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**Original Article** 





# Comprehensive Analysis and Epidemiology of High-Risk HPV Genotypes in Suspected Cervical and Uterine Cancer Cases in Iranian Women Using Specific Hybridization: Implications for Policy Makers and Public Health

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#### Abstract

**Background:** Human papillomavirus viruses (HPV), most commonly transmitted through sexual contact with an infected individual, are the primary cause of cervical cancer. This study aimed to evaluate the prevalence of high-risk and low-risk HPV types among Iranian women suspected of cervical cancer.

**Methods:** Vaginal samples were collected by gynecologists in university hospitals in Tehran from June 2023 to January 2024 from women suspected of having cervical cancer. Viral DNA was extracted from the samples and analyzed using real-time PCR. Positive samples were further analyzed using manual specific hybridization technique to determine the HPV genotypes.

**Results:** Out of 101 samples, 61 tested positive for HPV. The analysis of high-risk and low-risk HPV types revealed that 16.1% were high-risk (16, 18, and 52), 21% were low-risk (6, 11, and 62), and 9.7% had both low-risk and high-risk types. Additionally, other HPV types were detected in 53.2% of the positive samples. Among the 61 positive cases, one case had only type 16, four cases had type 52, thirteen cases had types 6, 11, and 52, nine cases had types 16, 18, and 52, five cases had types 6, 11, 62, 16, 18, and 52, and twenty-nine cases had other types.

**Conclusion:** Although low-risk HPV types were more frequent than high-risk types, the overall prevalence of HPV infection in the studied population is significant. This underscores the necessity of implementing a screening program for women to detect HPV and reduce its transmission and the associated cancer risk.

Keywords: human papillomavirus viruses (HPV), Cervical cancer, Genotype, Real-time PCR

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## Introduction

Human papillomavirus (HPV) is a significant cause of cervical cancer, altesring the shape, activity, and proliferation of cells in the affected organ (1). The virus is primarily transmitted through sexual contact (STD, sexually transmitted disease) with an infected person. Over 100 types of HPV have been identified and classified into high-risk and low-risk categories. High-risk strains, such as types 16 and 18, and low-risk types, such as 6 and 11, are among the most common viruses (2). Notably, types 16 and 18 are responsible for 70% of cervical cancer cases. HPV is most prevalent among sexually active women of reproductive age (3).

Individuals infected with HPV may be asymptomatic

carriers, which may lead to severe conditions such as uterine and cervical cancers in these individuals. This highlights the importance of timely diagnosis using molecular techniques (4). Cervical cancer is a significant global health issue, ranking fourteenth among all cancers and fourth among women (5). Prevention strategies focus on primary prevention and screening to reduce the disease burden and mortality rates (6). HPV is transmitted through skin-to-skin contact, including sexual intercourse, hand-to-genital contact, and oral sex. Risk factors for HPV and cervical cancer include early onset of sexual activity, multiple sexual partners, smoking, herpes simplex infection, HIV, co-infection with other genital infections, and use of oral contraceptives (7). More than



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75% of cervical cancer cases are caused by high-risk HPV types 16 and 18. Other types can also cause malignancies, while low-risk types, particularly 6 and 11, are known to cause anogenital warts (condyloma acuminatum) (8,9). The HPV genome is a small, highly conserved, double-stranded DNA molecule, approximately 8000 base pairs in length (10). Sequence variations, such as single nucleotide polymorphisms or genetic mutations in the L1, LCR, E6, and E7 regions, help determine the families, relatedness, and phylogeny of HPV types. HPV types are classified based on a difference of more than 10% in the DNA sequence of the L1 gene (11).

Given the strong association between HPV infection and cervical cancer, examining the frequency and genotyping of HPV has significant diagnostic and therapeutic value. Fast screening and diagnosis can prevent the progression of primary lesions to malignancy. This study aimed to determine the genotype and frequency of HPV in the Iranian population using real-time PCR and manual specific hybridization methods.

#### Methods

## Clinical samples

In this cross-sectional study, biopsy samples, along with vaginal and cervical secretions, were collected from 101 women between 20 and 70 years old. They visited gynecologists between June 2023 and January 2024 in university hospitals in Tehran and were suspected of having uterine cancer. A specialist conducted the sampling procedure after obtaining patient consent. The samples were stored in dedicated ThinPrep containers and sent to the laboratory for further analysis. In this study [code of ethics: IR.SBMU.RETECH.REC. 1402.617], we prepared consent forms and questionnaires to obtain the necessary information for our research. The participants were fully informed and aware of the content and reasons for the examination, and they participated in the study with their full consent.

# Screening of individuals by real-time PCR

For the initial screening of the samples, the real-time PCR (Applied Biosystems StepOnePlus\*, USA) technique was employed. Viral DNA was extracted using the LabPrep kit, Vendor) from the Czech Republic, and all extraction processes were based on manufacturer protocol. Following the protocol provided with the real-time PCR Kit (Simbio Lab Company, Simreal HPV MLA Screening Kit, Iran), the PCR reaction components (including 10  $\mu L$  of SYBR Green Master Mix, 2  $\mu L$  of HPV general primers (F: 5'TTTGTTACTGTGGTAGATACTAC3', R: 5' GAAAAATAAACTGTAAATCATATTC3') (12) 2  $\mu L$  of sterile distilled water, and 5  $\mu L$  of DNA sample) were dispensed into specialized microtubes and placed in the real-time PCR instrument.

All reactions were conducted with a final volume of 20

 $\mu L$ . The temperature-time program of the device involved an initial activation at 95 °C for 15 minutes, followed by 45 cycles comprising denaturation (95 °C for 30 seconds), annealing (53 °C for 30 seconds), elongation (72 °C for 30 seconds), and a final melting curve analysis performed from 58 °C to 99 °C.

# Human papilloma virus genotyping

The virus genotyping process consisted of two main steps: PCR amplification of the viral DNA's L1 region using specific primers and specific hybridization to detect the desired genotypes, which are mentioned below.

## HPV DNA amplification by PCR

Samples that tested positive with real-time PCR were further analyzed for genotyping. The manual specific hybridization protocol, involving PCR and hybridization techniques, was employed as per the manufacturer's instructions using the Master Diagnostica kit from Spain (13).

In the PCR step, the viral DNA sample was mixed with the necessary components (15 µL sterile distilled water, 1 μg DNA sample, 1 unit Taq polymerase, 1 μM dNTP, 1 mM MgCl2, 1 pmol each of primer F and R, and 5.5 μL PCR buffer) and run in a thermocycler (Applied Biosystems MiniAmp™ Plus, USA). The temperature-time program followed the specified conditions. The Initial activation phase involved heating the sample to 94 °C for 3 minutes to activate the necessary enzymes. Next, in the denaturation step, the sample was maintained at 94 °C for 30 seconds to separate the DNA strands. Then, the temperature decreased to 47 °C for 30 seconds. After this time, the temperature increased to 72 °C for 30 seconds. After repeating this process for 15 cycles, other amplification steps occur at 94 °C, 65 °C, and 72 °C for 90 seconds (30 seconds for each temperature). This protocol is cycled through 35 times to amplify the DNA sequences efficiently.

# HPV typing by specific hybridization test

The PCR product from the previous step was mixed with the hybridization solution and applied to the designed chips for specific hybridization. The mixture was then incubated at 41 °C for 8 minutes for pre-hybridization.

This method involved using PCR products and specialized nylon membrane chips with probes representing various HPV genotypes by dedicated link between single-stranded DNA and specific single-stranded probes for human HPV (pre-hybridization product), a purple precipitate formed at the hybridization site. The chip images were processed and interpreted using Hybrisoft software (Master Diagnostica, Spain). Each HPV chip was applied for a sample infected with the HPV virus and examined using the Hybrispot scanner (Master Diagnostica, Spain).

## Statistical analysis

If the data distribution was normal, t test and ANOVA statistical tests were used to compare two or more groups. If the data were not normal, equivalent non-parametric tests were used. The statistical significance level was set at 0.05. Statistical analyses were performed in GraphPad Prism.

#### Results

# Patients' demographic information

An analysis of the age distribution in the studied population is shown in Figure 1a. It revealed that most of the referents were 31-40, and the fewest were aged 61–70. Since the majority of the reproductive population falls within the 20-40 age range, the overrepresentation in these groups could raise concerns regarding reproductive health in society. In the surveyed population, one case had no formal education, 41 cases had a high school diploma or lower qualification, 42 cases held a bachelor's degree, 14 cases had master's degrees, and 3 cases possessed doctorates. Of the total, 53 cases had not undergone any treatment, while 48 cases were undergoing treatment. Among those receiving treatment, 20 sexual partners had been treated for HPV infection, whereas 42 sexual partners had not yet received treatment. Additionally, 38.6% of individuals did not disclose their stance on this matter. Among individuals with a history of HPV infection, 39 cases (38.6%) tested negative, and 62 cases (61.4%) tested positive.

## Analysis of samples by real-time PCR

In this study, the real-time PCR method was used to screen the collected samples for HPV. The melting curve analysis was utilized to confirm the proper functioning of the primers. In contrast, the amplification curve was employed to detect positive samples, ensuring the accuracy of the PCR reaction. The negative samples did not exhibit a melting curve on the amplification plot. Of 101 collected samples, 61 tested positive for HPV, and 40 were negative. For each sample, we included one positive control, one negative control, and one no-template control. According to the analysis guidelines provided with the kit, we checked the melting and amplification curves for all

mentioned controls to ensure proper kit performance and extraction.

#### HPV genotypes frequencies

Samples confirmed as positive by the real-time PCR method were further analyzed using the manual specific hybridization technique to determine the HPV genotype. According to Figure 1b, the most common types obtained in the reference population of women were types 6, 11, 62, 16, 18, and 52.

Based on these findings, 10 referrals were infected with high-risk types (16, 18, and 52), 13 female patients were involved with low-risk types (6, 11, and 62), and in six cases (9.7%) had both low-risk and high-risk types.

# Co-relationship of age and virus type in HPV-positive patients

As shown in Figure 2, the isolated high-risk genotypes within the age group of 20 to 30 years are 16, 18, and 52. The research highlighted a significant correlation between virus types and age groups. HPV typing results indicated a noticeably higher prevalence of other virus types in the 31-40 age group compared to the 20-30 age group (P=0.015). Similarly, a statistically significant disparity in the prevalence of other virus types was observed between the 31–40 age group and the 41–50 age group (P = 0.046). Furthermore, the findings illustrated a substantial contrast in the prevalence of other virus types between the 31-40 age group and the 51-60 age group (P=0.002). The analysis also revealed that the prevalence of types 6, 11, 62, 16, 18, and 52, as well as other virus types, was greater in the 31-40 age group compared to the 61-70 age group (P = 0.01).

#### Discussion

Over 99% of cervical cancer cases are caused by HPV infection, with more than 500 000 new cases and approximately 250 000 deaths occurring annually worldwide. Notably, 80% of uterine cancer instances are found in developing nations. Persistent HPV infection can progress to cervical cancer and intraepithelial neoplasia (14-16).

Several tests detect HPV DNA, such as sequencing,

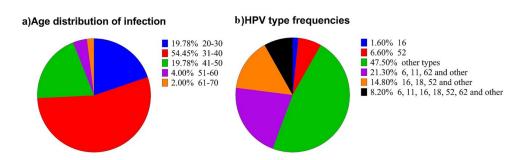
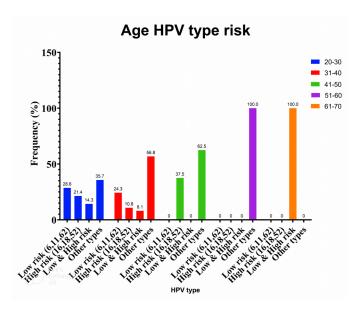


Figure 1. Women's demographic information. Frequencies of age (a) and HPV types (b) in the surveyed population



**Figure 2.** The frequency of different HPV types in age ranges. The low-risk (6, 11, and 62) and high-risk (16, 18, and 52) HPV types were compared with others in the 20–30, 31–40, 41–50, 51–60, and 61–70 age ranges

probe hybridization, or PCR amplification of L1 using different primers. GP5+/GP6+primers increase sensitivity by amplifying a 140–150 bp L1 fragment, enhancing PCR precision even though the test might not reliably detect numerous HPV genotypes (17). Given the unreliability of serological and culture methods in HPV detection, accurate and rapid tests are needed to pinpoint the virus and its type (18-20).

Relying solely on the Pap smear method for detecting HPV is insufficient, necessitating the use of PCR and molecular techniques. However, using urine and semen samples can compromise the accuracy of HPV identification in various methods (7,21). Consequently, this study employed vaginal secretions and biopsy samples to identify HPV infection through real-time PCR and hybridization techniques. Initially, samples were screened using real-time PCR, followed by typing positive samples with the specific hybridization method.

According to the findings of this study, 61 out of 101 samples of women suspected of HPV infection tested positive, accounting for 60.4% of the cases. Keyhani et al reported a 73% prevalence rate of HPV infection among Iranian women, consistent with these results (22). In a study by Mostafapour Badpey et al, the frequency of HPV infection in the Iranian population was shown to be 34.2%, while Jamshidi Makiani found a prevalence of 38.75% in Iranian women between 2014 and 2015 (16,23). Chalabiani et al reported a 29.3% prevalence of HPV in women across various regions of Iran (23).

Regarding age, the highest frequency of affected individuals was observed in the 31–40 age group (54.5%), while the lowest frequency was seen in the 61–70 age group (2%). Mostafapour Badpey et al indicated that the most affected women with HPV infection were aged 26–35 years, a finding consistent with the results of this

research (24). Similarly, Taghizadeh et al reported that the highest prevalence of affected women fell within the 30–40 age group (25). However, Coser et al (26) has shown a trend of high prevalence and persistence of HPV infection in women under 30 years of age, particularly in regions facing financial hardship.

Furthermore, findings from this study indicate that older age groups exhibit lower rates of virus infection, with prevalence diminishing with advancing age. Studies by Monsefi et al and Trottier et al (27,28) documented similar outcomes.

An analysis of the prevalence of high-risk and low-risk HPV types in the studied population revealed that 16.1% (10 cases) displayed high-risk types (16, 18, and 52), 21% (13 cases) had low-risk types (6, 11, and 62), and 9.7% (six cases) had both low-risk and high-risk types. The research indicated a higher incidence of low-risk types over high-risk types (29).

In a study by Jamshidi Makiani et al, low-risk genotypes (6, 11, 40, 42, 43, 44, 55, 54, 61, 62, 81, and 89) and highrisk genotypes (16, 18, 31, 35, 39, 45, 51, 52, 53, 56, 58, 66, 68, and 73) were examined (14). Among the low-risk types, HPV-6 levels were found to be higher than other genotypes. Shahi et al reported genotype 6 as the most prevalent in the city of Mashhad (30). Jamshidi Makiani et al also identified HPV16 as the most common type (20.2%) (14). HPV type prevalence varies by region, with HPV 16 being the most common cancer-causing type (31). Studies in Iran have shown HPV 16 to be more prevalent than other types (32,33). Malary found an overall HPV prevalence of 9.4% among 7655 women in Iran, with type 16 and 18 rates of 2.03% and 1.7%, respectively (34). In our research, besides genotypes 16 and 18, we also identified genotypes 39, 52, 53, and 58. Discrepancies between different studies may be attributed to variations in patient

samples studied. Given the high prevalence of high-risk genotypes (16.1%) found in our study and the diverse genotypes of HPV across different global regions, regional epidemiological factors, age of infection, social culture, and people's awareness of effective prevention methods, investigations on this virus hold substantial importance.

#### Conclusion

In the present research, genotypes 16 and 18 were documented, as well as genotypes 39, 52, 53, and 58. Thus, it is imperative to use accurate methods like hybridization in screening programs to detect at-risk populations.

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#### **Authors' Contribution**

Conceptualization: Mojgan Bandehpour. Data curation: Mobina Bandehpour. Formal analysis: Mobina Bandehpour. Funding acquisition: Mojgan Bandehpour. Investigation: Mahya Nasrollahi.

**Methodology:** Mahya Nasrollahi.

Project administration: Mojgan Bandehpour.

Resources: Mahya Nasrollahi.
Software: Mahya Nasrollahi.
Supervision: Mojgan Bandehpour.
Validation: Mojgan Bandehpour.
Visualization: Mojgan Bandehpour.
Writing-original draft: Mahya Nasrollahi

#### **Competing Interests**

The authors declare that they do not have any conflict of interest.

# **Ethical Approval**

All human experiments were conducted according to the ethical principles of the Medical Ethics Committee of Shahid Beheshti University of Medical Sciences [code of ethics: IR.SBMU.RETECH. REC.1402.617].

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