Co-infection of torque teno virus (TTV) and human papillomavirus (HPV) in cervical samples of women: a case-control study

Running Title: TTV/HPV Co-infection in Cervical Specimens

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Abstract

Objective: This study aimed to investigate the prevalence of TTV in cervical samples from HPV-infected women and its distribution with HPV genotypes.

Materials and Methods: In this case-control study, 240 women (120 HPV-positive cases and 120 HPV-negative controls) attending routine cervical screening in Hamadan were enrolled. DNA was extracted from cervical samples, and quality was assessed using the GAPDH gene. TTV detection was performed using nested PCR targeting the 5'UTR region. Data were analyzed using chi-square and Fisher's exact tests.

Results: Overall, TTV was detected in 36.3% (87/240) of samples, with 45% of HPV-positive women and 27.5% of controls testing positive (p=0.007). In both groups, the highest TTV prevalence was observed in women aged 26-30 years (47.7%). TTV positivity was highest in the High+Low Risk HPV group (65.5%), followed by High Risk (46.3%) and Low Risk (27.0%) groups (p=0.007). Moreover, high-risk HPV genotypes 52, 56, and 68 showed higher rates of TTV co-etection (p=0.032, 0.012, and 0.039, respectively). In the HR group, the highest frequencies were found in women older than 40 and 26-30 years, while LR and H-L groups showed more even distributions across ages.

Conclusion: TTV infection is prevalent among HPV-positive women, particularly those with multiple or high-risk HPV genotypes. The co-detection of TTV and HPV suggests potential shared transmission routes and raises the possibility that TTV may influence HPV persistence or cervical pathogenesis. These findings provide a foundation for future studies on TTV-HPV interactions and their implications for cervical disease progression.

Keywords: Cervical cancer, Genotype, Prevalence, Papillomavirus, Torque Teno virus

1. Introduction:

Cervical cancer is the fourth most frequently diagnosed cancer in women worldwide, with approximately 660,000 new cases and around 350,000 deaths recorded in 2022 [1]. Human papillomavirus (HPV) infection is the most widespread sexually transmitted viral infection globally and serves as the primary cause of cervical cancer, which continues to pose a significant public health challenge [2]. Today, more than 200 genotypes of HPV have been described. HPV genotypes are classified into high-risk (HR-HPV) and low-risk (LR-HPV) types based on their oncogenic potential [3]. While most infections, particularly those caused by LR-HPV types, such as HPV 6 and 11, are transient and may lead to benign lesions such as genital warts. There are at least 12 high-risk genotypes, such as HPV 16, 18, 31, and 45, are associated with progression to cervical intraepithelial neoplasia (CIN) and invasive carcinoma [4]. Although most HPV infections are transient and cleared by the host immune system, a subset of infections persist and progress to cervical intraepithelial neoplasia and invasive carcinoma [5]. The mechanisms determining HPV persistence and progression are multifactorial and include host immune status, viral genotype, and possible interactions with other microorganisms [6].

Torque teno virus (TTV), a small, non-enveloped, single-stranded circular DNA virus from the *Anelloviridae* family, is commonly found in the general population and is regarded as a component of the human virome [7]. TTV was initially considered a potential cause of non-A-G hepatitis [8]. Although TTV is not currently associated with a specific disease, its viral load has been proposed as a marker of immune competence, with higher levels observed in immunocompromised individuals such as transplant recipients and HIV-infected patients [9]. Importantly, TTV has been detected in various biological compartments, including blood, saliva, genital secretions, and

cervical samples [10]. Finding any epidemiological association is complicated by the fact that TTV is ubiquitous, with reported prevalence among blood donors ranging from 3.7% to 65% [11].

Emerging evidence suggests that TTV may influence local immune responses and could play a role in modulating the outcome of co-infections [12]. In the context of cervical health, TTV has been reported more frequently in women with cervical lesions and HPV infection than in healthy women, raising the hypothesis that TTV might act as a co-factor in HPV persistence or pathogenesis [13,14]. However, data remain limited and inconsistent, and the exact relationship between TTV and HPV in the female genital tract is not yet clear.

Given the high prevalence of both viruses and the clinical relevance of persistent HPV infection, understanding the interaction between TTV and HPV is of potential importance. Investigating whether TTV infection or viral load differs between HPV-positive women and HPV-negative controls may provide insights into viral co-infection dynamics and the potential utility of TTV as a biomarker of susceptibility to HPV persistence. Therefore, the present study aimed to investigate the prevalence of TTV in cervical samples from women infected with HPV compared to HPV-negative healthy controls, and to explore the distribution of TTV detection among different HPV genotypes.

2. Material and methods:

2.1. Study Population

This case-control study was conducted on women attending Hamadan laboratories for routine gynecological examination and cervical cancer screening between May 2024 and December 2024. The case group included women who tested positive for HPV DNA, while the control group

consisted of women with negative HPV results. Inclusion criteria were adequate sample volume and quality for DNA extraction. Written informed consent was obtained from all participants.

2.2. Sample Collection and DNA Extraction

Cervical samples had originally been collected by physicians during routine gynecological examinations. Each specimen was placed into liquid-based cytology medium after collection. Samples were stored at -20 °C until use for HPV testing. Based on the results, HPV-positive samples were assigned to the case group and HPV-negative samples to the control group. After confirmation of HPV status, all samples were transported under appropriate conditions to the Virology Laboratory, School of Medicine, and stored at -20 °C until further analysis.

DNA was extracted from 200 µL of each specimen using a commercial nucleic acid extraction kit (sinaclon, Iran), following the manufacturer's protocol. Each sample was treated with a buffer supplied by kit and Proteinase K. DNA quantity and purity were evaluated Nanodrop spectrophotometer (Nabi, South Korea). Also, the quality evaluation of the extracted genome involved amplifying the human GAPDH gene using primers (Table 1).

2.3. Nested PCR for TTV Detection

The primers used for the first (NG133/147) and second (NG134/132) rounds were selected from previously published studies targeting the 5′UTR region of the TTV genome and are summarized in Table 1. The reaction mix contained 5 µL of template DNA or controls, 12.5 µL of master mix 2x (sinaclon, Iran), 1 µL of each primer, and 5.5 µL of sterilized DW. Cycling conditions included an initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 40 seconds, with a final extension at 72 °C for 5 minutes. The second-round was performed using 1 µL of the first-round amplicon as a template for 25 cycles

under the same conditions. The expected product size was approximately 110 bp. Amplified products were analyzed by electrophoresis on a 3% agarose gel and visualized under UV illumination (Figure 1). One PCR-positive sample was subjected to sequencing analysis for confirmation of the amplification product.

Table 1: Primers used for nested PCR amplification of the TTV

Primer name	Polarity	Nucleotide sequence (5′-3′)	Product size (bp)	Reference
GAPDH	Forward	GTCTCCTCTGACTTCAACAGCG	131	[15,16]
GAPDH	Reverse	ACCACCCTGTTGCTGTAGCCAA		
NG133	Forward (91-115)	GTAAGTGCACTTCCGAATGGC TGAG	143	
NG147	Reverse (211-233)	GCCAGTCCCGAGCCCGAATTGCC		
NG134	Forward (114-136)	AGTTTTCCACGCCCGTCCGCAGC	110	
NG132	Reverse (204-223)	AGCCCGAATTGCCCCTTGAC		

2.4. Statistical Analysis

Data were analyzed using SPSS software version 26. Categorical variables such as prevalence of TTV and HPV were compared using chi-square or Fisher's exact tests. A p-value <0.05 was considered statistically significant.

3. Results:

3.1. Study Population

A total of 240 women were enrolled in the study, including 120 HPV-positive cases and 120 HPV-negative controls. The mean age of participants was 33.29 ± 8.048 years (range: 18-53), with no statistically significant difference between cases and controls (p=0.881).

3.2. The results of TTV detection in Cervical Samples

Amplification of the GAPDH confirmed the quality of extracted DNA (Figure 1). As shown in Figure 2, TTV was detected by PCR and analyzed on agarose gel. Positive bands were observed in lanes 1-6. The overall prevalence of TTV was 36.3% (87/240). TTV DNA was detected in 45% (54/120) of HPV-positive women compared to 27.5% (33/120) of controls. The difference in prevalence between the two groups was statistically significant (p=0.007). HPV-positive women had more than twice the odds of being TTV-positive (OR = 2.16, 95% CI: 1.29-3.61).

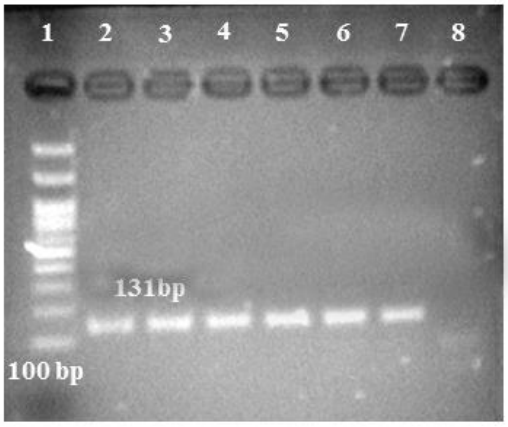

Figure 1: Agarose gel electrophoresis of GAPDH. Products: Lane 1: 100 bp DNA marker; Lanes 2-7: extracted samples; Lanes 8, negative control.

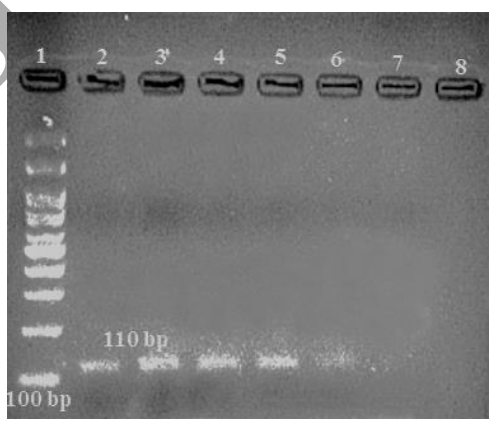

Figure 2: Torque Teno virus detection by electrophoresis on agarose gel. Gel-electrophoresis of second-round PCR products (5'UTR region) using 3% agarose. Lane1: 100 bp DNA marker; Lanes 2-6: positive samples; Lanes 7, negative sample; Lanes 8, negative control.

Based on age data, this study revealed a high rate of TTV infection, with no statistically significant difference in TTV positivity across age groups (p=0.226). In both groups, the highest rate of TTV infection was observed in the age group 26-30, with 21 out of 44 individuals (47.72%) affected (Table 2).

Table 2: Frequency and Percentage of TTV Positivity by Age Group

Age group	Positive/Total (n)	%
<20	2/13	15.38
21-25	15/34	44.11
26-30	21/44	47.72
31-35	18/50	36
36-40	15/47	31.91
>41	16/52	30.76

3.3. TTV and HPV Co-Detection

Table 3 shows the prevalence of TTV infection according to HPV risk groups. A statistically significant difference in TTV positivity was observed among the HPV genotype risk groups (p= 0.007). In High-Risk group, among 54 individuals, 25 (46.3%) were TTV-positive. In Low-Risk group, among 37 individuals, 10 (27.0%) were TTV-positive. In combined High+Low Risk group, among 29 individuals, 19 (65.5%) were TTV-positive. This group showed a significantly higher prevalence, indicating that it accounted for the largest share of TTV-positive cases in the comparison. TTV detection rates stratified by HPV genotypes are summarized in Table 4.

Statistical analysis revealed significant differences between TTV-positive and TTV-negative groups in the distribution of HR-genotypes 68 (p=0.039), 52 (p=0.032), and 56 (p=0.012).

Figure 3 shows distribution of TTV-positive cases according to age categories and HPV risk groups. The highest counts in HR were observed in the >40 and 26-30 age groups, while LR and H-L groups showed more even distributions across ages. No statistically significant difference in the distribution of low-risk and high-risk HPV genotypes was observed across age groups (p=0.665). A borderline significant difference among age groups was observed for genotypes 52 (p = 0.074) and 66 (p = 0.077).

Table 3: Prevalence of TTV infection according to HPV risk groups

Genotype	TTV Positive (n)	TTV Negative (n)	Total
High Risk	25	29	54
Low Risk	10	27	37
High+Low Risk	19	10	29
Negative	33	87	120
Total	87	153	240

Table 4: Prevalence of TTV according to HPV genotypes.

Genotype	Total (n)	TTV Positive (n)	TTV Negative (n)	*P* value
6	58	25	33	0.215
11	48	18	30	0.868
16	10	6	4	0.176
18	5	3	2	0.356
31	2	2	0	0.130
33	4	1	3	1.000
35	1	1	0	0.362
39	21	10	11	0.342
45	7	4	3	0.259
51	10	5	5	0.503
52	12	8	4	0.032
56	9	7	2	0.012

58	11	5	6	0.533
59	4	0	4	0.300
66	7	3	4	0.706
67	4	2	2	0.622
68	10	7	3	0.039

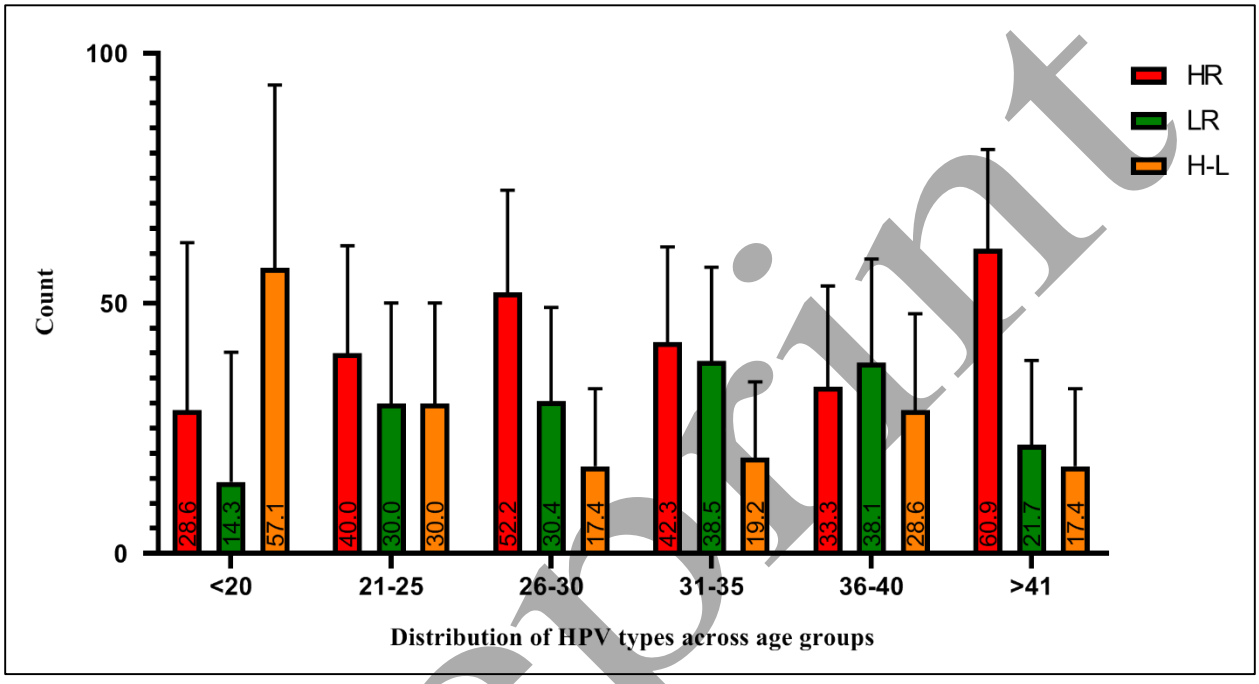

Figure 3: Distribution of TTV-positive cases according to age categories and HPV risk groups. The bar chart shows the number of TTV-positive individuals across different age categories stratified by HPV risk groups: High Risk (HR), Low Risk (LR), and combined High+Low Risk (H-L). Data are presented as percentage with 95% confidence intervals.

4. Discussion:

TTV is present in nearly all populations worldwide, with prevalence varying by geographic region from 4% in Iran to 96% in Jordan and may reach 100% in immunocompromised patients [10]. TTVs were initially discovered in the serum of a patient with non-A-E hepatitis, but further research has found them in a variety of human tissues. Several potential transmission routes for

TTV have been proposed in previous studies, including airborne transmission, fecal-oral route, blood transfusions, transplacental transmission during pregnancy, breastfeeding, and sexual contact [13,17-19]. Given its widespread presence and multiple transmission routes, TTV has been tentatively associated with a range of diseases, including pancreatic and laryngeal cancers, diabetes, arthritis, and several central nervous system (CNS) disorders, such as Alzheimer's disease [20]. Given the co-detection of HPV and TTV in cervical smears, and considering the established role of HPV in cervical cancer, we aimed to investigate the presence of TTV in cervical samples.

In the present study, we investigated the prevalence of TTV infection in cervical samples according to HPV infection. The overall prevalence of TTV in our study population was 36.3% (87/240). TTV DNA was detected in 45% (54/120) of HPV-positive women compared to 27.5% (33/120) of HPV-negative controls. This difference was statistically significant (p=0.007). The findings of this study indicate that the odds of TTV detection were approximately two times higher in HPV‑positive women compared with HPV-negative controls, suggesting a possible association between HPV infection and increased susceptibility to TTV co-infection.

The observed co-detection underscores the importance of considering TTV as a potential co-factor in studies of HPV-associated cervical pathology. Our findings are consistent with previous studies reporting frequent co-detection of TTV and HPV. Siahpoush et al. demonstrated a significantly higher prevalence (58%) of TTV in women infected with HPV compared to HPV-negative individuals, supporting the hypothesis of overlapping transmission routes and potential interactions between these two viruses [14]. In another study, Saláková et al. found TTV in 74.7% of cervical smears from women with cervical lesions, which was notably higher than in healthy women (52.7%) [13].

According to age, this study found no statistically significant difference in the rate of TTV infection across age groups (p=0.226). These findings contrast with studies suggesting that the rate of TTV infection may increase with age due to a weakened immune response associated with immunosenescence [21]. Similarly, Siahpoush et al. also found no significant association between age and TTV infection, suggesting that age may not be a key factor influencing TTV positivity [14].

As shown in Table 2, the highest prevalence was observed in women aged 26–30 years (47.7%) and 21–25 years (44.1%), while the lowest was in women under 20 years (15.4%) and over 41 years (30.8%). These results suggest that TTV infection is more frequent in sexually active women in their mid-20s to early 30s, which aligns with the peak age of HPV infection reported in multiple studies. The lower prevalence in younger <20 and older >41 women may reflect differences in sexual exposure, immune status, or other behavioral factors. Further studies are needed to investigate the role of sexual behaviors in age groups with high sexual activity, as the existing evidence is still limited and insufficient to draw definitive conclusions. Väisänen et al. has shown that TTV infection results from a combination of sexual and non-sexual transmission routes. Almost all children (99%) had tested positive for TTV-DNA at least once, and their viral loads fluctuated over the follow-up period [22].

According to Table 3, TTV positivity was highest in the High+Low Risk group (65.5%), followed by the High Risk group (46.3%), whereas the Low Risk (27.0%) showed lower prevalence. These results suggest that TTV infection may be more frequently associated with the presence of multiple HPV genotypes or with high-risk HPV infections, indicating a potential shared transmission route or synergistic interaction between these viruses in cervical tissue. This finding is consistent with the report by Siahpoush et al., who observed a significantly higher prevalence of TTV DNA in

individuals infected with HPV, both high-risk and low-risk types [14]. Additionally, Suzuki et al. demonstrated TTV DNA prevalence was significantly higher among the HPV positive patients with cervical cancer [23]. In a recent study, TTV DNA was detected in 12% of cervical samples, with the highest prevalence observed in high-grade squamous intraepithelial lesions (HSIL). No statistically significant differences were observed between lesion types, suggesting a potential role of TTV in pre-neoplastic cervical changes, but not in cancer progression [24]. Although TTV is generally considered non-pathogenic, its frequent detection in HPV-positive individuals suggests it may influence local immune responses or viral persistence, highlighting the need for further mechanistic studies.

Analysis of TTV prevalence according to individual HPV genotypes (Table 4) demonstrated that certain high-risk genotypes, notably HPV 52, 56, and 68, exhibited significantly higher rates of TTV co-infection (p=0.032, 0.012, and 0.039, respectively). Other genotypes, including HPV 16 and 18, 31, 35, and 45 showed elevated TTV positivity but without statistical significance (p=0.176, 0.356, 0.130, 0.362, 0.259 respectively). The observed significant associations with specific genotypes may indicate genotype-specific susceptibility or preferential viral interactions, although the underlying mechanisms remain unclear. Notably, longitudinal studies on HPV vaccination programs, such as the 12-year girls-only bivalent HPV vaccination study in the Netherlands, have demonstrated significant reductions in vaccine-targeted genotypes (HPV-16/18) alongside relative increases in certain non-vaccine high-risk genotypes including HPV-39, HPV-52 and HPV-56, suggesting shifts in genotype prevalence over time [25]. These findings may have implications for TTV co-infection patterns, as the changing distribution of high-risk HPV types could influence the epidemiology of concurrent viral infections.

Beyond the observed epidemiological association, several biological mechanisms may explain the relationship between TTV and HPV co-detection. TTV has been proposed as an immunomodulated virus whose replication reflects host immune competence. Elevated TTV levels have been associated with impaired cellular immune responses and altered T-cell activity [26]. Since effective clearance of HPV relies heavily on cell-mediated immunity and interferon-mediated antiviral pathways [27], subtle immune modulation reflected by TTV persistence may facilitate HPV persistence or reduce viral clearance [28].

This study has some limitations. First, the limited sample sizes for certain genotypes (e.g., HPV 35 and 31) may have reduced statistical power, which should be taken into account when interpreting the results. Second, we did not have access to patients' marital status, occupation, smoking status, immune status, HPV vaccination, or clinical history. Third, given the substantial genetic heterogeneity of TTV, use of a single primer pair may not comprehensively detect all circulating genotypes, potentially resulting in under-detection of certain variants. Future studies incorporating multiple primer sets or employing next-generation sequencing approaches would provide a more thorough characterization of TTV genetic diversity and improve detection sensitivity. Fourth, the use of qualitative PCR for TTV detection; without quantitative viral load assessment by qPCR, the ability to accurately interpret immune status and evaluate the quantitative relationship between TTV and HPV infection was constrained.

5. Conclusion:

In conclusion, our study indicates that TTV infection is prevalent among HPV-positive women, particularly those with multiple or high-risk HPV genotypes. Certain genotypes, specifically HPV 52, 56, and 68, are significantly associated with TTV co-infection. TTV, although generally

considered non-pathogenic, frequently co-occurs with HPV, particularly in high-risk genotypes and sexually active age groups. The co-detection of TTV and HPV suggests possible shared transmission routes. These findings provide a basis for future research on the potential interactions between TTV and HPV and their implications for cervical disease progression.

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Authors' Contribution

Conceptualization: Somayeh Shokri; Sample collection: Reza Nayebzadeh and Seyed Adel Sharifi Fard and Shamim Pilehvari; Methodology: Somayeh Shokri and Shahab Mahmoudvand; Analysis: Sheida Behzadi Sheikhrobat; Writing original draft preparation: Somayeh Shokri and Shahab Mahmoudvand; Review and editing: Farid Azizi Jalilian and Ali Teimoori; Supervision: Shahab Mahmoudvand. All authors have read and agreed to the published version of the manuscript.

Ethics

This study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Research Ethics Committee of Hamadan University of Medical Sciences, Hamadan, Iran (Ethical approval: IR.UMSHA.REC.1403.564).

Conflict of Interest

The authors declare that they have no conflict of interest.

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Data Availability

All of the data underpinning the work are available to others upon request.

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