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Liquid-Based Urine Cytology as a Tool for Detection of Human Papillomavirus, *Mycoplasma* spp., and *Ureaplasma* spp. in Men

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Liquid-based urine cytology (LB-URC) was evaluated for cytological diagnosis and detection of human papillomavirus (HPV), *Mycoplasma*, and *Ureaplasma*. Midstream urine samples were collected from 141 male patients with urethritis and 154 controls without urethritis, and sediment cells were preserved in liquid-based cytology solution. Urethral swabs from urethritis patients were tested for the presence of *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. Papanicolaou tests were performed for cytological evaluation. HPV, *Mycoplasma*, and *Ureaplasma* genomes were determined by PCR-based methods, and localization of HPV DNA in urothelial cells was examined by *in situ* hybridization (ISH). The β-globin gene was positive in 97.9% of LB-URC samples from urethritis patients and in 97.4% of control samples, suggesting that high-quality cellular DNA was obtained from the LB-URC samples. HPV DNA was detected in 29 (21.0%) urethritis cases and in five (3.3%) controls ($P < 0.05$). HPV type 16 (HPV 16) was most commonly found in urethritis patients. Cytological evaluations could be performed for 92.1% of urethritis patients and 64.3% of controls. Morphological changes suggestive of HPV infection were seen in 20.7% of the HPV-positive samples, and ISH demonstrated the presence of HPV DNA in both squamous and urothelial cells in HPV-positive samples. *Mycoplasma genitalium*, *Mycoplasma hominis*, *Ureaplasma parvum*, and *Ureaplasma urealyticum* were detected in 14.5%, 10.9%, 6.5%, and 12.3% of urethritis patients, respectively. The prevalence rates of these microorganisms (except *Ureaplasma parvum*) were significantly higher in urethritis cases than controls ($P < 0.05$). LB-URC is applicable for detection of HPV, *Mycoplasma*, and *Ureaplasma*. HPV infection occurs in urothelial cells, especially in gonococcal urethritis.

Human papillomavirus (HPV) infection is known to cause uterine cervical cancer and precancerous lesions in women (14, 29). HPV infection usually occurs through sexual intercourse, and it has been reported that the prevalence of HPV infection in sexually active healthy young women ranges from 20% to 60% (12, 19, 26). Thus, cervical HPV infection is thought to be one of the most common sexually transmitted infections (STIs) in women.

Prophylactic HPV vaccine has become available worldwide to prevent cervical cancer, and the prevalence and sites of HPV infection in the male genital tract have been studied. Some studies indicated that the external male genitalia, including the penile shaft, glans, coronal sulcus, and prepuce, are the most common sites of HPV infection and that the prevalence of HPV among healthy young men is as high as that among healthy young women (9, 10). However, a systematic review indicated that the HPV detection rate in urine was less than 7% and that urine is unsuitable for HPV detection in epidemiological studies (9). Indeed, Giuliano et al examined the presence of HPV DNA in multiple genital sites of 186 healthy men and reported that HPV was detected most commonly on the penile shaft (49.9%), followed by the glans (35.8%), scrotum (34.2%), perianal area (20.0%), anal canal (17.6%), urethra (10.1%), and semen (5.3%). The HPV detection rate was the poorest in urine samples (0.8%) (10).

We have recently reported a high (24%) prevalence of HPV infection in urine samples from 142 Japanese men with urethritis (22). However, the β-globin gene used as an internal control was detected in only 65% of cases ($n = 92$), and we considered that poor quality of DNA or some inhibitory factors for PCR may have been associated with the failure of PCR tests in urine samples. Urine samples are suitable for large-scale studies of not only HPV but also other microorganisms that cause urethritis because of the ease and noninvasive nature of sampling. Therefore, we have attempted to improve the analysis methods using urine samples.

In the present study, we used liquid-based cytology (LBC) samples of urine sediment to detect not only HPV DNA but also DNA of other microorganisms, such as *Mycoplasma* spp. and *Ureaplasma* spp. In addition, we evaluated the cytological findings of urine sediment tests based on Papanicolaou staining and performed *in situ* hybridization (ISH) analyses to confirm the localization of HPV DNA in urothelial cells.

MATERIALS AND METHODS

Subjects. A total of 141 male patients with urethritis (urethritis group) and 154 male patients without urethritis (controls) who visited the Urology Department of Kanazawa University Hospital, Kanazawa, Japan, and Ishikawa Prefectural Central Hospital, Kanazawa, Japan, and a sexually transmitted disease (STD) clinic in Osaka, Japan, between April 2009 and April 2010 were enrolled in this study. The control group consisted of male patients with urinary stones, infertility, or hypogonadism. Subjects with urogenital tumors or with histories of STD within the previous 12 months were excluded as controls. The clinical diagnosis of urethritis was

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based on microscopic detection of more than five white blood cells/high-power field from urethral swabs and the presence of definite histories of sexual intercourse with men or women. After obtaining written informed consent, according to the protocol approved by the Ethics Committee of Kanazawa University Graduate School of Medicine, each patient provided a midstream urine specimen. Urine specimens (15 ml) were centrifuged at 1,500 rpm for 10 min, and the sediment was placed into a separate tube containing 2.5 ml of preservative solution for LBC (LiquiPrep; LGM International Inc, Melbourne, FL) and stored at 4°C until testing.

The patients with definite genital lesions, such as condyloma acuminata, were excluded. All patients with urethritis were tested for the presence of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in urethral swabs, based on Amplicor STD-1 PCR (Roche Diagnostics, Basel, Switzerland).

HPV DNA analysis and genotyping. Aliquots of 700 μl of well-agitated samples were centrifuged at 5,000 rpm for 5 min, and the supernatants were discarded. The cell pellets were washed with 300 μl of 10 mM Tris-HCl buffer solution (pH 8.0). DNA was extracted using a DNA extraction kit (SMI test; G&G Science Co, Fukushima, Japan). The β-globin gene was first amplified to confirm the adequacy of extracted DNA in all samples. In β-globin-positive samples, HPV DNA was tested by modified GP5+/GP6+ PCR as described previously (22, 28).

In the HPV-positive samples, HPV genotyping was performed using HPV GenoArray test kits (HybriBio Limited, Central Hong Kong, Hong Kong) (22). This test is capable of detecting 21 HPV types, including 5 low-risk types (HPV 6, 11, 42, 43, and 44), 14 high-risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), and 2 undetermined-risk types (HPV 53 and HPV 81). Some samples that were positive on PCR analysis and negative on the genotyping test were defined as representing an unknown HPV type(s).

Cytological examination based on Papanicolaou staining and *in situ* hybridization. Aliquots of 700 μl of each sample were subjected to microtube centrifugation at 5,000 rpm for 10 min, and the supernatants were decanted. The residual cell pellets were mixed with 50 μl of Cellular Base Solution (LiquiPrep; LGM International Inc) and pipetted onto glass microscope slides. The slides were air dried for more than 60 min and then subjected to Papanicolaou staining. All slides were randomly checked by a cytopathologist certified by the Japanese Society of Clinical Cytology without previous knowledge of the molecular findings. HPV infection-associated morphological signs were judged based on the presence of the two classic signs (koilocytosis and dyskeratocytosis) and the nine nonclassic signs (mild koilocytosis, mild dyskeratocytosis, cleared cytoplasm, keratohyalin granules, condensation of filaments, spindle cells, nuclear hyperchromatism, multinucleation, and perinuclear halos) described previously by Schneider et al (21).

Furthermore, ISH was performed in all high-risk HPV-positive samples to determine the localization of HPV DNA in urothelial cells according to the manufacturer's instructions (Dako GenoPoint System [catalog no. K0620]; Dako, Carpinteria, CA). After being fixed with 95% ethanol for 60 min, the slides were placed into Target Retrieval Solution (catalog no. S1700; Dako, Glostrup, Denmark) at 95°C for 40 min, followed by digestion with proteinase K (catalog no. S3004; Dako) at room temperature for 10 min. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 20 min. Denatured DNA was hybridized with a wide-spectrum biotinylated HPV DNA probe (catalog no. Y1404; Dako) for 13 high-risk HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) at 90°C for 5 min. After hybridization with the HPV DNA probe in a humid chamber at 37°C for 16 to 18 h, the slides were reacted with primary streptavidin-horseradish peroxidase (streptavidin-HRP), biotin tyramide solution, and secondary streptavidin-HRP for 15 min each. Then, a diaminobenzidine reaction was performed for 5 min. HPV DNA signals were visualized as brown staining. All slides were counterstained with hematoxylin. As controls, ISH was also performed in 10 HPV-negative samples.

TABLE 1 Adequacy rates and detection rates of HPV, *Mycoplasma* spp., and *Ureaplasma* spp. in urine samples by liquid-based processing[a]

Gene or organism	No. of samples (% prevalence)		*P* value
	Urethritis group ($n = 141$)	Control group ($n = 154$)	
β-Globin gene	138 (97.9)	150 (97.4)	0.906
Any HPV type	29 (21.0)	5 (3.3)	<0.001
High-risk HPV type	18 (13.0)	1 (0.7)	<0.001
Low-risk HPV type	4 (2.9)	1 (0.7)	0.319
M. genitalium	20 (14.5)	5 (3.3)	0.002
M. hominis	15 (10.9)	3 (2.0)	0.004
U. parvum	9 (6.5)	7 (4.7)	0.668
U. urealyticum	17 (12.3)	4 (2.7)	0.003

[a] The ages (in years) of the members of the urethritis group ranged from 20 to 63 (37.6 ± 9.2) ($P = 0.209$); the ages (in years) of the members of the control group ranged from 19 to 59 (39.0 ± 9.8) ($P = 0.209$). HPV, human papillomavirus.

Analysis of *Mycoplasma* spp. and *Ureaplasma* spp. The detection rates of *Mycoplasma genitalium*, *Mycoplasma hominis*, *Ureaplasma urealyticum* (biovar 2), and *Ureaplasma parvum* (biovar 1) were also investigated by multiplex PCR assay in samples positive for β-globin as described previously (25, 27). Primer pair MG1 and MG2, primer pair RNAH1 and RNAH2, and primer pair UMS125 and UMA226 were used to identify *M. genitalium* (282 bp), *M. hominis* (334 bp), and *U. parvum* (403 bp) and *U. urealyticum* (448 bp), respectively (27).

Statistical analysis. The χ^2 test was used compare the positive rates determined for the two groups, and the Mann-Whitney *U* test was used to compare the numbers between two groups. In all analyses, $P < 0.05$ was taken to indicate statistical significance.

RESULTS

HPV infection in urothelial cells. To investigate the usefulness of liquid-based urine cytology (LB-URC), age-matched male patients with urethritis (37.6 ± 9.2 years of age) and male patients without urethritis as controls (39.0 ± 9.8 years) participated in this study. Of 141 patients with urethritis, 17 had gonococcal urethritis (GU), 24 had nongonococcal chlamydial urethritis (NGCU), and 100 had nongonococcal and nonchlamydial urethritis (NGNCU). None of the patients showed mixed infections with *N. gonorrhea* and *C. trachomatis*. None of the patients with urethritis had histories of sexual intercourse with men.

The β-globin gene was positive in 138 specimens (97.8%) of LB-URC samples from the urethritis group and in 150 specimens (97.4%) from the control group, and high β-globin-positive rates were observed in both groups (Table 1). HPV of any type and HPV high-risk types were detected in 21.0% ($n = 29$) and 13.0% ($n = 18$) of urethritis patients, respectively. In contrast, HPV of any type and HPV high-risk types were detected in 3.3% ($n = 5$) and 0.7% ($n = 1$) of controls, indicating a significantly higher prevalence of HPV in the urethritis patients than in the controls ($P < 0.05$) (Table 1). As shown in Table 2, HPV 16 was the most common type ($n = 6$; 20.0%) in the urethritis group, followed by HPV 58 ($n = 4$; 13.3%). High-risk HPV types, including HPV 16, 18, 31, 33, 39, 52, 58, 59, and 68, were detected in 67% of the HPV-positive samples in urethritis patients. One low-risk HPV type (HPV 6) and one undetermined-risk HPV type (HPV 66) were detected in control patients. HPV of an unknown type was detected in five (16.6%) urethritis patients and three (60%) controls.

Cytological findings using LB-URC samples could be evaluated in 130 (92.1%) cases and 99 (64.3%) controls. Eleven urethri-

TABLE 2 Type-specific frequency of HPV in urine samples[a]

HPV type	No. (%) of samples	
	Urethritis group	Control group
High risk		
HPV 16	6 (20.0)	ND
HPV 18	2 (6.7)	ND
HPV 31	1 (3.3)	ND
HPV 33	2 (6.7)	ND
HPV 39	1 (3.3)	ND
HPV 52	1 (3.3)	ND
HPV 58	4 (13.3)	ND
HPV 59	2 (6.7)	ND
HPV 68	1 (3.3)	ND
Risk undetermined	ND	ND
HPV 66	ND	1 (20.0)
HPV 53	1 (3.3)	ND
HPV 81	1 (3.3)	ND
Low risk		
HPV 6	2 (6.7)	1 (20.0)
HPV 11	1 (6.7)	ND
HPV 42	1 (6.7)	ND
Unknown	5 (16.6)	3 (60.0)

[a] HPV, human papillomavirus; ND, not detected.

tis samples and 55 control samples could not be evaluated because of the low quantity of cell samples. Of 29 HPV-positive samples from urethritis patients, HPV infection-related morphological changes, such as koilocytosis, dyskeratocytosis, and multinucleation, were observed in six (20.7%) cases (Fig. 1). ISH analysis was performed to clarify the localization of HPV DNA in the urothelium. Only a limited number of samples could be evaluated, because the cells were lost from the slides during the antigen activation process. Only seven (36.8%) HPV-positive and three (30%) HPV-negative samples could be evaluated. However, HPV DNA signals were successfully observed in the nuclei of either squamous cells or transitional cells in three HPV-positive samples (Fig. 2), while no such signals were seen in the HPV-negative samples. These findings suggested that HPV can infect both squamous cells and urothelial cells in urethritis patients.

***Mycoplasma* spp. and *Ureaplasma* spp. as causes of urethritis.** *M. genitalium*, *M. hominis*, *U. parvum*, and *U. urealyticum* were detected in 14.5%, 10.9%, 6.5%, and 12.3% of men with urethritis, whereas the species were detected in 3.3%, 2.0%, 4.7%, and 2.7% of controls, respectively (Table 1). *M. genitalium*, *M. hominis*, *U. parvum*, and *U. urealyticum* were detected in 14.2%, 12.2%, 9.2%, and 11.2% of patients with NGNCU, respectively (Table 3). The prevalence rates of *M. genitalium*, *M. hominis*, and *U. urealyticum* in men with NGNCU were significantly higher than in the control group ($P < 0.05$), suggesting that these microorganisms may cause urethritis independently. It was noted that HPV was detected in 47.0% of GU but in 13.0% of NGCU and in 18.4% of NGNCU patients. The prevalence of HPV in GU patients was significantly higher than in NGCU or NGNCU patients ($P < 0.05$) (Table 3).

DISCUSSION

The present study demonstrated that LB-URC is a promising method for molecular analysis of microorganisms in the urinary tract. The results of the present study indicated that a high positive rate of β-globin could be obtained in LB-URC samples from men both with and without urethritis. Some previous studies have demonstrated failure of amplification of DNA extracted from urine sediment cells by PCR. Giuliano et al reported that the β-globin gene was detected in 51.3% of urine samples collected from 226 healthy men and showed a low prevalence of HPV infection (0.8%) in analysis of adequate samples positive for the β-globin gene (10). A systematic review showed that the HPV detection rate from urine samples was less than 7%, although higher HPV-positive rates were observed in samples scraped from the male external genitalia, suggesting that urine may not be suit-

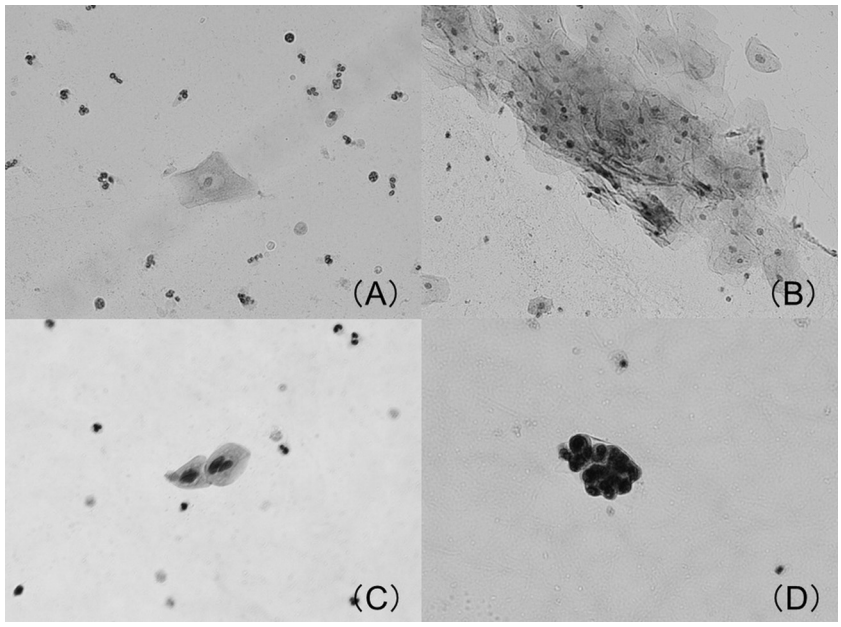

FIG 1 Cytological findings of HPV-positive samples. (A) Koilocytosis. (B) Dyskeratocytosis. (C) Binucleate cells. (D) Mildly atypical transitional cells.

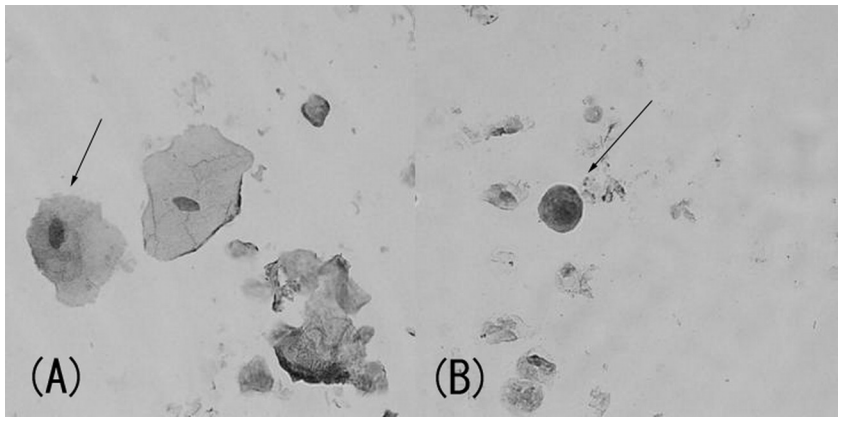

FIG 2 *In situ* hybridization findings of high-risk HPV-positive samples. (A) HPV DNA signals were predominantly observed in the nuclei of squamous cells. (B) HPV DNA signals were also observed in urothelial cells.

able for detection of HPV (9). Our study also demonstrated a lower detection rate of β-globin in urine sediment samples (22). The β-globin gene was detected in only 65% of the urine sediment samples but in 89% of scraped cell samples from the penis or in 93% of those from the urethra (22).

LBC is widely used for uterine cervical cancer screening in women, as cell morphology is well preserved in LBC for a long time (2). Furthermore, cell quantity on the slides can be manipulated for LBC samples in the laboratory. LBC generally has good sensitivity for diagnosis of high-grade cervical intraepithelial neoplasia or squamous cell carcinoma in the screening of cervical cancer (13, 17), although its use in such screening is still controversial (5). The most useful application for LBC is analysis of various molecular markers. LBC specimens are used for immunohistochemical analysis of expression of some proteins or genes and for gene detection of various microorganisms as diagnostic aids or for identifying causative agents. LBC is not commonly used for urinary tract cytology. Piaton et al compared the conventional and LBC tests (ThinPrep processing) in urine cytopathology and concluded that conventional methods remained appropriate for urinary cytology testing but that LBC was more suitable for cytology-based molecular studies (18). PCR analysis is most commonly used for various laboratory tests due to its high sensitivity and ease of performance in any laboratory. However, the β-globin detection rate is low in urine samples compared with rubbed cell samples in PCR analysis. Urine samples may contain some inhibitors of PCR amplification, such as salts, minerals, or other as-yet-unknown inhibitors (4). Therefore, some additional procedures are necessary to remove such substances from DNA samples from urine sediment in PCR-based analysis. In the present study, only cellular pellets of urine samples were preserved in the solution when liquid-based cytology was used immediately after collecting the samples from the patients, which may have contributed to the removal of salts and minerals from the solvent and to good preservation of the urinary cellular pellet.

Cell numbers in urine sediment are generally limited in comparison with scraped cell samples collected from the urinary tract. An additional advantage of LB-URC is that cell quantity can be manipulated to the required levels in the laboratory. However, cell numbers were not sufficient for Papanicolaou testing and ISH analysis in the present study. Samples of only 15 ml of urine were used for the analysis. Although further trials are needed, it may be possible to overcome this issue by collection of larger volumes of urine samples.

HPV prevalence in urine samples from urethritis patients was high in the present study, whereas it was significant lower (3.4%) in controls, which is consistent with results reported previously (9, 10). The precise reason for the high prevalence of HPV infection in the urinary tract among men with urethritis is not yet clear. A possible explanation is that inflammation of the urinary tract may induce cell exfoliation, which leads to easier detection of asymptomatic HPV infection (11). Alternatively, urethritis itself may increase the risk of HPV infection in the urinary tract. HPV infection requires the availability of a basal layer cell and usually occurs in microlesions of skin or mucosa (30). Thus, microinjury to the urothelium caused by urethritis may allow HPV to access the basal cells of the urothelium, which is necessary for the establishment of HPV infection. Indeed, it was reported previously that *N. gonorrhoeae* infection was an independent risk factor for HPV infection in men with urethritis (25). In particular, gonococcal urethritis can induce severe inflammation in the urethra. We also found that the prevalence of HPV was significantly higher in patients with GU who had more severe inflammation in the urethra than in those with urethritis caused by other microorganisms, such as *C. trachomatis*, *Mycoplasma* spp., and *Ureaplasma* spp. Another possibility is that men with urethritis are generally a high-risk group for STIs, including HPV infection (16).

With regard to the distribution of HPV types, high-risk genotypes were detected in 67% of HPV-positive samples, and the

TABLE 3 Comparison of HPV, *Mycoplasma* spp., and *Ureaplasma* spp. detection by urethritis type[a]

Sample category	No. (%) of samples			
	GU	NGCU	NGNCU	Control
Adequate	17	23	98	150
HPV positive	8 (47.0)	3 (13.0)	18 (18.4)	5 (3.3)
M. genitalium positive	1 (5.9)	5 (21.7)	14 (14.2)	5 (3.3)
M. hominis positive	1 (5.9)	2 (8.7)	12 (12.2)	3 (2.0)
U. parvum positive	0	0	9 (9.2)	7 (4.7)
U. urealyticum positive	1 (5.9)	5 (21.7)	11 (11.2)	4 (2.7)

[a] HPV, human papillomavirus; GU, gonococcal urethritis; NGCU, nongonococcal chlamydial urethritis; NGNCU, nongonococcal and nonchlamydial urethritis.

most common genotype was type 16 (20.0%), followed by type 58 (13.3%), which is consistent with our previous studies regarding HPV prevalence in the penis and urethra of men with urethritis (22). Both HPV 52 and HPV 58 are common HPV types among young Japanese women, and the distribution of HPV types detected in the urine of men with urethritis was approximately similar to that in the uterine cervix of Japanese women (3). HPV types 70, 72, 84, and 91 were also detected in samples from the Japanese female cervix as unusual HPV types, and unknown HPV types that might have been classified as undetermined in the present study may have included these HPV types (3, 20).

M. genitalium, *M. hominis*, *U. urealyticum*, and *U. parvum* have been detected in patients with urethritis. *M. genitalium* and *U. urealyticum* are thought to be especially important pathogens of nongonococcal urethritis (NGU) (6, 7). On the other hand, *U. parvum* and *M. hominis* are less common pathogens for male urethritis, although these microorganisms have been reported as important antecedents of preterm birth in women (1, 8). In the present study, the prevalence rates of *M. genitalium*, *M. hominis*, and *U. urealyticum* were significantly higher in urethritis patients than in controls and were higher in NGNCU patients, suggesting that species of bacteria other than *U. parvum* are likely to be the causative agents of NGNCU. Deguchi and Maeda reported that *M. genitalium* and *U. urealyticum* were significantly more prevalent in patients with NGNCU compared to healthy men in Japan based on analysis of first-voided urine specimens (6). Previous reports also suggested that the prevalence rates of *M. genitalium* and *U urealyticum* in men with NGNCU were significantly higher than those in men without urethritis, whereas there were no significant differences in the prevalence rates of *M. hominis* and *U. parvum* between the two groups (6, 7). However, the present results showed that the prevalence of *M. hominis* was also significantly higher in men with urethritis than in controls. One possible reason for this discrepancy in the results is that we recovered urothelial cells from the urinary bladder, which may be infected with HPV, by collecting midstream urine specimens (24). In fact, a previous study demonstrated that *M. hominis* was detected in 57.2% of the bladder mucosa specimens obtained from 60 patients with leukoplakia and concluded that *M. hominis* could frequently infect the bladder mucosa (15). The numbers of subjects in this study were too small to allow us to draw definitive conclusions regarding the causative role of *M. hominis*, and further studies are required to investigate this hypothesis.

It is important to determine whether HPV plays a causative role in the development of tumors in the urinary tract. Therefore, we performed ISH analysis for HPV DNA by the use of LB-URC samples to confirm the localization of high-risk HPV DNA in the urothelial epithelium. Of 19 high-risk HPV-positive samples, high-risk HPV DNA signals were successfully observed in some squamous cells (Fig. 2A) and transitional cells (Fig. 2B) in three HPV-positive cases. These observations suggest that HPV can infect both squamous and transitional cells in the urinary tract, including the urethra and urinary bladder. Papanicolaou test results also demonstrated that some morphological changes of cells related to HPV infection occurred in 20.7% of the HPV-positive samples, suggesting that HPV could replicate in some urothelial cells. Furthermore, we reported previously that HPV is associated with the development of inverted papilloma and low-grade cancers in the bladder (23, 24). Further studies to clarify the natural history of urinary HPV infection are needed to investigate the associations between HPV infection and the development of various tumors of the urinary tract.

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