

# Pyridine Extraction of Nocardial Acid Fastness

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Several strains of *Mycobacterium* and *Nocardia* were stained for acid fastness by using the method of Kinyoun. Duplicate, glutaraldehyde-fixed smears were extracted with fresh pyridine prior to staining. The results clearly demonstrated that both in vivo- and in vitro-grown *Nocardia* differed from *Mycobacterium* by becoming nonacid fast after extraction. This pyridine extraction of nocardial acid fastness should have diagnostic and taxonomic application.

The acid-fast staining property of members of the genus *Nocardia* is variable. Some strains are reported as being strongly acid fast (5-7), whereas others are readily decolorized with acid-alcohol (5-7). Furthermore, it is known that the acid fastness can be enhanced by growth in certain media such as milk (5). We found that many strains of *N. asteroides* that were not acid fast when grown in brain-heart infusion (BHI) became acid fast when grown in mice or on Middlebrook 7H10 agar supplemented with 1% glycerol (Beaman, unpublished data). Since nocardial filaments tend to fragment into rods and cocci that may be acid fast, especially in vivo, they may easily be mistaken for mycobacteria (9, 12). In fact, this has resulted in misdiagnoses (9, 12). Therefore, a rapid and simple method for distinguishing between the acid fastness of mycobacterium and that of nocardia would be of clinical and taxonomic value.

It is well established that the acid fastness of the leprosy bacillus, as well as nocardia, is not the same as that observed in most mycobacteria (2-6, 11). Fisher and Barksdale (3) demonstrated that the acid-fast staining of the leprosy bacillus could be removed by extracting the cells with pyridine, although this did not occur with other acid-fast mycobacteria. Later Convit and Pinardi (2) confirmed these findings, and Fisher and Barksdale extensively expanded the number of strains tested (4). Therefore, it appears that the acid fastness of the leprosy bacillus can be distinguished from that of other mycobacteria by using pyridine extraction (2-4).

The present investigation was undertaken to determine whether pyridine removed the acid fastness of nocardia. The bacterial strains used and their source are listed in Table 1. All strains

of *Nocardia* were maintained on BHI agar; the *Mycobacterium* were grown on Middlebrook 7H10 agar. To obtain in vivo-grown nocardiae, Swiss Webster mice (4 weeks old) were injected intraperitoneally with approximately  $10^8$  organisms suspended in saline. The bacteria used for the inoculum were grown in BHI broth for 24 h at 34 C in a Psychotherm environmental incubator (New Brunswick). Acid-fast organisms were obtained in vitro by growing them on Middlebrook 7H10 agar supplemented with 1% glycerol. Smears from either animal lesions or agar slants were air-dried and fixed for 30 min in 3% glutaraldehyde in Kellenberger buffer at pH 6.5 (8). The smears were rinsed in deionized water and dried. Duplicate sets of slides were either extracted in fresh pyridine (Baker analyzed reagent) at room temperature for 4 h or remained unextracted. All slides were stained by the Kinyoun acid-fast method (11) by using 1% concentrated hydrochloric acid in 70% ethanol (vol/vol) as the decolorizing agent. The smears were counterstained for 30 s in aqueous methylene blue. The results are shown in Tables 2 and 3.

Pyridine removed the acid fastness of all members of the genus *Nocardia*; however, *Mycobacterium* were not visibly affected by pyridine. Therefore, nocardiae can be distinguished from most mycobacteria by pyridine extraction of the acid fastness of these organisms. We encountered one problem with *N. farcinica* C. This organism was never acid fast when grown on BHI, but it was strongly acid fast when grown in mice or on Middlebrook 7H10 agar. This acid fastness was not always removed by pyridine extraction. In fact, some of the smears resisted extraction for 18 h, as did all of the strains of *Mycobacterium*. No other nocardial strain behaved in this manner. Many

TABLE 1. Bacterial strains tested for pyridine extraction of acid fastness

Organism	Source
<i>N. rubra</i> 721-A	Isolated from the air (Univ. of Kansas, Lawrence)
<i>N. asteroides</i> -mahvi	Fatal human infection (T. A. Mahvi, South Carolina Medical College, Charleston)
<i>N. asteroides</i> -GUH-1	Human infection (V. Garagusi, Georgetown Univ. Hospital, Washington, D.C.)
<i>N. asteroides</i> 10905	ATCC culture obtained from J. Rozanis, Univ. of Western Ontario, London, Canada.
<i>N. asteroides</i> 14759	ATCC <sup>a</sup>
<i>N. asteroides</i> 19247	ATCC
<i>N. braziliensis</i> 19296	ATCC
<i>N. braziliensis</i> 337	J. Rozanis, University of Western Ontario
<i>N. corallina</i> 4273	ATCC
<i>N. pellegrino</i> 159	J. Rozanis, University of Western Ontario
<i>N. farcinica</i> C	Originally from a bovine infection in Africa (From J. Rozanis, University of Western Ontario)
<i>M. rhodochrous</i> 4277	ATCC
<i>M. smegmatis</i> 14468	ATCC
<i>M. smegmatis</i> KU	University of Kansas Stock Culture
<i>M. fortuitum</i> 6841	ATCC
<i>M. intracellulare</i> GU	Georgetown University Stock Culture
<i>M. kansasii</i> GU	Georgetown University Stock Culture
<i>M. tuberculosis</i> H37Rv	ATCC
Tuberculous sputum	Confirmed active tuberculosis, VA Hospital, Washington, D.C.

<sup>a</sup> American Type Culture Collection, Rockville, Md.

investigators believe that *N. farcinica* is really a mycobacterium because of the kinds of mycolic acids it contains (10), and most researchers agree that it is at best a nomen dubium (1, 7, 10, 13). Even though the strain we used exhibited typical nocardial morphology, from the data presented in Tables 2 and 3 we take it to be more closely related to mycobacterium. *Mycobacterium rhodochrous* 4277 behaved more like a typical nocardia, and indeed, most studies have shown that many strains of *M. rhodochrous* were *Nocardia* (1, 10, 13). We found that many of the rapidly growing, soft colony nocardiae represented by *N. corallina* and *N. pellegrino* were not acid fast by any of

TABLE 2. Effect of pyridine on the acid fastness of *Nocardia* and *Mycobacterium* grown on Middlebrook 7H10 agar supplemented with 1% glycerol

Organism	Unextracted (acid fastness)	4-h Pyridine extraction (acid fastness)
<i>N. rubra</i> 721-A(R)	+	-
<i>N. asteroides</i> -mahvi	+	-
<i>N. asteroides</i> GUH-1	+	-
<i>N. asteroides</i> 10905	+	-
<i>N. asteroides</i> 14759	+	-
<i>N. asteroides</i> 19247	+	-
<i>N. braziliensis</i> 19296	+	-
<i>N. braziliensis</i> 337	+	-
<i>N. corallina</i> 4273	(±) <sup>a</sup>	-
<i>N. pellegrino</i> 159	-	(±) <sup>a</sup>
<i>N. farcinica</i> C	+	(±) <sup>a</sup>
<i>M. rhodochrous</i> 4277	+	-
<i>M. smegmatis</i> 14468	+	+
<i>M. smegmatis</i> KU	+	+
<i>M. fortuitum</i> 6841	+	+
<i>M. intracellulare</i> GU	+	+
<i>M. kansasii</i> GU	+	+
<i>M. tuberculosis</i> H37Rv	+	+

<sup>a</sup> Sometimes acid fast but frequently not.

TABLE 3. Effect of pyridine on the acid fastness of *Nocardia* and *Mycobacterium* grown in vivo

Organism	Unextracted (acid fastness)	4-h Pyridine extraction (acid fastness)
<i>N. asteroides</i> GUH-1 (mouse)	+	-
<i>N. asteroides</i> -mahvi (mouse)	+	-
<i>N. asteroides</i> 10905 (mouse)	+	-
<i>N. braziliensis</i> 337 (mouse)	+	-
<i>N. rubra</i> 721-A (S) (mouse)	+	-
<i>N. farcinica</i> C (mouse)	+	(±) <sup>a</sup>
<i>Nocardia</i> sp. DCV (human sputum) <sup>b</sup>	+	-
<i>M. tuberculosis</i> (human sputum)	+	+

<sup>a</sup> The removal of acid fastness by pyridine was variable in both smears and paraffin sections.

<sup>b</sup> The patient had undefined pulmonary problems with the production of sputum containing acid-fast organisms. An organism having most of the physiological properties of *N. asteroides* was repeatedly isolated from this sputum; however, we were never able to demonstrate acid fastness in vitro. No *Mycobacterium* were isolated (supplied by D.C. Village Hospital, Washington, D.C.).

the methods employed. They served, therefore, as negative controls.

The data presented here seem to indicate that

pyridine extraction offers a simple method by which acid-fast nocardiae may be distinguished from most cultivatable mycobacteria. This should have both clinical and taxonomic application. We are presently investigating more nocardial strains, and we are attempting to determine the nature of the substance(s) removed by pyridine.

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