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Reliable Urease Test for Identification of Mycobacteria

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A simple, modified formulation for urea broth gave consistent, reliable results to aid in the differentiation of *Mycobacterium* species. In a study of 1,346 isolates representing 17 different species, tests read after 7 days were distinct and reproducible. The use of this test facilitates the identification of *Mycobacterium* species, such as *M. scrofulaceum*, the *M. avium* complex, the *M. terrae* complex, and *M. triviale*.

Urease production has been a key test in the identification of microorganisms for many years. Söhngen reported urease activity in mycobacteria in 1913 (as noted by Urabe and Saito [8]). With the classification of mycobacteria in the 1950s by Timpe and Runyon, medical and taxonomic interest in mycobacteriology increased (5, 6). During the following decade, biochemical and morphological findings clearly established mycobacteriology as a distinct area for development (3, 10, 12). As investigators were searching for new biochemical tests for identification of *Mycobacterium* species, interest in urease production by mycobacteria was revived with the development by Toda et al. of a simple buffered urea solution for demonstration of this enzyme (7). The publication by Toda et al. gave only limited information on the then-recognized species of mycobacteria, but other investigators have utilized this basic procedure to compile data on the patterns of urease activity for classification (8, 11).

In recent years, with the recognition of many new species of mycobacteria, the need for a new and reliable test for urease production has developed. In an attempt to include this test in their investigative protocol, many investigators have utilized commercially available products, such as urea agar base concentrate, diluted 1:10 (11), or urea disks in sterile, distilled water (4). Attempts to implement both of these methods in our laboratory were unsuccessful due to inconsistent results. There may be several possible reasons for the poor results obtained with these procedures, such as (i) improper buffer system, (ii) volume of the test broth used, (iii) inoculum size, and (iv) time of incubation. All of these variables were considered in the preparation of the medium and the procedure discussed in this paper. Urease studies in this laboratory have indicated that consistent and reliable data are obtained when the amount of the buffer is adjusted, the pH of the medium is lowered (for better contrast in reading), a surface-active agent is added, the volumes of the broth and the inoculum are controlled, and the time of incubation is lengthened. The reliability of this urease test has allowed better differentiation of *Mycobacterium* species which previously had been difficult to identify.

MATERIALS AND METHODS

Test organisms. With the exception of the rare organisms listed below, mycobacterial cultures tested were received by the Texas Department of Health Bureau of Laboratories for identification during the period from January 1978 through November 1978. Because of the rarity of some species received by this laboratory, results included in the tabulation for *M. szulgai*, *M. simiae*, *M. xenopi*, and *M. gastri* were from the period from January 1977 through November 1978. All organisms were submitted to a complete battery of tests, using the protocol of the Center for Disease Control (9), and identifications were made in this laboratory. The identification of rare or unusual organisms was confirmed by the Center for Disease Control, Atlanta, Ga. A number of the organisms tested were known strains of mycobacteria or confirmed strains from national evaluation programs.

Media. All cultures were grown and maintained on Lowenstein-Jensen (L-J) medium. The urea broth for mycobacteria was prepared at this laboratory; Table 1 lists the components and their amounts. The ingredients were mixed well to insure complete solution. Application of heat was not necessary and is not recommended. The final pH was 5.8 ± 0.1. Although rarely necessary, pH adjustment with sodium hydroxide before sterilization did not affect our results. The broth was filter sterilized by passage through a membrane filter (0.22 μm), and 1.5-ml of amounts of the sterile broth were then dispensed into sterile screw-capped tubes (18 by 125 mm). The caps on the test tubes were tightened, and the medium was stored in a refrigerator at 4°C until used. Medium stored at this temperature is stable for up to 2 months with no loss in the specificity or sensitivity of the test. The urea broth described in this paper is not commercially

available, but all of the components are.

Procedure. Young, actively growing cultures on L-J medium were used for testing. A sterile applicator stick or a sterile spade was used to remove a moderate amount of the growth from the L-J medium. Inclusion of some of the L-J medium may be necessary with some of the cultures. It has been the experience of this laboratory that inclusion of small amounts of the L-J medium with the inoculum does not adversely affect the test. The inoculum was suspended in the urea broth for mycobacteria described above. Sufficient inoculum was added to each tube to make the broth noticeably turbid or contain moderately sized, granular clumps of cells. The tubes were incubated at 35 to 37°C without carbon dioxide, and the tests were read after 1 and 7 days. A change in the color of the broth from bright yellow to dark pink or red was an indication of the breakdown of urea and was read as a positive reaction. The following gradation of this reaction was used: light pink, 1+; dark pink, 2+; light red, 3+; dark red, 4+. Standards were prepared by using the same procedure used for preparation of standards for the nitrate reduction test (9). Only a 2+ or greater intensity was considered positive. Any questionable results were repeated. Quality control included *Mycobacterium fortuitum* CDC-196 as a positive control and an uninoculated tube of medium as a negative control with each week's tests.

TABLE 1. *Components for urea broth for mycobacteria*

Component	Amt
Peptone	1.0 g
Dextrose	1.0 g
Sodium chloride	5.0 g
Potassium phosphate (monobasic)	0.4 g
Urea	20.0 g
Phenol red (sodium; 1.0% solution)	1.0 ml
Tween 80	0.1 ml
Distilled water	1,000.0 ml

RESULTS

Results from the tests were distinct; most positive reactions were 3+ to 4+ after 7 days, whereas negative tests were bright yellow, and only rarely was a questionable slightly pink reaction encountered. In these rare instances, the test was repeated.

As Table 2 shows, a high percentage of the following *Mycobacterium* species gave positive results at 7 days: *M. tuberculosis*, *M. kansasii*, *M. marinum*, *M. scrofulaceum*, *M. flavescens*, *M. fortuitum*, *M. chelonei*, and *M. vaccae*. Others of the more commonly isolated *Mycobacterium* species gave predominately negative results. The data on the more rarely isolated organisms are not sufficient but may indicate possible trends for future studies.

Initially, tests were read after 1 and 3 days of incubation, but as Table 3 shows, a high percentage of the *Mycobacterium* species which should have produced positive results were negative or questionable at 3 days. By increasing the incubation time to 7 days, more consistent results were obtained in this preliminary study with stock strains and clinical isolates. Positive reactions that were noted at 1 or 3 days remained positive through 7 days, so a single reading after 7 days would be acceptable. Other tests for identification of mycobacteria, such as Tween 80 degradation, semiquantitative catalase, and others, require incubation for at least 1 week or

TABLE 2. *Urease activity of mycobacterial isolates in urea broth for mycobacteria*

Organism	No. of isolates tested	No. showing:		
		Negative reaction at 7 days	Positive reaction at:	
			1 day	7 days
M. tuberculosis	525	36 (7)[a]	19 (4)	489 (93)
M. kansasii	206	2 (1)	1 (0.5)	204 (99)
M. marinum	8	0	3 (37)	8 (100)
M. gordonae	186	138 (74)	16 (9)	48 (26)
M. scrofulaceum	67	5 (7)	6 (9)	62 (93)
M. flavescens	13	1 (8)	7 (54)	12 (92)
M. szulgai	3	1 (33)	1 (33)	2 (67)
M. avium complex	184	172 (94)	2 (1)	12 (6)
M. xenopi	2	2 (100)	0 (0)	0 (0)
M. simiae	7	0 (0)	0 (0)	7 (100)
M. terrae complex	23	21 (91)	0 (0)	2 (9)
M. triviale	19	18 (95)	0 (0)	1 (5)
M. gastri	1	0 (0)	0 (0)	1 (100)
M. fortuitum	70	0 (0)	13 (19)	70 (100)
M. chelonei	22	1 (4)	12 (55)	21 (96)
M. phlei	2	0 (0)	2 (100)	2 (100)
M. vaccae	8	0 (0)	8 (100)	8 (100)

[a] The numbers in parentheses indicate the percentages of isolates tested giving those results.

TABLE 3. *Preliminary evaluation of urea broth for mycobacteria*

Organism	No. of isolates tested	No. showing:			
		Negative reaction at 7 days	Positive reaction at:		
			1 day	3 days	7 days
M. tuberculosis	56	1 (2)[a]	0 (0)	15 (27)	55 (98)
M. kansasii	22	0 (0)	0 (0)	10 (45)	22 (100)
M. avium complex	26	24 (92)	0 (0)	2 (8)	2 (8)
M. scrofulaceum	7	0	0	4 (57)	7 (100)

[a] The numbers in parentheses indicate the percentages of isolates tested giving those results.

longer, so this increased incubation should present no problem.

DISCUSSION

Our laboratory experienced difficulty in obtaining satisfactory, consistent results in the urease test when the method of Wayne (11) was used, so attempts were made to find a better and simpler method. The use by Murphy and Hawkins of Difco urea disks at first appeared to be an acceptable procedure (4). However, we also obtained inconsistent results with this method during our early evaluation of the test. We often had problems of false-positive reactions, probably due to improper buffer, which were accentuated by inadequately cleaned glassware. To resolve the problem, components of previous test media and assumptions regarding the pH of the medium and enzyme activity should be evaluated.

Most urea media have been routinely adjusted to a pH of approximately 6.8 for at least two reasons. First, it was thought that if the pH of the medium was close to the pK, or the pH at which the indicator changed, the minimum amount of urea hydrolysis would cause a color change. Second, it was assumed that the enzyme urease would be more active at a neutral pH. The pH of our urea broth for mycobacteria is 5.8. Table 2 shows, the enzyme system(s) of the mycobacteria can sufficiently break down the urea to overcome the pH gradient and thus change the indicator. In addition, the lower pH of the medium allows better and easier differentiation between positive and negative results.

The buffer system used in this study was twice as concentrated as that used in urea R broth of Edwards and Ewing (1). Our laboratory found it necessary to have a more concentrated buffer system to preclude spontaneous changes in our broth due to hydrolysis of urea or from extraneous materials. It has been our experience in reusing glass test tubes for media that improperly cleaned glassware can cause difficulties. On the other hand, the buffer system used by Wayne et al. (12) was too concentrated to be consistently overcome by many of the *Mycobacterium* species. The use of potassium phosphate (monobasic) with peptone present in the medium supplies the proper buffering system.

Generally, the nutrients present in our broth were not sufficient for growth of many of the mycobacteria, but extended incubation may enable some reproduction and thus additional urease production. Tween 80, a surface-active agent, was added to aid in the dispersion of cell clumps and, possibly, facilitate faster access of urease to urea.

A satisfactory urease test is needed to aid in the distinction between the *M. avium* complex and *M. scrofulaceum*, which are morphologically and biochemically similar. As Table 2 shows, 62 of 67 *M. scrofulaceum* isolates tested gave positive reactions for urease. Because of initial results inconsistent with methods described previously (4, 11), parallel studies were not performed. At the present time, many laboratories are relying upon the semiquantitative catalase test as the key reaction for the distinction between pigmented isolates of the *M. avium* complex and *M. scrofulaceum*. Studies in our laboratory show a high correlation between semiquantitative catalase test results and positive urease tests, so that the 93% of the *M. scrofulaceum* isolates that gave positive urease reactions also produced a column of foam >45 mm in the semiquantitative catalase test. Conversely, 34 of 38 pigmented isolates of the *M. avium* complex with semiquantitative catalase test readings of less than 45 mm had negative urease tests. The colonial morphology of *M. gordonae* resembles that of *M. scrofulaceum*, but the urease reaction in combination with the Tween 80 degradation test, is helpful in differentiating the two species; however, the data indicate that there is a urease-positive variant of *M. gordonae*.

Although our one isolate of *M. gastri* will not verify the reaction, other studies have indicated that this species is urease positive (4, 11), whereas over 90% of the isolates of the *M. terrae* complex and *M. triviale* are negative. *M. xenopi*

and 94% of the *M. avium* complex isolates are negative (4), but a fairly recently described species, *M. simiae*, appears to be positive. It is extremely difficult to demonstrate a positive niacin test for many isolates of *M. simiae*, which closely resemble the *M. avium* complex; so urease production could be a good key to suspect *M. simiae* cultures. Therefore, closer examination of the slow photochromogenicity feature and additional incubation time for the niacin test may be warranted. All rapid growers with rare exception are urease positive and, frequently, positive reactions are noted at the 1-day reading. In some cases, due to improper inoculum or other reasons, the growth rate is difficult to determine, so the urease reaction may be a key to possible grouping of rapid growers and slowly growing, nonchromogenic mycobacteria. Overall, the inclusion of data from urease tests would have aided in correct identification of *Mycobacterium* species in the study of Kubica (2).

The nonchromogenic and scotochromogenic mycobacteria do present a problem to most mycobacteriologists. Most laboratories have little difficulty in identifying *M. tuberculosis* or *M. kansasii*, or even *M. fortuitum*, but beyond that point every tool is needed. A good, reliable urease test is a valuable tool. Our method has been used routinely at the Texas Department of Health Bureau of Laboratories and several of the local health department laboratories for approximately 2 years with excellent results. The Mycobacteriology Branch of the Center for Disease Control has evaluated the medium and has found it to be acceptable and useful (R. C. Good, personal communication). This is evidence that the procedure can be performed satisfactorily and consistently in other laboratories.

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