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Human Papillomavirus Detection in Urine Samples from Male Patients by the Polymerase Chain Reaction

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Human papillomavirus (HPV) detection was performed using the polymerase chain reaction technique on urine samples from 17 male patients with condylomata acuminata in the meatus urethrae. Urine samples from 14 male laboratory volunteers were analyzed as controls. The DNA was extracted and purified from urine sediments, centrifuged at 1,800 and 100,000 × *g*, and subjected to 40 cycles of amplification with HPV 6 and HPV 11 type-specific anticontamination primers and the heat-stable Taq DNA polymerase. HPV was detected in the urine of 15 (88%) patients. In all positive patients the urine sediments of both the 1,800 and 100,000 × *g* centrifugation steps contained HPV DNA. Eight patients were found to be positive for HPV 6 DNA, six were positive for HPV 11 DNA, and one was positive for both HPV 6 and HPV 11 DNA. None of the males in the control group was positive for either HPV 6 or HPV 11 DNA. The results demonstrate that HPV can be transported by the urine, probably in exfoliated HPV-infected cells. A similar mechanism may occur during ejaculation, allowing sexual transmission of HPV viruses harbored in the cells of the male genital tract.

Although the human papillomaviruses (HPVs) are regarded to be sexually transmittable, most clinical research on these viruses has been focused on females. However, penile as well as cervical cancer is strongly associated with HPV 16/18 (4, 10), and condylomata acuminata (mainly associated with HPV 6/11) are found in both males (3) and females (5). Several reports describe an increased risk of women developing cervical lesions if their sexual partners have condylomata acuminata on the penis (1, 2, 7, 11).

Grussendorf et al. (6) found a 5.8% HPV prevalence rate in swabs from the glans and sulcus corona glandis of men without clinically visible lesions in the genital area. Ostrow et al. (13) detected HPV in semen and suggested that semen may be an HPV reservoir. Both the sexual transmission of HPV and the existence of a male reservoir for these viruses are suggested. Therefore, screening males for HPV may be important in the prevention of cervical neoplasia. However, prevalence and transmission studies on males are limited, probably because of sampling problems resulting in insufficient clinical material for reliable HPV detection by routine methods.

With the recently described polymerase chain reaction (PCR) technique (15), it is theoretically possible to detect one HPV molecule in a clinical sample. Recently we described an increased sensitivity for HPV detection in cervical scrapes by this technique as compared with Southern blot analysis and the modified filter in situ hybridization technique (11a).

Since condylomata acuminata are often found in the lower urinary tract (12), HPV may well be present in the urine of infected patients. We investigated this possibility by using the PCR method and HPV 6 and HPV 11 type-specific primers.

MATERIALS AND METHODS

Study groups. The patient group consisted of 17 male outpatients attending the Clinic for Sexually Transmittable Diseases at the Academic Hospital, Rotterdam, The Netherlands, for treatment of condylomata acuminata in the meatus urethrae. The control group consisted of 14 male volunteers from the Department of Pathology at the Diagnostic Centre SSDZ, Delft, The Netherlands.

Sample collection. Samples (100 ml) of urine were collected from all patients when visiting the Sexually Transmittable Diseases clinic. Urine samples (100 ml) were also collected from the 14 male volunteers. Half of each sample (50 ml) was centrifuged for 1 h at 1,800 × *g* at 4°C, and the other half was centrifuged for 1 h at 100,000 × *g* at 4°C. The urine sediments were suspended in 0.5 ml of phosphate-buffered saline (pH 7.4).

Sample processing. Volumes of 25 μl of 10% sodium dodecyl sulfate (SDS) were added to the samples, and the samples were then treated for 30 min at 37°C with Pronase (150 μg/ml of sample). DNA was isolated by one phenol extraction, followed by one phenol-chloroform–isoamyl alcohol (24:1) extraction, one chloroform-isoamyl alcohol extraction, and an ethanol precipitation (8). The DNA was collected by centrifugation and suspended in 100 μl of distilled water.

The extracted DNA was purified and desalted using Geneclean (as described by the manufacturer, Bio 101 Inc., La Jolla, Calif.) and precipitated with ethanol. The purified DNA was suspended in 130 μl of distilled water.

The urine sediments contained between 100 and 500 ng of chromosomal DNA, as estimated by agarose gel electrophoresis with ethidium bromide staining (8).

HPV detection. For screening, HPV 6 and HPV 11 type-specific anticontamination primers were used, flanking the plasmid cloning site (*Bam*HI) to prevent amplification of contaminated plasmid HPV clones (A. van den Brule, H. Claas, W. Melchers, M. du Maine, T. Helmenhorst, W. Quint, C. Meijer, and J. Walboomers, J. Med. Virol., in press) (Table 1). Oligonucleotide sequences were selected using the sequence analysis program of Queen and Korn (14) and synthesized on a DNA synthesizer (Applied Biosystems 380 A) by the methoxy-phosphoramidite method.

Besides the use of these anticontamination primers, a spatial partition of the different technical steps of the PCR

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TABLE 1. PCR primers and oligonucleotide probes for HPV detection

Primer or probe	Sequence	Length (bp)[a] of amplification product
HPV 6 primer 1	+5′-TAGTGGGCCTATGGCTCGTC-3′	280
HPV 6 primer 2	−5′-TCCATTAGCCTCCACGGGTG-3′	
HPV 6 probe	5′-CATTAACGCAGGGGCGCCTGAAATTGTGCC-3′	
HPV 11 primer 1	+5′-GGAATACATGCGCCATGTGG-3′	360
HPV 11 primer 2	−5′-CGAGCAGACGTCCGTCCTCG-3′	
HPV 11 probe	5′-CGCCTCCACCAAATGGTACACTGGAGGATA-3′	

[a] bp, Base pairs.

was introduced. The production of reaction mixtures and primer synthesis, the sample preparation, and the amplification and detection of the PCR products were all performed at different locations. By the use of these procedures, contamination of plasmid clones and PCR products can be excluded.

Amplification of the HPV 6 and HPV 11 target sequences was carried out in a 100-μl reaction mixture containing 50 to 250 ng of DNA (obtained from 25 ml of the original urine sample), 200 mM KCl, 10 mM Tris hydrochloride (pH 8.3), 2.5 mM $MgCl_2$, 0.01% gelatin, 200 μM each dATP, dGTP, dTTP, and dCTP, 100 pmol each of HPV 6 and HPV 11 primers, and 1 U of the thermostable Taq DNA polymerase (Perkin-Elmer, Gouda, The Netherlands). The samples were overlaid with mineral oil (~100 μl) to prevent condensation and subjected to 40 cycles of amplification using a PCR processor (Biomed, Ditfurth, Federal Republic of Germany). Each cycle involved heating to 92°C for 1 min (DNA denaturation), followed by cooling to 37°C for 2 min (primer annealing) and again heating to 72°C for 3 min (chain elongation).

Determination of the PCR amplification products was performed by gel electrophoresis on a 2% agarose gel with ethidium bromide staining (8). For Southern blot analysis (16), the agarose gel was denatured for 20 min in 0.25 N HCl and the amplification products were transferred to a nylon membrane (Hybond, Amersham International plc, Amersham, England) by diffusion blotting in 0.4 N NaOH. The Southern blots were air dried and baked for 2 h at 80°C in a vacuum oven.

(Pre)hybridization was performed at room temperature in a solution containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate), 5× Denhardt solution (100× Denhardt solution is 2% bovine serum albumin, 2% Ficoll, 2% polyvinylpyrrolidone), 0.5% SDS, 0.075 M EDTA, and 0.1 mg of denaturated, sonicated herring sperm per ml. The Southern blots were hybridized for 16 h with a γ-^{32}P 5′-end-labeled HPV 6 type-specific oligonucleotide probe directed against the internal portion of the amplified product (Table 1).

After hybridization, the Southern blots were washed twice for 15 min with 2× SSC–0.1% SDS at 37°C and once for 15 min with 0.5× SSC–0.1% SDS at 60°C. The Southern blots were autoradiographed for 4 h on Kodak Royal X-Omat R film between intensifying screens at −80°C.

After autoradiography, the Southern blots were incubated at 45°C for 30 min in 0.4 N NaOH to remove the bound HPV 6 oligonucleotide probe. The blots were washed in a solution containing 0.1× SSC, 0.1% (wt/vol) SDS, and 0.2 M Tris hydrochloride (pH 7.5) at 45°C for 30 min. The Southern blots were successively hybridized with an HPV 11 type-specific oligonucleotide probe as described.

RESULTS

Both the urine sediments of 1,800 and 100,000 × *g* were used for HPV 6 and HPV 11 DNA detection by the polymerase chain reaction. After 40 cycles of amplification, HPV 6- and HPV 11-specific amplification products were analyzed by agarose gel electrophoresis and by Southern blot analysis (Fig. 1). Because of the differences in amplimer length (Table 1) it was easy to differentiate between HPV 6- and HPV 11-positive patients (Fig. 1A). In 13 patients (76%) HPV 6 or HPV 11 DNA, or both, could be detected. In addition to the gel electrophoresis results, two more patients were found to be positive for HPV (one for HPV 6 DNA and one for HPV 11 DNA) by using Southern blot hybridization with HPV 6 (Fig. 1B) and HPV 11 (Fig. 1C) type-specific radiolabeled oligonucleotide probes. In all HPV-positive patients, HPV was detected in both the 1,800 and the 100,000 × *g* urine sediments.

In the control group, no HPV 6 or HPV 11 DNA could be detected. As negative controls, human liver DNA and PCR mixtures without DNA were used for the amplification of HPV 6- and HPV 11-specific sequences. No amplification was observed. Table 2 shows the occurrence of HPV in the urine sediments of both patient and control groups.

DISCUSSION

HPV 6 and/or HPV 11 DNA could be detected by the PCR method in the urine of 15 (88%) male patients with condylomata acuminata in the meatus urethrae, whereas no HPV 6 or HPV 11 DNA could be detected in the control group (i.e., laboratory volunteers). The association between these HPV types and condylomata acuminata has already been established in both males and females (3, 5).

Grussendorf et al. (6) detected HPV in 5.8% of swabs from the glans penis of males without any clinical signs of HPV infection. However, they found HPV in 10% of women with no cytological abnormalities, and they suggested that the lower HPV detection rate in males could be explained by the fact that fewer epithelial cells are obtained by taking swabs from the penis. To override this sensitivity problem, the PCR can be used. The PCR is a very sensitive and highly specific method requiring only small amounts of target DNA for accurate detection of the virus (11a).

Because HPV is a sexually transmittable agent, it would be expected that the prevalences of HPV are comparable in males and females. The fact remains, however, that although cervical and penile cancer are both strongly HPV associated, the prevalence of cervical cancer is much higher than the prevalence of penile cancer. The reason for this phenomenon is not clear but could be explained by the fact that certain cells in the female are more susceptible to infection

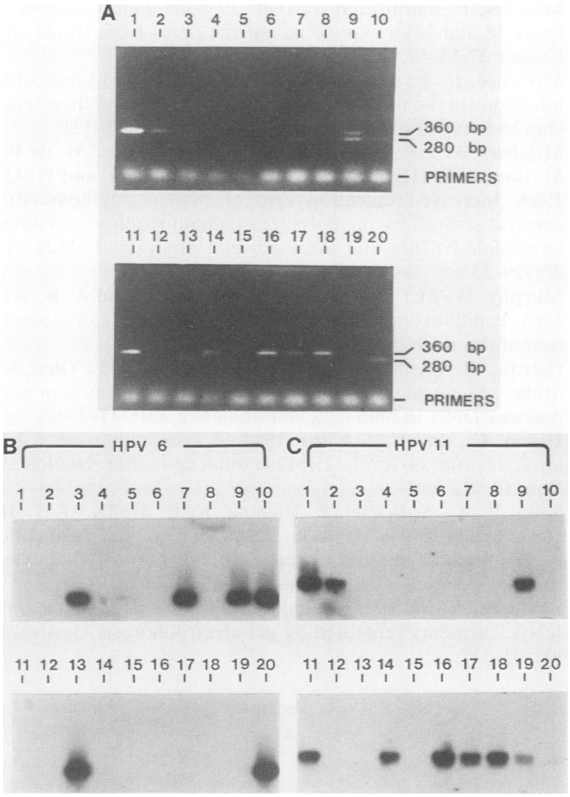

FIG. 1. The DNA extracted from 20 randomly selected urine sediments from patients and controls was subjected to 40 cycles of amplification using HPV 6 and HPV 11 type-specific primers. The reactions were analyzed by agarose-gel electrophoresis with ethidium bromide staining (A), transferred to a nylon membrane, and successively hybridized with an HPV 6 (B) and HPV 11 (C) type-specific oligonucleotide probe. In samples 7, 10, 13, and 20 an HPV 6-specific fragment of 280 bp can be seen on both the gel (A) and the HPV 6 hybridization (B). In samples 1, 2, 11, 14, and 16 through 18, an HPV 11-specific fragment of 360 bp can be seen on both the gel and the HPV 11 hybridization (C). Sample 3 only showed positivity for HPV 6 by hybridization (B), sample 19 only showed positivity for HPV 11 by hybridization (C), and in sample 9 both HPV 6 and HPV 11 fragments are synthesized. In samples 4 through 6, 8, 12, and 15, no HPV 6 or HPV 11 could be detected. For details of HPV occurrence in the different samples, see Table 2.

on contact with the virus (9). Cervical intraepithelial neoplasia (from which invasive carcinoma develops) arises in the area of immature metaplasia between the mature squamous epithelium of the ectocervix and the columnar epithelium of the endocervical canal, the so-called transformation zone. It seems that the transformation zone is the most susceptible site for HPV infection (9). This is supported by the fact that other types of malignancy are comparatively rare, such as vaginal and vulvar cancer. The lower prevalence of HPV lesions in males may also be related to the absence of such a typical transformation zone, since the meatus urethrae and the penis are covered with squamous epithelium.

TABLE 2. HPV detection in urine sediments of males

Group	No. of samples that were HPV:				% Positive
	Negative[a]	6	11	6+11	
Patients	2	8	6	1	88
Controls	14				0

[a] Negative for HPV 6 or HPV 11.

HPV could be detected in the 1,800 × *g* urine sediments. The sedimentation by centrifugation at this speed indicates cell-associated virus. Therefore, the detection of HPV in urine most likely reflects the presence of exfoliated HPV-infected cells from the epithelium of the meatus urethrae.

If urine can wash cells from the epithelium, it is likely that ejaculation can produce the same effect. The seminal fluid, contaminated with HPV-infected epithelial cells, comes into contact with the immature cells of the cervical transformation zone after ejaculation and could cause infection of the female with HPV.

Ostrow et al. (13) detected HPV 2 and HPV 5 in the semen from patients with severe chronic wart disease and epidermodysplasia verruciformis, respectively. Although these particular HPV types are not associated with genital cancer, Ostrow et al. suggested that cancer-associated HPV types could be sexually transmitted via semen in a similar way. There is a high correlation between the presence of oncogenic HPV types in men with penile intraepithelial neoplasia and the occurrence of cervical intraepithelial neoplasia in their female partners (1, 2, 7, 11). This strongly supports the idea that HPV can be sexually transmitted and that males can harbor HPV without any clinical signs. Male patients infected with cancer-associated HPV types should be regarded as high-risk partners, and screening men for HPV may be important in the prevention of cervical cancer.

The method we describe using urine samples is a way of obtaining information about the occurrence of HPV in males. However, it must be noted that HPV detection in urine samples may only identify those men with HPV infections in the meatus urethrae, the urethra, or the urinary bladder, but not those with superficial penile HPV infections.

In this paper a noninvasive method to detect HPV in males is described. Using HPV type-specific anticontamination primers and a spatial partition of the PCR technique in different steps, contamination of plasmid clones or amplified PCR products could be excluded. Virus detection in urine samples facilitates large epidemiological studies and the investigation of the mechanisms of virus transmission. Women involved in routine screening program or attending their gynecologist could bring their partner's urine for HPV detection concomitant to investigation of their own cervical scrape. In this way this procedure may also prove its potential value for the detection of HPV types which are more clearly associated with cervical intraepithelial neoplasia and cervical cancer. These studies may give a broader insight into HPV prevalence in males and females and the mechanisms of virus transmission.

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