

Highly Reproducible Techniques for Use in Systematic Bacteriology in the Genus *Mycobacterium*: Tests for Pigment, Urease, Resistance to Sodium Chloride, Hydrolysis of Tween 80, and β -Galactosidase

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The International Working Group on Mycobacterial Taxonomy has undertaken a series of cooperative studies to standardize and establish reproducibility of tests that are useful for classifying and identifying mycobacteria. To date 25 techniques have been examined, and 5 of these met our rigorous criteria for reproducibility and differential power. The properties determined by these tests are urease activity, pigment production, tolerance to 5% NaCl, hydrolysis of Tween 80, and β -galactosidase activity.

The International Working Group on Mycobacterial Taxonomy (IWGMT) is an informal confederation of investigators who have undertaken a series of cooperative studies into the taxonomy of the genus *Mycobacterium* (3, 6). The members also serve in an advisory capacity to the Subcommittee on *Mycobacterium* of the International Committee for Systematic Bacteriology. The data derived from the cooperative studies have not only contributed to a definition of species (clusters) of the mycobacteria, but have identified those properties that are most definitive of species and, thus, of the greatest potential determinative value. However, these investigations were conducted under a permissive philosophy, with each participant free to select the specific technique to be employed for determining a given property. The principles and logistics of the IWGMT studies have been discussed previously (6).

To contribute to greater standardization of methods applied to the systematic bacteriology of mycobacteria, a new series of studies has been undertaken to evaluate the reproducibility of specific techniques. This is a report on the

first IWGMT pilot cooperative study of standardization of techniques for use with slowly growing mycobacteria.

MATERIALS AND METHODS

Selection of tests for study. The tests were chosen on the basis of prior cooperative studies of the IWGMT that dealt with slowly growing mycobacteria. These cooperative studies were concerned with the taxonomy of scotochromogens of Runyon group II (6), non-photochromogens of group III (Meissner et al., *J. Gen. Microbiol.*, in press), photochromogens of group I (Tacquet et al., manuscript in preparation), and members of the *M. tuberculosis-M. bovis* complex (Kleeberg et al., manuscript in preparation). Characters were defined as of potential systematic value if over 85% of the strains of one or more cluster recognized in these studies exhibited the character and if over 85% of the strains of one or more of the remaining clusters failed to exhibit the character.

A total of 69 characters, derived from 25 tests, met the criteria defined above. Participants in the prior cooperative studies were asked to provide details of the techniques they had employed, and from these responses a working manual was developed in which the techniques were described in detail.

Cultures. The 18 cultures listed in Table 1 were selected from a collection that had been deposited previously with the American Type Culture Collection and distributed to members who had agreed to perform some or all of the selected tests. No attempt was made to select strains that were most characteristic of their species. On the contrary, some atypical strains were included to provide a broad range of reaction patterns.

Participation. Participants were not required to perform all of the tests described in the working manual. They were, however, to examine all 18 cultures by the tests they had elected to perform. Participants were asked to perform each test three times on each strain, if possible. The tests were to be carried out exactly as described in the working manual. Data were submitted to the study coordinator (L. G. Wayne) for transcription and analysis.

The descriptions of techniques for five of the tests which were evaluated are presented below, since the present authors believe that they yield highly reproducible data with taxonomic and determinative value.

Urease (1; Bönicke, personal communication)

1. Media and reagents

a. Löwenstein-Jensen (L-J) egg medium is prepared according to conventional formulation. (Minor differences in ingredients available to the participants did not appear to detract from the reproducibility of this test.)

TABLE 1. Cultures examined in the cooperative study^a

ATCC no.	Culture label	Comments
12478	<i>Mycobacterium kansasii</i>	
14470	<i>M. gordonae</i>	
14474	<i>M. flavescens</i>	
19981	<i>M. scrofulaceum</i>	
23285	<i>M. gordonae</i>	Ureolytic strain
23292	<i>M. triviale</i>	
23432	<i>Mycobacterium</i> spp.	Scotochromogen, see ref. (6)
25039	<i>M. marinum</i>	
25041	<i>M. kansasii</i>	
25046	<i>M. kansasii</i>	Nonpigmented strain
25145	<i>M. nonchromogenicum</i>	
25158	<i>M. gastri</i>	
25163	<i>M. xenopi</i>	
25167	<i>M. avium</i>	
25169	<i>M. intracellulare</i>	
25269	<i>M. terrae</i>	
25275	<i>M. simiae</i>	
27373	<i>M. bovis</i>	BCG

^a These cultures were not specifically selected as very typical of their species.

b. The substrate solution is prepared by dissolving 10 mg of urea in 100 ml of sterile water, in a sterile bottle. The solution is not further sterilized and is stable for at least 2 months under refrigeration.

c. For reagent 1, 67 mg of $MnSO_4 \cdot 4H_2O$ is dissolved in 100 ml of distilled water. If a different hydrate of the salt is employed, the amount is adjusted accordingly. This reagent is stable indefinitely.

d. For reagent 2, 25 g of phenol (reagent grade) is suspended, with shaking, in 10 ml of distilled water. A 54-ml volume of 5 N NaOH is added with shaking, until the solution clears; then an additional 36 ml of distilled water is added. This solution is not stable.

e. For reagent 3, commercially available sodium hypochlorite solution (approximately 15% NaOCl, and containing at least 5% available chlorine) is diluted by adding 1 part to 10 parts of distilled water. This solution is stable in the refrigerator for about 4 weeks.

2. Inoculum

Cultures are grown on L-J medium and harvested while they are actively growing. Growth is carefully scraped from medium (avoid carrying any medium into the inoculum). The cells are weighed in a sterile tube or bottle. There must be at least 20 mg (wet weight) of cells for this test. The weighed cells are suspended in sterile saline and centrifuged. The supernatant is discarded, and the cells are suspended in sufficient sterile 0.067 M phosphate buffer (pH 7.2) to yield a cell concentration of 10 mg/ml. A 1-ml volume of suspension is distributed to each of two sterile test tubes. The urea substrate solution (1 ml) is added to one tube of cell suspension and 1 ml of sterile water is added to the other.

3. Time and conditions of incubation

The tubes are capped and incubated at 37 C for 16 h. The following are added to each tube: reagent 1, 0.1 ml; reagent 2, 1.0 ml; reagent 3, 0.5 ml. The rack of tubes is then placed in a 100 C steam bath for 30 min.

4. Method of reading results

A blue color which appears after heating of the test mixtures constitutes a positive reaction. It may be helpful to establish some arbitrary scale of degree of intensity of color (i.e., 0 to 4+), but generally a result is recorded simply as positive or negative. Each time the test is run, a tube of substrate without cells should be run as a control for spontaneous hydrolysis.

5. Special problems or pitfalls

This test is based on a very sensitive assay for ammonia released by hydrolysis of the amides. It is essential, therefore, that ammonia-free water be used for all solutions. Avoid ammonia-laden atmospheres such as may occur in the proximity of animal rooms.

Pigment (7, 8)*1. Media and reagents*

L-J medium is prepared from standard formulation and dispensed in 3- to 3.5-ml amounts to 15-ml screw-capped bottles. These are laid flat and inspissated.

2. Inoculum

A small amount of growth is scraped from a stock culture (any medium) and suspended in distilled water. For colonies that resist suspending, Tween 80 is added to 0.02%. This suspension is added dropwise to another tube of the same diluent until a very slight, barely perceptible turbidity is seen. Three drops of this dilute inoculum are placed on the surface of each of two bottles of L-J medium.

3. Time and conditions of incubation

The cultures are incubated with loose caps, at 25 to 30 C. One of the tubes is unshielded; the other is placed in a light-proof box beside the unshielded one. Both are placed under a "daylight" fluorescent lamp (combined wattage of 30) at a distance of 40 cm. Incubation is continued until definite growth is first seen, usually about 2 weeks.

4. Method of reading results

When definite colonies are seen in the unshielded culture bottle, the presence or absence of pigment is noted. The shielded culture is then removed from its box, and the presence or absence of pigment is noted; this tube is then exposed to continuous light and reexamined after 8, 24, and 48 h for development of pigment. Several combinations of results are possible: (i) no pigment in any tube, before or after light exposure; (ii) pigment in the unshielded tube, none in the shielded one; pigment develops after exposing the shielded one to light, i.e., photochromogenicity; (iii) pigment of equal intensity in both tubes at first examination, i.e., scotochromogenicity; (iv) pigment in both tubes at first examination, but color is more intense in the unshielded one, i.e., scotochromogenicity with intensification by light. In the case of pigmented cultures, actual color should be noted, i.e., yellow, orange, pink, red, salmon, or coral.

5. Special problems or pitfalls

Proper development of pigment requires adequate air; be sure caps on bottles are loose throughout incubation period. Photochromogenic response may not occur in old cultures. When the unshielded culture is yellow and the shielded one is white, the shielded one should be removed from its box and exposed to light when less than 3 weeks old for confirmation of

photochromogenicity. Some cultures may be erratic producers of pigment and give an impression of photochromogenicity. This is sometimes true with unusual pink or coral-colored strains, so indication of actual color seen is important, although fine distinctions of hue are not. Some cultures show no pigment when first definite growth is seen, even under continuous light exposure, but may become yellow on prolonged incubation; these are recorded as nonpigmented.

Tolerance to 5% sodium chloride (2)*1. Media and reagents*

L-J medium is prepared from standard formulation. To 300 ml of L-J, 15 g of NaCl is added and mixed well. The medium is dispensed in 3- to 3.5-ml amounts of 15-ml screw-capped bottles. These are laid flat and inspissated. Similar slants of L-J control medium without NaCl are also prepared.

2. Inoculum

A barely turbid suspension of cells is prepared as described for the test for pigment production. A 0.1-ml volume of this dilute suspension is added to 10 ml of sterile water, and 3 drops each of this very dilute suspension are inoculated to a bottle of L-J with 5% NaCl and to a bottle of L-J control medium.

3. Time and conditions of incubation

Cultures are incubated with loose caps at 35 C. When surface moisture has evaporated, the caps may be tightened. Cultures need be incubated no longer than 6 weeks.

4. Method of reading results

Time of first definite growth on L-J control is noted. The inoculum described should ultimately yield innumerable colonies or confluent growth. The NaCl L-J bottles are examined weekly for 3 weeks after the first appearance of growth on control medium. If more than 50 colonies are seen in that time on the NaCl medium, the culture is recorded as tolerant if fewer than 50 colonies develop, it is sensitive.

Hydrolysis of Tween 80 (8)*1. Media and reagents*

A 500-mg amount of Tween 80 (polyoxyethylene sorbitan monooleate) and 2.0 mg of neutral red are dissolved in 100 ml of 0.067 M phosphate buffer (pH 7.0). The solution is dispensed in 4-ml amounts to screw-capped test tubes (16 by 125 mm) and

autoclaved for 15 min at 121 C. It is critical that the solution contain the full amount of actual neutral red dye specified. Inasmuch as many batches of dye are less than 100% pure, a correction should be made in weighing out the dye to compensate for the impurity present. For example, if the label on the batch being used indicates an 80% dye content, it would be necessary to use $100/80 \times 2.0$, or 2.5 mg, of the impure dye to get a true 2.0 mg of neutral red. The Tween neutral red solutions must be kept refrigerated and protected from light. Prolonged exposure to light causes the dye to fade.

2. Inoculum

A large loop of growth is scraped from an actively growing culture on solid medium and suspended well in a tube of Tween-neutral red reagent solution. The final concentration of cells should be between 1 and 10 mg (moist weight) per ml.

3. Time and conditions of incubation

The suspension is incubated in the dark at 35 C for 10 days. It is removed and read daily; the tube is shaken briefly after each examination.

4. Method of reading results

The tubes are held up to a fluorescent desk lamp, with the top level of fluid just against the light shield. The solution is viewed at such an angle that the eye is not in a direct line with the light, but slightly above the light shield. Uninoculated reagent and suspensions of cultures that fail to hydrolyze Tween 80 are an amber-yellow color. Hydrolysis of Tween 80 results in a gradual change of color from pale salmon, to pink, and often to bright red. The first definite change to the salmon color is the positive reaction. The day when this occurs is noted and the incubation is continued to confirm deepening of the pink color. Results are expressed as positive within 5 days, positive within 10 days, and negative after 10 days.

5. Special problems or pitfalls

The need for full dye concentration and protection from light was discussed above. One should also be aware that it is the color of the solution one is interested in, not the color of the suspended cells. Some cells may take up the dye and the sediment will appear pink, but if the supernatant is amber, the test is negative. This is the reason the tubes should not be shaken until after each daily reading.

β -Galactosidase (E. Kubala, personal communication)

1. Media and reagents

a. Modified Dubos medium base containing: KH_2PO_4 , 1.0 g; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 6.25 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$,

0.6 g; sodium citrate, 1.5 g; asparagine, 2.0 g; and Tween 80, 5 ml of a 10% aqueous solution. The ingredients are dissolved separately, each in 100 ml of water, and then combined, and the volume is brought to 1,000 ml (pH 7.2). The basal solution is dispensed in 100-ml amounts and autoclaved at 121 C for 20 min.

b. For the albumin solution, 9 g of albumin fraction V is dissolved in 100 ml of saline, and the solution is heated to 56 C for 30 min. This solution is sterilized by filtration.

c. 2-Nitrophenyl- β -D-galactopyranoside.

d. For complete medium, 100 mg of 2-nitrophenyl- β -D-galactopyranoside is dissolved in 100 ml of modified Dubos medium base. To this is added 4 ml of albumin solution. The complete medium is filter-sterilized and distributed aseptically in 5-ml amounts to sterile, screw-capped tubes.

2. Inoculum

A 0.5-ml aqueous suspension of 1 mg of bacteria per ml, prepared from an actively growing L-J culture, is added to a tube of medium.

3. Time and conditions of incubation

Four to six weeks; 35 C.

4. Method of reading results

A positive reaction is indicated by the suspension turning yellow.

RESULTS

Investigators from 16 laboratories examined the test cultures and submitted data before the study cutoff date. The data were transcribed and analyzed in the following manner.

The test result obtained by a majority of participants for each strain was considered the "consensus" result for that strain. The total number of results in agreement with strain consensus obtained for a test was divided by the total number of responses submitted for that test and designated mean percent agreement.

The number of laboratories showing over 90% agreement with consensus results were also tabulated.

The number of strains yielding over 90% positive and over 90% negative results in pooled data for a given test were also tabulated.

Internal consistency of labs was determined only for those laboratories that performed tests in triplicate. The number of cultures on which triplicate results were obtained in a given test

was designated *T*. The number of cultures in which all three replicate results were in agreement with each other was designated *C*. The mean percent internal consistency is thus $(C/T) \times 100$. This is not a measure of "correctness" of answer, but only of reproducibility within a lab.

It must be pointed out that the values for mean percent agreement with strain consensus in Table 2 are not strictly comparable to the scores for internal consistency within labs. In determining internal consistency of laboratories, a single disagreement among three replications was recorded as a replication failure for that set. Thus, if a laboratory examined all 18 strains in triplicate for a given test, there would have been 54 bits of data. If there was one disagreement with strain consensus, it would reduce mean agreement by one in 54, or approximately 2%. When, however, one of three replications in a single strain disagreed with the other two, that triplet was recorded as inconsistent. That is, that one set among 18 triplicate sets would reduce internal consistency by approximately 6%. If the one disagreement in a triplicate set were treated as a single score rather than as a set, disregarding correctness of the response in a given laboratory, then mean internal consistency of tests would have ranged from 97 to 99%, and the range of laboratories would have been 89 to 100% for the five tests reported.

In the present report we present results only for those techniques which met all four of the following rigorous criteria. (i) Ten or more replicate data (combined from all laboratories) were received for each test strain subjected to the test. (ii) The mean percent agreement for that test was equal to or exceeded 90%. (iii) At least one strain was recorded as positive in at least 90% of 10 or more replications of the test. (iv) At least one additional strain was recorded as negative in at least 90% of 10 or more replications of the test.

Eight tests met these criteria, but three of these were tests for drug susceptibility in agar. Because of discrepancies observed in results of other drug susceptibility tests and a need for uniform methods of testing susceptibility to many drugs, we are not presenting the drug susceptibility technique until further studies have been conducted. The analyses of results with the remaining five tests are presented in Tables 2, 3, and 4.

DISCUSSION

The data on urease activity in Tables 2 and 3 are based on an average total of 25 replications per strain of bacteria. Although a very high mean percent agreement is seen in this test, a disproportionate number of the few disagreements occurred in 1 of the 11 laboratories in which this test was performed. That laboratory

TABLE 2. *Reproducibility and differential consistency of five tests*

Property	Agreement with strain consensus			No. of strains $\geq 90\%$		Internal consistency of labs				
	No. of labs	Mean % agreement	Variance ^a	No. of labs 90% Agrmt.	Positive	Negative	No. of labs	Mean of labs (%)	Range of labs	
									Low	High
Urease	11	96.7	0.034	10	10	7	7	93	72	100
Pigment	13	93.4	0.068	9	<i>b</i>	<i>b</i>	10	92	69	100
Growth on 5% NaCl	13	92.1	0.081	9	1	10	10	95	67	100
Tween 80 hydrolysis, 5 day 10 day	15	90.0	0.101	7	7	4	11	95	83	100
	15	95.7	0.052	13	11	4	11	96	89	100
β -Galactosidase	6	90.0	0.107	5	2	7	3	96	93	100

^a Variance (s_1^2) was calculated from formula 14 of Sneath and Johnson (5). The value for degrees of freedom ($N-1$) in that formula were based on average values of N per strain for each test since not all strains were subjected to exactly the same number of replications for a given test.

^b Pigment was expressed as photochromogenic (P), scotochromogenic (S), or nonchromogenic (N), rather than as positive or negative. Two strains were $\geq 90\%$ P, four strains were $\geq 90\%$ S, and eight strains were $\geq 90\%$ N.

TABLE 3. *Distribution of replicate test responses by culture*

ATCC no.	Replications (%) positive for:							β -Galactosidase
	Urease	Pigment			5% NaCl	Tween hydrolysis		
		Photochromogenic	Scotochromogenic	Nonchromogenic		5 Day	10 Day	
27373	95	0	0	100	0	3	12	9
25158	95	0	0	100	16	100	100	18
12478	100	100	0	0	0	100	100	14
25046	100	0	0	100	0	100	100	14
25041	100	0	100	0	3	88	100	0
25039	100	93	7	0	25	91	100	0
25275	96	80	3	17	11	42	60	21
23285	100	10	90	0	9	49	87	18
14470	96	0	94	6	0	98	100	21
14474	97	0	100	0	18	84	92	14
25145	0	0	0	100	29	100	100	100
25269	0	0	0	100	14	88	98	100
23292	7	0	0	100	97	93	100	0
23432	0	14	77	9	0	89	97	14
19981	100	11	86	3	14	5	7	0
25169	19	20	0	80	3	0	0	0
25167	8	0	10	90	0	0	0	0
25163	4	0	7	93	3	11	11	36

TABLE 4. *Distribution of disagreements in interpretation of pigment*

Consensus interpretation	No. of strains	Total no. of entries	% of entries		
			Photochromogenic	Scotochromogenic	Nonchromogenic
Photochromogenic	3	99	91	3	6
Scotochromogenic	6	204	6	91	3
Nonchromogenic	9	285	2	2	96

had a mean agreement with consensus in this test of 87% and contributed 12% of the urease data in the tables, but accounted for 47% of the total disagreements. The remaining 10 laboratories had a mean agreement of 98.0%. For only one strain (25169) was there a mean agreement of less than 90%.

The data on pigment are based on an average total of 33 replications per strain. One of the 13 laboratories performing this test had a mean agreement score of only 78% and contributed only 7.7% of the data on this test, but accounted for 25.6% of the total disagreements. The strains that, on consensus, were nonchromogenic accounted for only about half as many disagreements, proportionately, as did the consensus photochromogens or scotochromogens (Table 4).

The data on growth on 5% NaCl are based on an average total of 34 replications per strain. Three laboratories contributed 25% of the data,

but accounted for 72% of the total disagreements. The remaining 10 laboratories had a mean agreement of 97.1%. The mean agreement with consensus was less than 90% for seven strains.

The data on hydrolysis of Tween 80 are based on an average total of 38 replications per strain. The criterion for a positive reaction in this test was originally the appearance of a color change after 5 days of incubation (8). However, it was found that when the cutoff date was selected at 10 days instead of 5, nine strains yielded higher mean agreement scores, and only two had lower scores. The overall mean agreement was 5.7% higher when the criterion for positivity was a color change after 10 days (Table 2). Only one strain, 25275, yielded highly variable results by either criterion. At 10 days, this strain contributed only 5.8% of the data for this test, but accounted for 53% of the disagreements. On the other hand,

eight strains were 100% positive by day 10 and two strains were negative in 100% of the reports at this time. The mean agreement with 10-day consensus was less than 90% for only three strains.

The data on β -galactosidase are based on an average total of 13 replications per strain. One laboratory, which contributed 15% of the data on the test, had a mean agreement of only 56% and accounted for 67% of the disagreements. The remaining five laboratories had a mean agreement of 96.1%. The mean agreement with consensus was less than 90% for nine strains.

Because of the limited number of strains employed, taxonomic inferences may not be made from the results of this study. Furthermore, the fact that a test yielded poor reproducibility cannot be taken as indication that the test is unsatisfactory; one may not even conclude that the particular detailed technique was not optimal for the given character. The only definitive positive statements that can be made are those concerning tests that did yield very producible results in a number of participating laboratories.

There are a number of possible sources of disagreement (error?) in a study of the kind reported here. For convenience, they may be classified into the following four types.

Type 1 consisted of labeling errors and cross-contamination of cultures. Such errors are obviously not inherent in the tests themselves. On examination of distribution and reproducibility of errors in tests as straightforward as pigment production, there is reason to believe that such errors did occur and contribute to lowering mean agreement scores. Therefore, many of the tests in the study are probably more reliable than they appeared in this analysis.

Type 2 consisted of deviations in performance of the test due either to inexperience with the test procedure or careless errors in performance. Some participants were experienced in performing most of the tests, and others were performing certain tests for the first time. The simpler tests are straightforward enough that experience plays only a small role in performance, whereas others require more skill. In general, young, actively growing bacteria should be used for all tests.

Type 3 consisted of variability in certain strains or species. Many of the tests included in this study had mean percent agreement scores too low to permit them to meet the rigorous criteria for inclusion in this report, but did show very high resolving power between certain pairs of strains. In those cases, the low mean

percent agreement suggests that some strains (or probably some species) are characterized by a high degree of variability in the test in question. This could reflect, for example, different degrees of uniformity in permeability and/or rates and conditions of enzyme induction. Other species may be very uniform in response to the given test. It is probably unrealistic to hope to find a series of tests (and techniques and conditions for performing them) that will provide uniform results for all species in the genus *Mycobacterium*. Thus, a given test may represent information in some cases and noise (6) in others, and application of a carefully assembled series of tests could provide enough information for a consistent numerical taxonomic analysis of the genus.

Type 4 consisted of variability inherent in the technique due to unknown and uncontrolled factors that affect test responses of most or all organisms. Basic studies are needed into the mechanisms of the various tests to at least provide an understanding of the reasons for variability of some strains or species.

The analyses of the five tests described in detail in this report were based on between 240 and 689 bits of data per test. All five of them had a mean reproducibility of 90% or better. On the basis of a mathematical model, Sneath and Johnson (5) have concluded that a probability of error exceeding 10% for a test is unacceptably large for numerical taxonomic use. In view of these criteria and of the demonstrated differential power of these tests in prior cooperative studies, it is recommended that the five tests described in this paper be included in a list of minimal standards for members of the genus *Mycobacterium* in accord with the proposed revision of the International Code of Nomenclature of Bacteria (4).

Nineteen tests, in addition to the five described, had mean agreement scores equal to or greater than 90% but failed to meet other criteria for inclusion in this report. Some of the tests might have been included if more replicate data were available. In other cases, it appeared that the restricted selection of organisms tested excluded tests that might be highly definitive for a single species (e.g., niacin test). Future studies are planned, or have been initiated, in which broader representation of cultures, greater replication, and some modifications in test procedures will be assured. It is anticipated that a battery of highly reproducible tests will be defined which will permit greater confidence to be placed in numerical taxonomic analysis of mycobacteria than has been possible to date. Furthermore, availability of such standardized

tests should permit more efficient identification of individual isolates and thus contribute to diagnosis and to our knowledge of epidemiology of mycobacterial disease.

Some of the participants in this study objected to certain details of the techniques presented in this report, primarily on the basis of practicality of performance of certain procedures in routine laboratory use. Specifically there were objections to the need for weighing inocula and for a 16-h incubation period in the urease test. It is anticipated that modifications will frequently be proposed for test performance, especially for those tests that are most useful for routine determinative studies in clinical practice. The five techniques described here can serve as standards against which such modifications can be evaluated before adoption in a given laboratory. It should be mentioned that the IWGMT has initiated a study to determine comparability of turbidimetric and gravimetric standardization of inoculum suspensions with a variety of mycobacteria.

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REPRINT REQUESTS

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