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Opacification of Middlebrook Agar as an Aid in Distinguishing *Nocardia farcinica* within the *Nocardia asteroides* Complex

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Among 58 aerobic actinomycetes isolated from different sources and geographical locations, none of 23 *Nocardia asteroides* isolates, all 18 *N. farcinica* isolates, 1 of 5 *N. otitidiscaviarum* isolates, and 1 of 4 *Rhodococcus* species isolates opacified Middlebrook 7H10 medium. Within the *N. asteroides* complex, this characteristic, together with growth at 45°C and resistance to each of erythromycin, cefotaxime, and tobramycin, provides a simple means of distinguishing *N. farcinica* from *N. asteroides*.

Within the *Nocardia asteroides* complex, it has been suggested that *Nocardia farcinica* may be more pathogenic (2, 3), that it shows greater antibiotic resistance than *N. asteroides*, and that it is a significant clinical isolate, often found in disseminated disease (10). Attempts by clinical laboratories to differentiate these two species appear to be warranted. The taxonomy of *N. asteroides* and *N. farcinica* presents difficulties, since the standard Gordon tests are unable to separate them (5). Wallace et al. (10) have reported that resistance to cefotaxime, tobramycin, and erythromycin is a useful adjunct to biochemical tests in the identification of *N. farcinica*. More recently, opacification of Middlebrook 7H10 agar (Difco Laboratories, Detroit, Mich.) as an aid in distinguishing *N. farcinica* from *N. asteroides* has been described by Flores and Desmond (3), and these authors awaited confirmation of their results by other groups working with strains from diverse geographical regions.

We sought to determine whether a simple scheme readily available to diagnostic laboratories, which included opacification of 7H10 agar, growth at 45°C, and resistance to erythromycin, tobramycin, and cefotaxime, could differentiate *N. farcinica* from *N. asteroides* and whether other *Nocardia* species or related actinomycetes could opacify Middlebrook agar.

Forty-one *N. asteroides* complex strains included 22 and 11 human clinical isolates from the United Kingdom and Australia, respectively; 4 isolates from fish (Australia); 2 soil isolates from Surtsey (Iceland); and another 2 soil isolates from Anak Krakatau (Indonesia). Other *Nocardia* species isolates tested were three *N. brasiliensis*, one *N. carnea*, and five *N. otitidiscaviarum* isolates, which, apart from *N. otitidiscaviarum* ATCC 14629, were all human clinical isolates from Australia. Additional actinomycetes tested included one *Streptomyces* species (human clinical isolate from Australia), a *Micropolyspora* species (fish isolate from Australia), one clinical isolate each of *Mycobacterium fortuitum* and *Mycobacterium chelonae*, and four *Rhodococcus* species isolates (*R. rhodochrous* ATCC 13808, *R. terrae* ATCC 25594, *R. coprophilus* ATCC 29080, and *R. rhodnii* ATCC 35071).

The amounts of whole-cell amino acids and sugars and the presence and type of mycolic acids were determined for all organisms by thin-layer chromatography as described in previously published methods (6, 7). Standard tests for the identification of isolates to the species level included those for decomposition of casein, tyrosine, xanthine, and hypoxanthine; reduction of nitrate; hydrolysis of esculin and urea; microscopic morphology; and presence of partial acid fastness by the modified Kinyoun stain (4, 5).

Isolates were identified as *Nocardia* species when they possessed *meso*-diaminopimelic acid, arabinose and galactose, and nocardiomycolic acid in whole-cell extracts. Further, isolates were assigned to the *N. asteroides* complex when they exhibited the following characteristics: branching and/or fragmenting, partially acid-fast, gram-positive rods; reduction of nitrate; hydrolysis of urea and esculin; and lack of decomposition of casein, tyrosine, xanthine, or hypoxanthine (4, 5).

All isolates in this study were tested for the production of milky-white opacification of Middlebrook 7H10 agar, which was prepared according to the instructions of the manufacturer and incubated at 28 and 35°C, with readings at 5 and 10 days.

Organisms within the *N. asteroides* complex were tested for growth at 45°C on horse blood agar at 3 days and for susceptibility to tobramycin, cefotaxime, and erythromycin. Disc diffusion susceptibility testing was performed by the method described by Wallace and Steele (8) on Mueller-Hinton agar with incubation for 48 to 72 h at 28 and 35°C. We incubated at 28 and 35°C because preliminary studies showed that the majority of our *N. asteroides* isolates from soil and all of the isolates from fish do not grow at 35°C, thus necessitating all work with these isolates to be at 28°C, whereas all clinical isolates grew equally well at both temperatures. Zone sizes were interpreted as susceptible, intermediate, or resistant according to criteria published by Wallace and Steele (8).

Eighteen isolates of 41 *N. asteroides* complex organisms grew at 45°C in 3 days, with growth being equal to that at 35 and 28°C, and were designated *N. farcinica*. These isolates opacified Middlebrook 7H10 agar within 10 days at both temperatures. After 5 days of incubation, 11 of the 18 isolates were positive at 28°C and 13 of 18 isolates were positive at 35°C. The five isolates that were negative in 5 days at 35°C showed opacification in 10 days that was stronger at 28°C than at 35°C. The 23 *N. asteroides* isolates yielded no growth at 45°C, and none produced opacification at either temperature. One clinical isolate of *N. otitidiscaviarum* produced strong opacification in 5 days at both temperatures, and the isolate of *R. terrae* produced weak opacification, but only at 35°C after 10 days.

All 18 isolates of *N. farcinica* were clearly resistant to cefotaxime and tobramycin, producing no zone of inhibition. They were also all resistant to erythromycin, with zones of 10 to 12 mm, compared with the cutoff zone diameter of ≤19 mm (8).

Results of susceptibility testing for *N. asteroides* were variable, as has been reported elsewhere (9). Cefotaxime resistance was found in 4 of 9 human clinical isolates from Australia, the 4 fish isolates, and the 4 soil isolates from

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Iceland and Indonesia; 11 of these had no zone of inhibition, and 1 had a zone of 16 mm, where ≤19 mm is resistant. This finding may reflect a diversity of source or geography, since it differs from the report of Wallace et al. (10). Boiron and Provost (1) reported that all 15 *N. asteroides* isolates and all 4 *N. farcinica* isolates were cefotaxine resistant by disc diffusion testing. An unspecified number of their strains were from environmental sources. Six of our 12 cefotaxime-resistant isolates were also tobramycin resistant but were markedly susceptible to erythromycin, with zones in excess of 30 mm. One clinical isolate, one isolate from soil, and the four fish isolates were susceptible to tobramycin as well as to erythromycin. All other *N. asteroides* isolates were tobramycin susceptible. Erythromycin resistance was found in 8 of the 23 *N. asteroides* isolates, with 13 isolates being susceptible and 2 isolates being classified as intermediate.

Incubation at 28 and at 35°C produced only one minor discrepancy in a clinical isolate of *N. asteroides*, which fell into the intermediate category, with a zone size of 23 mm for tobramycin at 35°C and the susceptible category with a zone size of 25 mm at 28°C (the criterion for susceptible being ≥25 mm).

We agree with Desmond and Flores (2) that opacification of Middlebrook 7H10 agar is a useful adjunct to the identification of *N. farcinica* once the organism being tested has been placed in the *N. asteroides* complex by routine methods. The choice of incubation temperature for isolation as well as opacification and susceptibility testing may be important, since we have found that not all environmental strains will grow above 28°C. The consistent discrimination (100%) among isolates of *N. asteroides* complex strains by opacification, growth at 45°C, and resistance to each of erythromycin, tobramycin, and cefotaxime may provide a simple means of confirming the presence of isolates of *N. farcinica* in the routine clinical laboratory; however, the utility of cefotaxime resistance as a single taxonomic marker requires further investigation, since we have shown that some *N. asteroides* strains are resistant to this antimicrobial agent upon repeated testing.

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