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## An Improved Reagent for Mycobacterial Nitrate Reductase Tests

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A new crystalline reagent for nitrate reductase tests was compared with standard liquid reagents on 437 strains of mycobacteria. The results for isolates of *Mycobacterium avium* complex, *Mycobacterium kansasii*, *Mycobacterium gordonae*, *Mycobacterium scrofulaceum*, *Mycobacterium fortuitum*, and *Mycobacterium chelonae* agreed 100% with the expected results. Of the 177 *Mycobacterium tuberculosis* isolates, 4 were negative by the conventional method. Two of these four isolates were positive with the new reagent. Of the positive nitrate tests carried out with liquid reagents, 42% flashed instantly or faded in color; none of the tests carried out with the new crystalline reagent flashed or faded. A stronger color reaction was seen for 28% of the positive tests with the new reagent.

The nitrate reductase test is one of a battery of biochemical tests used to identify members of the genus *Mycobacterium* (5). In conjunction with other tests, nitrate reductase is useful to distinguish *Mycobacterium szulgai* from *Mycobacterium scrofulaceum* and *Mycobacterium gordonae*, *Mycobacterium kansasii* from *Mycobacterium marinum*, *Mycobacterium fortuitum* from *Mycobacterium chelonae*, and *Mycobacterium tuberculosis* from *Mycobacterium simiae* and *Mycobacterium bovis*. However, the test is not without problems. Reagents used in the conventional test have a short shelf life and must be prepared in-house. The test can be difficult to read because the positive reaction color can flash instantly or quickly fade (6). Commercially available paper strip reagents (2) have a longer shelf life, but they usually are positive only for mycobacteria with strong nitrate reductase activity (3, 4).

Recently, Lampe (1) described a crystalline reagent that has a long and stable shelf life and that was used to detect nitrate reductase. Although reliable results were obtained with 135 bacterial strains, only 8 strains of mycobacteria, representing four species, were tested. In addition, the method used in that study differed from the conventional mycobacterial nitrate reductase test. The purpose of this study was to use conventional mycobacterial nitrate reductase test procedures to compare the crystalline and liquid reagents.

### MATERIALS AND METHODS

**Mycobacteria used.** The Mycobacterial isolates used consisted of stock cultures and clinical isolates. Mycobacteria recovered from clinical specimens (*M. tuberculosis*, *M. kansasii*, *M. gordonae*, *M. scrofulaceum*, *M. avium* complex, *M. terrae*, *M. trivale*, *M. fortuitum*, *M. chelonae* subsp. *chelonae*, and *M. chelonae* subsp. *abscessus*) were identified by standard methods (3, 4). Reference cultures of *M. tuberculosis*, *M. bovis*, *M. kansasii*, *M. gordonae*, *M. scrofulaceum*, *M. flavescens*, *M. szulgai*, *M. avium* complex, *M. terrae*, *M. trivale*, *M. nonchromogenicum*, *M. chelonae* subsp. *chelonae*, and *M. chelonae* subsp. *abscessus* were obtained from the Trudeau Mycobacterial Culture Collection (Curator, National Jewish Hospital, Denver, Colo.); College of American Pathologists, Proficiency Testing Program, Chicago, Ill.; the Centers for Disease Control, Atlanta, Ga.; and the Virginia Division of Consolidated Laboratory Services Stock Culture Collection, Richmond, Va. All cultures were maintained by subculturing on Lowenstein-Jensen medium. For nitrate reductase tests, isolates were grown in Middlebrook 7H-9 broth and inoculated onto Lowenstein-Jensen medium. Nitrate reductase activity for *M. fortuitum* and *M. chelonae* was tested after 2 weeks, for *M. tuberculosis* after 3 weeks, and for other mycobacteria after 4 weeks. *M. marinum* was incubated at 30°C, whereas other mycobacteria were incubated at 36°C. All cultures were incubated in 5 to 10% carbon dioxide atmosphere.

**Nitrate reductase test reagents.** The conventional liquid nitrate reagents are a 1:2 dilution of concentrated hydrochloric acid, a 0.2% solution of sulfanilamide in distilled water, and a 0.1% solution of *N*-(one-naphthyl)ethylenediamine dihydrochloride in distilled water. The new reagent described by Lampe (1) consists of one part sulfanilic acid, one part *N*-(one-naphthyl)ethylenediamine dihydrochloride, and 10 parts L-(+)-tartaric acid. All chemicals were obtained

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TABLE 1. Expected nitrate-positive mycobacteria

Species	Source	No. tested	No. (%) that test positive for the following reagents:	
			Conventional <sup>a</sup>	Powdered <sup>b</sup>
<i>M. tuberculosis</i>	Clinical isolates	166	162 (97.6)	164 (98.8)
<i>M. tuberculosis</i>	Reference cultures	10	10 (100)	10 (100)
<i>M. kansasii</i>	Clinical isolates	7	7 (100)	7 (100)
<i>M. kansasii</i>	Reference cultures	5	5 (100)	5 (100)
<i>M. fortuitum</i>	Clinical isolates	14	14 (100)	14 (100)
<i>M. fortuitum</i>	Reference cultures	2	2 (100)	2 (100)
<i>M. szulgai</i>	Reference cultures	3	3 (100)	3 (100)
<i>M. flavescens</i>	Reference cultures	3	3 (100)	3 (100)

<sup>a</sup> A total of 206 (98.1%) isolates tested positive with the conventional reagent.

<sup>b</sup> A total of 208 (99.0%) isolates tested positive with the powdered reagent.

from Sigma Chemical, Co., St. Louis, Mo. The chemicals were put in a dark bottle and were mixed by vigorous manual shaking about 30 times. The mixture had a heterogeneous crystalline appearance. The shelf life of the reagent was reported to be 6 months (1); our mixture was stable for 6 months, at which time the supply was exhausted.

**Nitrate reductase tests.** All mycobacteria were tested for nitrate reductase activity by using a slight modification of the conventional combined niacin-nitrate test (3, 4). Two milliliters of the conventional sodium nitrate solution was added to an actively growing mycobacterial culture that was then incubated at 36°C for 2 h. After incubation, a 0.5-ml portion of the liquid was withdrawn and placed in a sterile, screw-capped tube. With a spatula, a small amount of the crystalline reagent was added to the solution (the quantity of reagent was not critical). The test was read as positive when a pink to deep red color developed. The remaining 1.5 ml of substrate solution was used for the conventional niacin and nitrate tests. All positive reactions were compared with color standards (3, 4). A "flash in color" was defined as instantaneous color loss, and "fading" was defined as a gradual loss of color within 10 min. For either nitrate test, zinc powder was added to the tubes that developed no

color to confirm the negative test result. Isolates of *Mycobacterium* that had a nitrate reductase test result that differed from the expected result were retested.

## RESULTS

The results of nitrate reductase tests with the conventional and crystalline reagents are grouped according to the anticipated results (Tables 1-3). Table 1 lists the mycobacteria that were positive for nitrate reductase. The test results for isolates of *M. kansasii*, *M. fortuitum*, *M. szulgai*, and *M. flavescens* and reference cultures of *M. tuberculosis* agreed 100% with the expected results. Four clinical isolates of *M. tuberculosis* were negative by conventional reagent tests; of these isolates, two strains were positive when the crystalline reagent was used. The remaining two isolates were confirmed as nitrate reductase-negative *M. tuberculosis* by the Centers for Disease Control. The results of tests on nitrate reductase-negative strains are listed in Table 2. No mycobacteria were falsely

TABLE 2. Expected nitrate-negative mycobacteria

Species	Source	No. tested	No. (%) that test negative for the following reagents:	
			Conventional	Powdered
<i>M. avium</i> complex	Clinical isolates	70	70 (100)	70 (100)
<i>M. avium</i> complex	Reference cultures	10	10 (100)	10 (100)
<i>M. bovis</i>	Reference cultures	9	9 (100)	9 (100)
<i>M. marinum</i>	Reference cultures	12	12 (100)	12 (100)
<i>M. chelonae</i> subsp. <i>chelonae</i>	Clinical isolates	6	6 (100)	6 (100)
<i>M. chelonae</i> subsp. <i>chelonae</i>	Reference cultures	1	1 (100)	1 (100)
<i>M. chelonae</i> subsp. <i>abscessus</i>	Clinical isolates	8	8 (100)	8 (100)
<i>M. chelonae</i> subsp. <i>abscessus</i>	Reference cultures	3	3 (100)	3 (100)
<i>M. scrofulaceum</i>	Clinical isolates	5	5 (100)	5 (100)
<i>M. scrofulaceum</i>	Reference cultures	5	5 (100)	5 (100)
<i>M. gordonae</i>	Clinical isolates	60	60 (100)	60 (100)
<i>M. gordonae</i>	Reference cultures	2	2 (100)	2 (100)

TABLE 3. Nitrate-variable mycobacteria

Species	Source	No. tested	No. (%) that test positive with the following reagents:	
			Conventional <sup>a</sup>	Powdered <sup>b</sup>
<i>M. terrae</i>	Clinical isolates	26	18 (69)	20 (77)
<i>M. terrae</i>	Reference cultures	5	5 (100)	5 (100)
<i>M. triviale</i>	Clinical isolates	2	0 (0)	1 (50)
<i>M. triviale</i>	Reference cultures	2	2 (100)	2 (100)
<i>M. nonchromogenicum</i>	Reference cultures	1	1 (100)	1 (100)

<sup>a</sup> A total of 26 (72%) isolates tested positive with the conventional reagent.

<sup>b</sup> A total of 29 (81%) isolates tested positive with the powdered reagent.

positive for nitrate reductase when either the liquid or the crystalline reagents were used. Variable reactions were observed with members of the *M. terrae* complex (Table 3). Of 36 isolates, 26 (72%) gave a positive reaction with the liquid reagent, whereas 29 of 36 isolates (81%) were positive when the crystalline reagent was used.

Of the 226 tests that were positive with the conventional liquid reagent, 34 (15%) flashed and 60 (27%) faded in color, which made the test difficult to interpret 45% of the time (Table 4). None of the tests carried out with crystalline reagent flashed or faded. The color reaction was noted to be more intense 28% of the time when the crystalline reagent was used.

#### DISCUSSION

For a biochemical test to be useful for identifying bacteria, it should exhibit a high degree of sensitivity, specificity, and ease of interpretation. Additionally, reagent preparation should be as convenient as possible. The conventional nitrate reductase test has been shown to be reliable and specific (3, 4), but difficulties with the procedure have been observed. Conventional reagents have a short shelf life which require frequent preparation. If the reaction is not watched carefully by the microbiologist per-

forming the test, the color reaction can be missed. When a number of tests are being carried out, fading of the positive reaction to a negative reaction can cause confusion when the technologist reviews the results. Use of the crystalline reagent eliminated these technical difficulties. Preparation was easy, the shelf life was found to be at least 6 months, and reading the test was easier. Positive color reactions were always stable which was not the case when the conventional liquid reagents were used. For example, all of the *M. szulgai* isolates and one-half of the *M. terrae* isolates were observed flashing or fading in color when the liquid reagents were used (Table 4).

With the conventional reagents, unexpected negative results were observed in four isolates of *M. tuberculosis*; repeat tests of these isolates were also negative. Of these four isolates, two were positive with the crystalline reagent and two were negative. These latter two isolates were retested and were found to be negative. These results were confirmed by the Centers for Disease Control. No false positive results were obtained with either reagent. Specificity of the crystalline reagent was determined to be 100% (Table 2). Sensitivity of the test was found to be 99% or slightly higher than the 98.1% obtained for the conventional reagent (Table 1).

TABLE 4. Problems encountered in reading conventional nitrate tests

Species	No. positive	No. color flashed with the following reagents <sup>a</sup> :		No. color faded with the following reagents <sup>b</sup> :		No. with a stronger reaction with powdered reagent <sup>c</sup>
		Conventional	Powdered	Conventional	Powdered	
<i>M. tuberculosis</i>	172	19	0	50	0	52
<i>M. kansasii</i>	12	1	0	0	0	3
<i>M. fortuitum</i>	16	0	0	8	0	4
<i>M. szulgai</i>	3	3	0	0	0	0
<i>M. terrae</i>	23	11	0	2	0	5

<sup>a</sup> Color flashed is the instantaneous color change from pink (positive) to colorless (negative). A total of 34 (15%) color flashed with the conventional reagent, and none color flashed with the powdered reagent.

<sup>b</sup> Color faded is the gradual color change from pink to colorless. A total of 60 (27%) color faded with the conventional reagent, and none color faded with the powdered reagent.

<sup>c</sup> A total of 64 (28%) underwent a stronger reaction with the powdered reagent.

In summary, the data obtained in this evaluation of the crystalline reagent for the nitrate reductase test showed it to be highly sensitive and specific. This reagent also offers several additional advantages: (i) ease in reagent preparation, (ii) simplicity in performing the test, (iii) stability of test results, and (iv) prolonged shelf life.

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