Modified Metabolic Inhibition Test for Serotyping Strains of *Ureaplasma urealyticum* (T-Strain Mycoplasma)

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Antisera prepared against the eight recognized serotypes of *Ureaplasma urealyticum* were tested against the homologous and heterologous antigens by a modified metabolic inhibition test that used bromothymol blue broth in microtiter plates. The method gives clear endpoint determinations which are usually maintained on continued incubation. Antisera against serotype 8 showed cross-reactions with types 2 and 4. The ninth strain, Vancouver, when tested by this method, did not fit the recognized serotyping scheme and may represent a new serotype.

The metabolic inhibition test (MIT) is useful for serotyping strains of *Mycoplasma* and *Ureaplasma* (10). Its basis is the ability of strain-specific antiserum, in the presence of complement in a liquid medium, to inhibit the hydrolysis of a specific substrate by an homologous strain. After this procedure, the antiserum titer is defined from an endpoint taken as soon as a change of at least 0.5 pH unit is detected in the mycoplasma (antigen) control.

In applying this test to *Ureaplasma urealyticum* (11) we found that, even with high dilutions of antiserum, nonspecific antimicrobial effects sometimes retarded growth and resulted in erroneously high inhibition titers in the presence of heterologous antiserum. To circumvent this problem, we have modified the original method by introducing a more reliable endpoint and facilitated its recognition by using an improved indicator broth (12).

MATERIALS AND METHODS

Organisms. All strains of *U. urealyticum* examined in this study were obtained in lyophilized form from D. K. Ford, Department of Medicine, University of British Columbia, Vancouver, British Columbia, Canada. The strains had been cloned three times by Ford (4) and, for the purpose of this study, were not cloned further before their propagation for antigen. All strains were reidentified by the urease spot test (13).

Preparation of antigen. All strains were within three transfers of the lyophilized preparations described above. Each strain was grown serially in bromothymol blue (B) broth (12) in which increasing volumes of normal rabbit serum were substituted for the normal horse serum otherwise present. After a given strain had been passed at least twice in B broth with 10% normal rabbit serum as the only serum supplement, an inoculum volume of 1% of a logarithmic

mic-phase culture was introduced into a large volume of this medium. The culture was incubated at 35°C without agitation until the color change from yellow to green and subsequent pH determinations indicated that the culture had reached near-maximum titers (12). After examination of a Gram stain of the sediment from 10 ml of this culture showed the absence of contaminating bacteria, the cultures were chilled, and the cells were collected by centrifugation in a type 19 rotor in a Beckman L5-50 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) at 14,000 rpm for 20 min. The pellets were washed in sterile 0.85% (wt/vol) NaCl until no trace of the indicator could be seen and then combined, resedimented, and suspended in a small volume of sterile, pyrogen-free, physiological saline. The cell suspension was further diluted with this saline to contain 2 mg of protein per ml, as determined by the method of Lowry et al. (8), using bovine serum albumin as a standard. After Gram staining and inoculation to genital mycoplasma agar (12), blood agar, and Vacutainer culture tubes with supplemented peptone broth (Becton-Dickinson, Rutherford, N. J.) so that its purity could be ascertained, the suspension was mixed by a 1-min exposure in a sonicator water bath; this is four times the length of the pulse required for maximum dispersal of more diluted cell suspensions (12). Merthiolate was added to a final concentration of 1:10,000. For primary injections, the antigen was emulsified in a homogenizer (model 45; The VirTis Co., Inc., Gardiner, N. Y.) with an equal volume of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). For secondary injections, the cell suspension was diluted with the pyrogen-free saline to contain 1 mg of protein per ml. Two liters of culture usually provided sufficient antigen to immunize one rabbit.

Preparation of antiserum. Three female San Juan rabbits between 6 and 8 weeks old were used to prepare antiserum against each strain. For primary immunization, each rabbit received 0.2 ml of emulsified antigen in each toe pad and 2.0 ml subclavically. After 3 weeks secondary immunization was begun,

with the animal receiving doses of antigen twice weekly for 3 weeks, increasing from 0.3 to 0.8 ml. The animal was bled before the primary injections and within several days of the secondary immunization series. In the former instance, no inhibition was demonstrated. If the antibody titer was sufficiently high on the latter occasion, the animal was exsanguinated. Otherwise, the secondary immunization was repeated after 3 weeks. This one additional course of immunization was sufficient in nearly all instances. Sera were sterilized by Seitz filtration, inactivated at 56°C for 30 min, assayed, combined, and then reassayed.

MIT. For assessment of antisera the following procedure was followed. A 0.25-ml volume of each antiserum, diluted 1:10 in B broth, was placed in the first two wells of each horizontal row of a microtiter plate with U-shaped wells (Cooke Engineering Co., Alexandria, Va.) and in the wells for the antiserum controls. Microdilutors calibrated to carry 0.025-ml volumes (Cooke Laboratory Products, Alexandria, Va.) were used to make doubling dilutions of antiserum in 0.025 ml of B broth from wells 2 to 12 of each row. Then 0.15 ml of B broth to which 10% (vol/vol) pooled fresh guinea pig sera had been added (and the pH of which had been readjusted to 6.0) was put in each test well and in the antiserum, *Ureaplasma*, and broth controls. A rise in pH resulting from the degradation of urea changes the bromothymol blue indicator in B broth from yellow to green. A culture which is just turning green is in the logarithmic phase of growth and contains about 10^7 color change units₅₀ (CCU₅₀) per ml (12). An inoculum of 0.025 ml of a 1:10,000 dilution of such a culture was added to each test well and to the *Ureaplasma* control; CCU₅₀ determinations performed on actual inoculum preparations showed that each well received between 30 and 300 CCU₅₀. The controls received additional B broth without added complement to make up the 0.2-ml volume of the test wells. The plates were covered with adhesive plastic tape, and the wells were vented by pricking with a sterile needle. Venting was required to prevent the released ammonia from lifting the tape off the plate and effecting a color change in the other wells. Evaporation during incubation was judged insignificant.

The plates were incubated in air at 35°C and examined for color change until at least 1 day had passed after the inoculum control had changed color and there had been no alteration in the endpoint of a given strain. These conditions were usually met within 2 days, after which breakthrough (i.e., further color change) rarely was noted. In the first well the final antibody concentration was 1:80. The titer is the reciprocal of the highest dilution of an antiserum in which no color change occurred. All tests and controls were run in duplicate. If endpoints differed by one dilution, the lower dilution was taken. In the unusual event that the difference exceeded one dilution, the test was repeated. Titers also showed little variation in independent experiments.

RESULTS

The results of the MITs of the eight recognized serotypes of *U. urealyticum* (1, 15) tested with antisera raised against them are shown in Table 1. Also included in this table are the results of tests with a ninth antigen, Vancouver, and antiserum prepared against it. Neither this antigen nor this antiserum showed significant cross-reactions with the serotype strains.

The first serum prepared was against serotype 8 (anti-8a). The antigen preparation used for the primary injections was not emulsified properly and, because of this, 6 months were needed to reach maximum MIT titers instead of the 6 to 12 weeks required for the other sera. Because this serum reacted with a number of heterologous strains, new antigen was prepared, and the immunization was repeated. The second serum (anti-8b) still showed clear cross-reactions with heterologous strains 2 and 4 but decreased reactivity with several others; it reacted similarly with clinical isolates we have typed as 8. Any heterologous reactions with other antisera occurred at three or more dilutions below the homologous reaction; CCU₅₀ determinations

TABLE 1. MIT titers of homologous and heterologous strains of *Ureaplasma*

Antigen serotype ^a	Strain designation ^b	MIT titers with the following antisera:									
		1	2	3	4	5	6	7	8a ^c	8b	Vancouver
1	7	40,960	80	160	160	<80	<80	80	320	80	80
2	23	<80	5,120	640	160	<80	<80	80	2,560	2,560	80
3	27	320	160	10,240	<80	<80	<80	80	640	80	80
4	58	<80	160	160	163,840	<80	<80	80	640	1,280	320
5	354	<80	80	80	320	40,960	<80	160	640	320	160
6	Pi	160	<80	80	<80	<80	20,480	80	80	<80	160
7	Co	<80	80	80	80	<80	320	2,560	640	160	160
8	T-960	<80	80	<80	80	<80	<80	80	2,560	5,120	160
9?	Vancouver	<80	160	<80	80	<80	80	80	320	160	81,920

^a For convenience, the roman numeral designations used by Black (1) have been replaced by arabic numerals.

^b Strain T-960 was designated by M. C. Shepard; the others were designated by D. K. Ford. See references 1, 4, and 15.

^c The antigen emulsion used for primary injection of the rabbits was unstable, and the immunization schedule was prolonged.

confirmed that test dilutions which showed no color change (i.e., metabolic inhibition) also had reduced populations.

With regard to the method itself, several comments may prove useful. First, commercially available, lyophilized guinea pig sera often contain a microbial inhibitor (e.g., sodium azide) in either the freeze-dried or diluent portion. The former is clearly unsuitable for use, and in our hands, when the latter was replaced by broth, antisera titers were below those obtained with fresh guinea pig serum which had been stored at -70°C . The minimum concentration of fresh guinea pig serum which allowed maximum MIT titers was 10% (vol/vol) in broth (final concentration, 7.5%). Reduced concentrations resulted in lower titers. For example, a serum in the anti-2 pool tested with guinea pig serum at final concentrations of 0, 0.37, 0.75, 3.75, and 7.5% showed titers of <80 , <80 , 1,280, 5,120, and 10,240, respectively. Titers obtained using 7.5 and 10% complement were identical. In tests carried out both in the usual and in inactivated (56°C , 30 min) B broth, guinea pig serum was required for inhibition, ruling out the presence of complement in the horse serum supplement as a contributing factor.

For convenience, sets of inocula containing about 10% added glycerol may be stored at -70°C and thawed at about 35°C just before use. However, these dilute cell suspensions lose viability rapidly, and after storage for 1 month their titers should be checked before use.

Titers were dose dependent only at low dilutions. For instance, one of the antisera in the anti-3 pool was tested using 1:400, 1:4,000, and 1:40,000 dilutions of the inoculum culture as the test antigen; the resulting MIT titers were <80 , 10,240, and 10,240, respectively. The same pattern was exhibited by the other two sera in the pool. Finally, in wells containing low dilutions of antiserum (1:80 and, sometimes, 1:160), a slight color change was sometimes noted; this was never seen in the corresponding antiserum controls and appears to be a prozone phenomenon.

DISCUSSION

The MIT has been instrumental in both the initiation (4, 10, 11) and the development of the presently recognized serotyping scheme for strains of *U. urealyticum* of human origin (1, 15). We think that the changes described here are improvements and increase convenience. For example, the proper use of B broth insures that inocula consist of healthy cells from logarithmic growth rather than of cells of indeterminate condition from rapidly declining cultures. Furthermore, B broth allows for more rapid growth

of the organisms than was formerly possible (12) so that, even with the delay in the endpoint readings, the results are generally available within the 48-h incubation period reported for the original method (11). Although B broth contains half of the serum supplement of the broth used by Purcell et al. (11) (10 versus 20% vol/vol), when in combination with the guinea pig serum used as the complement source and the variable amounts contributed by the rabbit antiserum dilutions, the test wells contain at least 17.5% animal serum. Although such serum concentrations can be somewhat inhibitory to the growth of certain strains of *U. urealyticum* (12), they have not interfered with our ability to determine serotypes. The replacement of phenol red by bromothymol blue indicator allows the use of a lower medium pH (6.0 versus 7.2) and one which yields better growth in terms of both generation time and cell yields (12). Any effects of this lowered pH on complement activity appear not to be detrimental to the test. During preliminary experiments we found that metabolic inhibition sometimes occurred when heated antiserum was tested against the homologous strain in the absence of guinea pig serum, although its presence (as a complement source) gave higher titers. The enhancing effect of complement on mycoplasma MIT titers was noted by Purcell et al. (10), whereas in the studies of Coleman and Lynn with *Mycoplasma pneumoniae* complement dependence was found only early in the immune response (3). When the MIT is carried out as described here, guinea pig serum is required for inhibition. The need for such a high concentration of guinea pig serum for maximum titers is not understood, nor, for that matter, is the role of complement in metabolic inhibition.

B broth contains less added urea (0.025 versus 0.01%, wt/vol) than the earlier medium (11), and this may explain the greatly reduced likelihood of breakthrough with B broth. We found that a discernible color change in B broth requires about the same number of milli-equivalents of alkali (NH_3OH) as did the medium used in the original work with *Ureaplasma* MITs (11).

The antisera raised for this study gave MIT titers which are comparable to those previously reported (4, 10). The greatest cross-reaction we observed, and the only one which might jeopardize an unambiguous scheme for subdividing the strains, is between types 2, 4, and 8. Purcell has reported cross-reactions of antigen 2 with anti-8 antiserum (10). The other cross-reactions shown in Table 1 do not correlate with those reported with these strains when tested by the original method (10) and may represent differ-

ences in the immunization procedure or in the genetic makeup of the responding animals.

Since relatively few clinical isolates of *U. urealyticum* have been serotyped by either the MIT (4, 9) or other methods (2, 14) and untypable strains have been found, it is not unlikely that additional types exist. Strain Vancouver, isolated by Ford and Smith from a patient with nongonococcal urethritis that was refractory to tetracycline therapy (6), may be such a strain or may be homologous with one of the subtypes reported by Lin et al. (7) which have yet to be correlated with the recognized scheme. We hope to find further wild strains of the Vancouver type.

Antibody levels in sera from highly immunized rabbits (Table 1) greatly exceed those which have been detected in human sera by the MIT (5, 11). For serodiagnosis we therefore recommend using undiluted serum and increased volumes of the test components and performing the test in tubes.

The modified micromethod, as described here, provides an easy and reproducible means of typing clinical isolates. Such methodology is required so that many isolates of *U. urealyticum* from patients with and without nongonococcal urethritis in the absence of *Chlamydia trachomatis* and other possible etiological agents can be typed. Only in this way can the relationship between serotype and pathogenicity be properly examined.

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ADDENDUM IN PROOF

We have tested the 11 subtypes of Lin et al. (7) with anti-Vancouver serum by this modified MIT method and have been unable to demonstrate significant inhibition. These results confirm that strain Vancouver is unique and the ninth serotype to be identified.

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