



Prevalence and Genotype Distribution of Human Papillomavirus Among a Subpopulation of Jordanian Women

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Abstract

Objectives: Human papillomavirus (HPV) infection is the leading sexually-transmitted infection (STI) worldwide and the main etiology of cervical cancer. HPV infection rates are important in directing vaccination policies and screening for cervical cancer. Unfortunately, no recent reports have evaluated the prevalence of this infection among Jordanian women despite major globalization-driven changes in sexual behavior. Accordingly, this study aimed to determine the prevalence of HPV infection and its genotypic distribution in the cervical samples of Jordanian women.

Materials and Methods: The study was carried out at Prince Hamza Hospital (Amman, Jordan) during 2016-2017. Women (15-75 years old) were randomly selected for cervical cell collection. DNA was extracted and then amplified using MY09/11 and GP5+/6+ consensus primers. Finally, positive samples were genotyped by applying real-time- polymerase chain reaction and reverse line blotting.

Results: Fourteen out of 348 women tested positive for HPV with a prevalence rate of 4%. In addition, multiple HPV genotypes were observed in 36% (5/14) of infected women while single HPV genotype infection was found in 64% (9/14) of infected women. Further, high-risk (HR), potential high-risk (pHR), and low-risk (LR) HPV genotypes were detected in most cases with a 78.6% (11/14) infection rate, 42.9% (6/14), and 7.1% (1/14) of infected women, respectively. Eventually, 10 different genotypes were detected in infected women and HPV 16 was the most common type (42.9%, 6/14).

Conclusions: Our data suggest that the prevalence of HPV infection among Jordanian women is below the global and regional rates. It is hoped that these data should facilitate the implementation of appropriate cervical cancer screening and future HPV vaccination programs.

Keywords: Human papillomavirus, Real-time PCR, HPV 16, Prevalence, Jordan, Cervical cancer

Introduction

Cervical cancer represents the fourth most frequently diagnosed malignancy in women and the fourth leading cause of cancer-related demise worldwide (1-3). In addition, human papillomavirus (HPV) infection is the leading sexually-transmitted infection (STI) globally (4). Multiple clinical and epidemiological reports have clearly established the role of HPV infection as the primary etiologic factor of cervical cancer (5-7). According to their oncogenicity, HPVs are classified into high-risk (HR), potential high-risk (pHR), and low-risk (LR) groups. The oncogenic HR-HPV genotypes 16 and 18 are the most prevalent types in individuals with cervical carcinoma or cervical intraepithelial neoplasia and are implicated in more than 70% of cervical carcinoma cases (8, 9). Conversely, the LR group genotypes 6 and 11 are implicated in most cases of condylomata and genital warts (10, 11). Given that HPV prevalence and distribution differ geographically and within different populations,

there is a constant need for conducting HPV genotype screening routinely.

In Jordan, the annual incidence of cervical cancer is estimated at 50-60 cases with a 50% mortality rate (12). These low figures could be largely attributed to predominant conservative sexual practices in Jordan. However, in recent years, globalization has tremendously affected several aspects of social life. Unfortunately, no recent report has examined whether this change has affected HPV infection rates. Furthermore, these changes in sexual practices might still be accompanied by the underutilization of mechanical protection (condoms), which significantly decreases the risk of STIs, which is still a main concern in the Middle East region (13). In this pilot study, Jordanian women attending the Gynecology Clinic at Prince Hamza Hospital were screened to identify the frequency of the HPV infection in this population. Moreover, the distribution of HPV genotypes and the extent of multiple HPV infections were studied in



Key Messages

- ▶ HPV prevalence among women in Jordan is 4%.
- ▶ HPV 16 is the most common subtype identified in 43% of infected Jordanian women.
- ▶ The introduction of the HPV bivalent vaccine as part of the national vaccination program is recommended.

women from the region of Amman, Jordan. To the best of our knowledge, this is the first report that provides epidemiologic data on HPV infection and highlights its prevalence and genotype distribution in a general female subpopulation from a local area in Jordan.

The identification of specific HPV genotypes in the clinical setting is critical for accurate diagnostic workup, treatment planning, and prediction of the prognosis of HPV infection-related pathologies in certain populations. Accordingly, our results provide essential information to guide the future planning of public prevention programs and the potential use of HPV type-specific vaccines, ultimately, leading to an overall reduction in the incidence of cervical cancer in Jordan.

Materials and Methods

Specimen Collection

Cervical samples were collected from 380 healthy, married Jordanian females aged 18-70 years. Women attending the Gynecology Clinic at Prince Hamza Hospital as part of a routine gynecological exam were recruited during 2016-17. Single unmarried women, pregnant women, and those with previous hysterectomy or previously diagnosed with cervical cancer were excluded from this study. The samples were obtained using a Digene cervical brush, and then transferred into sterile tubes containing 2 mL of phosphate buffer saline, and finally, stored at 4°C for DNA extraction (14). The Institutional Review Board approvals of this study were obtained from the Hashemite University and Prince Hamzah Hospital according to the established guidelines. All participating individuals signed an informed consent form, along with a structured questionnaire.

Nucleic Acid Extraction

Cervical samples were vortexed to dissociate the cells and centrifuged at 10000 rpm for 5 minutes. Then the supernatant was discarded and DNA was extracted from cervical samples using the DNeasy Blood and Tissue Kit (Qiagen, USA) according to the manufacturer's protocol. Next, DNA was eluted in 200 µL of the Adams–Evans buffer and stored at -20°C for subsequent processing. Eventually, DNA concentration was measured using the Qubit 3.0 Fluorometer (Thermo Fisher Scientific, USA) as detailed by the manufacturer.

HPV Detection Through Conventional Polymerase Chain Reaction

A touchdown Polymerase Chain Reaction (PCR) was used to amplify the conserved sequences of the HPV L1 gene by employing the consensus GP5+/GP6+ primer set. As previously mentioned, touchdown PCR enhances the detection of HPV-positive samples compared to the conventional PCR (15). The applied primer sets in this study are listed in Table 1. The PCR for the GP5+/GP6+ primer set was carried out in a 25 µL final volume using the HotStarTaq Master Mix (Qiagen, USA) as reported previously (15). To increase the yield of HPV-positive sample detection, the MY09/MY11 primer set was also used to screen cervical samples for HPV infection (16). Moreover, PCR for the MY09/MY11 primer set was conducted in a 25 µL final volume by utilizing HotStarTaq Master Mix (Qiagen, USA) under the conditions of initial denaturation at 95°C for 15 minutes, followed by 40 cycles at 94, 55, and 72°C for 45, 45, and 45 seconds, respectively, with a final extension step of 72°C for 5 minutes. All PCR runs included a negative control (no DNA template) and a positive control (HPV 16 or 18) thankfully provided by MedLabs Consultancy Group (Amman, Jordan). Additionally, the human Beta-globin gene was employed as an internal control to check for the quality and adequacy of the extracted DNA. Then, the electrophoresis of PCR products was conducted using a 2% agarose gel stained with ethidium bromide, and finally, visualized using UV transillumination (Alpha Innotech, USA).

Table 1. Primer Sequences Used for HPV Amplification

Primer Name	Target Gene	Primer Sequence	Band Size
MY09	HPV L1	5'-CGTCCMARRGGAWACTGATC-3'	450 bp
MY11		5'-GCMCAGGGWCATAAYAATGG-3'	
GP5+	HPV L1	5'-TTTGTTACTGTGGTAGACTAC-3'	150 bp
GP6+		5'-GAAAAATAAACTGTAAATCATATTC-3'	
BG-F	β-globin	5'-GAAGAGCCAAGGACAGGTAC-3'	268 bp
BG-R		5'-CAACTTCATCCACGTTACC-3'	

The table includes a detailed illustration of all the applied primer sequences for detecting positive HPV samples. Consensus HPV primers were used to detect the HPV L1 gene sequence.

Note. HPV: Human papillomavirus.

HPV Genotyping

Two methods were used for HPV genotype identification, including the real-time PCR (RT-PCR) kit and a reverse line blot kit (AB ANALITICA, Italy). REALQUALITY RQ-Multi HPV detection is an *in vitro* diagnostic kit which is capable of identifying 28 HPV types including 14 HR (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), 6 pHR (26, 53, 67, 70, 73, and 82), and 8 LR (6, 11, 40, 42, 43, 44, 55, and 83) genotypes (17). This kit amplifies a fragment in the regions of E6 and E7 in the HPV genome and detects different genotypes as a pool except for HPV 16 and HPV 18 which are detected individually. In addition, the kit includes an internal control (human beta-globin) to monitor for the quality of DNA extraction and the amplification process. Further, it provides a negative control (nuclease-free water) and a positive control (plasmid DNA contains the fragments of HPV 16, 18, and 33 for HR-HPV, along with the fragments of HPV 6, 11, and 26 genotypes for LR-HPV).

The inconclusive results of a single genotype were further analyzed with AMPLIQUALITY HPV-TYPE EXPRESS (version 3.0, AB ANALITICA, Italy) according to the manufacturer's instructions (18). This kit can identify 40 HPV individual genotypes based on the amplification of a 145-bp fragment in the L1 region of the HPV genome, followed by a reverse line blot assay. Furthermore, the kit includes a housekeeping gene control and a plasmid DNA with fragments of the HPV 61 genotype as a positive control.

Statistical Analysis

All statistical calculations were performed using SPSS software, version 22 (IBM, USA), and the chi-square test was the main tool for testing differences in proportions. In this study, a $P < 0.05$ was used as the point of statistical significance.

Results

This cross-sectional study recruited 380 females attending the Gynecology Clinic at Prince Hamza Hospital as part of a routine gynecological exam. Of 380 collected samples collected, a few had no cells, looked turbid, or were extremely bloody, and thus were excluded from the study. Some cervical samples yielded no housekeeping gene (β -globin) and thus were not further processed. However, most samples tested positive for internal control (Figure 1A). The remaining 348 samples were further analyzed by the PCR using MY09/11 and the GP5+/6+ primers (Figure 1B-C). MY09/11 and the GP5+/6+ are L1 consensus primer sets that are routinely used for detecting HPV DNA using the PCR (16). Samples that demonstrated a strong positive (a sharp band) or a possibly positive (a faint band) were further analyzed by the RT-PCR in order to confirm or rule out the presence of HPV infection (Figure 2A). Moreover, the indeterminate HPV genotype in the RT-PCR was further tested for using the reverse line blot

assay (Figure 2B), and the prevalence of HPV infection in the studied population was 4%.

Table 2 summarizes the demographic data and risk factors for the studied group. Based on the data, the mean age of women in the studied population was 42.61 ± 10.95 , and there was no difference in the mean age between HPV positive and negative women. Furthermore, other demographic data such as educational level, marital status, and body mass index revealed no significant differences between the two populations. Finally, risk factors such as the onset of sexual activity, pregnancy, abortion, alcohol, and tobacco use also demonstrated an insignificant variability between HPV positive and negative groups.

As mentioned earlier in Table 3, 14 women were infected with HPV with a prevalence of 4%. Additionally, 36% (5/14) of women were found to be infected with multiple HPV genotypes while 64% (9/14) of them showed single HPV genotype infection. Based on the results, HPV low-risk infection was detected in 7.1% (1/14) of women whereas the pHR HPV infection was observed in 42.9% (6/14) of women. Similarly, infection with high-risk HPV was detected in the majority of cases with a 78.6% (11/14) infection rate. A total of 10 different HPV genotypes were detected as well (Table 3). HPV type 16 was the most common type (42.9%, 6/14), followed by HPV 53 and 73

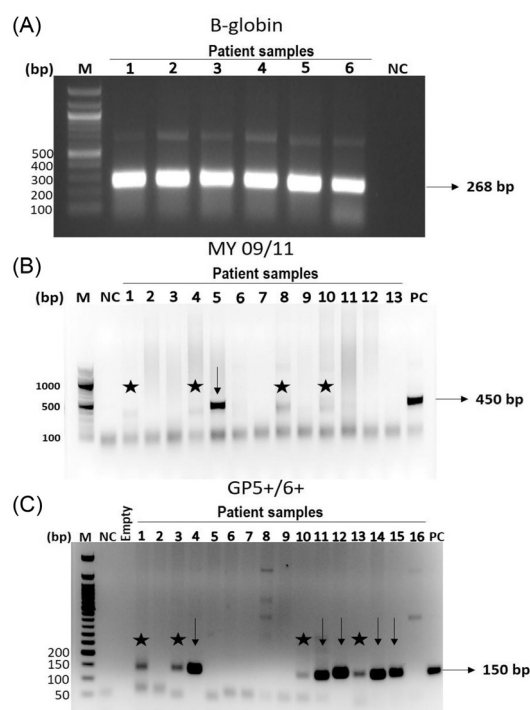


Figure 1. PCR of β -globin and HPV consensus primers MY09/11 and GP5+/6+. **A.** A 268 bp band representing the presence of β -globin genes in all six cervical samples is shown, samples with negative β -globin were excluded from the study. **B.** PCR amplification with the MY09/11 primer yielded a 450 bp band. **C.** GP5+/6+ primer amplified a 150 bp band. Arrows represent HPV positive samples, while stars represent a possible positive HPV sample that needs further testing, unmarked samples represent an HPV negative sample. M: DNA ladder, NC: Negative control, PC: Positive control.

(21.4%, 3/14), HPV 18, 68, 70, and 82 (14.3%, 2/14), and HPV 6, 35, and 53 (7.1%, 1/14).

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Discussion

The pathogenic role of HPV infection in cervical cancer is well identified. It is expected that sexually active men and women eventually acquire an infection with a strain of HPV during their lifetimes with an associated increased

risk of cancer development in both populations (19). The increasing rates of HPV-associated malignancies have guided the implementation of rigorous vaccination protocols against HPV and the development of screening protocols for cervical cancer (20). On the contrary, cultural and religious conservatism in the Middle East is expectedly a major contributor to the lower prevalence of HPV infection in comparison to Western societies, particularly among females (21). However, certain sexual behaviors are gradually being adopted in Arab countries due to globalization, suggesting the possibility of changes in HPV infection rates, especially in the last few years. These changes would certainly require the reassessment of the currently implemented strategies to lower the risk of cervical cancer among women living in the Middle East, including Jordan. Unfortunately, there are no recent reports estimating the prevalence of HPV infection, and so far, this has been the first study that has directly reported this issue among Jordanian women.

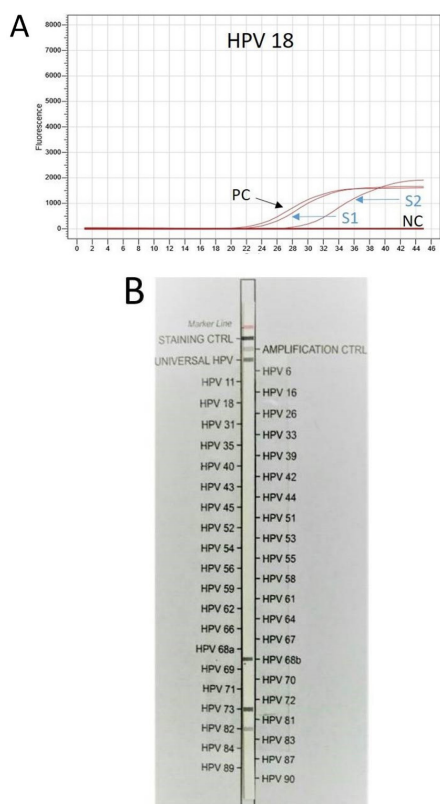


Figure 2. A Representative RT-PCR and Reverse Line Blot Strip Analysis (A) A RT-PCR run using REALQUALITY RQ-Multi HPV detection kit. A single demonstrative run for 20 samples using the cy5 channel is shown which detects an individual genotype (HPV 18). The 3S-shaped curves represent the positive control (PC) and two positive samples (S1 and S2) while the remaining samples and the negative control (NC) run along the baseline. (B) A reverse line blot HPV genotyping test strip showing colored bands corresponding to HPV68 73 and 82. The strip is coated with a staining control, an amplification control, and a universal HPV band. Note. RT-PCR: Real-time polymerase chain reaction; HPV: Human papillomavirus.

Table 2. Demographic Data and the Risk Factors of Women Included in the Study

Variable	HPV (-)	HPV (+)	P Value
Age (y) (n=348)	(334)	(14)	-
42.61 ± 10.89	42.73 ± 10.95	39.71 ± 9.38	
Marital status			
Married (n=331)	91.4% (318)	3.7% (13)	0.689
Separated (n=17)	4.6% (16)	0.3% (1)	
Education			
≤ 9 years (n=123)	33.9% (118)	1.4% (5)	0.976
≥ 9 years (n=225)	62.1% (216)	2.6% (9)	
Onset of sexual activity			
≤ 18 years (n=77)	21.6% (75)	0.6% (2)	0.471
≥ 18 years (n=271)	74.4% (259)	3.4% (12)	
Pregnancy			
None (n=20)	5.5% (19)	0.3% (1)	0.819
One or more (n=328)	90.5% (315)	3.7% (13)	
Abortions			
No (n=227)	62.4% (217)	2.9% (10)	0.619
Yes (n=121)	33.6% (117)	1.1% (4)	
Body mass index			
≤ 29 (n=183)	50.3% (175)	2.3% (8)	0.727
≥ 30 (n=165)	45.7% (159)	1.7% (6)	
Tobacco			
Yes (n=80)	22.1% (77)	0.9% (3)	0.887
No (n=268)	73.9% (257)	3.1% (11)	
Alcohol			
Yes (n=4)	1.1% (4)	0% (0)	0.680
No (n=344)	94.8% (330)	4% (14)	

The table describes several demographic parameters of the participating subjects, including age, marital status, education, and the onset of sexual activity. It also provides the quantification of several risk factors associated with cervical cancer.

Note. HPV: Human papillomavirus.

Table 3. HPV Genotypes Distribution Among HPV Positive Women (n=14)

Sample Number	HPV Genotype		
	HR	pHR	LR
1	16, 35, 56	70, 73	
2	18, 68		
3		73	
4		70	
5		53	
6	18		
7	16		
8	16		
9	16		
10	68	73, 82	
11	16		6
12	56		
13	56	82	
14	16		

Samples numbered 1-14 represent HPV positive women. In addition, samples 1, 2, 10, 11, and 13 show mixed HPV infection while the others demonstrate single HPV genotype infection. Further, genotypes 16, 18, 35, 56, and 68 represent high-risk HPV while 53, 70, 73, and 82 are considered as pHR genotypes. Finally, HPV 6 is a low-risk genotype.

Note. HPV: Human papillomavirus; HR: High-risk; pHR: Potential high-risk; LR: Low-risk.

In our study, the prevalence of HPV infection was 4% (14/348) in women attending the Gynecology Clinic at Prince Hamza Hospital (Amman, Jordan). This prevalence rate was in accordance with that of a recent study done at King Hussein Cancer Center in Amman, showing a 3.8% abnormal Pap smear rate among a total of 5,529 routine tested smears (22). Our results showed slightly higher HPV infection rates when compared to other local studies that demonstrated a low prevalence of cervical epithelial cell abnormality as an indicator of HPV infection (23). This favors the expectation that the number of HPV-positive and thus individuals at the risk of cervical cancer is increasing.

It should be noted that the HPV infection rate varies in different countries, with a global prevalence of 11.7% (24). The highest infection rates were recorded in Africa (21%), followed by Central America and Mexico (13%), and then North America, Europe, and Asia with 4.7%, 14.2%, and 9.4%, respectively (24). In the Middle East, HPV prevalence studies are scarce. However, in recent years, a number of studies have emerged in Iran, Kuwait, Qatar, Iraq, Saudi Arabia, Bahrain, and Palestine, reporting 10.3%, 9.3%, 7.6%, 12.5%, 9.8%, 9.8%, 13%, and 4.9% prevalence rate of HPV infection, respectively (25-31). In comparison with these numbers, the infection rate in this study is the lowest in the region. Several reasons might account for this difference and are mainly attributed to cervical sample collection bias. This is because these samples were collected only from women attending one gynecology clinic. Furthermore, HPV infections might be

Table 4. HPV Genotypes in Infected Women

HPV Prevalence/Genotype	n	%
Single infection	9	64.3
Multiple infection	5	35.7
Any LR-HPV	1	7.1
6	1	7.1
Any pHR-HPV	6	42.9
53	1	7.1
70	2	14.3
73	3	21.4
82	2	14.3
Any HR-HPV	11	78.6
16	6	42.9
18	2	14.3
35	1	7.1
56	3	21.4
68	2	14.3

Single HPV genotype infection was noticed in 64.3% of HPV positive women while 35.7% of them had mixed HPV infection. The frequency of infection for each genotype is displayed as the number of HPV positive cases (n) and percentage (%).

Note. HPV: Human papillomavirus; HR: High-risk; pHR: Potential high-risk; LR: Low-risk.

transient, and thus the infection rate might change with time and reflects only the current time-point.

Our results further showed that 36% and 64% of infected women had mixed or single HPV infection, respectively, which is in agreement with the findings of Martins et al (32) representing mixed infection in 113 cases (35%) compared to 210 cases (65%) of single genotype infection (Table 4). Moreover, 71% (10/14) of HPV infections were reported in women with a mean age of 34 years while the prevalence reduced to 4 cases only with a mean age of 54 years (the data are not presented). Our findings are consistent with those of other studies which demonstrated that the frequency of HPV infection decreases with age (31-33). On the other hand, AlObaid et al reported an increase in the frequency of HPV infection with age, suggesting community-specific differences (27).

The most prevalent HPV in this study was genotype 16 at 42.9%, which is in line with the findings of most studies conducted in the region (28,31,33,34), and other areas of the world where the HPV vaccine was not implemented effectively (32,35). Other results in this study might deviate from the findings reported elsewhere due to a difference in the age group compared to other studies. In the present study, the mean age was almost 43 years, which is 20-25 years more than the peak age incidence of HPV infection (36). Another reason might be sexual and socio-behavioral differences governed by cultural and religious values in a conservative community.

Finally, factors such as women's immune status, parity, smoking, and alcohol play a role in the progression of

HPV infection into pre-cancerous or cancerous lesions (37). Nonetheless, the above-mentioned factors had no significant association with HPV infection in the current study.

Bivalent and quadrivalent vaccines provide coverage against the HR HPV 16 and 18. Moreover, some recent studies have reported cross-protection against HR non-vaccine genotypes (37,38). Considering that most HPV infections in Jordan are caused by HPV 16, Jordanian women might benefit from the introduction of the bivalent vaccine to provide protection against cervical cancer.

Eventually, this study had some limitations. First, the samples were taken from women visiting a gynecology clinic in a single hospital in Jordan, and this renders our samples non-representative of the Jordanian population. In addition, the interpretation of the genotyping data might be affected by the limited sample size and the low number of HPV positive women (n=14). Finally, single women with no previous history of sexual intercourse were excluded from sample collection due to the inability to perform Pap smears on patients with intact hymens. Unfortunately, the current screening practice in the country excludes the inclusion of single women from routine cervical swab collection. Thus, these data represent HPV prevalence in married or separated Jordanian women. Accordingly, future studies are directed toward estimating the prevalence of the HPV of a larger, more representative population of Jordanian women. This includes collecting cervical samples from several health institutions providing gynecological care spread over all Jordanian governorates of both private and public sectors. We also believe that it is important to investigate the use of new tools that allow the detection of HPV infection in single women since their exclusion continues to be a major drawback for a more accurate estimation of the HPV infection prevalence rate in Jordan.

Conclusions

To the best of our knowledge, this study is the first one to evaluate the prevalence of HPV infection in Jordan. It has been also shown that the HPV infection rate in Jordan is considered the lowest in the Middle East and Gulf countries. However, the current numbers suggest a potential increase in infection rates when compared to previous reports on the prevalence of cervical dysplasia. This study has enabled us to obtain a rough estimation of the HPV prevalence in Jordan and to realize circulating genotypes in the local community. Finally, the finding suggests that future vaccination programs should include HPV bivalent vaccine since HPV 16 is the most common subtype among infected Jordanian women.

Authors' Contribution

Conceptualization, validation, formal analysis, original draft preparation, supervision, funding acquisition: AIK. Methodology: JAAR, NMH, MAS, and GHAG. Investigation, and sample collection: RMK and FFA. Review and editing: TS.

Conflict of Interests

Authors declare that they have no conflict of interests.

Ethical Issues

The research project was approved by the institutional review boards of The Hashemite University (ethics no. 7/2.2015/2016) and Prince Hamza Hospital (ethics no. MH/32/112).

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