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Ureaplasma urealyticum Serotypes in Urinary Tract Disease

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Ureaplasma urealyticum **cultures from 124 patients with urinary tract disease were serotyped by indirect immunofluorescence, using antisera to serotypes I to VIII. A similar range of serotypes was recovered from first-voided, midstream, and bladder-aspiration (SPA) urine, upper urinary tract samples, and vaginal swabs. Serotype VI was predominant (44/124) among the samples, whereas serotypes V (1/124 samples) and VII (0/124 samples) were uncommon. Twenty of 124 cultures contained more than one serotype, and three cultures were untypeable. Serotypes cultured from bladder urine were also present in vaginal and urethral samples, although these samples often carried additional serotypes. Consecutive SPA samples from the same patient invariably contained the same serotype, whereas some consecutive midstream urine samples showed a loss or gain of serotypes with time. One patient carried the same serotype in SPA urine over a period of 13 months. The pattern of serotypes recovered from the urinary tract was similar irrespective of the sampling site, the site of infection, the clinical diagnosis and renal function of the patient, and the presence or absence of other microorganisms. Colonization above the urethra and association with urinary tract disease appeared to be serotype independent.**

A pathogenic role for *Ureaplasma urealyticum* has been postulated in a range of diseases of the human genitourinary tract (30). With the exception of nongonococcal urethritis (29), however, a causal relationship between ureaplasmal colonization and urogenital disease has not been convincingly demonstrated. One difficulty has been the finding that *U. urealyticum* is present as part of the vaginal and urethral flora of asymptomatic individuals (14, 15), and this has prompted the suggestion that only a proportion of ureaplasmal strains are pathogenic.

The recognition of 14 serotypes within the species (24) has led to attempts to link ureaplasmal pathogenicity to particular serotypes. A wide range of serotypes has been recovered from the urogenital tracts of patients and from healthy controls (3, 8, 9, 11, 16–18, 28, 34, 35). Despite a single report claiming an association between serotype IV and "disease of the genito-urinary tract" (27), no simple relationship has been found.

Ureaplasmal colonization is dependent on sexual activity (14, 15), giving rise to the possibility of multiple infection. As a result, cultures often contain a mixture of serotypes, and repeated samplings at short intervals from a single individual may contain different serotypes (35).

U. urealyticum has been isolated from the bladder and upper urinary tract (1, 4, 13, 31–33), sites from which genital and urethral contamination can be rigorously excluded and which may be less susceptible to repeated exposure resulting from sexual contact. Ureaplasmas have been recovered more often from patients with reflux nephropathy than from patients with other renal diseases (1), which suggests that ureaplasmal colonization contributes to progressive renal disease.

In this study, isolates of *U. urealyticum* from the bladder and upper urinary tract were serotyped to determine whether particular serotypes were associated with infection at different levels within the urinary tract or particular urinary tract diseases, with emphasis on reflux nephropathy.

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MATERIALS AND METHODS

Patient selection. Midstream (MS) urine or bladder-aspiration (SPA) urine yielding ureaplasmas was obtained from 112 patients (105 female, 7 male) referred for investigation of a range of urinary tract diseases. Samples of ureaplasma-positive urine or tissue were obtained from the kidneys of two patients. In one of these, urine was collected by direct aspiration from the renal pelvis, and in the second patient, culture of tissue obtained for routine renal biopsy yielded a growth of ureaplasmas.

Level of colonization of urinary tract. Patients with a ureaplasmal count of $>10^3$ CFU/ml in bladder urine were recalled for a localization test (1) to determine the site of ureaplasmal colonization in the urinary tract.

Ureaplasmal agar. Ureaplasma differential basal agar medium, A7 (GIBCO Laboratories), with 20% horse serum (not inactivated), 0.1% urea, 0.25% yeast extract, 0.5% Vitox, 0.01% cysteine hydrochloride, 0.002% phenol red, 20 μg of flucloxacillin per ml, 0.5 mg of carbenicillin per ml, and 40 μg of ampicillin per ml was used for ureaplasmal culture.

Ureaplasmal broth. Ureaplasmal broth was prepared as described by Birch et al. (1).

Ureaplasmal culture. Volumes (0.02 ml) of urine and 10^{-2} saline dilutions of urine were placed on ureaplasmal agar without mechanical spreading and incubated anaerobically for 48 h. Ureaplasmal counts were expressed as CFU/ml.

For broth cultures, 0.5 ml of urine was added to 5 ml of ureaplasmal broth and incubated. Broths showing a color change were subcultured onto ureaplasmal agar in 10-fold saline dilutions.

Aerobic and anaerobic culture. The methods of Birch et al. (1) were followed for aerobic and anaerobic culture of urine.

Microbial identification. *U. urealyticum* was identified by its colony size and morphology, urease production, and oxidation of manganese salt on ureaplasmal agar. *Mycoplasma hominis* was identified by colony size and morphology, failure to hydrolyze urea or oxidize manganese salt on ureaplasmal agar, and arginine hydrolysis. Other microbial species were identified by standard microbiological methods (6).

TABLE 1. Numbers of patients harboring different serotypes and the site of recovery within the genitourinary tract

Sample source	Serotype recovery	No. of patients	No. of patients with the following ureaplasmal serotype:							
			I	II	III	IV	V	VI	VII	VIII
Upper urinary tract	Single serotype	3			1	1				1
	Mixture of 2 serotypes	0								
	Mixture of >2 serotypes	0								
	Untypeable	0								
Bladder (SPA urine)	Single serotype	57	7	9	11	7	0	18	0	5
	Mixture of 2 serotypes	11	7	0	1	2	1	9	0	2
	Mixture of >2 serotypes	0								
	Untypeable	3								
MS urine	Single serotype	34	6	3	7	4	0	12	0	2
	Mixture of 2 serotypes	4	2	0	0	3	0	2	0	1
	Mixture of >2 serotypes	3								
	Untypeable	0								
VB1 urine	Single serotype	4	0	3	0	0	0	1	0	0
	Mixture of 2 serotypes	1	1	0	0	1	0	0	0	0
	Mixture of >2 serotypes	0								
	Untypeable	0								
Vaginal (vaginal swab)	Single serotype	3	1	1	0	0	0	1	0	0
	Mixture of 2 serotypes	1	1	0	0	0	0	1	0	0
	Mixture of >2 serotypes	0								
	Untypeable	0								

Standard ureaplasmal strains. Strains of *U. urealyticum* serotypes I to VIII (ATCC strains 27813 to 27819 and 27618) were obtained from the American Type Culture Collection, Rockville, Md.

Preparation of antisera. For the initial experiments, rabbit antisera to *U. urealyticum* serotypes I to VIII were supplied by M. C. Shepard (Camp Lejeune, N.C.). When the American Type Culture Collection *U. urealyticum* strains were obtained, rabbit antisera to serotypes I to VIII were prepared by the method of Shepard and Lunceford (27).

Indirect immunofluorescence. *U. urealyticum* isolates were serotyped by indirect immunofluorescence of unfixed colonies on agar by the method of Rosendal and Black (25) with modifications.

For ureaplasmal counts of 50 to 10^4 CFU/ml in urine, the primary isolates on ureaplasmal agar were serotyped. If the primary plate failed to yield ureaplasmal growth (<50 CFU/ml) but the ureaplasmal broth culture showed a color change, serotyping was done on the first agar subculture from the broth.

Agar blocks carrying ureaplasmal colonies were glued to glass slides with clear nail varnish, with up to four blocks on each slide. The slide surface was scratched with a diamond pencil to improve adherence. Blocks were embedded in large drops of wet varnish on the slide and dried in air for at least 30 min. Washing and incubations were done in glass Coplin jars. Immobilization of blocks prevented damage and inversion during washing and allowed for economy of reagents. Each block was incubated with 5 μl of rabbit antiserum for 30 min, washed twice in phosphate-buffered saline (0.01 M [pH 7.2]), and incubated with 5 μl of goat anti-rabbit immunoglobulins, fluorescein conjugated (GAR/FITC; Nordic Immunological Laboratories), for 30 min. Blocks were then rinsed in phosphate-buffered saline for 24 h. For each test, two blocks were incubated with normal rabbit serum and 0.9% saline as controls.

The optimal dilutions for rabbit antisera and conjugated antiserum were determined by titration in the homologous and heterologous reactions, using the American Type Culture Collection strains. Dilutions were chosen which gave strong fluorescence in the homologous reaction with minimal fluorescence in the heterologous reactions and fell within the range of 1/100 to 1/200.

Fluorescence microscopy. Colony fluorescence was studied by using a Leitz Dialux microscope with a fluorescence attachment (magnification, ×100). The intensity of fluorescence was assessed on the scale of Rosendal and Black (25).

RESULTS

Serotype frequency and site of recovery. Ureaplasmas were recovered from 3 upper urinary tract samples (2 direct renal samples and 1 postwashout urine from a localization test), 71 SPA urines, 41 MS urines, 5 samples of the first 10 ml of voided urine (VB1), and 4 vaginal swabs from different patients. The serotypes detected in these samples are shown in Table 1.

A single serotype was recovered from 101 (81%) patients, and a mixture of two serotypes was recovered from 17 (14%) patients. Cultures from three (2%) patients showed no fluorescence with any of the antisera used. They were classed as untypeable and may have belonged to one of the serotypes IX to XIV. Cultures from three patients showed strong fluorescence with several antisera. These were classed as mixtures of more than two serotypes. Several clones were obtained from each of eight cultures, which had been classified as mixtures of two or more serotypes. Each clone was tested and belonged to a single serotype. Of 48 clones obtained from cultures for other purposes, 46 were identified as single serotypes, and 2 gave strong fluorescence with two antisera, I and VI.

A range of serotypes was recovered from each of the sampling sources. Serotype VI was most frequently recovered, occurring in 44 of 124 patients (35%), and serotypes V (1 patient) and VII (no patients) were uncommon. The most

TABLE 2. Ureaplasmal serotypes recovered from vaginal, urethral, and bladder samples from three patients

Patient	Time (mo)	Serotypes recovered from			
		Vagina (vaginal swab)	Urethra (VB1)	Bladder (SPA urine)	Urethra of sexual partner
A	0	I, VI	VI	NT[a]	NT
	1	VI	VI	VI	
	2	I, VI	VI	VI	
B	0	II	II	II	II
	1	II	II	II	II
	3	II, IV	II	—[b]	II
C	0	I	I, IV	—	NT
	1	I	Mixture of >2 serotypes	—	
	3	I, VI	I, VI	—	

[a] NT, Not tested.
[b] —, No ureaplasmas recovered.

common two-serotype mixture was I:VI, occurring in 8 of 17 patients. Two of the I:VI mixtures were separated by cloning into pure serotype I and VI isolates. There was no significant difference in the frequencies of single serotypes or serotype mixtures obtained from SPA and MS urines (chi-square test, Yates correction) or in the distribution of serotypes recovered (Friedman two-way analysis of variance). Patient numbers in other specimen categories were too low for statistical analysis.

The serotypes recovered from vaginal, urethral, and SPA samples from three patients over a 3-month period are shown in Table 2. The patients received no antibiotic treatment during this period. Patient A harbored serotype VI throughout the test period, and on two occasions serotype I was also recovered from vaginal swabs. Similarly, patient B yielded serotype II from 8 of 9 samplings, and on one occasion serotype IV was also present in the vagina. Serotype II was also recovered from the urethra of her sexual partner. Vaginal and urethral samples from patient C consistently yielded serotype I, and in addition other serotypes were recovered intermittently. Throughout the study period, however, ureaplasmas were not detected in the bladder.

Persistence of serotypes. The serotypes recovered from initial and subsequent samplings from four patients with ureaplasma-positive SPA urine and 13 patients with ureaplasma-positive MS urine are shown in Table 3.

In patients giving SPA urine, the serotype recovered on initial testing was also present in subsequent samples. Two of these patients received antibiotic treatment which resulted in failure to recover ureaplasmas from SPA urine. However, ureaplasmas of the original serotype were later reisolated. The longest period over which consecutive samplings yielded ureaplasmas of the same serotype was 5 months (patient A).

Three of 13 patients with ureaplasma-positive MS urine received effective antibiotic treatment after which ureaplasmas could not be recovered from the urine. When ureaplasmas were later isolated from these patients, two carried a serotype different from that in the initial sample, and one carried the same serotype.

In patients receiving no antibiotic treatment, some consecutive MS urine samplings carried different serotypes (patients I, J, K, M, N, and L), and some showed a gain of additional serotypes with time (I, Q, and R) or a loss of serotypes (I). Two patients (O and P) retained the serotype detected in their initial samples. The longest period over which the same serotype could be recovered from consecutive samples was three months (patient Q).

Serotype frequency and site of infection. Twenty patients with ureaplasmal counts of $>10^3$ CFU/ml in SPA urine underwent localization tests to determine the site of colonization in the urinary tract. In six patients, the results were equivocal due to low ureaplasmal counts in the bladder

TABLE 3. Serotypes recovered from multiple urine samples collected from 17 patients[a]

Sample	Patient	Serotype recovered at the following time after initial sample (mo)																			
		0	1	2	3	4	5	6	7	8	9	10	11	13	14	17	18	19	20	21	23
SPA urine	A	VI	VI	VI	VI[b]	VI	VI	—[b]	VI	—	VI	VI	—	VI							
	B	II	—[c]					II	II												
	D	VI		VI																	
	E	I, VI	I																		
MS urine	F	VI	—[d]							—						—	—	—		—	I
	G	VIII	—[d]	—[d]	—[e]	—[d]	—[d]	—[e]	—[e]	—[e]	—[e]	—[e]	—[d]	—[d]	—[d]	—			IV	II	
	H	III	—[d]	III																	
	I	III	—				III, IV	>2[f]	>2	>2		U	II								
	J	III				II															
	K	III		U																	
	L	III						I													
	M	IV			VI																
	N	IV					VI		U												
	O	II	II																		
	P	I		I																	
	Q	VI			I, VI																
	R	VI	>2																		

[a] Symbols: —, no ureaplasmas recovered; >2, >2 serotypes recovered; U, untypeable.
[b] Patient was receiving antibiotic treatment (amphotericin [topical]).
[c] Patient was receiving antibiotic treatment (rosaramicin).
[d] Patient was receiving antibiotic treatment (erythromycin).
[e] Patient was receiving antibiotic treatment (erythromycin), but sample was not taken.
[f] Patient was receiving antibiotic treatment (doxycycline).

TABLE 4. Numbers of patients harboring different serotypes in bladder urine and their clinical diagnoses

Major diagnosis	No. of patients	No. of patients with the following ureaplasmal serotypes[a]									
		I	II	III	IV	V	VI	VII	VIII	Untypeable	>2 Serotypes
Chronic atrophic pyelonephritis (reflux nephropathy)	61	13	4	14	8	1	20	0	6	1	2
Glomerulonephritis (biopsy proven)	15	3	2	1	1	0	7	0	0	1	1
Recurrent urinary tract infection	15	4	4	2	1	0	5	0	0	1	0
Renal papillary necrosis (analgesic nephropathy)	2	1	0	0	1	0	1	0	0	0	0
Renal calculi	2	0	0	1	1	0	0	0	0	0	0
Polycystic kidney disease	5	0	0	0	1	0	3	0	1	0	0
Miscellaneous renal disease	12	1	2	1	3	0	5	0	3	0	0

[a] Data are total numbers of patients harboring each serotype, either alone or as part of a mixture of serotypes.

urine. Eight of the remaining 14 patients were judged to have upper tract infection. A similar distribution of serotypes was recovered from patients with bladder and upper urinary tract infections.

Serotype frequency in different patient groups. The serotypes recovered from different patient groups classified according to their major clinical diagnoses are shown in Table 4. The table includes results obtained from both SPA and MS urine.

There was no significant difference between the serotype distribution in any patient group when compared with the other six groups (Spearman's rank correlation coefficient). Serotype VI was the predominant serotype recovered in each group. A similar range of serotypes was recovered from patients with impaired renal function (plasma creatinine >0.11 mmol/liter) and those with normal function.

Serotype frequency and recovery of other microorganisms. Eighty-four of 148 ureaplasma-positive bladder urine samples yielded ureaplasmas alone, and 64 of 148 gave a growth of other microorganisms. These showed similar patterns of serotype recoveries, with serotype VI the predominant serotype recovered in both groups. Of the 64 samples with mixed growth, 4 yielded *Streptococcus* species, 5 yielded *Staphylococcus* species, 7 yielded *Escherichia coli*, 1 yielded *Proteus mirabilis*, 15 yielded *Gardnerella vaginalis*, 1 yielded *Corynebacterium* species, 12 yielded *M. hominis*, and 19 yielded a mixture of two or more of these microorganisms.

DISCUSSION

The difficulties involved in serotyping ureaplasmas from the human urogenital tract were not fully appreciated when such studies were first undertaken. Consequently, many reports should be interpreted with caution, taking into consideration the serotyping method used, the sampling sources, the specimen collection procedures, and the numbers of patients tested and their clinical characterization.

Growth inhibition (2), metabolic inhibition (19), and mycoplasmacidal (11) and enzyme-linked immunosorbent assay serotyping tests (34) are performed with broth cultures. When cultures are cloned before serotyping, mixtures may not be detected. If, however, samples containing multiple serotypes are directly tested by these methods, predominant or faster-growing strains may overgrow and mask other reactions in the tests (28). Indirect immunofluorescence is a more sensitive test than growth inhibition, resulting in fewer untypeable cultures (18). It was chosen for this study because it can be performed on primary culture plates, thereby identifying both the serotypes present and their relative numbers in the sample.

Cultures showing strong fluorescence with several antisera were classed as mixtures of more than two serotypes. Colonies on individual blocks showed a range of fluorescence intensities, which we interpreted as a mixture of homologous reactions and cross-reactions. Because cloned isolates from 8 serotype mixtures and from 46 other cultures were identified as single serotypes, intertypes were not thought to be a major factor in our study. However, two clones that gave strong fluorescence with both antisera I and VI may indicate an intertype, although not one of those noted by Stemke and Robertson (28). It is suggestive that I:VI was the major two-serotype mixture, with an intertype possibly accounting for 6 of 8 of these.

In most serotyping studies to date, ureaplasmas were recovered from vaginal or urethral samples. Because there is a high probability of multiple colonization at these sites, serotypes of clinical interest cannot be identified with certainty. This problem also exists with urine samples, which may be contaminated with genital or urethral flora during voiding. Similarly, the use of closed-end catheters may introduce urethral organisms into the bladder (26). However, by using VB1 urine, SPA urine, and upper urinary tract samples, it was possible to investigate ureaplasmas from defined levels of the urinary tract.

Our study indicated that a similar range of serotypes colonizes each level within the urinary tract. In three patients, the same serotype was recovered from bladder, urethral, and vaginal samples collected simultaneously. Vaginal and urethral samples, however, were more likely to carry additional serotypes. Not all urethral organisms ascended to the bladder, although the selection did not appear to be serotype related. Identical serotypes were recovered from one patient and her sexual partner. Sharing of serotypes between marital partners occurs because urethral and vaginal colonization depends on sexual activity (14, 15).

In patients who gave SPA urine, consecutive ureaplasma-positive samples invariably carried the same serotype. In contrast, consecutive MS urine samples frequently showed a loss or gain or both of serotypes with time. The inconstancy of serotypes in MS urine samples probably reflects the addition of bacteria from the urethra during collection. Viarengo et al. (35) have demonstrated serotype inconstancy in VB1 urine samples collected over 12 weeks from healthy men. Only four of eight men carried a common serotype in every sample tested, and six of eight showed a change of serotype in consecutive samples.

In the present study, one patient (A) carried the same serotype in consecutive SPA urine samples for 5 months and, intermittently, for a further 8 months. It has previously been reported that individuals may harbor ureaplasmas in SPA urine for up to 18 months (1). However, isolates were not serotyped, and it was not possible to determine whether long-term carriage was due to persistence of a particular strain. Ureaplasmas have been recovered from meatal swabs collected over 13 months (12) and 2.5 years (10) from Antarctic base personnel, indicating that chronic urethral infection may occur without the need for reinfection by sexual contact.

Treatment of our patients with antibiotics usually resulted in failure to recover ureaplasmas from the urine. In all such patients, ureaplasmas were reisolated, and this may have been due to inadequate clearance or to reinfection. MacLeod et al. (12) demonstrated that, after adequate antibiotic treatment of Antarctic base personnel, urethral infection recurred only after further sexual contact. This suggests that any serotype recovered after treatment should be that carried by the sexual partner, and this was confirmed with our patient B.

Among patients with renal disease, ureaplasmas are recovered more frequently from those with reflux nephropathy and impaired renal function than from other patient groups (1). However, neither reflux nephropathy nor impaired renal function was associated with increased recovery of any particular serotype in our study. Serotype VI was most frequently recovered from our patient group. Because comparable samples from healthy individuals were not available to us during the study period, we cannot exclude the possibility that the range of serotypes recovered from these patient groups is similar to that occurring in the general population in Victoria. Our three most common serotypes, I, III, and VI, were those which are not inhibited by the manganese in ureaplasmal agar (23), and this may have affected the serotype frequencies obtained. It is of interest to note that serotypes I, III, and VI form one of the two genetic clusters of *U. urealyticum* (5, 22).

Previous serotyping studies have not dealt specifically with urinary tract disease. However, a range of serotypes has been recovered from several patient groups and from healthy controls (3, 8, 9, 11, 16–18, 28, 34, 35). In other studies, the predominant serotypes have been III (3, 9, 34), II (17), VI (16), and XIII (28), which may indicate geographical variations in serotype frequencies.

Shepard and Lunceford (27) found that serotype IV was predominant in several patient groups, including those with nongonococcal urethritis. However, assessment of the proposed serotype-disease association was difficult because of the variety of clinical conditions associated with serotype IV and the imprecise definition of clinical categories. In contrast, Stemke and Robertson found no association between nongonococcal urethritis and serotype IV (28). Elevated mean antibody titers have been demonstrated to serotypes IV and VIII in mothers with pregnancy loss, to serotypes VI and VIII in their infants, and to serotypes IV, VII, and VIII in neonates with respiratory disease, suggesting that serotypes IV, VI, VII, and VIII are pathogenic (20, 21).

This study showed that the pattern of serotypes recovered from the urinary tract was independent of the sampling site, the site of infection, the clinical diagnosis and renal function of the patient, and the presence or absence of other microorganisms. It has been difficult to reconcile the association of ureaplasmas with several urogenital diseases (30) and their ability to cause nongonococcal urethritis (29), with their presence in asymptomatic individuals. The existence of virulent and avirulent strains has been proposed to account for this anomaly. Differences in the persistence of human strains after intrarenal inoculation into mice (7) and in sensitivity to antisera in the growth inhibition test (27) have been suggested as criteria for classifying ureaplasmas according to their virulence. Whereas pathogenic and nonpathogenic *U. urealyticum* strains may occur in the human urinary tract, colonization above the urethra and association with urinary tract disease do not appear to be serotype related.

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