42 and 28.16, respectively), indicating the highest viral loads. In contrast, NILM and LSIL groups showed significantly higher mean Ct values (35.34 and 35.41), indicating low viral loads. Furthermore, the individual distribution of Ct values identified a potential diagnostic threshold. All cases of HSIL and AGC clustered below a Ct value of 30 (High Viral Load zone). Conversely, positive cases within the NILM and LSIL categories were predominantly clustered between Ct 34 and 37, As shown in Figure 2.

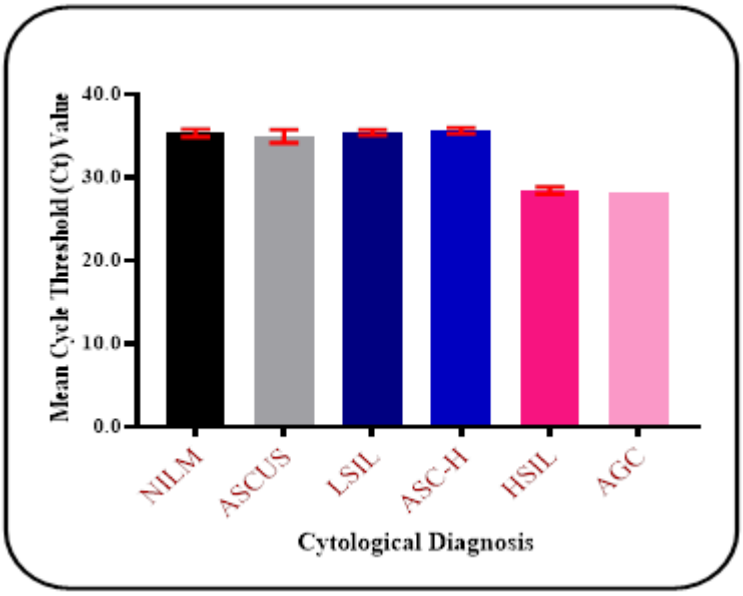

Figure 3: Mean Cycle Threshold (Ct) values of HPV-16 across different cytological diagnoses.

Discussion

The current study gives solid reasons to confirm that HPV-16 infection, viral load, and the extent of cervical cytological abnormalities in Iraqi women have a statistically significant association. The reported HPV-16 prevalence of 15.7% is in line with the recent global and regional levels reporting that HPV-16 is the most prevalent high-risk genotype that causes cervical cancer[23, 24]. These results also support the fact that HPV-16 continues to be the major oncogenic agent in different geographic and demographic settings. An important finding in the given study is that all the lesions of high grade (ASC-H and HSIL) were HPV-16 positive. This finding is very similar to the international data on cervical cancer prevention efforts and cancer monitoring efforts, which have continually reported a high genotype-specific relationship between HPV-16 and high-grade cervical lesions and invasive disease [25, 26]. The overall lack of HPV-16 negative cases in the high-grade lesions in this cohort indicates a causal relationship and the predictive predominance of HPV-16 in the carcinogenesis of the cervix. Several past studies have shown that HPV-16 detection rates are rising with the severity of the cervical lesions, with low-grade abnormalities being at the low end to the high end, including HSIL and cervical cancer[25, 27]. Current research findings on the subject matter are consistent with this trend, which proves the idea that chronic HPV-16 infection is one of the key factors in multistage cervical epithelial transformation. The chronic infection and not the random exposure is the determinant

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that seems to be the most important factor in the progression of disease. In addition to qualitative HPV detection, this study has made an emphasis on the significance of the viral load as a clinically significant parameter[28]. The association of low Ct values (i.e. high viral load of HPV-16) with high-grade cytological abnormalities was dominant. The observation has been in line with past reports that reveal that high HPV-16 viral load correlates with viral persistence, augmented oncogenic activity and augmented risk of progression to high-grade lesions[27, 29]. It is believed that the high viral load is a symptom of improved viral replication and continuous transcription of oncogenic viral genes. Molecular studies that reveal that high levels of gene expression of HPV-16 E6 and E7 lead to sustained silencing of the p53 and retinoblastoma (Rb) tumor suppressor pathways, provide good biological plausibility of this association. The result is uncontrolled progression of the cell cycle, genomic instability, and progressive acquisition of genetic changes that favor malignant transformation [30]. Thus, viral load is not only a quantitative measurement, but also an indirect measure of cellular-level oncogenic activity. Clinically, measurement of the HPV-16 viral load could be of great benefit in comparison with qualitative HPV testing[31-33]. Conventional screening approaches can detect a high percentage of non-progressive transient infections that will not lead to subsequent follow-ups, causing patients psychological stress[34]. It has been suggested that viral load quantification is a feasible triage method that can be used to differentiate between clinically important infections and those of low oncogenic potential[27]. The results of this study are in favor of the possibility of a role of viral load stratification in enhancing clinical decision-making. To some extent, the overall idea of introducing Ct-based viral load estimation into cervical cancer screening programs can be of specific use in resource-limited environments. Kyrgiou et al. emphasized that molecular biomarkers such as HPV viral load might help to increase screening effectiveness and increase the use of resources in low- and middle-income countries[20]. In the environment where access to repeated cytology or colposcopy is scarce, the viral load assessment may be used to prioritize high-risk women to monitor them with increased attention or to initiate treatment earlier. Regardless of the strong points of this research, some drawbacks are to be admitted[35]. A cross-sectional design does not permit the examination of time changes in viral load and building a causal association between HPV-16 viral load and lesion progression[36]. Moreover, the fairly limited quantity of high-grade lesions can be a limitation to the generalizability of the results. The longitudinal research needs to be prospective to assess the changes in the viral load with the course of time and understand whether HPV-16 viral load can be trusted to predict or regress the cervical lesions [37]. In general, this paper presents useful molecular epidemiological information on HPV-16 viral load and its relationship with cervical cytological severity among Iraqi women. The findings back up the increasing literature indicating that HPV-16 viral load is a biologically and clinically relevant biomarker that can be utilized in promoting cervical cancer screening and risk-stratification interventions.

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