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Serological Typing of *Ureaplasma urealyticum* Isolates from Urethritis Patients by an Agar Growth Inhibition Method

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An agar growth inhibition method for serotyping *Ureaplasma urealyticum* is described, and the results of applying this method to serotyping 338 strains of the organism are presented. The serotyped strains consisted of cloned isolates from male patients with primary and recurrent nongonococcal urethritis (NGU), isolates from symptomatic patients with other genitourinary tract infections and disorders, and isolates from asymptomatic carriers of *U. urealyticum* in the genitourinary tract (controls). Among 122 male patients with NGU, serotype 4 was associated most frequently (52%) with this disease at Camp Lejeune, N.C. Seventeen percent of the isolates were type 2. The remaining isolates consisted of types 1, 3, 6, and 8 and accounted for 6 to 9% each of the serotypes isolated from the NGU group. Types 5 and 7 were not isolated. Among 91 symptomatic patients with other genitourinary tract infections and disorders, *U. urealyticum* type 4 also was associated most frequently (37%) with these disorders. The remaining isolates, represented by types 1, 2, 3, 6, 7, and 8, accounted for 9 to 15% each of the types isolated from this group. Type 5 was not isolated. Among 125 symptom-free carriers of *U. urealyticum* in the genitourinary tract, type 8 was recovered most frequently (30%), whereas type 4 was isolated next most frequently (24%). The remaining isolates consisted of types 1, 2, 3, 5, and 6 and accounted for 2 to 15% each in this asymptomatic control group. Type 7 was not isolated. Of the present eight serotypes of *U. urealyticum* studied in this investigation, type 4 was associated most frequently with disease (NGU) and certain other disorders of the genitourinary tract at Camp Lejeune. A previously unknown association of *U. urealyticum* with frequently abacteriuric, unexplained pyuria (with or without urethral pruritis and dysuria) is reported, suggesting the existence of asymptomatic *Ureaplasma* urethritis.

Human isolates of *Ureaplasma urealyticum* (23) (T-mycoplasmas) are antigenically distinct from all other recognized species of *Mycoplasmatales* and represent a serologically hetrogeneous group. The existence of antigenic differences among strains of *U. urealyticum* was first reported over a decade ago (D. K. Ford, Arthritis Rheum. **9**:503-504, 1966). Using the metabolism inhibition test of Purcell et al. (15), Ford initially identified four distinct serotypes of *U. urealyticum* isolated from 35 male patients with nongonococcal urethritis (NGU) and Reiter's syndrome. Two additional serotypes were identified the following year (8), and a seventh serotype (strain 58) was subsequently demonstrated (D. K. Ford, personal communication).

Ford's seven serotypes of *U. urealyticum* were used in a comparative serological study by Black (2), who utilized four different serological methods: a growth inhibition test, an indirect immunofluorescent test, an indirect hemagglutination test, and a metabolism inhibition test. The existence in 1970 of at least seven different serotypes of *U. urealyticum* was confirmed by Black, and on the basis of his comparative studies he proposed a serological classification of the human ureaplasmas, consisting of serotypes I through VII (2). Shepard's *U. urealyticum* strain 960 (19, 23), cloned three times in Purcell's laboratory and five additional times in Black's laboratory in 1972, was designated the type strain of the species: *U. urealyticum* strain 960-(CX8), ATCC 27618 (23).

Lin et al. (12) studied the serological characteristics of 47 isolates of *U. urealyticum*, using a complement-dependent mycoplasmacidal test (11). Antisera were prepared against 14 strains of *U. urealyticum* that had been isolated from the genital tracts of pregnant women and from infants. Cross-reactivity of these antisera with the 47 original strains (including the immunizing strains) showed the presence of 5 serogroups on

the basis of shared common antigens and of 11 serotypes among these groups. The precise antigenic relationships among Lin's 11 serotypes of *U. urealyticum* (12) and the 8 serotypes proposed by Black (2) are unclear and remain to be clarified.

The growth inhibition test originally used by Black (2) was further modified by him (3) and applied to the serotyping of 24 *U. urealyticum* strains isolated from various sources. Ten of these strains were isolated from the urethras of male patients with NGU. Subsequently, Black and Krogsgaard-Jensen (4) described an indirect immunofluorescence technique for the identification and classification of *U. urealyticum*, using unfixed agar colonies, and compared this method (and others) with the agar growth inhibition test (3). The indirect immunofluorescence technique was found to possess the same degree of specificity as the agar growth inhibition test. If the growth inhibition test was performed at an incubation temperature of 37°C (unlike a temperature of 27°C formerly recommended by Black [3]), it proved to be the most type specific of the various procedures used (4).

We report a further modification of Black's growth inhibition test (3) and the application of this new modification to serotyping strains of *U. urealyticum* isolated from male patients with primary and recurrent NGU, patients with genitourinary tract infections and other disorders, and asymptomatic carriers of *U. urealyticum* in the genitourinary tract.

MATERIALS AND METHODS

Organisms. A total of 338 cultures of *U. urealyticum* was used in this study. The orgainsms were obtained from the following sources: 122 strains from the genitourinary tract of male patients with primary and recurrent NGU; 91 strains from both males and females with a variety of symptomatic genitourinary conditions (primarily nonurethritis); and 125 strains (controls) from the genitourinary tract of asymptomatic carriers of *U. urealyticum*. The controls included 74 strains isolated from urine specimens from Marine Corps personnel undergoing final physical examinations before release from service, 41 strains isolated form Marine Corps personnel in an infantry training regiment whose cultures had been stored in the frozen state at −85°C for 1 to 3 years, and 10 strains isolated from urine specimens collected from a healthy group of children less than 10 years of age at a pediatric clinic. Cultures from all groups were identified as *U. urealyticum* by means of previously published descriptions (9, 16, 18, 20–23), and all cultures were cloned three times by the cloning method described below.

Culture media. (i) Standard fluid medium 10-B. Standard fluid medium 10-B was used for the general cultivation strains of *U. urealyticum* and for the cloning of freshly isolated strains from clinical material. Medium 10-B basal medium was first prepared and contained: PPLO broth without crystal violet (Difco, no. 0554), 1.47 g; deionized distilled water, 73 ml. The broth powder was completely dissolved, and the reaction of the basal broth was adjusted to pH 5.5 with 2 N HCl. Sterilization was accomplished by autoclaving at 121°C for 15 min. The basal broth (70 ml) was cooled to 37°C and converted to complete standard fluid medium 10-B by addition of the following sterile supplements: unheated normal horse serum, 20 ml; yeast extract, pH 6.0 (fresh 25% aqueous extract of pure dry yeast), 10 ml; 2% filter-sterilized L-cysteine hydrochloride stock solution, 0.5 ml; 10% filter-sterilized urea stock solution prepared from ultrapure grade no. 9200 urea (Schwarz/Mann Division, Becton-Dickinson and Co., Rockville, Md.), 0.4 ml; CVA enrichment (GIBCO chemically defined enrichment contianing cofactors, vitamins, and amino acids), 0.5 ml; 1% autoclave-sterilized sodium phenol red solution, 0.1 ml; and penicillin G potassium (100,000 U/ml of stock solution), 1.0 ml. It was convenient to combine aseptically (in the manner previously described [22]) all of the seven sterile supplements (32.3 ml) in a separate, sterile, screw-cap bottle and to then add the combined supplements at one time to the sterilized basal broth to yield a final unit volume of 102.3 ml. The final reaction of complete medium 10-B should be approximately pH 6.0. The medium was aseptically dispensed in convenient small volumes in screw-cap tubes, vials, or bottles and stored in the frozen state at −20°C. For use, the stored, frozen medium was thawed at 37°C. The final concentrations of the various supplements were: horse serum, 20%; yeast extract, 10%; L-cysteine hydrochloride, 0.01%, urea, 0.04%; CVA enrichment, 0.5%; phenol red (sodium), 0.001%; and penicillin, 1,000 U/ml. When inoculated with *U. urealyticum*, the standard incubation time at 36 to 37°C for medium 10-B is 16 to 20 h or until appearance of color change (pale orange-red).

(ii) Special fluid medium U-17B. Special fluid medium U-17B was formulated from freshly prepared tryptic digest broth (BBL, no. 11754). (This product is no longer available commercially. BBL Spirolate broth no. 11636 may be substituted at half-strength. BBL tryptic digest broth [no. 11754] is still available gratis from one of us [M.C.S.].) A half-strength basal broth was first prepared as follows: tryptic digest broth powder, 8.0 g (or Spirolate broth, 15.0 g); sodium chloride, 5.0 g; 2-(*N*-morpholino)ethanesulfonic acid buffer, 0.1 M; magnesium chloride, 0.5 g; deionized distilled water, 1,000 ml. After all the ingredients were dissolved, the reaction was adjusted to pH 6.0 with 2 N sodium hydroxide solution. The basal medium was then sterilized in the autoclave at 121°C for 15 min. After cooling, 930 ml of the sterile broth was converted to complete medium U-17B by the addition of the following sterile supplements: unheated normal horse serum, 50 ml; L-cysteine hydrochloride (2.0% filter-sterilized stock solution), 5.0 ml; urea (10% filter-sterilized stock solution), 5.0 ml; phenol red, sodium salt (1.0% autoclave-sterilized stock solution), 1.0 ml; penicillin G potassium (100,000 U/ml of stock solution), 10.0 ml. The final concentrations of the various enrichments were: horse serum, 5.0%; L-cysteine hydrochloride, 0.01%; urea, 0.05%; phenol red, 0.001%; peni-

cillin, 1,000 U/ml. Horse serum for use as enrichment in media for *U. urealyticum* was stored frozen at −20°C at all times and thawed for use at a temperature no higher than 37°C in a water bath. Partial bottles of thawed serum were promptly refrozen for further storage. It is emphasized that medium U-17B is a low-serum medium containing only 5.0% horse serum in contrast to the usual 10 to 20% concentration used for most species of mycoplasmas. This low level of horse serum is desirable in a medium designed for antigen production where good growth titers are essential.

(iii) Differential agar medium A7. Differential agar medium A7 for identification of *U. urealyticum* was prepared as described by Shepard and Lunceford (22). (Ureaplasma differential basal agar medium [A7] is now available commercially from GIBCO Diagnostics, Madison, Wis., catalog no. M52650).

Cloning procedure. All isolates of *U. urealyticum* were purified by means of a standard coloning method (7) adapted to *U. urealyticum* as follows. After primary isolation from clinical material, secondary cultures were initiated in medium (i) and incubated at 37°C overnight (18 to 20 h) until color change (pale orange-red) was noted. A small volume (3.0 to 5.0 ml) of this overnight broth culture was blended in a Vortex mixer and filtered through a sterile Nuclepore polycarbonate membrane filter of 0.2-μm porosity (Arthur H. Thomas Co., Philadelphia, Pa.). Serial 10-fold dilutions of the filtrate were made in medium (i). By means of a sterile Pasteur pipette, small drops from each of the respective dilutions of filtrate were placed on the surface of agar plates. For this purpose, agar plates of standard medium A5H (22) and differential medium A7 (medium iii) were used. The agar cultures were incubated for 24 to 48 h in an atmosphere provided by a single cycle of air evacuation and gaseous replacement with a mixture of 10% carbon dioxide and 90% nitrogen, or anaerobically in a BBL GasPak anaerobic jar, using the BBL no. 70304 disposable generator packet producing hydrogen and carbon dioxide. (Colonies of *U. urealyticum* on differential agar medium A7 are deep brown in color by transmitted light through the substage condenser.) A small agar block or chip bearing a single *U. urealyticum* colony was carefully excised and transferred to a tube of medium (i). This microexcision technique was facilitated by use of a no. 7 surgical knife handle fitted with a no. 11 pointed surgical knife blade whose tip was broken off 1 to 2 mm from the point, thus making a small truncate cutting instrument. Some workers prefer a small ear curette. The agar chip was mascerated against the inner wall of the broth tube, and the broth was incubated at 36°C overnight (18 to 20 h) until the time of the first color change to pale orange-red of the phenol red indicator. At this time a small volume (3.0 to 5.0 ml) of culture fluid was filtered as before. The cloning process was repeated three times. This procedure is considered superior to the limit-dilution method alone.

Preparation of antigens. *U. urealyticum* serotypes 1 through 8 were used as immunizing antigens. The history, including source, strain number, and American Type Culture Collection accession numbers of these strains has been published elsewhere (23). All strains were grown in 3-liter volumes of fluid medium (ii) and incubated at 36°C for 18 to 24 h or until appearance of color change (orange-red) of the phenol red indicator. The medium was inoculated with 30 ml of an overnight starter culture of the immunizing strain grown in medium (ii), diluted 10^{-2} in the same medium. Immunizing strains should be adapted to yield good growth in medium (ii) before large-volume cultivation for antigen. Organisms were harvested in a Beckman L5-40 preparative ultracentrifuge, using a type 30 angle rotor, at 30,000 rpm for 30 min (average 78,100 × *g*). We found it convenient to harvest the organisms by centrifugation in polycarbonate centrifuge tubes for good pellet adhesion. For each serotype, the sedimented organisms were pooled and washed twice in phosphate-buffered saline, pH 7.2, resuspended in 3.0 ml of phosphate-buffered saline by means of mechanical blending in a Vortex mixer, and sonically treated (in the same tube) for 1 min in the treatment cup of a Bronwill Biosonic III sonic oscillator operated at maximum power. Satisfactory suspensions were of uniform cloudiness and relatively free of clumps of organisms. The concentrated, sonically treated suspensions were then diluted in phosphate-buffered saline to yield a 30-ml volume (100× concentration from 3 liters of original antigen culture). The 100× antigen suspensions were further diluted in phosphate-buffered saline to yield a transmittance of 50% at 450 nm and stored in the frozen state at −20°C.

Preparation of antisera. Three approximately 3-kg albino rabbits were utilized for immunization with each of the respective immunizing strains of *U. urealyticum*, representing serotypes 1 through 8. The immunization schedule used (Table 1) was adapted from the method of Al-Aubaidi and Fabricant (1) and from M. F. Barile (personal communication). Serum titers of individual rabbits were measured by means of the metabolism inhibition test of Purcell et al. (15) and by the agar growth inhibition method described in this communication. The respective individual sera were stored in the frozen state at −20°C without preservative.

Agar growth inhibition test. Culture plates of A7 differential agar (medium iii) were prepared in standard plastic petri dishes (100 by 15 mm), using

TABLE 1. *Rabbit immunization schedule used*

Day	Step
0	Preimmunization bleeding
1	Prepared 5.0 ml of initial immunizing antigen (2.5 ml of antigen of 50% transmittance + 2.5 ml of complete Freund adjuvant); injected 1.0 ml into nape of the neck, 1.0 ml into the rump, 2.0 ml intraperitoneally (used half-volumes bilaterally for neck, rump, and peritoneum), and 0.25 ml into each of four center footpads
4	1.0 ml intravenously, no adjuvant
5	2.0 ml intravenously, no adjuvant
6	3.0 ml intravenously, no adjuvant
7	3.0 ml intravenously, no adjuvant
8	4.0 ml intravenously, no adjuvant
11	4.0 ml intravenously, no adjuvant
13	4.0 ml intravenously, no adjuvant
25	Trial bleeding; if titer was satisfactory, made final bleeding by exsanguination

approximately 20 ml of agar per plate. After solidification, the agar plates were placed in the inverted position in a dry 36°C incubator overnight to reduce excess moisture. This partial drying of the agar surfaces was essential to the satisfactory performance of the growth inhibition test. The same day the agar plates of medium (iii) were poured, a broth culture of *U. urealyticum* for serotyping (a cloned clinical isolate, for example) was initiated by inoculation of a tube of medium (i). The culture was incubated overnight (16 to 20 h) at 36°C until the first appearance of color change of the phenol red indicator from yellow to pale orange-red. The actively growing broth culture was thoroughly mixed by agitiation in a Vortex mixer, and a sample was diluted to 10^{-3} in medium (i). Two sets of eight small circles each were marked on the bottom of a culture plate of medium (iii) with a felt tip marking pen (using a template or other device to achieve uniform spacing) to serve as a position guide for subsequent cutting of the agar wells to receive antisera. Such an arrangement permitted the serotyping of two different antigens on the same agar plate, using two sets of antisera (types 1 through 8). By means of a 1.0-ml pipette, a drop of diluted culture (antigen) was placed on the agar surface over the first set of eight marker circles, and the drops were made to coalesce with the aid of the pipette tip to produce uniform distribution of the inoculum. The same procedure was followed for the second set of eight small marker circles, for inoculating the second antigen to be serotyped. Strict precautions were taken to avoid intermixing the two different antigens. The antigens were allowed to air dry for at least 30 min at room temperature in a horizontal position for absorption. (Unlike some other mycoplasmas, *U. urealyticum* does not produce a "lawn" of growth on agar surfaces but, rather, individual colonies whose size is a function of the degree of crowding.) After drying of the antigen inoculum, two sets of eight wells each were cut in the agar, using the "marker" circles on the bottom as position guides (Fig. 1). The wells were cut with short lengths of thin-wall brass tubing similar to that used in many ball-point pens. We used brass tubing of 0.125-inch (3.173-mm) inner diameter by 0.147-inch (3.731-mm outer diameter, obtained from Helenor Metals Corp., Tuckahoe, N.Y. We found it convenient to assemble a "well cutter" from a short length of such brass tubing, a 2/0 or 3/0 rubber stopper bored with a small hole, and a rubber bulb (Fisher Scientific Co., no. 14-065A) to suck out the cutout agar plug. Type-specific rabbit antiserum was applied to each of the eight respective, numbered wells in each set by means of small (2.0 ml), sterile, plastic syringes fitted with 21-gauge by 1.5-inch (3.8-cm) needles. The filled wells were allowed to stand at room temperature for 30 min and were refilled if necessary, using extreme care not to overfill the wells. The inoculated, antiserum-charged agar plates were then incubated right side up at 36°C in an atmosphere provided by a single cycle of evacuation (to 25 inches of Hg) and replacement with a gaseous mixture of 10% carbon dioxide and 90% nitrogen. (Incubation of the charged plates in the BBL GasPak anaerobic system, using the hydrogen-plus-carbon dioxide generator packet, gave equally satisfactory performance.) The plates were incubated overnight (18 h) and given initial examination without delay in the morning. If a zone of growth inhibition by a specific antiserum was detected, the serotype was recorded and the plate was reincubated aerobically to detect possible delayed cross-reactions. If no zones of growth inhibition were detected on the initial examination of the test (due to slower than usual colony development), the plate was reincubated and reexamined every 2 h during the day for evidence of growth inhibition by specific antiserum. This examination schedule was necessary to preclude rapid break-

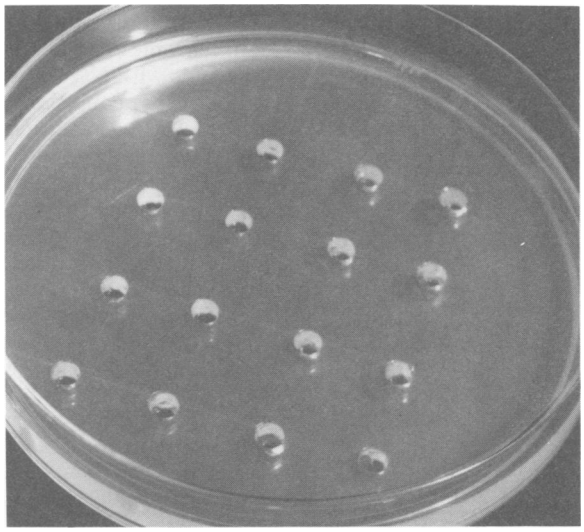

FIG. 1. *Culture plate of differential agar medium A7 in which two sets of eight wells each have been cut to receive antisera for serotyping two different U. urealyticum cloned cultures.*

through growth of *U. urealyticum* colonies which initially were inhibited by a specific antiserum. Failure to observe this precaution could result in reporting a so-called "untypable" isolate with these eight type-specific antisera. The recognition of *U. urealyticum* colony growth, as well as zones of growth inhibition, was greatly facilitated by the use of a differential agar (medium iii), which produces deep-brown colonies of *U. urealyticum* when examined under low power (×100) in the usual manner by transmitted illumination or white colonies when observed by indirect, oblique illumination (22).

RESULTS

Specificity. The specificity of the agar growth inhibition test was determined by testing the eight serotypes of *U. urealyticum* antigens against their homologous antisera prepared in rabbits. The eight serotypes easily could be separated by the agar growth inhibition method described (Table 2). Minor, one-way cross-reactions were observed between antiserum against type 5 and antigen type 2 and between antiserum against type 8 and antigen type 4 when individual rabbit antisera were used. These minor cross-reactions were reduced when pooled rabbit antisera were use.

NGU patients. A total of 122 cultures isolated from male patients with primary and recurrent NGU was serotyped by the method described. *U. urealyticum* type 4 was associated most frequently (52%) with this disease at Camp Lejeune, N.C. (Fig. 2). The remaining serotypes, listed in decreasing frequency of isolation, were type 2 (17%), type 6 (9%), types 3 and 8 (8% each), and type 1 (6%). Types 5 and 7 were not isolated.

Other symptomatic patients. Among 91 cultures isolated from symptomatic patients with other genitourinary tract infections and disorders, *U. urealyticum* type 4 also was found to be the predominant serotype (37%) associated with these disorders (Fig. 3). The remaining serotypes, listed in decreasing frequency of isolation, were type 3 (16%), type 6 (14%), type 2 (12%), type 8 (11%), type 1 (9%), and type 7 (1%). Type 5 was not isolated.

TABLE 2. *Serological relationships between eight serotype strains of U. urealyticum determined by the agar growth inhibition test*

Serotype no.	Inhibition zone[a] (mm) with antisera against serotype:							
	1	2	3	4	5	6	7	8
1	6	0	0	0	0	0	0	0
2	0	7	0	0	4	0	0	0
3	0	0	8	0	0	0	0	0
4	0	0	0	7	0	0	0	1
5	0	0	0	0	9	0	0	0
6	0	0	0	0	0	10	0	0
7	0	0	0	0	0	0	7	0
8	0	0	0	0	0	0	0	7

[a] Measurement from edge of the well.

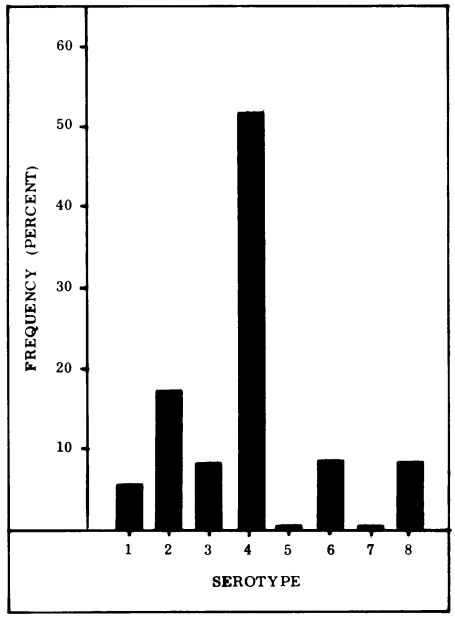

FIG. 2. *NGU patients. Frequency distribution of U. urealyticum serotypes isolated from 122 male patients with primary and recurrent NGU at Camp Lejeune, N.C.*

The 91 different strains of *U. urealyticum* from the above group were isolated from six different categories of symptomatic patients (Table 3) and included patients with renal calculi, chronic prostatitis, unexplained pyuria, husbands with NGU and their respective wives, various other conditions, and a five-colony cloning study. *U. urealyticum* type 4 was the predominant serotype isolated in four of the six different categories. Although the number of patients in each of the separate categories is small, it is interesting, for example, that type 1 organisms were isolated most frequently (40%) from the renal stone patients. Among the chronic prostatitis, unexplained pyuria, and NGU husband and wife groups, *U. urealyticum* type 4 again was the predominant serotype isolated (31, 50, and 50%, respectively). Patients with various other conditions yielded type 3 and type 8 organisms (24% each) as the predominant serotypes. The five-colony cloning study isolates came from male NGU patients, and *U. urealyticum* type 4 was the predominant serotype (67%).

Among 21 patients in the symptomatic group

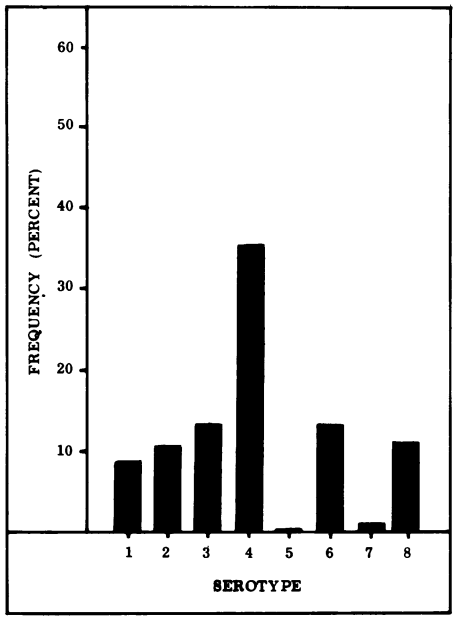

FIG. 3. *Symptomatic, primarily nonurethritis patients. Frequency distribution of U. urealyticum serotypes isolated from 91 patients with various genitourinary tract disorders.*

TABLE 3. *Predominant serotypes of U. urealyticum isolated from 91 symptomatic, primarily nonurethritis patients*

Category	No. of strains	Predominant serotype(s)	Frequency (%)
Renal stone patients[a]	10	1	40
Chronic prostatitis[a]	16	4	31
Unexplained pyuria	22	4	50
NGU husbands and wives[b]	10	4	50
Various other conditions	21	3, 8	24, 24
Five-colony cloning study[c]	12	4	67

[a] Male patients only.
[b] Wives of respective NGU husbands.
[c] From 12 NGU patients, single isolates.

with a variety of other conditions (Table 4), the serotypes were as diverse as the variety of conditions. Types 3 and 8 were the predominant serotypes in this group (24% each). Two male patients with Reiter's syndrome yielded types 4 and 7, respectively. A knee joint aspirate from a patient with arthritis yielded *U. urealyticum* type 4. The sputum from a child with a chronic respiratory infection yielded a type 3 organism. The "Boston T strain," isolated in 1967 from a woman with a spontaneous middle-trimester abortion (10), also was a type 3 strain of *U. urealyticum*.

Asymptomatic carriers (controls). Among 125 asymptomatic carriers of *U. urealyticum* in the genitourinary tract, including 115 Marine Corps enlisted men and 10 healthy children under 10 years of age from a pediatric clinic, *U. urealyticum* type 8 was associated most frequently (30%) with these clinically symptom-free individuals (Fig. 4). However, type 4 organisms were the next most frequently isolated serotype (24%) from asymptomatic carriers. A possible explanation of the relatively high isolation rate of type 4 *U. urealyticum* from the controls will be discussed below. The remaining isolates, listed in decreasing frequency of isolation, were type 3 (15%), type 1 (11%), type 2 (10%), type 6 (8%), and type 5 (2%). Serotype 7 was not isolated.

DISCUSSION

Before the present study, the number of *U. urealyticum* isolates from NGU patients and asymptomatic carriers serotyped by other investigators was relatively small. Black (2) serotyped 12 *U. urealyticum* isolates from NGU patients, using an immunofluorescence technique and an agar growth inhibition test modified from a method described by Clyde (6). No predominant serotype was demonstrated with antisera prepared against the seven antigenic types then known (1970). Two isolates were not typable. Shepard's strain 960-(CX8) (23) was designated

TABLE 4. *Clinical status and serotype of U. urealyticum isolated from 21 patients with a variety of genitourinary tract infections and other disorders*

Clinical status	Serotype isolated
Wife of NGU husband[a]	8
Wife of NGU husband[a]	4
Wife of NGU husband[a]	6
Wife of NGU husband[a]	3
Reiter's syndrome (male urethral culture)	4
Reiter's syndrome (male urethral culture)	7
Pyuria with hematuria (male)	3
Wife of above hematuria patient	3
Spontaneous abortion (Boston T-strain)	3
Sputum from a child with chronic respiratory infection	3
Chronic prostatitis	8
Hematuria (male)	4
Knee joint aspirate (arthritis)	4
Tender ovarian tube	1
Urology referral	8
Urology referral	6
Urology referral	6
Genital culture (England)	8
Genital culture (Canada)	8
Genital culture (England)	2
Genital culture (England	2

[a] *U. urealyticum* failed to be isolated in primary culture from the respective NGU husband.

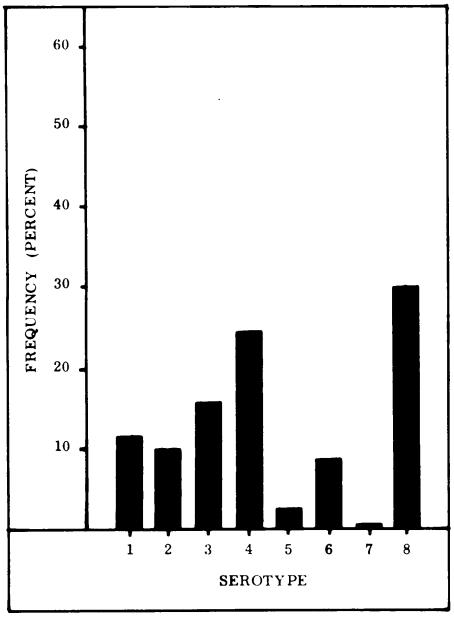

FIG. 4. *Asymptomatic carriers (controls). Frequency distribution of U. urealyticum serotypes among 125 individuals without clinical symptoms who were carriers of U. urealyticum in the genitourinary tract.*

serotype 8 by Black.

Using a modification of his earlier growth inhibition test, Black (3) subsequently serotyped 10 different strains of *U. urealyticum* isolated from NGU patients. Four isolates were type 3, and three were type 1. One each of types 5, 7, and 8 were demonstrated, but types 2, 4, and 6 were not found. Seven patients with gonorrhea also yielded *U. urealyticum* in culture. Three of the seven isolates were type 4, and the remaining isolates were types, 2, 3, 6, and 7, respectively.

Piot (13) used the modifed growth inhibition method of Black (3) to serotype 46 *U. urealyticum* isolates from 19 NGU patients, 12 gonorrhea patients, and 15 asymptomatic carriers (controls). No difference in distribution of eight serotypes among these 46 isolates was demonstrated, and no particular serotype could be correlated with disease symptoms. Fifteen (33%) of the 46 cultures tested from the three different groups were untypable. Recognition of colony growth and inhibition zone boundaries was facilitated in Piot's study by application of Shepard's urea-manganese direct (spot) test reagent for urease in colonies of *U. urealyticum* (18). Piot (14) extended his serotyping studies to include a comparison of agar growth inhibition and indirect immunofluorescence tests in serotyping 39 clinical isolates of *U. urealyticum*. These strains originated from 17 men with NGU, 10 patients with gonorrhea, and 12 healthy men. Using the agar growth inhibition test, 27 of 39 isolates could be serotyped with the available eight antisera. Twelve isolates (30.8%) could not be serotyped. The indirect immunofluorescent test was more sensitive but yielded more cross-reactions. Thirty-four of 39 isolates were serotyped by the latter method, and five isolates (12.8%) could not be serotyped. Piot (14) recommended that clinical isolates of *U. urealyticum* be screened with the indirect immunofluorescence test and that all isolates with strong cross-reactions or doubtful results be examined additionally by the agar growth inhibition method.

Black's modified growth inhibition test (3) was further modified in our laboratory in the following manner: (i) use of a differential agar medium (medium iii) in place of a standard agar as used by Black (the differential agar medium [22], which contains a urea-manganese indicator system [18], greatly facilitated recognition of *U. urealyticum* colonies and demonstration of growth inhibition zones; growth of *U. urealyticum* colonies was generally rapid, requiring only 16 to 20 h for development of small, but easily recognizable colonies and 24 to 48 h for fully mature colonies); (ii) use of an incubation temperature of 36°C in contrast to 27°C as used by Black (3) or 32°C as used by Piot (13, 14); (iii) use of a short incubation period of only 16 to 18 h at 36°C before initial examination of the test agar plates for first evidence of zones of growth inhibition by antiserum. Early examination of the test after overnight incubation of only 16 to 18 h, in our opinion, was the key to the success of the serotyping method described. Early appearing zones of growth inhibition by antiserum may be overgrown by rapid breakthrough growth of *U. urealyticum* colonies on continued incubation. Delayed reading of the test thus may result in failure to observe any zones of growth inhibition. An isolate of *U. urealyticum* tested under such conditions of delayed reading might falsely be labeled "untypable."

Of almost equal importance to the short incubation period noted above in performance of the serotyping method described is the proper seeding of agar plates with antigen. A 10^{-3} dilution of *U. urealyticum* antigen is specified. However, since strain difference and differing multiplication rates in broth cultures occur, optimal colonial density is not always achieved on seeded agar plates. Under such circumstances the test should be repeated, using antigen dilutions of 10^{-2}, 10^{-3}, and 10^{-4} of overnight broth culture to seed the agar plates. Standardization of the inoculum was important, as emphasized by Piot (14).

During the early phase of our serotyping studies, we encountered approximately 20% of *U. urealyticum* isolates that were untypable with the eight antisera we used. During this same early phase, the test was incubated 48 h at 36°C before examination, at which time *U. urealyticum* colony size was maximal. These same untypable isolates were subsequently retested against the same eight antisera, but the tests were given only 16 to 18 h of incubation before first readings were made. All such isolates were successfully serotyped by using the short incubation period.

It was regularly observed that *U. urealyticum* strains isolated from controls (asymptomatic carriers, young children, and patients with non-NGU genitourinary tract infections and other disorders) appeared to be less sensitive to antisera (i.e., yielded smaller growth inhibition zones) than strains isolated from male patients with NGU, using the same serotyping antisera. These differences in response to type-specific antiserum in vitro are probably related to strain differences and may suggest the existence of virulent and avirulent strains of *U. urealyticum*.

In the present study 338 strains of *U. urealyticum* were serotyped by the method described. Of these, 213 strains were isolated from clinically symptomatic patients (122 male patients with primary and recurrent NGU and 91 patients with genitourinary tract infections and other disorders). *U. urealyticum* type 4 was associated most frequently with disease of the genitourinary tract and other symptomatic conditions at Camp Lejeune (Table 3, and Fig. 2 and 3). However, among 125 asymptomatic genitourinary tract carriers of *U. urealyticum* (controls), the predominant serotype isolated was type 8, with type 4 the next most frequently isolated serotype. The relatively high isolation rate of type 4 organisms from asymptomatic carriers of *U. urealyticum* was puzzling. However, several of the men in this control group were found to have unexplained pyuria. As noted below, this finding may explain the elevated recovery rate of type 4 organisms from the asymptomatic carrier controls.

A separate group of 64 clinically asymptomatic Marine Corps enlisted men was cultured for *U. urealyticum* because of (frequently abacteriuric) unexplained pyuria, often with pruritis urethras and dysuria, at the time of physical examination. *U. urealyticum* was recovered from 40 (63%) of the 64 men. This was more than twice the isolation rate (30%) from 580 similar Marine Corps enlisted personnel (controls) cultured in a separate study at Camp Lejeune (17). Of 22 additional *U. urealyticum* isolates which were serotyped from the unexplained pyuria group, 11 (50%) were found to be type 4 organisms (Table 3). These observations suggest a possibly significant association of *U. urealyticum* with unexplained pyuria in males and further suggests the existence of asymptomatic *Ureaplasma* urethritis. The significance of these observations is supported by recently reported studies demonstrating an etiological role for *U. urealyticum* in NGU (5, 24–26). In one study, clinical urethritis was experimentally produced in two human volunteers by the intraurethral inoculation of purified, live *U. urealyticum* organisms. Koch's postulates were fulfilled, thus clearly establishing a pathogenic role for *U. urealyticum* in NGU (25).

In a comparative study of *U. urealyticum* isolates from men with NGU and from their respective wives at camp Lejeune (Table 3), it was of interest to learn whether both partners carried the same serotype at the time of initial culturing. In this small group, the identical serotype organism was isolated from both marital partners. However, we have cultured other marital partners in whom the husband suffered from NGU, with different findings. *U. urealyticum* was often recovered from the genitourinary tract of the wife but not from her husband. In a conjugal relationship such as this, one would normally expect the husband to harbor the same organism, although possibly of a different (more than one) serotype if sexual promiscuity was involved. Occasionally, repeated cultures from the husband eventually yielded *U. urealyticum*, or the organism was isolated by blind serial agar passage. The explanation for the failure to isolate the organism from the husband of a *U. urealyticum*-positive wife is incompletely understood. The presence of local antibody (24) against the husband's strain of *U. urealyticum* could inhibit or prevent its outgrowth in primary cultures. We have obtained evidence (unpublished findings) which supports this hypothesis. Other possibilities are: (i) the husband's urethritis may have been caused by a different agent, such as *Chlamydia trachomatis*; (ii) the husband may have been under the influence of a broad-spectrum antibiotic for some unrelated infection; (iii) for reasons presently unknown, certain strains of *U. urealyticum* appear to be very fastidious and difficult to isolate in primary cultures from the genitourinary tract of men with NGU, and those with NGU whose wives are *U. urealyticum* carriers. Such strains of *U. urealyticum* may undergo specific alterations in metabolic requirements, for example, when proliferating in the male genitourinary tract (NGU), which preclude subsequent outgrowth on artificial culture media. This type of behavior has previously been observed in *Ureaplasma*-in-

fected established cell monolayer cultures (M. C. Shepard, unpublished findings).

The serotyping method described provides a reliable procedure for typing strains of *U. urealyticum* isolated from a variety of sources and should furnish important information concerning the etiology and epidemiology of *Ureaplasma*-associated urethritis and other infections in which *U. urealyticum* may be implicated.

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