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Diagnostic Key to Mycobacteria Encountered in Clinical Laboratories

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A diagnostic key has been developed which will permit identification of most mycobacteria encountered in clinical laboratories. The key is based on performance of a few simple tests. The efficiency and accuracy of the key was evaluated in terms of correlation between identifications based on the few tests and those arrived at through application of the techniques of numerical taxonomy, which involves a large battery of tests. Of 679 cultures of mycobacteria other than *Mycobacterium tuberculosis*, 86.5% were correctly identified by use of the key, and only 1.8% of the cultures were erroneously identified. The remaining cultures required further examination.

Although the mycobacteria have been studied extensively for well over half a century, in the past few years the slow-growing members of the genus *Mycobacterium* have been re-examined systematically and a number of new species have been defined. The detailed definition of species (the taxonomic phase) was necessary before it was possible to evaluate the relative efficiency of tests which could be used for identification of individual cultures (the determinative phase) (15). The intensive studies of these problems in recent years were largely the result of the recognition that a number of mycobacteria other than *M. tuberculosis* and *M. bovis* are capable of causing pulmonary diseases in man. These diseases closely resemble classical tuberculosis, but the differential diagnosis is most important, because most of these other mycobacteria are resistant to the chemotherapeutic agents which are effective against tuberculosis. The early studies of Runyon and his co-workers provided a systematic basis for recognizing some of these organisms (7, 12). Subsequent detailed analyses of the genus by means of the techniques of numerical taxonomy have provided further information on the composition of the four Runyon groups and have permitted definition of a number of species and subgroups possessing different degrees of clinical significance (4, 16, 17, 20).

Further application of numerical analysis to the problem has made it possible to evaluate the reliability of a number of tests which might be used for identification of acid-fast bacilli encountered in clinical laboratories. From these analyses, it has been possible to develop a diagnostic key for identification of these organisms and to present a mathematical statement of the efficiency and accuracy of the key. This key is designed to yield a useful identification of the majority of cultures encountered, and to direct attention to those cultures which will require special efforts for identification.

MATERIALS AND METHODS

The tests to be described were performed on 679 cultures, representing a broad variety of mycobacterial species, which are maintained in the culture collection in this laboratory.

Niacin test. The test is employed in the modification described by Runyon et al. (9). A primary culture or subculture on Lowenstein-Jensen egg medium is employed. It is essential to have plentiful growth; a culture containing only a few colonies may give a false-negative reaction. The surface of the culture is flooded with 0.5 ml of sterile water. The niacin occurs in the medium as well as in the colonies. After 5 min of extraction at room temperature, the water is removed to a small test tube. To this is added 0.5 ml of a 4% solution of analine in 95% ethyl alcohol, and the contents of the tube are mixed. A 0.5-ml amount of a 10% solution of cyanogen bromide in water is added, and the mixture is observed for development of a yellow color, which indicates the presence of niacin. This test must be performed in a hood and a rubber bulb must be used for pipetting of reagents, as the cyanogen bromide produces extremely noxious vapors.

Tween hydrolysis (21). This test is performed by suspending a large loopful of actively growing bacilli from a slant into a tube containing 4 ml of a sterile (autoclaved) solution of 5 mg of Tween 80 per ml and 20 μg of neutral red per ml in 0.067 M phosphate buffer at *p*H 7.0. This preparation is amber to yellow, and hydrolysis of the Tween 80 by the mycobacteria

causes the solution to turn pink. The suspension is incubated at 37 C and examined daily for the first evidence of the shift to a pink color. If this occurs within the first 5 days, the test is recorded as positive. If the color change occurs between the 5th and 10th days of incubation, the test should be repeated. Suspensions which have failed to change color within 10 days are recorded as negative reactors. It is important that the original solution contain the full 20 μg of neutral red dye per ml; i.e., correction should be made for dye content of individual batches employed, as recorded on the labels. It is also important to store this reagent in the cold and protect it from light. During the 37 C incubation, the tubes should also be protected from light to minimize spontaneous decolorization of the neutral red.

Nitrate reduction (*19*). This test is based on quantitative estimation of the amount of nitrite produced when a culture is grown to a preselected optical density in liquid media containing sodium nitrate. Dubos broth base is prepared from commercial dehydrated medium to which is added 0.01 M sodium nitrate. The base is autoclaved and cooled; Dubos medium albumin is added, and the medium is distributed aseptically in 7-ml amounts to sterile screw-capped test tubes (16 × 125 mm). Three drops of a barely turbid suspension of the test organism are inoculated to a tube of liquid medium, and the optical density is determined at 580 mμ. The culture is incubated at 37 C; three times a week the culture is shaken, and the optical density is determined. When the optical density reaches 0.10, a 1.5-ml sample is removed and added to 5.0 ml of a 0.006% solution of sulfanilamide in 0.1 N hydrochloric acid; then 0.5 ml of 0.02% *N*-(1-naphthyl)ethylenediamine dihydrochloride is added, the solution is mixed, and the optical density of the solution is determined at 530 mμ in a 16-mm diameter cuvette. In our laboratory, an optical density of 0.30 or higher represents a positive reaction in this test. Many organisms produce smaller amounts of nitrite which yield a weaker pink color in this test (i.e., optical density less than 0.30), but for purposes of this key these are considered negative. The criterion for a positive reaction is production of more than 14 mμmoles of nitrite per ml. Laboratories intending to use this test should set up their own standard curves to establish the optical density at this end point.

Catalase. This test is a semiquantitative measure of catalase activity of mycobacteria (14). A 5-ml amount of Lowenstein-Jensen medium is inspissated in screw-capped tubes (16 × 150 mm) in an upright position, i.e., *not* slanted; the surface is inoculated with a drop of barely turbid aqueous suspension of the organisms, and the culture is incubated at 37 C for 14 days. Grossly visible growth should be evident by this time. A 1-ml amount of a mixture of equal parts of 10% Tween 80 solution and 30% hydrogen peroxide is pipetted to the tube, which is then allowed to stand exactly 5 min in a hood. The height, in millimeters, of the column of foam produced is recorded. Cultures which produce over 45 mm of foam are considered strongly reactive, and are thus distinguished from cultures which produce less than 45 mm of foam.

Aryl sulfatase (*13*). In this diagnostic key, the 3-day aryl sulfatase test on solid medium is employed. The medium is prepared by incorporating 1 ml of glycerol and 65 mg of the tripotassium salt of phenolphthalein disulfate in 100 ml of melted Dubos oleic agar base. This medium is distributed in 2-ml amounts to screw-capped vials (18 × 60 mm), autoclaved, and permitted to harden in an upright position. A drop of barely turbid suspension of the organism to be tested is inoculated to the surface of the agar, and the culture incubated at 37 C for 3 days. A 1-ml amount of 1 M sodium carbonate is then added, and the tube is observed for development of a pink color indicative of release of free phenolphthalein.

Pigmentation. Two tubes of Lowenstein-Jensen egg medium are seeded with a few drops of a barely turbid suspension of the culture to be examined. The cultures are incubated at 25 to 30 C. One of the pair of cultures is exposed to continuous light while growing, and the other is placed in an opaque container. The cultures are examined twice a week, and when definite colonies are visible the color of the growth on both tubes is recorded. If both tubes exhibit yellow or orange colonies at this point, the organism is recorded as being able to produce pigment in the dark, i.e., it is scotochromogenic. If the culture which was grown in the light is brilliant yellow, but the culture in the opaque box is white or buff, the organism is photochromogenic. This should be confirmed by removing the latter culture from its opaque container and exposing it to continuous light overnight. If these colonies become brilliant yellow within 48 hr after such exposure, their photochromogenicity is confirmed. It is important that this confirmatory test be performed on young cultures 3 weeks or less in age, and that the caps on the culture tubes not be too tight, as limitation of aeration inhibits the pigment production (18). For the purposes of this analysis, only a brilliant yellow color is acceptable for definition of the Runyon group I photochromogens. Occasionally, some cultures produce a pink or coral color under the influence of light, but these colors are not characteristic of *M. kansasii* or *M. marinum* (*balnei*), the two significant photochromogens.

Iron uptake. This is a test based on a variation of the method of Szabo and Vandra (11). A slant of Lowenstein-Jensen egg medium is inoculated with a drop of barely turbid aqueous suspension of the organism and incubated at 37 C until definite growth has appeared. To the slant are then added a few drops of a sterile (autoclaved) solution of 20% aqueous ferric ammonium citrate solution. For a slant which contains 4 ml of Lowenstein-Jensen medium, we employ four drops of this solution. If commercially prepared egg medium slants are used which contain more than 4 ml of medium, then proportionately greater amounts of ferric ammonium citrate solution should be used. The cultures are reincubated for a maximum of 21 days. The appearance of a rusty brown color in the colonies and a tan discoloration of the medium is recorded as a positive reaction.

Susceptibility to isoniazid (*INH*) *and thiophene-2-carboxylic acid hydrazide* (*T2H*) (*3*). Dubos oleic agar

is prepared from commercial Dubos oleic agar base and Dubos oleic albumin complex according to the manufacturer's instructions. One batch is prepared to contain 1 μg of INH per ml; a second batch, to contain 10 μg of T2H per ml; and a third, to contain no drug and to serve as the control. The media are dispensed in 4-ml amounts, aseptically, to 15-ml sterile prescription bottles, and are slanted. The organism to be tested is suspended in sterile water to a barely turbid concentration and then diluted 1:1,000 in sterile water. Three drops of this 1:1,000 suspension are inoculated to one tube each of the INH, the T2H, and the control medium, and the slants are incubated at 37 C. The time when definite growth is observed on the control slant is recorded, and the two drug-containing slants are maintained for an additional 3 weeks, or until definite growth has appeared. The organism is recorded as resistant if the growth on the drug-containing medium is greater than 1% of the growth on the control. This can usually be determined readily when one assumes that the growth in the control tube, even if confluent, does not contain over 10^4 organisms, and usually contains considerably fewer than this number when the inoculum is prepared as described.

RESULTS

The use of the diagnostic key is based on comparison of results obtained on the unknown organism, with the patterns indicated in Table 1. In preparing the table, correlations were made between the patterns obtained with the tests described above and patterns obtained with a larger series of 38 characters employed in taxonomic studies essentially like those described in an earlier publication (17). An organism was assigned to a given species or group if it matched the hypothetical median strain pattern for that species or group at a level in excess of 81% in the large series of tests. Approximately 4% of the cultures in our collection could not be so assigned, and these are included in a heterogeneous group of unclassified organisms.

After analysis of the distribution of properties within the various species and groups, a selection was made of tests which were simple to perform, yielded most consistent results, and provided greatest differential power. These are the tests described in the preceding section. The expected pattern in these selected tests was then coded for each species, as either positive (+), negative (−), or, in certain cases, as not important for identification of members of the particular group, and so to be disregarded (D) in the analysis. All organisms which fitted one of the key patterns *perfectly* were assigned to the appropriate species. It was then possible to determine the degree of correlation between identification made by the short diagnostic key, and that made by use of the extended, 38-test, taxonomic screen. This correlation could be expressed in terms of efficiency (the proportions of members of a group that were recognized by use of the key) and accuracy (the proportions of cultures that were correctly and incorrectly assigned to a given group by the use of the key).

The results of this analysis are presented in Table 1. Because the key was designed to provide information for clinical use, with a minimum of effort in the unspecialized laboratory, certain species with similar properties and clinical significance are grouped together. In most cases, this is sufficient to permit use of the data in clinical diagnosis. If the bacterial identifications were needed for epidemiological purposes, more detailed information would be required, and such studies would best be performed in special reference or research laboratories.

In preparation of the key, cultures of *M. tuberculosis* were excluded on the basis of slow growth, lack of pigmentation, and a strongly positive niacin reaction. All mycobacterial cultures which failed to meet these criteria were included in the analysis.

The identifications achieved by application of the diagnostic key to 679 mycobacterial cultures other than *M. tuberculosis* are presented in Table 1. Of 600 identifications which were made by this means, only 12 were in disagreement with the identity ascribed to the cultures in the larger taxonomic study. Among the 79 cultures (11.6%) which did not fit one of the key patterns exactly, 49 (7.3% of the total examined) could be assigned to a species or group if a large series of tests was employed. That is, these were deviant strains of established species. The remaining 30 cultures did not fit any of the taxonomic units under consideration at the 81% level of similarity.

On the basis of these observations, the following procedure is proposed for routine application in diagnostic laboratories which isolate mycobacteria from clinical materials:

(i) Perform a niacin test on all acid-fast isolates, and note the rate of growth and color of the colonies. If the cultures require over 1 week for growth, are white to buff in color, and yield a distinctly positive niacin test, they may be reported as *M. tuberculosis*.

(ii) Subject all mycobacterial cultures which fail to meet the criteria for *M. tuberculosis* to the tests listed in Table 1.

(iii) Record the results obtained for a given culture with these tests in a form similar to that of Table 1 and compare the pattern to each of the tabulated patterns. If the unknown culture matches perfectly with one of the group or species patterns (disregarding those tests where a

TABLE 1. *Diagnostic key to mycobacteria other than Mycobacterium tuberculosis*[a]

Test or property	Runyon group											
	—	I		II		III			IV			—
	M. bovis	*M. kansasii*	*M. marinum*	"Tap water"	"Scrofula"	Battey *-avium* complex	*M. terrae* complex	*M. gastri*	*M. fortuitum*	*M. flavescens*	*M. phlei, smegmatis, rhodochrous*	Miscellaneous unclassified
Niacin	−	−	−	−	−	−	−	−	−	−	−	?
Tween hydrolysis within 5 days	D[b]	+	+	+	−	−	+	+	D	+	D	?
Nitrate reduction (>14 mμmoles of nitrite produced/ml)	D	+	−	−	−	−	+	−	+	+	+	?
Catalase highly reactive (> 45 mm of foam)	−	D	−	+	+	−	+	−	D	+	D	?
Aryl sulfatase agar positive in 3 days	−	D	D	D	D	D	D	D	+	D	−	?
Pigment produced in dark	−	−	−	+	+	−	−	−	−	+	D	?
Yellow pigment stimulated by light	−	+	+	D	D	−	−	−	−	D	D	?
Iron uptake (rusty colonies)	−	−	−	−	−	−	−	−	+	−	+	?
Resistant to 1 μg of INH per ml	D	+	+	+	+	+	+	−	+	−	+	?
Resistant to 10 μg of T2H per ml	−	+	+	+	+	+	+	+	+	+	+	?
No. of strains correctly identified	6	56	2	86	72	163	48	32	60	19	44	—
No. of strains wrongly identified	0	0	0	3	3	2	1	0	3	0	0	—
No. of strains not identified	0	4	2	9	8	9	9	1	5	1	1	30

[a] To be identified by use of the key alone, an organism must fit one of the listed patterns perfectly. Relative efficiency and accuracy of the key are indicated in terms of actual numbers of cultures which were correctly identified, incorrectly identified, or which could not be identified by use of the key.

[b] Disregard properties coded "D" when matching the properties of an unknown organism to a given species pattern.

"D" is indicated in the key), the identification is considered completed.

(iv) If the pattern of the unknown culture fails to match any of the key patterns, repeat all of the tests, and attempt to match results again, as in step iii. If, after the second attempt, a match is not achieved, the culture will require special tests which may require advice or services of special reference laboratories or consultants.

A brief discussion of the significance of the various identifiable groups follows.

M. bovis. This organism is rarely seen in the United States, but is still a significant pathogen in many parts of the world. When isolated from a clinical specimen, it is considered etiologically significant.

M. kansasii. In establishing the identity of a member of this species, the catalase results were disregarded in the diagnostic key. In terms of clinical significance, however, the catalase test is important. Most isolates of *M. kansasii* are considered to be etiologically significant in pulmonary disease and exhibit strong catalase activity (over 45 mm of foam). Occasionally strains are seen with low catalase activity, and these do not appear to be significant clinically (14).

Occasional strains of mycobacteria may produce a pink or coral pigment upon exposure to light. These are not *M. kansasii*, because *M. kansasii* strains turn bright yellow.

M. marinum. This organism (also known in the past as *M. balnei*) exhibits the same photochromogenic pattern as *M. kansasii*. *M. marinum*, however, when first isolated from clinical material, grows at 25 C but not at 37 C. It is the cause of skin lesions ("swimming pool granuloma") but has not been implicated in pulmonary disease (6). It is likely to be missed entirely if primary isolation cultures are incubated at 37 C only. On repeated subculture in the laboratory, some strains may acquire the ability to grow at 37 C.

Group II scotochromogens. This group presents the greatest difficulty in interpretation and, as can be seen in Table 1, the greatest proportion of failures and of errors in identification. In general, group II organisms of the "tap water" subgroup have rarely been implicated conclusively as pathogenic agents (4, 20). The members of the "scrofula" subgroup (also known as *M. marianum* and *M. scrofulaceum*), on the other hand, are frequently, but not always, associated with disease processes, especially cervical adenitis in children.

Group III Battey-avium complex. M. avium and the so-called Battey bacilli are very similiar (16), although some investigators prefer to apply a separate species designation, *M. intracellulare*, to the Battey organisms (8). In the United States, most human infections by members of this complex are caused by the Battey bacilli, although human infections caused by avian bacilli which are virulent for birds are known to occur in other countries (1). For purposes of clinical diagnosis, it is probably sufficient to identify these organisms as members of the Battey-*avium* complex, although more definitive studies, e.g., serotyping (2, 10), would be needed for epidemiological studies.

A related organism, *M. xenopei*, is commonly seen in Europe and the British Isles, and may be associated with disease (5). It has been seen only rarely in the United States. *M. xenopei* is sensitive to 1 μg of isoniazid per ml, and, although colonies frequently have no pigment when very young, they tend to become yellow, even in the dark, much sooner than is seen with some strains of Battey bacilli. They do not grow at 25 C, and they grow better at 45 C than at 37 C. We have not studied this species sufficiently to include it in the present diagnostic key.

Group III, other species. It must be stressed that "Runyon group III" is *not* synonymous with the "Battey-avium complex." The latter are pathogenic members of this group, but there are other species which meet the definition of group III (nonphotochromogenic, slow-growing, niacin-negative mycobacteria). These include members of the *M. terrae* complex (16, 17), as well as *M. gastri* (16). *M. terrae* and *M. gastri* are not generally considered to be implicated in human disease.

Group IV, rapid growers. The diagnostic key provides for division of group IV into three categories. Of these three, only *M. fortuitum* has been implicated in human pulmonary disease. It is important to remember, however, that *M. fortuitum* is a common soil organism which may be isolated fairly frequently from sputum specimens without necessarily being implicated in a disease process. Only occasionally is it possible to assign etiological significance to this species.

Members of the second category in group IV, *M. flavescens*, have not been implicated in human disease; nor have members of the third category, which is composed of *M. smegmatis*, *M. phlei*, and *M. rhodochrous*.

DISCUSSION

The values describing the efficiency and accuracy of this diagnostic key are dependent on performance of the tests exactly as described, and on the actual composition of the culture collection examined. Thus, because the authors are engaged in a research activity, it is likely that a relatively high proportion of cultures in this collection represent problem organisms which were

submitted for reference purposes. Depending on geographic location, and on the nature of the patient population submitting specimens to a given diagnostic laboratory, the proportions of members of the different groups and the overall efficiency of the key will vary among laboratories.

It is possible that some laboratory workers would prefer to use a different modification of a test from the one described. Whenever this is done, for use with this key, it is essential that correlation be made between the two modifications to assure that the results are comparable and the key applicable.

The incorporation of a property into the key was decided by balancing the effects that the inclusion of the property would have on both efficiency and accuracy. The choice was weighted in favor of accuracy. Thus, 11.6% of the cultures could not be identified by use of the key alone, but the investigators were made aware that further work on these cultures was needed. On the other hand, "definite" identifications were made in error on only 1.8% of the 679 cultures examined. The proportion of *significant* errors was even lower, because 3 of the 13 errors involved assigning the name of one nonpathogen to a culture that actually belonged to another nonpathogenic species. Of the remaining nine errors, four involved confusion between intermediate forms of the "Battey-*avium*" and the "scrofula" groups, and three involved confusion between *M. fortuitum* and other group IV mycobacteria. In one case, a rare form of nonpigmented *M. kansasii* was incorrectly identified as *M. terrae*, and in another a tap water scotochromogen was erroneously identified as a scrofula variety. In all, then, for only 1.3% of the cultures examined was an identification made which could have contributed to a clinical diagnostic error.

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