Simple Amidase Test for Identification of Mycobacteria

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A modified amidase test for differentiation of mycobacteria is described. A total of 224 atypical mycobacteria, 154 Mycobacterium tuberculosis, and 26 M. bovis strains were classified by this procedure. Of the 404 strains of various species studied, 400 exhibited an amidase spectrum identical to the established pattern. The simplicity of this method may promote its application in routine examinations.

In 1961, Böncke introduced the amidase test for the identification of Mycobacteria (2). The method was well received but not widely applied, due to its tediousness. It required the weighing of large amounts of bacteria and their homogenization. After repeated washings of microorganisms, they were resuspended in phosphate buffer to yield a bacterial density of 10 mg (wet weight) per milliliter. During the preparation and distribution of the heavy suspension, a considerable portion of the microorganisms adhered to the glassware, jeopardizing the reliability of the test.

Other shortcomings of the method are the cumbersome preparation of the hypochlorite reagent from chlorine of lime and the laborious estimation of ammonia. The latter requires heating the bacteria-containing tubes at 100°C for 15 to 20 min, which is somewhat difficult under the prevailing safety precautions.

As the method of Böncke is a valuable diagnostic tool, an effort was made to modify it by omitting the preparation of the heavy bacterial suspension, by utilization of a commercially available hypochlorite solution, and a simpler procedure for the determination of ammonia.

MATERIALS AND METHODS

Buffer-substrate solution. Preparation of Sörensen phosphate buffer (pH 7.2) involved two solutions: (i) 5.96 g of Na₂HPO₄, dissolved in 630 ml of distilled water, and (ii) 2.27 g of KH₂PO₄, dissolved in 250 ml of distilled water. Then 540 ml of solution i was added to 210 ml of solution ii. The final pH was checked using a pH meter. The buffer solution was transferred in 40-ml volumes into 125-ml Erlenmeyer flasks and sterilized at 68 kg of pressure for 15 min.

Table 1 shows the weight of various amides added to 100 ml of distilled water in order to obtain 0.00164 M substrate solutions. The amide solutions 1, 2, 5, 6, 8, and 9 were dispensed in 5-ml quantities into screw-capped tubes (25 by 125 mm) labeled with corresponding numbers. The tubes were sterilized at 6.8 kg of pressure for 15 min. Urea was sterilized by filtration and transferred in volumes of 5 ml into sterile tubes marked "3." Prior to performing the test, 5 ml of the Sörensen phosphate buffer was added aseptically to the respective tubes containing the substrate solution.

Cultures. The 224 atypical mycobacterial strains studied were received from the Borstel Institute, Germany, the Trudeau Institute Inc., Saranac Lake, N.Y., the National Jewish Hospital, Denver, Colo., and the Centre for Disease Control, Atlanta, Ga. The 154 Mycobacterium tuberculosis and the 26 M. bovis strains used were either submitted by Canadian hospitals for identification or belonged to our stock cultures.

Culturing. In general, the organisms were grown on Löwenstein-Jensen medium for 7 to 12 days. Dysgonic mycobacterial strains needed an incubation period up to 30 days, whereas rapid growers required only 3 to 4 days.

Decantation of supernatant. Figure 1 demonstrates the apparatus used for decantation of the supernatant. Two 2-liter side-arm flasks (C and D) were joined by a rubber tubing. Flask C was connected to the barrel of a Mantoux syringe (A), having a 15-gauge needle, 15 cm in length. When not in use the needle was dipped in a test tube. The connecting rubber tubing was provided with a pinch-cock (B) to shut off the airflow when not required. About one-quarter of both the flasks was filled with 5% phenol solution. The tips of the glass tubes in flasks C and D were immersed well within the disinfectant solution. The side arm of D was connected to a small electric pump through a glass tube (E) filled with cotton. By operating this system, the supernatant from the bacterial suspensions could be easily removed with a considerable saving of time. Cross contamination of the strains was avoided by flame sterilization of the needle between consecutive tests.

Method. To each of the six small screw-capped tubes labeled 1, 2, 3, 5, 6, and 9, and containing 4 ml of sterile physiological saline, one loopful (diameter 3 mm) of bacteria was suspended and centrifuged for 10 min at 2,000 rpm. After removal of the supernatant using the apparatus described above, the sediment was washed in approximately 4 ml of saline delivered
AMIDASE TEST FOR MYCOBACTERIA IDENTIFICATION

Table 1. Quantity of amide dissolved in 100 ml of water to prepare substrate solution

<table>
<thead>
<tr>
<th>Substrate solution no.</th>
<th>Amide</th>
<th>Weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acetamide</td>
<td>9.68</td>
</tr>
<tr>
<td>2</td>
<td>Benzamide</td>
<td>19.85</td>
</tr>
<tr>
<td>3</td>
<td>Urea</td>
<td>9.84</td>
</tr>
<tr>
<td>5</td>
<td>Nicotinamide</td>
<td>20.00</td>
</tr>
<tr>
<td>6</td>
<td>Pyrazinamide</td>
<td>20.20</td>
</tr>
<tr>
<td>8*</td>
<td>Allantoin</td>
<td>25.90</td>
</tr>
<tr>
<td>9</td>
<td>Succinamide</td>
<td>19.00</td>
</tr>
</tbody>
</table>

*Substrate 8 is only used in identification of M. fortuitum.

FIG. 1. Device for decantation of supernatant.

into the tubes by a sterile plastic syringe. The washing fluid was subsequently removed by centrifugation and suction.

One milliliter of the buffer-substrate solution 1, 2, 3, 5, 6, or 9 was added to the bacteria sediment in the tube bearing the corresponding number. Tube 8 was only used to confirm identification of M. fortuitum. The tubes were incubated overnight (approximately 18 h) at the optimal temperature. The ammonia produced was then determined by adding 0.5 ml of a 20% solution of crystalline phenol in absolute alcohol (11), followed by 1 ml of 5% sodium hypochlorite solution (Fisher SO-S-290). The development of a blue color after 5 min indicated the presence of ammonia and was recorded as a positive reaction.

RESULTS

The amidase activity of various mycobacterial strains is delineated in Table 2. Of the 14 M. kansaii strains, 13 gave positive results with urea and nicotinamide, which is characteristic of this species.

However, one aberrant strain was only urease positive even on repeating the test three times. The two M. marinum strains of the photochromogen group were identified by their positive reaction with pyrazinamide in addition to urea and nicotinamide. Among the seven M. scrofulaceum strains, six were active against urea, nicotinamide, and pyrazinamide, while one (similar to M. gordonae) exhibited negative results with all the amides employed. Of the 23 M. gordonae strains, 22 gave negative reactions in the amide series, whereas one resembled M. scrofulaceum in its amidase spectrum.

From the 143 strains of Runyon group III, 142 followed the normal pattern. A deviation was noticed in one of the M. avium strains obtained from the Borstel Institute, Germany. It demonstrated positive urease activity in two consecutively repeated examinations. Nine M. terrae strains examined in this study produced negative amidase tests as anticipated. However, strains encountered in this group may also give positive reactions with substrates 5 and 6 (nicotin- and pyrazinamide) as observed by others (4, 10, 12). The 35 "rapid growers" could be differentiated into M. smegmatis, M. phlei, and M. fortuitum on the basis of the amidase spectrum. M. fortuitum was distinguished from M. phlei by its positive reaction to allantoin and from M. smegmatis by its negative results with benzamide and succinamide.

In addition to the above-mentioned atypical mycobacteria, 153 wild strains isolated from tuberculosis patients and one reference strain (H37Rv) were investigated. These organisms exhibited amidase activity against urea, nicotinamide, and pyrazinamide and were confirmed as M. tuberculosis. On the other hand, 22 BCG strains obtained from local lesions and/or lymph nodes of freshly vaccinated individuals, as well as the four laboratory reference strains, were only urease positive and could be distinguished from the M. tuberculosis isolates.

DISCUSSION

Our modified procedure omits the preparation and transfer of heavy bacterium suspensions (10 mg/ml). A standard loop is employed for the distribution of the microorganisms in the saline solutions. The method retains the double washing of the strains, although a single one in 6 ml of saline solution and thorough decantation of the supernatant would be sufficient. In daily routine, however, the double washings of the microorganisms would seem to be easier than a complete removal of the supernatant. Moreover, one may be sure that a possible small residual washing fluid will not influence the outcome of the test. As the resuspension of the bacteria with a syringe and decantation by means of the designed device is not time consuming, preference was given to double washing.
The revised method has been tested on 224 atypical mycobacteria, 154 *M. tuberculosis*, and 26 BCG strains. With the exception of four, all strains demonstrated the anticipated amidase pattern (2, 4). Two of the strains exhibiting a deviating amidase reaction belonged to the scotochromogen group. It is known that Bönicek did not differentiate between *M. scrofulaceum* and *M. gordonae* (aqua) but subdivided group II into urease positive (IIa) and negative (IIb). Studies on *M. scrofulaceum* as a distinct species of group II (5, 14) resulted in the investigation of its amidase spectrum. In our study, 6 *M. scrofulaceum* and 22 *M. gordonae* strains demonstrated the same amidase patterns as reported by Meissner (9), Zykov et al. (16), and Dionne et al. (4). On the other hand, as noted in Table 2, one strain labeled as *M. scrofulaceum* and another labeled *M. gordonae* evinced a reversed amidase spectrum. It is probable that some difficulty still prevails over the characterization of these species (8, 15), particularly in routine laboratory diagnosis, based solely on Tween 80 hydrolysis which may yield indefinite results (7).

For differentiation of *M. tuberculosis* and *M. bovis*, including BCG strains, both the niacin and amidase tests should be carried out. This is because some human strains may give a weak positive result in the niacin test, resembling that occasionally observed with BCG strains (2, 3). On the other hand, pyrazinamidase-resistant human tubercle bacilli may show markedly reduced nicotinamidase and pyrazinamidase activity similar to the bovine strains (6). In addition to these tests the nitrate reduction and the thiophen-2-carboxylic acid hydrazide test of Bönicek (1) should also be performed for confirmation of the results.

The simplification of the amidase test might well promote the application of this valuable method. In routine laboratory practice, the amidase test, employing substrates 3, 5, and 6 for slow growers, and 2, 3, 5, and 6 (8 when required) for group IV, could complement the published results of mycobacteria set forth in the diagnostic key of Wayne and Dubek (13).

**LITERATURE CITED**


