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Pyridine Extractability of Acid-Fastness from *Mycobacterium leprae*[1]

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It is a well-known fact that leprosy bacilli possess acid-fastness as well as an enzyme, *o*-diphenoloxidase, which converts 3,4-dihydroxyphenylalanine ([8, 9]) to a colored product that is readily discernible. Obviously, differentiating *M. leprae* from other mycobacteria using acid-fastness alone cannot be done because this is also an inherent trait of other mycobacterial species. On the other hand, the *o*-diphenoloxidase activity of leprosy bacilli harvested from lepromatous tissues (human as well as experimentally infected armadillo) ([6, 7]) is specific and differentiates *M. leprae* from other mycobacteria ([10]). Pyridine extraction of acid-fastness from the non-cultivatable mycobacterium *M. leprae* was reported as an additional means of identification because other species of mycobacteria retain their acid-fastness ([1, 2, 3, 4, 5]).

Recent reports suggest that the pyridine test is not as specific as was first thought ([12, 13]). Cultivatable mycobacterial species were reported to lose their acid-fastness. Slosarek, *et al.* ([15]) have claimed recently that only *M. leprae* in histologic sections exposed to pyridine show a loss of acid-fastness.

The present results indicate that the pyridine test, when properly done, differentiates *M. leprae* from cultivatable mycobacteria.

MATERIALS AND METHODS

M. leprae to be tested were provided primarily from tissues of 20 experimentally infected armadillos ([6, 7]). The 18 different species of cultivable mycobacteria were obtained from the American Type Culture Collection, Rockville, Maryland, and the cultivatable strain HI-75 was obtained from the Pharmacology Research Department, USPHS Hospital, Carville, Louisiana (which received the culture from Dr. Olaf K. Skinsnes, Hololulu, Hawaii, who gave his permission for use of the culture) ([15]). These cultivatable bacteria were maintained on Lowenstein-Jensen slants, and smears were prepared after 2 and 10 weeks of incubation at 37°C. Cultivatable bacteria were also separated from the lymph node of a feral armadillo that had not been experimentally infected. Bacilli from the skin biopsy of a lepromatous patient and known cultivatable acid-fast organisms were used as controls. Bacilli contained in armadillo tissues (e.g. spleen, liver, and lymph node) were separated by mincing the tissue into small pieces, followed by homogenation in 0.2 M sucrose, filtration, and differential and density gradient centrifugation ([11]).

The mycobacterial suspensions from armadillo tissue were prepared for testing by placing an inoculation loopful of each on numbered slides and heat fixing. Smears of the ATCC mycobacteria were prepared by first placing an inoculating loopful of 5% serum phenol on the slides, followed by the bacterial suspension and heat fixation. Slides of a known mycobacterial culture and the *M. leprae* from lepromatous biopsy tissue were prepared similarly.

Four separate slides of each organism to be tested together with the controls were prepared. Direct smears also were made from tissues of armadillos experimentally infected with *M. leprae* and from the one naturally infected with a cultivatable mycobacterium. Utilizing bacilli from liver and lymph nodes, four slides of each were also prepared. The smears were heat fixed. Two slides from each set of four were processed by the following method of Convit and Pinardi ([2]): a) fixing in Bouin's fixative for 1 hour; b) treating in 70% ethanol for 5 minutes; c) treating in 50% ethanol for 5 minutes; d) washing in running tap water for 2 minutes; e) laying the slides horizontally in

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TABLE 1. *Retention of acid-fastness by 18 species of cultivable mycobacteria.*

Mycobacterial species	ATCC No.	Pyridine Extraction Age of Culture 2 weeks	10 weeks
Mycobacterium microti	19530	Acid-fast	Acid-fast
Mycobacterium gastri	15754	+	+
Mycobacterium intracellulare	13950	+	+
Mycobacterium terrae	15755	+	+
Mycobacterium sp. (Duval)	4233	+	+
Mycobacterium chelonei	19977	+	+
Mycobacterium chitae	19627	+	+
Mycobacterium nonchromogenicum	19530	+	+
Mycobacterium bovis	19015	+	+
Mycobacterium phlei	11758	+	+
Mycobacterium smegmatis	11468	+	+
Mycobacterium fortuitum	68741	+	+
Mycobacterium vaccae	15483	+	+
Mycobacterium diernhoferi	19340	+	+
Mycobacterium avium	25291	+	+
Mycobacterium marinum	927	+	+
Mycobacterium tuberculosis	25177	+	+
BCG (Chicago)		+	+
Known acid-fast staining organism*		+	+
Bacilli separated from a skin biopsy of a lepromatous patient*		Lost acid-fastness (−)	(−)

* Used as controls during all tests.

a large staining dish and treating with pyridine for 2 hours at room temperature (initially spectrophotometric grade pyridine obtained from J. T. Baker Chemical Co., Phillipsburg, New Jersey; thereafter distilled pyridine was used for the procedure); f) washing in running tap water for 2 minutes; g) placing all four slides (the two untreated and the two treated slides) of each set in formol-calcium solution for 1 hour and washing in running tap water for 2 minutes. The pyridine was rejuvenated by placing the used solvent in a closed container that had a one inch thick layer of potassium hydroxide pellets (KOH) on the bottom. The solvent remained in contact with the KOH pellets for approximately 2 weeks. Refluxing of the solvent was then performed at a temperature of 115.5°C. The initial distillate was discarded, and the rest collected and placed in tightly sealed bottles for future use. After treatment with pyridine, all smears were stained with a modified Ziehl-Neelsen method: a) the slides were laid horizontally on a staining rack over a sink; b) carbol fuchsin solution was poured over the slides; the staining solution was then heated for 4 seconds with a Bunsen burner, and the solution allowed to remain there for 10 minutes; c) the slides were then washed in running tap water for 15 seconds; d) decolorized for 2 minutes with 2% HCl acid-alcohol solution; e) and then washed in running tap water for 15 seconds. Slides were read microscopically without counter-staining.

TABLE 2. *Pyridine extractability of acid-fastness of Skinsnes' cultivatable bacterium (14) and* M. leprae *in direct smears.*

Sample	Acid-fast staining results
Skinsnes' Strain HI-75 2-week old culture	Retained acid-fastness
Skinsnes' Strain HI-75 10-week old culture	Retained acid-fastness
Direct smears from lepromatous armadillo tissue	Lost acid-fastness
Known acid-fast staining organism*	Retained acid-fastness
Bacilli separated from a skin biopsy of a lepromatous patient*	Lost acid-fastness

* Used as controls during all tests.

TABLE 3. *Comparison of the DOPA oxidase test results with those of the pyridine extraction procedure.*

Source of bacilli	Acid-fast staining results	DOPA oxidase results*
Armadillos numbers 1–20	Lost acid-fastness	Positive
Culturable mycobacterium from a feral armadillo:		
Separated from lymph node	Retained acid-fastness	Negative
Grown in culture	Retained acid-fastness	Negative
Skin biopsy of a lepromatous patient**	Lost acid-fastness	Quantity insufficient for DOPA testing

* Provided by Dr. K. Prabhakaran.
** Used as a control on all tests.

RESULTS

The results showed that pyridine extraction leads to no loss of acid-fastness in all cultivatable mycobacteria tested. These results were the same both in the older (10 weeks old) and in the younger (2 weeks old) mycobacterial cultures (Table 1). Skinsnes' cultivatable bacteria ([14]) also produced the same results, i.e., no loss of acid-fastness after pyridine extraction (Table 2). Smears of cultivatable mycobacteria in a suspension prepared from feral armadillo tissue and grown in culture retained their acid-fastness as shown in Table 3.

Extractability of acid-fastness by pyridine was found in the smears of *M. leprae* separated from infected armadillo tissues (Table 2) and from the lepromatous patient. Direct smears of the liver and the lymph nodes of experimentally infected armadillos showed complete loss of acid-fastness after exposure to pyridine for 2 hours. As shown in Table 3, there was a perfect correlation between pyridine extraction of acid-fastness and DOPA oxidation.

DISCUSSION

It has been reported by Skinsnes ([12]) and by Skinsnes, *et al.* ([13]) that the pyridine extraction procedure was only effective on "aged, probably nonviable bacilli." Slosarek, *et al.* ([15]) reported that "the results of the experiments showed that, under the conditions used, pyridine extraction leads to a loss of acid-fastness in *M. leprae* in histologic sections only."

Smears prepared from suspensions of *M. leprae* separated from organs of experimentally infected armadillos contain an astronomical number of bacilli. Such suspensions contain mycobacteria of various ages and in various states of viability. When exposed to pyridine for 2 hours and stained by a modified Ziehl-Neelsen technic, the bacilli in smears prepared from *M. leprae* suspensions consistently lost their acid-fastness. The cultivatable mycobacteria and the cultivatable bacilli separated from the feral armadillo consistently retained their acid-fastness. It seems impossible to explain this as being related to "stages in the life cycle of the leprosy bacillus" ([12,13]).

Experiments dealing with the possible pyridine extraction of acid-fastness from cultivatable mycobacterial species (Table 1) were not confirmed in our laboratory. All cultivatable mycobacteria were grown on standard Lowenstein-Jensen slants, and smears were prepared at growth periods of 2 weeks and 10 weeks. The smears presumably contained mycobacteria of various generations. Staining with the Ziehl-Neelsen technic demonstrated that all of these organisms remained acid-fast after 2 hours exposure to pyridine. Dr. Skinsnes' alleged *M. leprae* culture ([14]) also retained its acid-fastness.

The claim of Slosarek, *et al.* ([15]) that pyridine extractability of acid-fastness from *M. leprae* occurs only after histochemical reactions during histological section preparation and staining was not confirmed. *M. leprae* in direct smears prepared from experimentally infected armadillo tissues lost their acid-fastness after exposure to pyridine. The bacterial controls were from a lepromatous patient's skin biopsy and a known cultivatable acid-fast staining organ-

ism. Leprosy bacilli from the skin biopsy lost their acid-fastness while the cultivatable mycobacteria retained theirs.

Convit and Pinardi's pyridine extraction procedure ([2]) stated that pyridine of the highest quality must be used. The solvent that has been recycled in our laboratory met this criterion when compared to pyridine received from the manufacturer. A two hour limit is sufficient to show the extractability of pyridine. Exceeding this time period will only demonstrate the eventual loss of acid-fastness in all mycobacteria. Counter-staining is not necessary because after exposure to pyridine, methylene blue may impart a purplish color to the slide which may be confusing.

Experiments in our laboratory have shown that the pyridine extraction method in conjunction with the D-DOPA oxidase test are reliable tests for the identification of *M. leprae*.

SUMMARY

Various mycobacteria were tested for their ability to retain acid-fastness after treatment with pyridine: a) *Mycobacterium leprae* separated from organs of 20 experimentally infected armadillos (which were sacrificed); b) *M. leprae* separated from a biopsy of a lepromatous patient; c) direct smears of lepromatous tissues from armadillos; d) eighteen cultivable mycobacteria obtained from the American Type Culture Collection (ATCC); e) cultivatable mycobacteria separated from the lymph nodes of a wild-caught armadillo and also the same organism grown in culture and Skinsnes' alleged *M. leprae* culture.

A loss of acid-fastness was observed microscopically from *M. leprae* separated from experimentally infected armadillo tissues, *M. leprae* separated from a lepromatous patient biopsy, and *M. leprae* found in direct smears prepared from infected armadillo tissues. The eighteen cultivatable mycobacteria from ATCC, cultivatable mycobacteria separated from the tissue of a wild-caught armadillo (and also grown in culture) and Skinsnes' alleged *M. leprae* culture retained their acid-fastness. Testing of pyridine extractability of acid-fastness combined with those of D-DOPA oxidase testing proved to be extremely reliable in our laboratory in differentiating *M. leprae* from other mycobacteria.

RESUMEN

Se probó la habilidad de las siguientes micobacterias para retener su ácido resistencia después de tratamiento con piridína: a) *Mycobacterium leprae* separado de los órganos de 20 armadillos infectados experimentalmente, b) *M. leprae* separado de una biopsia de un paciente lepromatoso, c) *M. leprae* encontrado en extendidos directos de tejidos de armadillos infectados, d) dieciocho micobacterias cultivables obtenidas de la Colección Americana de Cultivos Tipo (ATCC), e) micobacterias cultivables separadas de los ganglios linfáticos de un armadillo "salvaje" capturado, el mismo organismo crecido en cultivo, y el cultivo del microorganismo procalamdo como *M. leprae* por Skinsnes.

Microscópicamente, se observó una pérdida de la ácido resistencia del *M. leprae* separado de tejidos de armadillos infectados experimentalmente, del *M. leprae* separado de la biopsia del paciente lepromatoso y del *M. leprae* encontrado en los extendidos directos de los tejidos infectados de armadillo. Las siguientes micobacterias retuvieron su ácido-resistencia: las 18 micobacterias cultivables de la ATCC, las micobacterias cultivables separadas de los tejidos del armadillo "salvaje" capturado, y el organismo crecido en el medio de Skinsnes y proclamado como *M. leprae*. En nuestro laboratorio, la combinación de las pruebas de extractabilidad de la ácido-resistencia con piridina y de la D-DOPA oxidasa, ha resultado ser extremadamente confiable para la diferenciación del *M. leprae* en relación a otras micobacterias.

RÉSUMÉ

On a étudié diverses mycobactéries en ce qui concerne leur capacité à retenir l'acido-résistance après traitement par la pyridine. Ces mycobactéries ont consisté de: a) *Mycobacterium leprae* récoltés à partir des organes de 20 tatous infectés expérimentalement, qui ont été sacrifiés; b) *M. leprae* isolé à partir d'une biopsie de malade lépromateux; c) des frottis directs de tissus lépromateux de tatous; d) dix-huit mycobactéries cultivables obtenues à partir de l'American Type Culture Collection (ATCC); e) des mycobactéries cultivables recueillies à partir des ganglions lymphatiques d'un tatou capturé dans la nature, et également le même organisme après croissance en culture, de même que des cultures prétendûment de lèpre sur milieu de Skinsnes.

Une perte d'acido-résistance a été observée microscopiquement au niveau de *M. leprae* recueilli à partir de tissus de tatous infectés expérimentalement, de *M. leprae* isolé à partir d'une biopsie de malade lépromateux, et également de *M. leprae* mis en évidence dans des frottis directs préparés à partir de tissus de tatous infectés. Les dix-huit mycobactéries cultivables de l'ATCC, de même que les mycobactéries cultiva-

bles recueillies de tissus de tatous capturés dans la nature (et également de ces bacilles de tatous après croissance en culture), de même que les cultures prétendûment de *M. leprae* sur milieu de Skinsnes, ont conservé leur acido-résistance. L'épreuve d'extraction de l'acido-résistance par la pyridine, combinée avec les épreuves de D-DOPA oxydase se sont révélées extrêmement fiables dans notre laboratoire pour différencier *M. leprae* d'autres mycobactéries.

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