Volume 69, Number 1
Printed in the U.S.A.
(ISSN 0148-916X)

PCR-Restriction Fragment Length Polymorphism Analysis (PRA) of *Mycobacterium leprae* from Human Lepromas and from a Natural Case of an Armadillo of Corrientes, Argentina[1]

Martin Jose Zumarraga, Edmundo Hector Resoagli, Maria Elena Cicuta, Anibal Ramon Martinez, Maria Isabel Ortiz de Rott, Sonnia Gracia de Millan, Karina Caimi, Andrea Gioffre, Alicia Alito, Fabiana Bigi, Angel Adrian Cataldi, and Maria Isabel Romano[2]

The armadillo is a mammal of the order *Edentata* whose origin can be traced to the end of the Eocene epoch, some 40 million years ago, of which today there remain only three living families: *Bradypodidae* (sloths), *Myrmecophagidae* (anteaters) and *Dasypodidae* (armadillos). The last family includes six subfamilies. The subfamily *Dasypodidae* genus *Dasypus* comprises *D. novemcinctus*, *D. hybridus*, *D. sabanicola* and *D. kappleri*. There are 20 species of armadillos distributed for the most part in the southeastern United States, Central and South America. It is of interest to note that armadillos occur exclusively in the Western Hemisphere ([4]). It is a valuable animal model for the study of leprosy because of the facility to reproduce the illness and many of the animals, captured in the U.S.A., Mexico and Argentina have been found to be naturally infected with *Mycobacterium leprae* ([1, 10, 11, 14–16]).

Wild leprosy in the nine-banded armadillo (*Dasypus novemcinctus*) is characterized by the presence of multiple bacilli in the internal organs such as the lymph glands, spleen and liver with nerve and skin involvement. We could observe this in 9 out of 132 animals captured in Corrientes, Argentina, an area endemic for leprosy in humans with a prevalence of 6 per 100,000 ([14, 15]). *M. leprae* was recognized by 1982 in those naturally infected armadillos through specific stains (Ziehl-Neelsen, Fite-Faraco and King-Young), loss of the acid-fastness with pyridine extraction, positive D-dopa oxidase reaction, noncultivable on Löwenstein-Jensen and Stonebrink media, replication in the mouse foot pad (Shepard technique) ([8]) and reaction to the armadillo bacterin (AB), similar to the normalized-bacillary lepromin (NBL-40) in diseased persons with different forms of the illness ([6]).

Polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis (PRA), which relies on the amplification of a 439-bp portion of the *hsp*65 gene present in all mycobacteria, followed by two distinct digestions (with *Bst*EII and *Hae*III) of the PCR product, offers a rapid and easy alternative that permits the rapid identification of mycobacteria without the need for specialized equipment ([13, 17]). In the present investigation we attempted to use the same methodology to investigate if it was possible to conclusively identify *M. leprae* from two human lepromas and different tissues of the naturally infected nine-banded armadillo.

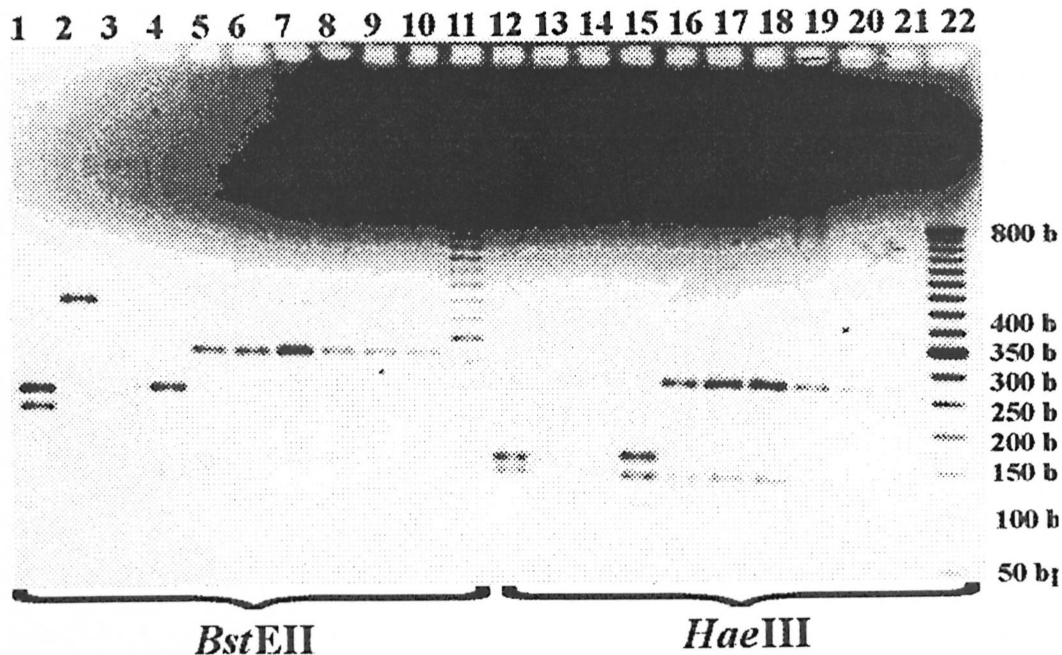
MATERIALS AND METHODS

DNA of *M. leprae* was extracted and purified from the liver, popliteal and axillary lymph nodes of a naturally infected ar-

[1] Received for publication on 27 November 2000. Accepted for publication in revised form on 13 February 2001.

[2] M. J. Zumarraga, K. Caimi, A. Giofre, A. Alito, F. Bigi, A. Cataldi, M. I. Romano, Instituto de Biotecnologia (CICV-INTA), Castelar, Buenos Aires, Argentina. E. H. Resoagli, M. E. Cicuta, A. R. Martinez, M. I. O. de Rott, S. G. de Millan, Programa Experimental en Armadillo (PEA), Facultad de Ciencias Veterinarias (FCV/UNNE), Sgto. Cabral 2.139-3400, Corrientes, Argentina.

Reprint requests to Prof. Maria Elena Cicuta at the above address or FAX 54-3783-425753; e-mail: cicuta@vet.unne.edu.ar



THE FIGURE. PRA gel after digestion with *Bst*EII (lanes 1 to 11) and *Hae*III (lanes 12 to 22) of 439-bp DNA fragments amplified from *M. scrofulaceum* (lanes 1 and 12), *M. szulgai* (lanes 2 and 13), *M. avium* (lanes 3 and 14), *M. bovis* (lanes 4 and 15) and *M. leprae* from armadillo (lanes 6, 7, 10, 17, 18 and 21) and from human lepromas (lanes 5, 8, 9, 16, 19 and 20); molecular weight marker (lanes 11 and 22).

madillo [captured during the Experimental Program in Armadillo (PEA)], and three samples from two human lepromas of the same region. Briefly, 0.5 g of each infected tissue was manually ground with micropestles. Once macerated it was suspended in 300 μl of buffer Tris-EDTA (TE) 1×, with 10 μl of proteinase K (PK; 10 mg/ml) and incubated overnight at 37°C. After adding 10 mg/ml lysozyme solution and incubating for 2 hr at 37°C, 100 μl of SDS 10% and 10 μl of PK were added and incubated 15 min at 65°C. Two extractions of the same volume with phenol-chloroform-isoamyl alcohol (24-24-1) were carried out and one with chloroform-isoamyl alcohol (24-1). Then, 100 μl of NaCl 5 M was added and precipitated overnight with absolute ethanol at –20°C. It was centrifuged at 12,000 *g* × 15 min and the supernatant was discarded. After two washes with ethanol 70%, it was resuspended in 40 μl of buffer TE 1×. It was used as 1 μl of pure DNA for PCR. A fragment of 439 bp was amplified with the primers Tb11 and Tb12 by the procedure described by Telenti, *et al.* ([17]), and the amplification product was subjected to *Bst*EII and *Hae*III enzyme digestions. The fragments obtained were separated by electrofocusing on 3% agarose plus ethidium bromide and visualized under UV. A 50-bp DNA ladder (Gibco BRL, Gaithersburg, Maryland, U.S.A.) was used as a molecular weight marker. DNA fragments were also amplified from *M. scrofulaceum, M. szulgai, M. avium* and *M. bovis.*

RESULTS

The results of the PRA are shown in The Figure. The patterns of the six samples of lepromatous tissues analyzed were identical and coincident with those of *M. leprae* ([9, 13]) that are characterized by uniformly obtaining two fragments of 315 and 135 bp with the digestion with *Bst*EII and two fragments of 265 and 130 bp with the digestion with *Hae*III that agree with the published sequences of the antigen of 65-kDa.

DISCUSSION

Since *M. leprae* are noncultivable mycobacteria, the diagnosis of leprosy is based

on the demonstration of at least two of the following: characteristic lesions of the skin, loss of sensibility, nerve involvement or the presence of acid-fast bacilli in smears of skin lesions ([13]). In this context, the quick diagnosis by molecular biology methods has renovated the interest in scientific groups that have described a variety of techniques, such as amplification of *M. leprae*-specific repetitive sequences ([19]), *in situ* hybridization ([2]), nested-primer gene amplification of a 347-bp product from a bacterial genomic library ([12]), amplification of the gene that encodes an antigen of 36-kDa size ([7]), PCR based on the selective amplification of a 530-bp fragment of the gene encoding the proline-rich antigen of *M. leprae* ([5]), a specific 360-bp DNA-probe encoding 80% of the 18-kDa protein gene of *M. leprae* ([18]), or PCR using a unique sequence of 16S ribosomal RNA ([3]).

We conclude that the PRA method is relatively simple and that it was able to compare a variety of mycobacterial species in a single experiment, allowing the conclusive identification of *M. leprae*. These studies, made for the first time in Argentina, corroborate the initial discoveries in South America made by our investigative group on the detection of naturally infected armadillos with the Hansen's bacillus ([8, 14, 15]). The similar reaction of the comparative test made in humans with armadillo bacterin (AB) obtained from this naturally infected animal and bacillary lepromin (LBN-40) confirm that it was always natural or wild leprosy acquired by this animal from the environment ([6]).

SUMMARY

Polymerase chain reaction (PCR)-restriction fragment length polymorphism analysis (PRA) which relies on the amplification of a 439-bp portion of the *hsp*65 gene present in all mycobacteria, followed by two distinct digestions (with *Bst*EII and *Hae*III) of the PCR product, offers a rapid and easy alternative that allows identification of the species without the need for specialized equipment.

Wild leprosy in the nine-banded armadillo (*Dasypus novemcinctus*) is characterized by the presence of multiple bacilli in internal organs such as lymph nodes, spleen and liver, as well as in nerves and skin. We could observe this in 9 out of 132 animals captured in Corrientes, Argentina, an area endemic for leprosy in humans. *Mycobacterium leprae* were recognized in those naturally infected animals through different techniques. Three samples of extracted DNA of the mycobacteria present in the spleen, liver and popliteal lymph node of a naturally infected animal during the Experimental Program in Armadillo (PEA) and three samples of human lepromas were processed by PRA.

The patterns of the six samples analyzed were identical and were characteristic of *M. leprae*. These studies, made for the first time in Argentina, corroborate the initial discoveries in South America made by our investigative group on the detection of armadillos naturally infected with the Hansen bacillus.

RESUMEN

El análisis por *PCR* del polimorfismo en el largo de los fragmentos restrictivos (*PRA*) del ADN, que se basa en la amplificación de una porción de 439 pb del gen *hsp*65 presente en todas las micobacterias, seguido de dos digestiones diferentes del producto de *PCR*, ofrece una alternativa rápida y fácil que permite la identificación de la especie, sin la necesidad de dificultosas pruebas bioquímicas.

La lepra salvaje en el armadillo de nueve bandas (*Dasypus novemcinctus*, Linné, 1758) cursa con la presencia de abundantes bacilos con localización preferentemente linfo-espleno-hepática, con compromiso nervioso y dérmico, tal cual se pudo observar en animales capturados en la provincia de Corrientes, Argentina, zona endémica de la enfermedad en humanos. Habiéndose reconocido *Mycobacterium leprae*, infectando naturalmente estos armadillos, a través de diferentes técnicas, se procesaron, por medio de la técnica de *PRA*, tres muestras de ADN extraído de las micobacterias presentes en bazo, hígado y linfoglándula patelar de un animal naturalmente infectado obtenido dentro del PEA, y de tres muestras de lepromas humanos de pacientes de la misma provincia.

Los seis patrones logrados coinciden con los de esta especie, caracterizándose por la obtención uniforme de fragmentos característicos de *M. leprae*. Estos estudios, efectuados por primera vez en Argentina, corroboran los hallazgos iniciales en Sudamérica hecho por este grupo de investigación, sobre la detección de armadillos naturalmente infectados con el bacilo de Hansen.

RÉSUMÉ

La réaction de Polymérase en chaîne (PCR) associée à l'analyse du polymorphisme des fragments obtenus après digestion par des enzymes de restriction (PRA), qui s'applique ici à l'amplification du segment

de 439 paires de base du gène *hsp65* qui est présent chez toutes les mycobactéries, siuvi de deux digestions distinctes par *BstEII* et *BaeIII* des produits du PCR, représente une méthode facile et rapide qui permet l'identification des espéces sans la nécessité d'un équipement spécialisé.

La lèpre chez le tatou à neuf bandes (*Dasypus novemcinctus*) est caractérisée par la présence de très nombreux bacilles dans les organes internes comme les nœuds lymphatiques, la rate et le foie, ainsi que les nerfs et la peau. Nous avons pu observer cela dans neuf parmi 32 animaux qui furent capturés à Corrientes en Argentine, une région endémique de lèpre humaine. *Mycobacterium leprae* fut identifiée par différentes techniques. Trois échantillons d'ADN isolés de mycobactéries présentes dans la rate, le foie et le nœud lymphatique poplité d'un animal naturellement infecté durant le programme expérimental chez le tatou (PHA) et trois échantillons de lépromes humains préparés pour une PRA. La distribution des bandes de restriction était identique et caractéristique de *M. leprae* dans les 6 échantillons testés. Ces études, menées pour la premiére fois en Argentine, corroborent la découverte initiale faite par notre groupe de la présence de tatous naturellement inféctés par le bacille de Hansen.

Acknowledgment. We are grateful to M. V. Juan Carlos Sampietro for his technical assistance.

REFERENCES

1. Amezcua, M. E., Escobar-Gutierrez, A., Storrs, E. E., Dhople, A. M. and Burchfield, H. P. Wild Mexican armadillo with leprosy-like infection. (Letter) Int. J. Lepr. **52** (1984) 254–255.
2. Arnoldi, J., Schluter, C., Duchrow, M., Hubner, L., Ernst, M., Teske, A., Flad, H. D., Gerdes, J. and Bottger, E. C. Species-specific assessment of *Mycobacterium leprae* in skin biopsies by *in situ* hybridization and polymerase chain reaction. Lab. Invest. **66** (1992) 618–623.
3. Cox, R. A., Kempsell, K., Fairclough, L. and Colston, M. J. The 16S ribosomal RNA of *Mycobacterium leprae* contains unique sequence which can be used for identification by the polymerase chain reaction. J. Med. Microbiol. **35** (1991) 284–290.
4. Cuba-Caparó, A. The armadillo in biomedical research. In: *The Armadillo as an Experimental Model in Biomedical Research.* Washington, D.C.: Pan American Health Organization, 1978, pp. 18–30. PAHO/WHO Sci. Pub. 366.
5. de Wit, M. Y .L., Faber, W. R., Krieg, S. R., Douglas, J. T., Lucas, S. B., Montreewasuwat, N., Pattyn, S. R., Hussain, R., Ponnighaus, J. M., Hartskeerl, R. A. and Klatser, P. R. Application of a polymerase chain reaction for the detection of *Mycobacterium leprae* in skin tissues. J. Clin. Microbiol. **29** (1991) 906–910.
6. Giménez, M. M., Molinari, M. L., Giménez, M. F., Resoagli, E. H. and Cicuta, M. E. Estudio comparativo del test cutáneo con lepromina bacilar (*M. leprae*) y con un antígeno (bacterina) de la lepra salvaje del armadillo (*Dasypus novemcinctus*), Lin., 1758. Dr. Guillermo Basombrío Prize given by the Sociedad Argentina de Leprología, Buenos Aires, 20 July 1985.
7. Hartskeerl, R. A., de Wit, M. Y. and Klatser, P. R. Polymerase chain reaction for the detection of *Mycobacterium leprae.* J. Gen. Microbiol. **135** (1989) 2357–2364.
8. Martínez, A. R., Resoagli, E. H., de Millán, S. G., Resoagli, J. P., Ramírez, M .M., Cicuta, M. E., de Rott, M. I. O. and Sandoval, A. Lepra salvaje en *Dasypus novemcinctus* (Linneo, 1758). Arch. Arg. Dermatol. **34** (1984) 21–30.
9. Mehra, V., Sweetser, D. and Young, R. A. Efficient mapping of protein antigenic determinants. Proc. Natl. Acad. Sci. U.S.A. **83** (1986) 7013–7017.
10. Meyers, W. M., Binford, C. H., Brown, H. L. and Walsh, G. P. Leprosy in wild armadillos. In: *Comparative Pathology of Zoo Animals. Proceedings of a Symposium, National Zoological Park, Smithsonian Institution, Washington, D.C., 2–4 October 1978.* Washington, D.C.: Smithsonian Institution Press, 1980, p. 247.
11. Meyers, W. M., Walsh, G. P., Binford, C. H., Storrs, E. E. and Brown, H. L. Indigenous leprosy in nine-banded armadillos. In: *The Armadillo as an Experimental Model in Biomedical Research.* Washington, D.C.: Pan American Health Organization, 1978, pp. 67–78. PAHO/WHO Sci. Pub. 366.
12. Plikaytis, B. B., Gelber, R. H. and Shinnick, T. M. Rapid and sensitive detection of *Mycobacterium leprae* using a nested-primer gene amplification assay. J. Clin. Microbiol. **28** (1990) 1913–1917.
13. Rastogi, N., Goh, K. S. and Berchel, M. Species-specific identification of *Mycobacterium leprae* by PCR-restriction fragment length polymorphism analysis of the *hsp65* gene. J. Clin. Microbiol. **37** (1999) 2016–2019.
14. Resoagli, E. H., Martínez, A. R., Resoagli, J. P., de Millán, S. G., de Rott, M. I. O. and Ramírez, M. M. Micobacteriosis natural en armadillos similar a lepra humana (comunicación del estado actual de las investigaciones en el NEA). Gaceta Veterinaria **45** (1982) 674–676.
15. Resoagli, E. H., Martínez, A. R., Resoagli, J. P., Morales, C. R., de Millán, S. G., Cao, E. A. and de Rott, M. I. O. Comunicación de un caso de micobacteriosis esplénica en el armadillo con características histopatológicas y tintoriales similares a lepra. Leprología **21** (1979) 18–25.
16. Storrs, E. The life and habitat of the *Dasypus novemcinctus*. In: *The Armadillo as an Experi-*

mental Model in Biomedical Research. Washington, D.C.: Pan American Health Organization, 1978, pp. 3–11. PAHO/WHO Sci. Pub. 366.

17. TELENTI, A., MARCHESI, F., BALZ, M., BALLY, F., BOTTGER, E. C. and BODMER, T. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J. Clin. Microbiol. **31** (1993) 175–178.
18. WILLIAMS, D. L., GILLIS, T. P., BOOTH, R. J., LOOKER, D. and WATSON, J. D. The use of a specific DNA probe and polymerase chain reaction for the detection of *Mycobacterium leprae.* J. Infect. Dis. **162** (1990) 193–200.
19. YOON, K. H., CHO, S. N., LEE, M. K., ABALOS, R. M., CELLONA, R.V., FAJARDO, T. T., JR., GUIDO, L. S., DELA CRUZ, E. C., WALSH, G. P. and KIM, J. D. Evaluation of polymerase chain reaction amplification of *Mycobacterium leprae*-specific repetitive sequences in biopsy specimens from leprosy patients. J. Clin. Microbiol. **31** (1993) 895–899.