Distribution of human papillomavirus (HPV) genotypes in genital warts from males in Slovenia

M. Potočnik, B.J. Kocjan, K. Seme, and M. Poljak

ABSTRACT

Background: Genital warts (GWs) are the most frequent benign tumors in the anogenital region of both males and females. Human papillomaviruses (HPV) are etiologically associated with the development of virtually all GWs. HPV-6 and HPV-11 are the most commonly detected HPV genotypes, but at least 20 other alpha-HPV genotypes have occasionally been found in GW tissue specimens.

Objective: There is limited knowledge of GWs in Slovenia. Thus in this study we tested 55 GW tissue specimens collected from the same number of male patients using 2 different PCR protocols to obtain the first data concerning HPV and GWs in Slovenia.

Material and methods: 55 GW tissue specimens were tested for the presence of HPV using PGMY09/PGMY11 and CPI/CPIIg polymerase chain reaction (PCR). HPV genotypes were determined using restriction fragment length polymorphism analysis of PGMY09/11 PCR products or by sequencing of the CPI/CPIIg PCR products. In some GWs, the genotyping results were also confirmed using the Linear Array HPV Genotyping Test.

Results: HPV DNA was detected in all 55 tissue specimens of GWs. HPV-6 or HPV-11 was detected in 53 cases of GWs, and HPV-44 and candHPV-91 in one GW each. HPV-6 was detected approximately 4 times more frequently than HPV-11. In addition, HPV-16, HPV-31, HPV-51, HPV-53, HPV-55, candHPV-62, HPV-66, HPV-70, HPV-73, and HPV-84 were detected in some GW specimens. According to the published data, our study is the first to report the presence of candHPV-62 and candHPV-91 in GW tissue specimens.

Conclusions: Our study showed that HPV can be found in virtually all GW tissue specimens obtained from male patients in Slovenia. Because HPV-6 or HPV-11 was detected in 96.4% of GWs studied, it seems that, if a quadrivalent HPV vaccine proves to be effective in males, this vaccine could prevent the great majority of incidental GWs in males in Slovenia.

KEY WORDS

genital warts, HPV, human papillomavirus, PCR, males, Slovenia

Introduction

Genital warts (GWs) are the most frequent benign tumors in the anogenital region (1, 2). GWs typically present as flesh-colored, exophytic lesions on the external genitalia, including the penis, scrotum, vulva, perineum, and perianal skin (3). Diagnosis of GWs is primarily clinical. Patients with GWs may have discom-

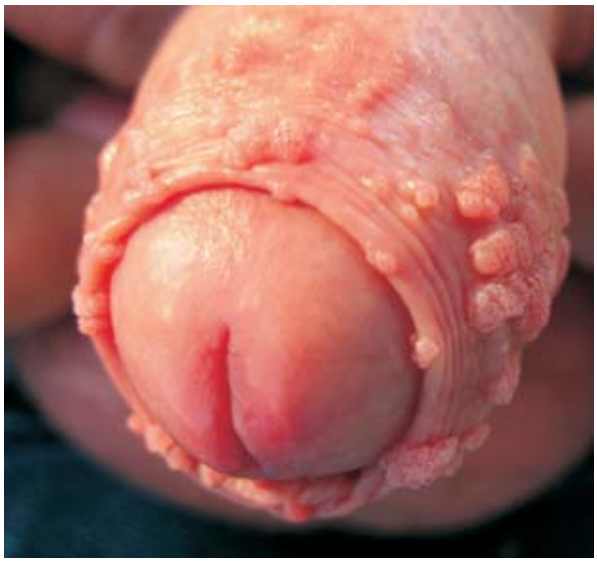

Figure 1. Multiple genital warts of the prepuce.

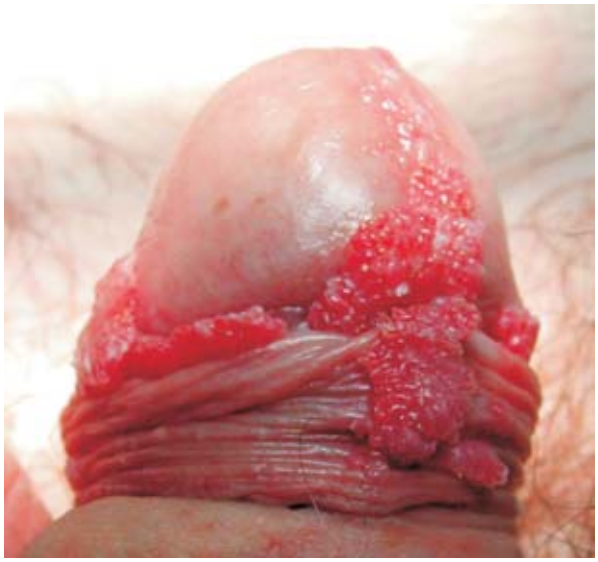

Figure 2. Multiple genital warts of the glans and prepuce.

fort, pain, bleeding, or difficulty with intercourse; these symptoms are more common in patients with larger, cauliflower-like lesions (2). Untreated GWs may resolve spontaneously, remain the same, or increase in size and number (4). The primary treatment goal is the removal of GWs. The choice of therapy is based on the number, size, site, and morphology of lesions, as well as patient preferences, cost, convenience, adverse effects, and the clinician's experience (1, 5). Podofilox, imiquimod, surgical excision, and cryotherapy are the most convenient and effective options; however, there is frequent recurrence of GWs after therapy (1, 5).

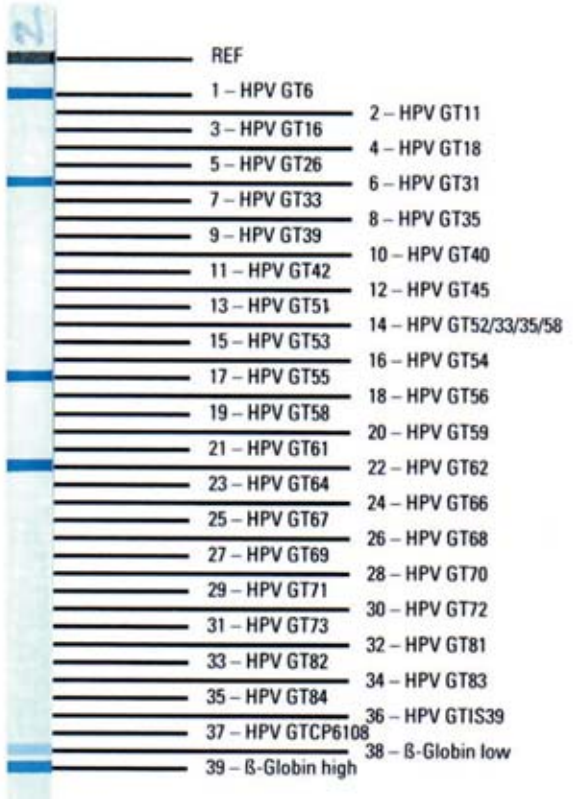

Figure 3. Determination of HPV genotypes in GW tissue specimen using the Linear Array HPV Genotyping Test (Roche Molecular Diagnostics) in a patient infected with 4 HPV genotypes: HPV-6, HPV-31, HPV-55, and candHPV-62 (Patient no. 5, Table 1).

Human papillomaviruses (HPV) are etiologically associated with the development of virtually all GWs (1). Since the first detection of HPVs in GWs in 1981 (6, 7), in the past 25 years several studies have examined the presence of HPV in GW tissue samples using various diagnostic methods (summarized in ref. 8). Thus, in early studies using immunohistochemistry, HPV antigens were detected in 20% to 80% of GW specimens (8). 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% (9) to 100% (10). However, when researchers started to use the most sensitive molecular method for detection of HPV – polymerase chain reaction (PCR) – HPV detection rates in GWs exceeded 90% in all published studies (summarized in ref. 8) and reached 100% in several studies (11–14). To the best of our knowledge, the study by Dianzani et al., in which 197 GW

specimens were tested for the presence of HPV infection, is the largest study published to date on this topic (14). In this study, HPV DNA was detected in all 197 specimens of GWs tested. In addition to HPV-6 and HPV-11, which were detected in 87% of GW specimens, HPV-16, HPV-18, and HPV-31 were identified in some GWs (14). In all HPV prevalence studies performed on GW specimens, two alpha-HPV genotypes (HPV-6 and HPV-11) were most frequently detected, but at least 20 other alpha-HPV genotypes have occasionally been found in GW tissue specimens. Thus, to the best of our knowledge, in addition to HPV-6 and HPV-11, the following HPV genotypes have been detected at least once in GW tissue specimens: HPV-16, HPV-18, HPV-26, HPV-31, HPV-33, HPV-39, HPV-42, HPV-44, HPV-45, HPV-51, HPV-52, HPV-53, HPV-54, HPV-55 (a subtype of HPV-44), HPV-56, HPV-57, HPV-58, HPV-59, HPV-61, HPV-66, HPV-68, HPV-70, HPV-73, HPV-82, HPV-83, and HPV-84. Brown et al. found the most: 23 different HPV genotypes were found in GWs obtained from both immunocompetent and immunocompromised persons when tested using very sensitive PGMY09/PGMY11 PCR coupled with reverse *line-blot* genotyping assay (15).

There is limited knowledge of GWs in Slovenia. A recent study that examined the associations between self-reported sexually transmitted infections and sociodemographic and behavioral factors among sexually active individuals in Slovenia aged 18 to 49 years, performed on a probability sample of the general population at respondents' homes by a combination of face-to-face interviews and anonymous self-administered questionnaires, determined that 0.4% of sexually active Slovenian males (CI 0.0-1.6) and females (CI 0.1-1.4) had experienced GWs at least once in their lifetime (16). To the best of our knowledge, there are no published data concerning the prevalence of HPV infection as well as HPV genotyping distribution in GWs obtained from Slovenian males or females. Thus this study tested 55 GW tissue specimens collected from the same number of male patients using two PCR protocols to obtain the first data concerning HPV and GWs in Slovenia.

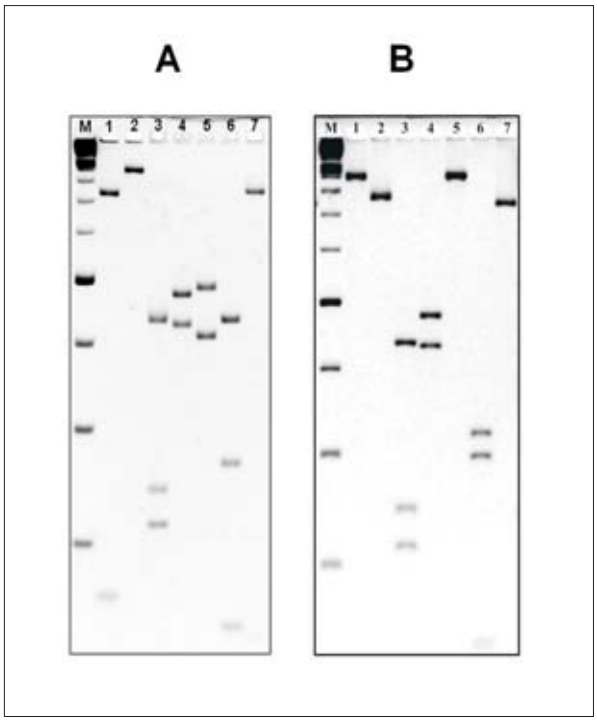

Figure 4. Determination of HPV genotypes by restriction analysis of the PGMY09/PGMY11 PCR products: A) agarose gel electrophoresis patterns of a PCR product of DNA extracted from a GW obtained from patient no. 4 cleaved with *Bam*HI (lane 1; 366 and 83 bp), *DdeI* (lane 2; 447 bp), *HaeIII* (lane 3; 217, 124, and 108 bp), *HinfI* (lane 4; 234 and 215 bp), *PstI* (lane 5; 242 and 207 bp), *RsaI* (lane 6; 216, 135, 72, and 26 bp), and *Sau3AI* (lane 7; 366, 63, and 20 bp). The restriction pattern is specific to HPV-11; B) agarose gel electrophoresis patterns of a PCR product of DNA extracted from a GW obtained from patient no. 2 cleaved with *Bam*HI (lane 1; 449 bp), *DdeI* (lane 2; 382 and 67 bp), *HaeIII* (lane 3; 217, 124, and 108 bp), *HinfI* (lane 4; 234 and 215 bp), *PstI* (lane 5; 449 bp), *RsaI* (lane 6; 161, 149, 72, and 67 bp), and *Sau3AI* (lane 7; 366, 63, and 20 bp). The restriction pattern is specific to the HPV-6 genotype. Lanes M: DNA 50 bp ladder as size marker (Roche Diagnostics).

Patients and methods

Sampling and DNA isolation

55 tissue specimens were collected from the same number of male patients with GW (17–36 years old; mean age 28.5 years). Patients included in the study were required to have at least 5 exophytic GWs on the glans of the penis, the coronal sulcus, or the foreskin (Fig. 1 and 2). GW tissue specimens were removed from all patients included in the study by curettage, collected, and stored frozen at -80 °C until DNA isolation.

A detailed clinical examination as well as careful review of the patient's medical history showed the absence of any apparent immune system deficiency in all patients. In addition, all patients included in the study tested negative for the presence of antibodies to human immunodeficiency virus (HIV) types 1 and 2. The study was approved by the Ethics Committee of the Ministry of Health of Slovenia, and informed consent was given by all participants.

DNA was extracted from fresh frozen GW tissue samples using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) and the protocol for nucleic acid isolation from mammalian tissue, strictly following the manufacturer's instructions. Briefly, tissue samples were placed in 200 µl of Tissue Lysis Buffer containing 40 µl of proteinase K and incubated overnight at 55 °C. Binding Buffer was added and samples were incubated a further 10 min at 72 °C. After the addition of isopropanol, each sample was transferred into a Mini column, centrifuged for 1 min at 8,000 rpm, and then washed once with Inhibitor Removal Buffer and twice with Wash Buffer. Bound DNA was eluted with 200 µl of Elution Buffer preheated at 70 °C.

Following DNA isolation, the quality of each DNA sample was verified by PCR amplification of 536 bp fragment of beta-globin gene on real-time PCR instrument LightCycler® (Roche Diagnostics) using LightCycler–FastStart DNA Master SYBR Green I kit and KM29/RS42 primers (17). Successful amplification of the beta-globin gene fragment indicated that the DNA sample was adequate for the HPV DNA analysis and that no PCR inhibitors were present.

HPV detection and genotyping

For detection of HPV, PCR amplification was performed on all samples using FastStart Taq DNA Polymerase (Roche Diagnostics) and consensus PGMY09 and PGMY11 HPV primers targeting approximately 450 bp fragments of L1 HPV gene, as described previously (18–20). All PGMY09/11 PCR–negative specimens were also tested using CPI and CPIIg HPV PCR primers targeting 188 bp fragment of E1 HPV gene, as described previously (21, 22). All known precautions to avoid a PCR product carry-over and sample-to-sample contamination were rigorously taken, as described previously (23, 24). To determine the HPV genotypes present in GW samples, the PGMY09/11 PCR products were digested using 7 restriction endonucleases (*Bam*HI, *Dde*I, *Hae*III, *Hinf*I, *Pst*I, *Rsa*I, and *Sau*3AI) and analyzed by agarose gel electrophoresis, as described previously (25–27) (Fig. 4). In PGMY09/11-negative samples, HPV genotypes were determined by sequencing of the CPI/CPIIg PCR products, as described previously (22). In all GW in which more than 1 HPV genotype was initially detected, genotyping results were additionally confirmed using the commercially available assay Linear Array HPV Genotyping Test (Roche Molecular Diagnostics, Pleasanton, CA, USA) capable of recognizing 37 different alpha-HPV genotypes, as described previously (28) (Fig. 3).

Results

The 536 bp fragment of beta-globin gene was successfully amplified from all 55 tissue samples of GWs, indicating that the DNA samples contained no apparent PCR inhibitors.

Using PGMY09 and PGMY11 HPV primers targeting the L1 HPV gene, the presence of HPV DNA was detected in 54 out of 55 GW specimens. The single initially negative PGMY09/11 sample obtained from a 21-year-old male was also tested for the presence of HPV using CPI/CPIIg HPV primers targeting the E1 HPV gene. The presence of the candHPV-91 genotype was detected in this sample (patient no. 15, Table 1). Thus, HPV DNA was detected in all 55 GW specimens tested. As summarized in Table 1, 14 different HPV genotypes were detected in 55 GW specimens. Infection with a single HPV genotype was found in 43 (78.2%) out of 55 GW specimens. Among the 43 samples with a single HPV genotype, HPV-6 was found in 33 specimens (76.7%), HPV-11 in 8 (18.6%) specimens, and HPV-44 and candHPV-91 in one GW specimen each. In contrast, 12 (21.8%) out of 55 GW specimens contained more than one HPV genotype. Out of 12 specimens with multiple HPV genotype infection, 7 samples contained 2 different HPV genotypes, 4 samples 3 different HPV genotypes, and one sample 4 different HPV genotypes. Out of 12 specimens with multiple HPV genotype infection, 10 and 2 samples contained HPV-6 and HPV-11, respectively. Thus, taking all GW specimens together (those with single and those with multiple HPV infections) HPV-6 or HPV-11 was detected in all but 2 GW specimens, or in 96.4% of GWs studied (Table 1).

Overall, 14 different HPV genotypes were detected in GW specimens obtained from 55 GW patients in Slovenia (Table 1). According to the recent epidemiological (29) and phylogenetic (30) classifications of HPV genotypes, HPV-6, HPV-11, HPV-44, HPV-55, candHPV-62, HPV-84, and candHPV-91 are low-risk HPV genotypes; HPV-16, HPV-31, HPV-51, and HPV-73 are high-risk HPV genotypes; and HPV-53 and HPV-66 are probable high-risk HPV genotypes. In epidemiological classification (29), HPV-70 was allocated to the low-risk group, but in phylogenetic classification (30) this genotype belongs to the high-risk HPV genotypes.

Discussion

To obtain the first data concerning HPV and GWs in Slovenia, 55 GW tissue specimens collected from the same number of male patients in Slovenia were tested for the presence of HPV DNA using 2 PCR protocols. Using both PCR protocols, HPV DNA was detected in all 55 GW specimens tested, which is in agreement with results of similar recent studies that showed that alpha-HPV are etiologically associated with the development of virtually all GWs (10–14). HPV-6 or HPV-11 was detected in all but two GW specimens, or in 96.4% of GWs studied, which is in agreement with the results of similar recent studies in which PCR was used as an HPV

Table 1. HPV genotypes present in 55 GW tissue specimens.

Patient no.	HPV genotype	Patient no.	HPV genotype
1	6, 84	29	6
2	6	30	11
3	6, 66	31	6
4	11	32	6
5	6, 31, 55, cand62	33	6
6	6, 51	34	6, 66, 73
7	6	35	6
8	6	36	6
9	6	37	6
10	6	38	6
11	6	39	6
12	6, 84	40	6
13	6	41	11
14	11, 73	42	6
15	cand91	43	6
16	6	44	11
17	6	45	6
18	11, 16, 53	46	11
19	6	47	6
20	6	48	44
21	6, 70	49	6
22	6	50	6, 16, 73
23	6	51	6, cand62
24	6, 53, 73	52	6
25	6	53	6
26	11	54	6
27	6	55	11
28	11		

diagnostic tool (11–14). Not surprisingly, HPV-6 was also detected in our study approximately 4 times more frequently than HPV-11.

According to the published data, our study is the first to report the presence of candHPV-91 as a single HPV genotype in GW tissue specimens, giving this particular HPV genotype a more prominent place in the etiopathogenesis of GWs than was supposed before. According to the recent phylogenetic classification (30), candHPV-91 belongs to the alpha-HPV species 8 (type species HPV-7) together with other low-risk HPV genotypes: HPV-7, HPV-40, and HPV-43. The most probable reason candHPV-91 was not detected previously in GWs is that in the majority of previous studies investigators did not use appropriate consensus primers that allow amplification of candHPV-91. Thus candHPV-91 also was not initially detected in this study when we used PGMY09 and PGMY11 primers targeting the L1 HPV gene. However, when this particular GW sample, obtained from a 21-year-old male, was also tested using CPI/CPIIg primers targeting the E1 HPV gene, it turned out to be HPV-positive. After sequencing the CPI/CPIIg PCR products, candHPV-91 was definitively determined as the single HPV genotype present in the GW sample. Our study also confirmed that, in all HPV prevalence studies, at least two PCR protocols using primers targeting different HPV genes should be used.

In our study, which was performed exclusively in individuals without any apparent immunodeficiency, 12 (21.8%) out of 55 GW specimens contained more than one HPV genotype. Up to 4 different HPV genotypes were detected in such specimens (Table 1, Fig. 3). Our results agree with the results of similar recent studies because, according to the published data, the prevalence of simultaneous infection with different HPV genotypes (mixed HPV infection), ranges between 19% and 100%, mainly depending on the immune status of the individuals included in the study and the selection of method used for HPV genotyping (14, 15, 31). In addition to HPV-6 or HPV-11, which were detected in all 12 samples with mixed HPV infection, 10 different HPV genotypes including 4 high-risk HPV genotypes (HPV-16, HPV-31, HPV-51, and HPV-73) were detected in GW specimens containing more than one HPV genotype. Nine out of these 10 HPV genotypes have been found in GWs earlier (mainly as one of the HPV genotypes in the content of mixed HPV infection), but to the best of our knowledge our study is the first to report, in addition to candHPV-91, the presence of candHPV-62 in tissue specimens of GWs. According to the recent phylogenetic classification (30), candHPV-62 belongs to alpha-HPV species 3 (type species HPV-61) together with other low-risk HPV genotypes: HPV-61, HPV-72, HPV-81, HPV-83, HPV-84, candHPV-86, candHPV-87, and candHPV-89.

In conclusion, our study showed that HPV can be found in virtually all tissue specimens of GWs obtained from male patients in Slovenia. HPV-6 or HPV-11 was detected in all but two GW specimens, or in 96.4% of GWs studied. This finding suggests that, if the recently approved quadrivalent HPV vaccine proves to be as effective in preventing HPV infections in males as it has proved in females (32), this vaccine could prevent the great majority of incidental GWs in males in Slovenia.

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