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Penile, Urethral, and Seminal Sampling for Diagnosis of Human Papillomavirus Infection in Men[▽]

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Methods that used specimens from three genital sites (penile brushing [PB], urethral brushing [UB], and the retrieval of semen [SE]) from 50 men were examined for human papillomavirus (HPV) DNA detection. The rates of detection by PB, UB, SE, PB and UB, and PB and SE were 88.9%, 50.0%, 33.3%, 100%, and 97.2%, respectively. The use of PB and UB appears to be the most accurate method; as an alternative to UB, the use of SE with PB could be used to improve the rate of HPV DNA detection in men.

Infection with high-risk human papillomavirus (HPV) is the main cause of cervical cancer (7). Since men have been suggested to play a likely role as reservoirs and vectors for HPV infection (8, 12, 28), screening of men may be relevant for the prevention of cervical cancer in women.

In the absence of clinical lesions, the most reliable diagnostic strategy for men is testing for HPV DNA (25). As recently reviewed (10), HPV infection rates in males range from 1.0% to 82.9%. In addition to the different profiles of the patients and the different HPV assays used, this wide range of rates may also be due to the variation in the clinical material analyzed (e.g., penile surface, preputial cavity, glans, scrotum, urethra, semen, and urine) because of a lack of agreement on the anatomical sites that should be sampled. Of the few studies that compared different specimens from men (1, 2, 11, 22), some reported that penile brushing (PB) represents the most sensitive method, while others selected urethral brushing (UB) or the retrieval of semen (SE). To assess the most adequate procedure, useful information could be obtained by comparing different samples from "high-risk" men, such as the sexual partners of HPV-positive women (8, 16, 25). To this aim, this study researched specimens from different sites for HPV DNA by three different methods, namely, PB, UB, and SE. The specimens were from 50 partners (age range, 23 to 58 years; mean age, 36.7 years) of HPV-positive women consecutively referred between May 2003 and May 2005 to the Department of Hygiene and Microbiology, University of Palermo, Palermo, Italy. The study was approved by the university ethical committee.

Patients were instructed not to wash their genitalia on the day before the examination and to have 2 days of sexual abstinence. Three types of genital specimens were collected in parallel. The first type was collected by PB, which consisted of the collection of cells from the dorsal and the ventral surfaces of the penile shaft, sampled with a standard-sized, dry cotton-tipped swab, and cells from the inner part of the foreskin, coronal sulcus, frenulum, and glans, sampled with a saline-prewetted cytobrush. Five to six backward and forward swab or brush movements were performed at each site, and all these samples were placed in the same tube containing 3 ml of phosphate-buffered saline (PBS). The second sample was obtained by UB, for which a very thin, saline-prewetted brush was inserted 1.5 cm into the urethra, rotated 360 degrees, and removed; when it was required by the patients, a 2% lidocaine gel was applied before sampling by UB. The cells obtained by UB were also placed in 3 ml of PBS. The third sample was obtained by SE, with the semen collected by masturbation and placed in sterile containers. If the sample was obtained at home, the sample was transferred to the laboratory within 2 h after ejaculation. All of the samples obtained by PB, UB, and SE were immediately processed for DNA extraction. Cells obtained by PB and UB were spun down at 13,000 rpm for 5 min, resuspended in 200 to 500 μl of 1 M Tris-HCl (pH 8.3)–0.45% Tween 20, 0.45% Nonidet P-40 containing 200 μg/ml proteinase K, and incubated at 60°C for 2 h and 95°C for 10 min. The samples obtained by SE were maintained at room temperature until complete liquefaction (if the sample was very viscous, it was maintained at 37°C for 10 to 15 min). Samples obtained by SE were processed with a commercial NucleoSpin Tissue mini column (Macherey-Nagel, Düren, Germany), and two 50-μl aliquots of the samples obtained by SE were analyzed. Samples obtained by SE were initially digested at 56°C for 15 min in the T1 buffer supplied with the kit, sperm cells were removed by centrifugation at 1,600 rpm for 10 min, and the supernatant was extracted by following the manufacturer's protocol. A final volume of 100 μl was obtained; the SE sample fraction analyzed was primarily composed of epithelial cells. Amplifications were carried out in a Mastercycler (Eppendorf, Germany) and the products of all PCR assays were analyzed in 8% polyacrylamide gel, as described elsewhere (14). Clinical samples were checked for DNA quality and the absence of inhibitors of amplification by analysis for the human β-globin gene (14); only β-globin-positive samples

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were examined for HPV DNA. The INNO-LiPA HPV genotyping system (version 1) kit (Innogenetics N.V., Ghent, Belgium) was used for HPV DNA amplification and genotyping by following the manufacturer's protocol. Briefly, 5 μl of each sample was amplified with biotinylated SPF_{10} primers, which amplify a 65-bp fragment of the L1 open reading frame and allow detection of at least 43 different HPV types (20). Thereafter, the amplicons were hybridized with 24 oligonucleotide probes, specific for 15 high-risk types (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, and 68) and 9 low-risk types (HPV types 6, 11, 40, 42, 43, 44, 54, 70, and 74). Due to the higher numbers of genotypes detected by the SPF_{10} primers (43 HPV types) than by the specific probes (24 HPV types), some samples yielded SPF_{10}-positive, hybridization-negative results. These HPV types not identifiable with the INNO-LiPA system were subsequently amplified by a nested PCR assay with the outer primer pair MY09-MY11 and the inner primer pair GP05+-GP06+, and genotyping was performed by direct cycle sequencing analysis, as described elsewhere (14, 15). A total of 10 μl of the extracted sample and controls was mixed with PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.4], 1.5 mM $MgCl_2$), 200 μM of each deoxynucleoside triphosphate, 50 pmol of the MY09-MY11 primer pair, and 1 U of AmpliTaq Gold polymerase (Perkin-Elmer Cetus, Berkeley, CA) in a final volume of 50 μl. Following *Taq* polymerase activation and template denaturation for 8 min at 95°C, the procedure included 4 cycles of 30 s at 95°C, 2 min at 60°C, and 45 s at 72°C (step 1) and then 6 cycles of 30 s at 95°C, 2 min at 57°C, and 45 s at 72°C (step 2) and 40 cycles of 30 s at 95°C, 30 s at 53°C, and 45 s at 72°C (step 3). The last cycle was extended by a 4-min elongation step at 72°C. In the second round of the amplification, 2 μl of the first PCR product was amplified with 30 pmol of the GP5-GP6 primer pair in the same reaction mixture described above, except that the concentration of $MgCl_2$ was 2 mM. After a first step of 10 min at 95°C, a total of 35 cycles of 30 s at 95°C, 30 s at 45°C, and 45 s at 72°C were performed, followed by a final 5-min extension step at 72°C. For the sequencing analysis, amplification products were purified by the use of Microcon YM-100 filter devices (Amicon; Millipore); when secondary amplification products were present, the PCR product was electrophoresed through 1% agarose and the band of interest was extracted with Ultrafree-DA filter devices (Amicon; Millipore). About 5 ng of the purified nested PCR product was added to 4 μl of the BigDye Terminator ready reaction mix (Perkin-Elmer Applied Biosystems), 4 μl of 0.8 μM primer, and deionized water to a final volume of 20 μl. The cycle incubator program consisted of 25 cycles of 30 s at 96°C, 45 s at 45°C, and 1 min at 60°C. Purification of the reaction mixtures and removal of free BigDye were performed with Centrisep spin columns (Princeton Separations, Adelphia, NJ). The reaction product was analyzed on an ABI Prism 310 analyzer (Perkin-Elmer Applied Biosystems). Alignments were obtained by use of the BLAST server (http://www.ncbi.nlm.nih.gov). HPV genotypes were considered low or high risk according to a recently published classification of HPV types (9).

Data were analyzed by using the SAS (Institute Cartz, NC) package and the χ^2 test (significance was a P value ≤0.05); in case of a significant χ^2 test result, the Student range Q of Tukey was used to assess any significant difference among groups (13).

TABLE 1. HPV DNA results by genital site for 50 men

No. of men	Genital site examined by:		
	PB	UB	SE
6	+	+	+
5	+	+	−
3	+	−	+
3	+	+	ND[a]
3	ND	+	+
11	+	−	−
1	−	+	−
2	+	ND	−
2	+	−	ND
14	−	−	−

[a] ND, not determined due to a negative β-globin result.

Warts were observed in three (8.3%) cases. Positive results for β-globin were obtained in 47 (94.0%) samples obtained by PB, 48 (96.0%) samples obtained by UB, and 45 (90.0%) samples obtained by SE ($P > 0.05$). Among the adequate samples, HPV was found in 32/47 (68.0%) samples obtained by PB, 18/48 (37.5%) samples obtained by UB, and 12/45 (26.6%) samples obtained by SE, for a total of 36/50 (72.0%) positive men (Table 1). Among the HPV-positive men, the HPV detection rate by PB was 32/36 (88.9%), that by UB was 18/36 (50%; $Q = 8.6$; $P < 0.01$), and that by SE was 12/36 (33.3%; $Q = 7.3$; $P < 0.01$). The detection rate by PB and UB combined was 36/36 (100%), and that by PB and SE combined was 35/36 (97.2%; $P > 0.05$). Of 32 samples obtained by PB, 6 (18.7%) harbored low-risk types (Table 2), 11 (34.3%) harbored high-risk types, and 15 (46.8%) harbored mixed types ($P > 0.05$); of 18 samples obtained by UB, the rates were 5 (27.7%) for low-risk types, 7 (38.8%) for high-risk types, and 6 (33.3%) for mixed types ($P > 0.05$); and of 12 samples obtained by SE, the rates were 3 (25.0%) for low-risk types, 5 (41.6%) for high-risk types, and 4 (33.3%) for mixed types ($P > 0.05$). Fourteen patients with adequate DNA at all sites had HPV infection at more than one site: for only nine (64.3%) of the patients the samples obtained by PB contained (all) the HPV type(s) infecting the patient, while for the remaining cases the samples obtained by UB and/or SE harbored types in addition to or different from those found in the samples obtained by PB. A total of 16 high-risk genotypes and 11 low-risk genotypes were detected, of which the most prevalent were HPV type 31 (HPV-31) (19.4%), HPV-53 (16.6%), HPV-6 (13.8%), and HPV-16 (11.1%). Mixed infections were found in 20 (57.1%) men.

In this analysis, sampling by PB, UB, and SE yielded satisfactory amplification results (a ≥90% rate of successful β-globin detection). The method used for sampling by PB is a critical issue, since some procedures may yield inadequate samples at a high frequency (5, 6, 17, 19). Of note, the method used in this analysis was also rated as easy to tolerate by all the men. For sampling by UB, some studies reported the detection of β-globin in only 13% and 65% of cases (2, 6); since the collection methods used in this study and in the previously reported studies were similar, inadequate results may be due to the laboratory techniques used for DNA analysis. Surprisingly,

TABLE 2. HPV type distribution among 36 HPV-positive men

Subject no.	HPV type obtained by[a]:		
	PB	UB	SE
1	6	6	6
2	33, 51, 53	51, 53	51, 53
3	33, 56, 74	52, 56	39, 52
4	51, 53, 54, 66	51, 53, 54, 66	51, 53, 54
5	62	62	62
6	68	68	68
7	16	11	−
8	31	31	−
9	58	42	−
10	56, 70	56, 70	−
11	66, 68	66	−
12	42	−	42
13	62	−	83
14	91	−	53
15	6[b]	6	ND[c]
16	53	53	ND
17	66, 70	66, 70	ND
18	ND	31	31
19	ND	16	16
20	ND	6, 43, 66	16, 43
21	6	−	−
22	11, 31, 33[b]	−	−
23	16, 52	−	−
24	31	−	−
25	31, 44, 68	−	−
26	31, 45, 70	−	−
27	31, 56	−	−
28	39, 59	−	−
29	53, 56, 58[b]	−	−
30	56	−	−
31	68	−	−
32	−	54	−
33	6, 51	ND	−
34	58	ND	−
35	53, 58	−	ND
36	54	−	ND

[a] High-risk HPV types are types 16, 31, 33, 39, 45, 51, 52, 53, 56, 58, 59, 62, 66, 68, 73, and 82. Low-risk HPV types are types 6, 11, 42, 43, 44, 54, 61, 70, 74, 83, and 91. −, negative result.
[b] Presence of penile warts.
[c] ND, not determined due to a negative β-globin result.

the use of controls for inhibitory material in semen before HPV testing was reported by only a few authors (2, 18, 26, 27). Because of the large quantity of PCR inhibitors present in semen (23, 24), testing for β-globin is strongly suggested to avoid the risk of false-negative HPV results.

HPV was found in 72.0% of the partners of HPV-positive women, in line with the findings of previous reports (16, 25). Of note, only 8.3% of the men had genital warts, confirming that mostly subclinical infections are found in men (6). Sampling by PB yielded the highest HPV rate (68.0%), followed by sampling by UB (37.5%) and SE (26.6%). Thus, sampling by PB, which is the only sampling method used in many studies, is confirmed to be the most adequate. In contrast, sampling by UB resulted in a low detection rate, as has also been found in other studies (4, 10), in which the rate of HPV DNA detection by UB ranged from 21% to 37%. Similarly, testing of semen for HPV was not efficacious, with only a 26.6% detection rate, which falls in the range of 23.4% to 39.0% reported in the literature (3, 18, 21). Even though sampling by UB and SE was less adequate than sampling by UB alone, when associated with the latter method, UB and SE each still contributed to HPV DNA detection; thus, the results from this study would result in the recommendation for parallel testing by PB plus UB or SE, which yielded 100% and 97.2% rates of HPV DNA detection, respectively. This finding is consistent with that of Nicolau et al. (25), who found that the HPV detection rate increased from 58.0% to 70.0% when the UB method was added to the PB method.

No difference in the prevalence of a specific genotype or group of HPV was found by PB, UB, and SE. For UB, this finding is in contrast to that of a study with a similar male population (4), in which infection of the urethra was mostly caused by low-risk HPV types. Among males infected at multiple sites, in only 64.3% of the cases the type detected by PB was representative of the type(s) infecting the patient. This finding is in contrast to those of Aynaud et al., who found the same type in penile/urethral biopsy specimens as in the specimens obtained by SE and UB (3, 4), suggesting possible contamination. In this analysis, the presence of a different type(s) in samples obtained by UB and SE than in samples obtained by PB suggested actual multifocal infection involving either the urethra or distant parts of the reproductive tract (21, 29). A high prevalence of mixed HPV infections (up to 57.1%) was found in the study group. Although the clinical significance of infection with multiple HPV types in men has still not been determined, a role in the acquisition or persistence of infection has been suggested (19).

In conclusion, data from the present analysis seem to indicate PB combined with UB is the best sampling method for the testing of men for HPV; as an alternative to UB, sampling by PB and SE combined could be applied to improve the rate of detection of HPV DNA in men.

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