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The impact of human papillomavirus (HPV) types 6, 11 in women with genital warts

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Abstract
Objective Human papillomaviruses (HPV) are etiologically associated with the development of virtually all genital warts. HPV-6 and HPV-11 are the most commonly detected HPV genotypes, but at least 20 other HPV genotypes have occasionally been found in genital wart tissue specimens.
Study design The aim of this study was to determine from 100 genital wart tissue specimens collected from female patients using multiplex gap-PCR technique the prevalence of various genital HPV among women with HPV genital warts in south of Iran. 100 genital wart tissue specimens were tested for the presence of HPV PG5/PG6 and also for HPV type using polymerase chain reaction (PCR).
Results Based on the collected data, 73 (73 %) samples were detected positive for HPV DNA and 23 (23 %) samples out of 100 samples were detected negative for HPV DNA. 49 (49 %) and 67 (67 %) of patients were detected positive for HPV type 6 and 11, respectively. There was a significant association between marital status and HPV genotype 6 (OR = 0.51, 95 % CI = 0.37–0.70, $P = 0.01$). Nevertheless, no significant association was found between marriage and HPV genotype 11 (OR = 0.85, 95 % CI = 0.58–1, 24, $P = 0.7$). Similarly, this result was demonstrated, in combined marriage and HPV-general (OR = 0.80, 95 % CI = 0.62–0.05, $P = 0.4$).
Conclusion Concerning the prevalence of HPV in our study, determination of genital HPV prevalence and multiple infections among the normal population of women of Hormozgan Province is recommended.

Keywords Human papillomavirus · Genital warts · PCR · Typing

Introduction

Human papillomavirus (HPV) is the most common sexually transmitted virus in young and sexually active people of both sexes [1]. Anogenital HPVs, which are primarily mucosotropic, are classified as high and low risk, according to their relationship with benign or malignant proliferative lesions [2]. Genital warts (GWs) are the most frequent benign tumors in the anogenital region [3]**.** Genital warts typically present as flesh-colored, exophytic lesions on the external genitalia, including the penis, scrotum, vulva, perineum, and perianal skin [4]. Diagnosis of GWs is primarily clinical. Patients with GWs may have discomfort, pain, bleeding, or difficulty with intercourse; these symptoms are more common in patients with larger, cauliflower-like lesions [3].

The primary treatment goal is the removal of GWs. The choice of therapy is based on the number, size, site, and

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morphology of lesions, as well as patient preferences, cost, convenience, adverse effects, and the clinician's experience. Podofilox, imiquimod, surgical excision, and cryotherapy are the most convenient and effective options; however, there is frequent recurrence of GWs after therapy [4]. Human papillomaviruses (HPV) are etiologically associated with the development of virtually all GWs (Center for Disease Control and Prevention). Since the first detection of HPVs in GWs in 1981 [5], in the past 25 years several studies have examined the presence of HPV in GW tissue samples using various diagnostic methods. Thus, in early studies using immunohistochemistry, HPV antigens were detected in 20–80 % of GW specimens [6].

However, this diagnostic approach did not permit differentiation among HPV genotypes. Later, when molecular techniques became easier to perform and more accurate, investigators started to use these methods by preference for detection and typing of HPVs. In earlier molecular studies, various hybridization techniques were used for detection of HPVs, such as filter in situ hybridization, Southern blot hybridization, and in situ hybridization. In 16 studies using traditional hybridization methods published between 1982 and 2000, the prevalence of HPV infection in GWs ranged from 58.8 % [7] to 100 % [8]. Based on the viral DNA sequence, more than 230 HPV types are known [9]; 118 genotypes are well-characterized according to biological niche, oncogenic potential and phylogenetic position [2]. Approximately 40 HPV types infect the anogenital region and 15 of them—16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82—are considered oncogenic or as high risk because they are associated with high-grade squamous intraepithelial lesions or cancer. Types 26, 53 and 66 are likely to be carcinogenic, whereas types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and HPV89/Cp6108 are considered to be low risk [10]. HPVs have been associated with many proliferative lesions, with condyloma acuminatum being the most common, as well as with different types of cancer, including cervical, vaginal, vulvar, penile, anal, oropharyngeal, buccal cavity and larynx [11, 12]. Among them, uterine cervix carcinoma is particularly important due to its high incidence and its high mortality rate. In most cases, tumors evolve slowly and can be prevented by identifying precursor lesions in the cervical epithelium as early as possible, allowing for effective treatment before local invasion and spread of the disease [13]. A strong association between HPV and cervical cancer stimulated the development of several diagnostic tests, particularly those based on molecular biology. There are currently two main approaches for molecular detection of HPV: PCR with generic primers to amplify part of the L1 gene of the viral capsid, which is highly conserved among anogenital HPVs and the hybrid capture test which detects the main types of HPV by forming DNA–RNA hybrids [14, 15, 16]. As PCR is more sensitive, it has been largely used worldwide [17, 18, 19].

The diversity of virus types and the incidence of multiple infections have made it necessary to develop reliable methods to identify the different genotypes, for epidemiological studies as well as for the patient follow-up [20]. As no test has officially been approved for HPV genotyping [21], several methods have been used to identify different virus types, including PCR with generic primers [17], restriction fragment length polymorphism [22], hybridization with specific probes [23], reverse hybridization line probe assay—HPV-LiPA [24], reverse line-blot hybridization [23], nucleotide sequencing [25, 26, 12] and DNA Chip [27]. PCR with specific primers for each virus type is another approach and is based on polymorphisms, mainly E6 and E7 [18, 20]. This is a highly sensitive method that is easy to interpret and can characterize virus types in cases of multiple infection [16, 18, 20, 25, 28]. Selecting virus types to be tested should be based on epidemiological and prevalence studies, as there is a wide variation in the genotype distribution in different regions around the world. Over the last few years, virus genotyping has become an important method to approach cervical cancer. Several groups have searched for an effective genotyping test for HPV, due to its great contribution in the diagnosis of infections and to a better understanding of the relationship of HPV with carcinogenesis, in addition to contributing to the development of type-specific vaccines. The overall prevalence of HPV infection and distribution of HPV genotype varies with patient age, cytology stage, and geographic region including regions within one country. However, in Asia, HPV18 is the fourth most frequent type after types 16, 52 and 58 [31]. The overall prevalence of HPV infection and distribution of HPV genotype varies with patient age, cytology stage, and geographic region including regions within one country. Previous studies have reported that two most prevalent types worldwide are HPV16 and HPV18, accounting for more than 70 % of cervical cancer [29, 30].

However, in Asia, HPV18 is the fourth most frequent type after types 16, 52 and 58 [31]. Infection with more than one HPV type is found in 20–50 % of infected women [32, 33, 34]. Infection with multiple HPV types has been observed frequently in patients with cytological abnormalities [35]. Some studies have reported multiple HPV types less frequently in cervical carcinoma than in normal cytology and in precancerous lesions [36], whereas other studies have found multiple HPV infection to be associated with a significantly increased risk of high-grade SIL/invasive cancer compared to infection with a single HPV type [37]. In this study, we determined the prevalence of various HPV type 6 and 11 among the women subjected to routine test in Jamshidy

Outpatient Clinic of Bandar Abbas, Iran, by PCR analysis.

Materials and methods

This study was performed on 100 patients presenting to the Dr. Jamshidy outpatient clinic during the period from June 2009 to August 2010, with common genital warts.

Skin biopsies and DNA extraction

Skin specimen was obtained from each patient; 4–6 mm was taken from the selected warts. The lesion was kept at −80 °C for DNA extraction and HPV detection. DNA extraction from 100 mg of each specimen was performed according to standard protocols [38].

PCR for HPV DNA

Broad-spectrum HPV DNA amplification was performed using the polymerase chain reaction (PCR) fragment (GP5 and GP6) primer set, which amplifies a 150-bp fragment in the L1 region of the HPV genome. Primer sequences and PCR conditions were done as previously described [13]. Each experiment was performed with positive and negative PCR controls. The amplicons were run on a 3 % agarose gel and the 150-bp product was visualized with ethidium bromide staining. All HPV-negative cases were confirmed by the second PCR assay using standard DNA concentration, as well as a 10× diluted DNA sample to exclude the presence of PCR inhibitors. Appropriate positive and negative PCR controls were run with all reactions. To control for DNA quality, the globin gene was amplified [39] in all samples. PCR was performed in a final reaction volume of 50 µL, containing 5 µL of DNA, 5 µL buffer 10× [100 mM Tris–HCl (pH 8, 8) 500 mM KCl], 3 µL MgCl2, 1 µL dNTPs (200 Mm), 2.5 µL of each primer at 10 pmol/µL and 2.5 U of Taq DNA polymerase. The PCR conditions were as follows: preheating for 1 min at 94 °C was followed by 40 cycles of 30 s at 90 °C, 2 min at 55 °C and 1 min at 72 °C and a final extension of 10 min at 72 °C.

Type-specific PCR-DNA was amplified with specific primers for the following HPV types: 6, 11, [39, 40] in independent reactions. PCR was performed in a final reaction volume of 50 µL, containing 5 µL of DNA, 5 µL buffer 10× [100 mM Tris–HCl (pH 8.8), 500 mM KCl], 3 µL MgCl2, 1 µL dNTPs (200 µM), 2.5 µL of each primer at 10 pmol/µL and 2.5 U of Taq DNA polymerase. Amplification conditions were the same as those for globin gene, except for the annealing temperatures, which were as follows: for HPV type 6, 2 min at 56 °C; for HPV type 11, 2 min at 61 °C. All PCR products were submitted to agarose gel electrophoresis in a 2 % gel, treated with ethidium bromide and analyzed under UV light.

Data analysis

The SPSS 16 program (Statistical Package for the Social Sciences) was used. Chi-square test (χ^2) was performed to test the association variables for categorical data. Fisher exact test was done in table containing value <5. Student's t test was also used, and differences were considered significant at $P < 0.05$.

Results

Hundred patients complaining of common and genital warts were included from south region of Iran in this study. Demographic data such as age, marriage status, mean of marriage; number of children; mean of menarche; smoking status and type of HPV for cases have been summarized in Table 1. The mean ± SD age was 26.12 ± 5.07 years in the study group. The participants in the study group were married at a younger age 22.09 ± 3.89 and the mean of menarche was 13.87 ± 1.35. The result of HPV DNA for collected samples revealed that there was positive 73/27 (73 %) samples with HPV DNA by using general primers for all genotypes of HPV. Distribution of general-HPV, HPV6 and HPV11 is shown in Table 2. The result of genotyping based on multiplex-PCR analysis that 49 samples out of 73 HPV DNA positive samples were detected as HPV-6, 67 samples were identified as HPV-11. The extracted DNA samples from all cases were positive for the β-globin gene indicating acceptable quality for HPV analysis. Prevalence of HPV general, HPV6 and HPV type-11 according to the patients' age have been that the most positives age group were between 23 and 36 and prevalence of HPV11 according to the patients' age is shown that the most positive group are between 31 and 34. Correlation between different variables and HPV DNA, and

Table 1 Characteristics of study participants

Parameters	
Mean age ± SD	26.12 ± 5.07
Mean of marriage	22.09 ± 3.89
Mean of menarche	13.87 ± 1.35
Married/single	91/9
Smoking: (yes/no)	16/84
General_HPV (+/−)	73/27
HPV6 (+/−)	49/51
HPV11 (+/−)	67/33

Table 2 Distribution of HPV general, type 6 and 11

Parameters	General-HPV		HPV6		HPV11		
	+	−	+	−	+	−	
Smoking							
Yes	10	6	9	7	8	8	16
No	63	21	40	44	59	25	84
Sum	73	27	49	51	67	33	
	100		100		100		100

HPV type 6, 11 are summarized in Table 3. There was a significant association between marriage and HPV genotype 6 (OR = 0.51, 95 % CI = 0.37–0.70, $P = 0.01$). Logistic regression analysis indicated highly significant association between marital status and HPV type 6 ($P = 0.000$). In this study significant association between marriage and HPV genotype 11 (OR = 0.85, 95 % CI = 0.58–1.24, $P = 0.7$) was not found. Likewise this result was demonstrated, in combined marriage and HPV-general (OR = 0.80, 95 % CI = 0.62–0.05, $P = 0.4$). The relationship between those with passive smoking and HPV-general no significant difference and also no increased risk of HPV DNA was noticed (OR = 0.83, 95 % CI = 0.56–1.24, $P = 0.3$). When we combined smoking statues with those having HPV genotype 6 the result revealed no significant association between two respective combination, but a borderline increased risk of HPV type 6 (OR = 1.18, 95 % CI = 0.73–1.92, $P = 0.7$) has been shown. Similar result has been detected in combination those exposed to passive smoking and HPV genotype 11 (OR = 0.71 95 % CI = 0.43–1.18, $P = 0.2$). The interaction between HPV genotype 6 and 11 has shown significant association with increased risk of HPV types (OR = 1.9 95 % CI = 0.43–2.54, $P = 0.000$).

Discussion

Cervical cancer is an invasive cancer affecting approximately 500,000 women each year of which 80 % live in developing countries. The vast majority of cervical cancer cases are caused by infection with certain genotypes of human papillomaviruses [41]. Molecular tests may accurately identify different types of HPV (of low and high cancer risk) in cells from cytological screening of cervical lesions and, due to their high sensitivities, has been the focus of attention of many studies [17, 18, 19]. There is international consensus that "high risk" genotypes, including genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66 can lead to cervical cancer. Infections with low-risk genotypes, including 6, 11, can cause benign or low-grade cervical tissue changes and genital warts [41].

HPV cannot be cultured in vitro, thus analysis of DNA sequences can be used to identify HPV genotypes. PCR and in situ hybridization are two of the most sensitive methods [42]. In this study, we used the PCR method for initial detection of HPV-general and therefore we performed multiplex gap-PCR in order to genotyping of HPVs types 6, 11. In our investigation the prevalence of HPV in genital warts tissue was 73 (73 %) in 100 samples, but in another study the HPV prevalence was 34.6 in Zagreb region and also the presence of HPV DNA was 99.7 % in women with histologically confirmed SCC [24]. In one study in Mazandaran Province (Iran) 33 (78 %) cases were HPV positive based on PCR screening in cancerous group [15]. In our research study by using PCR, it was revealed that out of 100 samples there were 10 (5.5 %) positive samples for HPV DNA positive and those having passive smoking; there were 15 (2 %) positive samples with HPV type 6, 11, respectively.

Garland et al. has indicated that among women who were negative by PCR for HPV-6 or HPV-11 from day 1

Table 3 Correlation between different variables and HPV DNA, and HPV type 6, 11

Variable	HPV general		HPV type 6		HPV type 11	
	+	−	+	−	+	−
	65 (89 %)	26 (96.3 %)	41 (83.7 %)	50 (98 %)	60 (89.5 %)	31 (94 %)
Marriage	OR = 0.80 (0.62–0.5)		OR = 0.51 (0.37–0.70)		OR = 0.85 (0.58–1.24)	
	$P = 0.43$		$P = 0.01$		$P = 0.71$	
Passive smoking	10 (13.6 %)	6 (22.3 %)	9 (18.4 %)	7 (13.8 %)	8 (12 %)	8 (24.2 %)

ORs were adjusted for marriage HPV types

CI Confidence interval, *OR* odds ratio

Significant set at $P < 0.05$

through month 7, the time to development of HPV-6 or HPV-11-related GW was approximately 2 years. However, the exact time from incident infection to the development of GW in these women could not be determined. The long latent period may reflect detection of low viral loads well before lesion development and clinical manifestation of GW [43].

Other, smaller studies have reported the contribution of HPV-6 and HPV-11 to GW. Two studies conducted in the United States found HPV-6 or HPV-11 in 100 % of GW analyzed (samples from 37 and 41 participants, respectively) [44]. In another US study, HPV-6 or HPV-11 was detected in 74 % of GW (samples from 42 participants) [45]. The HPV types 16, 18, 31, 33 and 35 are among the eight most prevalent types in cervical cancer worldwide [43] and therefore of great importance and types 6 and 11 were chose because they are of low risk, as they are found in up to 95 % of cases of condyloma acuminatum [46].

Ball et al. has shown that multiple infections in tissue are frequent and the subsequent analysis of HPV 6 and 11 E6 DNA viral loads suggested that other HPVs could also be causing lesions. Further analysis of HPV 6/11 E6 mRNA levels showed that there was no discernable relationship between HPV 6 E6 DNA viral load and relative HPV 6 or 11 E6 mRNA levels thereby questioning the relevance of viral load to lesion causality. Our investigation also showed a similar result that multiple infection with genital warts and subsequently HPV DNA positive 73 % all of tissue samples, therefore the relationship between HPV type 6 and 11 and the risk of any cervical malignancy remains unclear because in our region we could not to find any study research in field area of HPV 6/11 E6 mRNA levels [47]. Muller et al. [48] showed the prevalence of anogenital HPV among study participants was 78 % (166) and HPV DNA was detected in 100 % (108) of GW, 48 % (27) of men with urethral discharge syndrome and 62 % (31) of voluntary counseling and testing participants. HPV types 6, 11, 16 and 18 were prevalent as either single or combined infections in 81 % (134) of all HPV-positive study participants. HPV types 6 and/or 11 were significantly higher among GW patients ($P < 0.001$).

Our finding also has indicated the frequent prevalence of HPV DNA positivity in HPV type 6 and 11 infections. On the other hand our investigation analyzed multiple variable such marriage, passive smoking, age of marriage and from these parameters revealed that marriage has significant association with HPV positive also HPV type 6, 11 and multiple genital warts infection in women at the sexually active ages. The limitations of the present study are as follows: first, the study is clinic-based, which may introduce selection bias. Second, it did not include all patient with genital warts in our region in south of Iran. Over the past two decades there has been substantial research demonstrating the causal link between HPV infection and cervical neoplasia also genetic polymorphism of DNA repair genes such *NBS1*, cytokine polymorphism genes anti and pro-inflammatory interleukins seem to modulate the susceptibility to cervical cancer and the relatively high proportion of cervical cancers associated with certain HPV types, particularly types 16 and 18 [49, 50]. Epidemiologic studies to date have generally not been specifically designed to evaluate HPV infection or disease natural history in a manner ideal for infectious disease modeling. In summary, this present study describes additional data for modeling outcomes of incident HPV 6, 11, infections. However, further studies with rigorous disease ascertainment would be helpful, particularly for incident infections caused by other HPV types. With the arrival of new technologies, such as HPV tests, mRNA analysis and vaccines targeting specific HPV types, there has also arisen a need to accurately model the natural history of the infection for individual or groups of HPV types for policy evaluations.

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Conflict of interest The authors declare that there is no conflict of interest.

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