

A Simple Method for the Differentiation of *Mycobacterium leprae* from Other Mycobacteria Through Routine Staining Technics^{1,2}

J. Convit and M. E. Pinardi³

It has not been possible to obtain multiplication of *Mycobacterium leprae* in cultures through routine procedures. Some multiplication has been obtained *in vivo* in mouse foot pads⁽³⁾, but the rate of multiplication is low and several months are needed for its development. In fact, this low rate of multiplication, requiring more than six months for the development of the lesion has been considered as one of the characteristics used to identify *M. leprae*. Due to the impossibility of obtaining *M. leprae* completely pure and in sufficient amounts, methods for its identification are very few and difficult to carry out in the laboratory. The method most widely used is an indirect procedure, using the negative response obtained in the lepromatous leprosy patient when injected intradermally with a suspension of killed *M. leprae*. For this test, adequate patients are needed; and a period of at least three weeks must go by before the test can be read.

The determination of phenolase activity has been considered as a specific test⁽⁴⁾, but it is a procedure which cannot be done in a routine laboratory.

It would thus be extremely useful to have a simple method of differentiating between *M. leprae* by three routine procedures: Baker's method for phospholipids⁽²⁾, the Ziehl-Neelsen stain for acid-fast bacteria and the Truant fluorescent stain for acid-fast bacteria. Our main interest was in determining how the staining

with these methods was modified by previous treatment of the bacteria with pyridine⁽¹⁾.

MATERIALS AND METHODS

Mycobacterial strains. Six mycobacterial strains were studied, comparing them with respect to their staining properties:

- 1) *M. leprae*, obtained from lepromatous leprosy patients,
- 2) BCG, obtained from cultures kept in our laboratory,
- 3) *M. smegmatis*, obtained from cultures kept in our laboratory,
- 4) Strain 1582, which is an acid-fast bacillus obtained in our laboratory from the culture of a lepromatous leprosy lesion in Lowenstein-Jensen medium,
- 5) Strain L. D. hamster, an acid-fast bacillus obtained from lesions produced in a hamster through the inoculation of material obtained from a borderline leprosy patient,
- 6) *M. lepraemurium*, obtained from lesions produced in a mouse injected intraperitoneally with *M. lepraemurium*.

Suspensions of each of these mycobacterial strains were made in Hank's balanced salt solution. All the suspensions had at least 10⁵ bacteria per milliliter. For the smears, we used glass slides with a circle drawn on them with a diamond pencil. The smears were made by placing one drop of the suspension within the circle and spreading it with the tip of the pipette to cover all the marked area. With this method we were sure that all our smears were similar in thickness and thin enough for the pyridine to act.

For *M. leprae*, *M. lepraemurium* and L. D. hamster, we also prepared cryostat sec-

¹ Received for publication 22 October 1971.

² Requests for reprints should be directed to Dr. J. Convit, Instituto Venezolano de Dermatología, Esquina de San Nicolás, San José, Ministerio de Sanidad y Asistencia Social, Caracas 101, Venezuela.

³ J. Convit, M.D. and M. E. Pinardi, B.S. Division de Dermatología, Ministerio de Sanidad y Asistencia Social and Department of Dermatology, Escuela de Medicina "José Vargas," Universidad Central de Venezuela, Caracas.

tions of lesions which had been fixed previously either in Bouin's fixation or in formol-calcium.

The smears and sections were always stained in pairs, one of the slides having been treated with pyridine previously and one untreated.

The slides which were to be treated with pyridine were fixed in Bouin's fixative before treatment and in formol-calcium after treatment; the others were fixed directly in formol-calcium.

We tried several methods for treatment with pyridine, changing times and temperatures. After trying approximately 12 different methods we selected the following, since it was the only one that gave constant and reproducible results with all three staining methods, clearly differentiating *M. leprae* from the other five acid-fast bacteria.

Treatment with pyridine:

- 1) Fix in Bouin's fixative for one hour.
- 2) Treat in 70% ethanol for five minutes.
- 3) Treat in 50% ethanol for five minutes.
- 4) Wash in running water for two minutes.
- 5) Treat in new pyridine for two hours.⁴
- 6) Wash in running water for two minutes.
- 7) Fix in formol-calcium for one hour.

These procedures were carried out at room temperature.

After treatment, the smears and sections were stained by the three different procedures already mentioned. The staining times differed for smears as compared with sections but they followed the general routine procedures used by other workers.

Examination of the slides was done by a person who did not know to which group they belonged. When treatment with pyridine had destroyed the acid-fastness of a

bacillary smear, the slides were stained with Gram's method to see whether the bacilli were still there.

RESULTS

All the mycobacteria studied were easily stained by the methods used, except for Baker's stain, which stained *M. leprae* and *M. lepraemurium* very well, but did not give such good results with the other mycobacteria.

After treatment with pyridine, only *M. leprae* completely lost its staining capacities with the three methods used. The other five mycobacteria took the stains exactly in the same way as they did when not treated with pyridine. The slides with *M. leprae* treated with pyridine, which did not show any bacteria when stained with Ziehl-Neelsen, Truant's fluorescent stain or Baker's method for phospholipids, showed Gram positive bacilli when stained with Gram's method.

These experiments were repeated at least twenty times, using *M. leprae* obtained from several patients, some of whom had received treatment and some untreated. The results were always the same, both in smears and in sections.

DISCUSSION

The procedure described allowed the differentiation of *M. leprae* from the other acid-fast mycobacteria studied, using smears or cryostat sections of material that has an adequate number of bacteria, and in very thin smears.

It must be emphasized that the two-hour room temperature pyridine treatment described is the only one that gives these results, since longer treatments at room temperature (more than 16 hours) or 2-3 hour treatments at 60°C will erase acid-fast properties from all the mycobacteria studied.

These results suggest that the location of the components that combine with the dyes of the various methods used must be placed at different depths or their chemical bonds must be different, since *M. leprae* seems to be the one that has them either most superficially or with the weaker bonds so that they are easily removed by treat-

⁴The pyridine used should be of the best quality. We have used Merck pyridine, reagent grade, with excellent results. Since pyridine seems to lose potency after the bottle has been opened for some time, we only use pyridine which keeps its strong, sharp, characteristic odor. We feel this is of great importance since, when these conditions are not fulfilled, the results are very variable.

ment with pyridine for two hours at room temperature.

These investigations are continuing with the purpose of determining the chromatographic composition of the acid-fast determinant extracted by pyridine from *M. leprae*.

SUMMARY

A simple laboratory method for the differentiation of *M. leprae* from BCG, *M. smegmatis*, an atypical mycobacteria, a hamster lesion mycobacterium, and *M. lepraemurium* is described. The differentiation is accomplished by the use of Ziehl-Neelsen's, Truant's, and Baker's staining methods combined with treatment with pyridine.

Smears and cryostat sections were treated for two hours with pyridine at room temperature. *M. leprae* then lost its acid-fastness while the other mycobacteria did not. The method requires a suspension of at least 10^7 bacilli per milliliter. The stain-negative pyridine-extracted *M. leprae* slides revealed abundant mycobacteria when stained with Gram's method.

RESUMEN

Se describe un método simple de laboratorio para diferenciar el *M. leprae* de BCG, *M. smegmatis*, una micobacteria atípica, una micobacteria obtenida de una lesión de hamster y *M. lepraemurium*. La diferenciación se lleva a cabo a través de las técnicas de coloración de Ziehl-Neelsen, Truant y Baker, en combinación con tratamiento con piridina.

Los extendidos y los cortes hechos con criostato se trataron durante dos horas con piridina, a temperatura ambiente. El *M. leprae* perdió la ácido-resistencia, lo que no sucedió con las otras micobacterias. El método requiere

una suspensión de por lo menos 10^5 micobacterias por mililitro. Las láminas de *M. leprae* extraídas con piridina, que fueron negativas con las tinciones mencionadas, revelaron abundantes micobacterias cuando se tiñeron con el método de Gram.

RÉSUMÉ

On décrit ici une méthode de laboratoire simple pour distinguer *M. leprae* du BCG, de *M. smegmatis*, d'une mycobactérie atypique, d'une mycobactérie trouvée dans les lésions chez le hamster, et de *M. lepraemurium*. Le diagnostic différentiel est opéré grâce à l'utilisation de la coloration de Ziehl-Neelsen, de la coloration de Truant, et de la coloration de Baker, combinées avec un traitement par la pyridine.

Les frottis et les coupes congelées ont été traités pendant deux heures par la pyridine à la température ambiante. Dans ces conditions, *M. leprae* perd son acido-résistance, alors que les autres mycobactéries la conservent. Cette méthode exige que l'on dispose d'une suspension d'au-moins 10^5 bacilles par millilitre. Les lames de *M. leprae* extrait par la pyridine et non colorable, révèlent des mycobactéries abondantes lorsqu'elles sont soumises à la coloration par la méthode de Gram.

REFERENCES

1. BARKSDALE, LANE. Personal communication.
2. CAMPO-AASEN, I. and CONVIT, J. Identification of the noncultivable pathogenic mycobacteria *M. leprae* and *M. lepraemurium*. Internat. J. Leprosy **36** (1968) 166-177.
3. SHEPARD, C. C. Multiplication of *Mycobacterium leprae* in the foot pad of the mouse. Internat. J. Leprosy **30** (1962) 291-306.
4. PRABHAKARAN, K. and KIRCHHEIMER, W. F. Use of 3,4-dihydroxyphenylalanine oxidation in the identification of *Mycobacterium leprae*. J. Bact. **92** (1966) 1267-1268.