Mycobacterium leprae in six-banded (Euphractus sexcinctus) and nine-banded armadillos (Dasypus novemcinctus) in Northeast Brazil

Cristiane Cunha Frota¹/, Luana Nepomuceno Costa Lima¹, Adalgiza da Silva Rocha², Philip Noel Suffys², Benedito Neilson Rolim³, Laura Cunha Rodrigues⁴, Maurício Lima Barreto⁵, Carl Kendall⁶, Ligia Regina Sansigolo Kerr⁷

¹Departamento de Patologia e Medicina Legal ²Departamento de Saúde Comunitária, Faculdade de Medicina, Universidade Federal do Ceará, Fortaleza, CE, Brasil ³Laboratório de Biologia Molecular Aplicada a Micobactérias, Instituto Oswaldo Cruz-Fiocruz, Rio de Janeiro, RJ, Brasil ⁴Controle de Zoonoses/Vetores, Secretaria de Saúde do Estado do Ceará, Fortaleza, CE, Brasil ⁵London School of Hygiene and Tropical Medicine, London, UK ⁶Instituto de Saúde Coletiva, Universidade Federal da Bahia, Salvador, BA, Brasil ⁷Department of Global Community Health and Behavioral Sciences, Center for Global Health Equity, Tulane University School of Public Health and Tropical Medicine, New Orleans, LA, USA

Human beings are the main reservoir of the causative agent of leprosy, Mycobacterium leprae. In the Americas, nine-banded armadillos (Dasypus novemcinctus) also act as a reservoir for the bacillus. In the state of Ceará (CE), which is located in Northeast Brazil and is an endemic area of leprosy, there are several species of armadillos, including D. novemcinctus and Euphractus sexcinctus (six-banded armadillo). Contact between humans and armadillos occurs mainly through hunting, cleaning, preparing, cooking and eating. This study identified M. leprae DNA in the two main species of armadillos found in Northeast Brazil. A total of 29 wild armadillos (27 D. novemcinctus and 2 E. sexcinctus) were captured in different environments of CE countryside. Samples from the ear, nose, liver and spleen from each of these animals were tested by a nested M. leprae-specific repetitive element polymerase chain reaction assay. The samples that tested positive were confirmed by DNA sequencing. M. leprae was detected in 21% (6/29) of the animals, including five D. novemcinctus and one E. sexcinctus. This is the first Brazilian study to identify the presence of a biomarker of M. leprae in wild armadillos (D. novemcinctus and E. sexcinctus) in a leprosy hyperendemic area where there is continuous contact between humans and armadillos.

Key words: Euphractus sexcinctus - Dasypus novemcinctus - Mycobacterium leprae - eco-epidemiology - leprosy

Mycobacterium leprae, the causative agent of leprosy, is not cultivable in vitro. The lack of growth on standard mycobacterial isolation media differentiates this organism from other mycobacterial pathogens. Human beings are the only known reservoir of infection, except in the southern United States of America (USA), where nine-banded armadillos (Dasypus novemcinctus) are believed to also provide a reservoir (Truman et al. 2011).

In 1960, Shepard introduced the footpad mouse model to study experimental leprosy. In 1971, nine-banded armadillos were successfully infected with the bacillus and developed clinical symptoms and pathologies similar to the human disease (Kirchheimer et al. 1972, Kirchheimer & Sanchez 1977). Armadillos provide a much more effective animal model than the mouse footpad, producing 10^6 acid-fast bacilli/mL (Shepard 1985). Because armadillos do not breed well in captivity, the animals to be used in the laboratory need to be captured in the wild. While searching for these animals in 1975, Walsh et al. (1977) found armadillos that were naturally infected with M. leprae in the wild for the first time. The authors proposed that armadillos might have acquired leprosy infections from untreated human patients in the USA.

The exact mode of transmission of leprosy between humans and armadillos is not known, though cross-reactivity between IgM antibodies against phenolic glycolipid-I of humans and armadillos has been reported (Truman et al. 1991, Job et al. 1992). Infected nine-banded armadillos have been identified in the states of Texas and Louisiana and in Central and South America (Smith et al. 1983, Amezcu et al. 1984, Stallknecht et al. 1987, Zunarraga et al. 2001). Additionally, biomarkers of armadillo infection have been detected in Colombia and Brazil (Deps et al. 2007, Cardona-Castro et al. 2009). Several studies have shown an association between armadillo exposure through hunting, cleaning and eating the meat and the development of leprosy (Clark et al. 2008, Deps et al. 2008, Truman 2008). More than half of the leprosy cases that have been reported in the southeastern USA have described some direct or indirect exposure to armadillos (Bruce et al. 2000, Truman et al. 2011) and other studies have raised the hypothesis that exposure to these animals could be a significant risk factor for leprosy in Brazil (Kerr-Pontes et al. 2006, Deps et al. 2008).

Unlike Brazil, where there are other species of armadillos, only D. novemcinctus is found in the USA. Among the 21 species of armadillos found in Brazil, Euphractus sexcinctus (six-banded armadillo) is commonly found. This species is known to eat animal carcasses, justifying
the local name of “vulture or gravedigger” and, because of this, hunters keep the animal alive and caged for several days before eating (Dalponte & Tavares-Filho 2004, Armadillo Online! 2012). This type of “quarantine” is believed to “clean” the animal, but instead enhances its contact with humans. In the state of Ceará (CE), located in Northeast Brazil, armadillos are used as a source of meat and hunting is seen as a leisure pursuit.

Brazil is a high leprosy-burdened country, with 34,894 new cases detected in 2010 (WHO 2011). More than half (53.5%) of the cases are concentrated in areas where only 17.5% of the population live, which reflects that leprosy is a rural disease (MS/SVS 2008). A study conducted with cases reported to the Brazilian Ministry of Health between 1990-2007 shows that leprosy is geographically concentrated, and a spatial analysis shows 29 clusters of higher prevalence. In these clusters, the mean rate of detection was more than the double the rate of the rest of the country (56.2 vs. 20.6 cases per 100,000 inhabitants). CE is one of the poorest states in Brazil and more than half of the municipalities in CE (46 out of 84) reported local transmission of new cases. CE was also included in one of the 29 Brazilian leprosy clusters (Penna et al. 2009). However, in Brazil, epidemiological studies are still controversial in terms of whether armadillo meat intake and direct animal contact are associated with leprosy infection (Kerr-Pontes et al. 2006, Deps et al. 2008, Schmitt et al. 2010). Aiming to investigate the question further, we used molecular diagnostic tools to search for M. leprae DNA in the two main species of wild armadillos found in CE (D. novemcinctus and E. sexcinctus).

MATERIALS AND METHODS

Armadillos - A total of 29 wild armadillos from two species (27 D. novemcinctus and 2 E. sexcinctus), consisting of males and females weighing from 2.6-3.8 kg, were captured between July-August 2007. The animals were captured by local hunters under the supervision of a veterinarian in rural sites of 12 selected endemic municipalities from CE (Figure). Biopsy samples from the ear, nose, liver and spleen of each of these animals were studied.

Euthanasia - Animal captures were authorised by the Brazilian Institute of Environmental and Renewable Natural Resources. Before euthanasia, armadillos were anesthetised with tiletamine and zolazepam (5.0 mg/kg/IM) (Virbac, Brazil). Euthanasia was conducted in the place of capture and the animals were kept in ice until they arrived at the laboratory in Fortaleza, the capital city of CE.

Sample biopsies and DNA extraction - Stringent precautions were necessary to avoid cross-contamination. Clean protective clothing was worn and gloves were changed frequently. Before the tissue dissection, the animals were carefully rinsed with distilled water. For each desired tissue, a sterile blade was used to cut. Ear, nose, liver and spleen samples were kept frozen at -20°C in separate sterile plastic storage bags until DNA extraction was performed. The sampling extraction was conducted batch-wise, four samples at a time. The frozen sections were incubated with 50 µL of 100 mM Tris-HCl, pH 8.5, containing Tween-20 and 60 µg of proteinase K per mL for 18 h at 60°C. The digestion was conducted at 97°C for 15 min (de Wit et al. 1991). The extract was purified once with phenol/chloroform/isoamyl alcohol (25:24:1) and then precipitated with ethanol.

Polymerase chain reaction (PCR) and nucleotide sequencing - The M. leprae-specific repetitive element (RELP) PCR was amplified in a nested PCR reaction. The primers for RELP-2-1 (5’-ATATCgATGCGCCGCGT-GAG-3’) and RELP-2-2 (5’-GGATCATCGATGCACT-GTTCC-3’) amplified a 282-bp sequence of the RELP element. The second set of inner primers, RELP-2-3 (5’-GGTAGGGGCGTTTTAGTGT-3’) and RELP-2-2, amplified a 238-bp product. A 1 µL aliquot of the isolated DNA was added to 24 µL of PCR mix, which contained 15 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTP, 5% DMSO, 1.25 units of Taq DNA Polymerase and 0.2 µM of each primer. The mixture was denatured at 94°C for 4 min, followed by 35 PCR cycles (30 s at 94°C, 30 s at 59.6°C and 1 min at 72°C), with a final extension at 72°C for 10 min. Each run included negative and positive controls. For the nested PCR, 0.5 µL of product was used as the DNA template. The amplification reactions were visualised on a 1.5% agarose gel. Each PCR sample was double-blind tested by different researchers in Fortaleza and Rio de Janeiro. When the results for the same sample were different, a third PCR was performed for confirmation. Different amounts of purified DNA from M. leprae (kindly donated by Dr R. Truman, Louisiana State University, USA) were added to all negative PCR samples to assess the presence of inhibitory substances. A standard curve was constructed by serial dilution of purified M. leprae DNA ranging from 10 fg-1 µg. Purified M. leprae DNA was also used as a positive control for the amplifications.

The M. leprae gyrA region was amplified using primers gyrAF (5’-CCCGGACCGTATGCCAGCTAAAGTC-3’) and gyrAR (5’-CATCCTGCCTGGGTGGTCCATTAGTC-3’). The thermal profile involved an initial denaturation at 94°C for 5 min and six cycles of 94°C for 45 s, 68 to 63°C...
for 45 s and 72°C for 90 s, followed by 35 cycles of 94°C for
45 s, 62°C for 45 s and 72°C for 90 s. The final extension
was for 10 min at 72°C. The 187-bp PCR products were
purified using the Invitrogen ChargeSwitch PCR Clean-Up
kit prior to sequencing in an Applied Biosystems DNA
sequencer (Perkin-Elmer Applied Biosystems) using a
BigDye Terminator Cycle Sequencing kit. The sequences
were identified using SecScape software v2.7 (Applied
Biosystems). A reference gyrA sequence (GenBank acces-
sion NC002677) was used to align the sequences.

False-positive amplifications were addressed by using
individual sterile section-cutting blades and sterile
glassware for each biopsy sample. The armadillo biopsies
and extracted DNA samples were carefully identi-
fied and kept in separate boxes. A simple DNA extraction
protocol was established to minimise the risk of
contamination. The extraction method used one purifi-
cation step to reduce the accumulation of impurities that
would inhibit the polymerase reaction.

Ethical considerations - This project was submitted
to and approved by the Ethical Committee of the Fed-
eral University of Ceará.

RESULTS

A total of 116 liver, spleen, ear and nose tissue samples
from 29 armadillos were tested using the nested
RLEP PCR assay. M. leprae was detected in six (21%) of
the animals; five were from the species D. novemcinctus
(samples 8, 21, 22, 23 and 25) and one was from the spe-
cies E. sexcinctus (sample 9) (Table I). M. leprae DNA
was amplified in the ear biopsy samples of all six ani-
mals, but in only five of the liver or nose biopsy samples
and three of the spleen biopsy samples (Table II).

The PCR amplification of M. leprae with the primers
RLEP2-3 and RLEP2-2 was species-specific, targeting the
same region of the primers developed by Donoghue et al.
(2001). It generated a single band of 238 bp, a larger am-
plexion compared to the Donoghue primers that amplified a
99-bp product. In addition, the sequencing of the gyrA re-

TABLE I
Polymerase chain reaction (PCR) results
for the 29 wild armadillos analyzed according
to the armadillo species in the state of Ceará, Brazil

<table>
<thead>
<tr>
<th>Armadillo species</th>
<th>Total (n)</th>
<th>RLEP PCR positive (n) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dasypus novemcinctus</td>
<td>27</td>
<td>5 (19)</td>
</tr>
<tr>
<td>Euphractus sexcinctus</td>
<td>2</td>
<td>1 (50)</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>6 (21)</td>
</tr>
</tbody>
</table>

Additional, no blood samples were taken from the
animals; therefore, we cannot conclude that the PCR-
positive armadillos had clinical leprosy.

DISCUSSION

This is the first study to identify M. leprae in two
species of wild armadillos (D. novemcinctus and E. sex-
cinctus) in Brazil. The tested armadillos came from an
endemic leprosy area where the prevalence rate was re-
ported to be 2.99/100,000 inhabitants in 2008 and where
there is continuous contact between humans and these
animals (MS/SVS 2011). In this region, the hunting and
eating of armadillos is a popular and frequent practice, a
situation similar to that described in Colombia (Cardona-
Castro et al. 2009). It is noteworthy that previous studies
in the Americas and in the USA (Louisiana), Mexico,
Colombia and Brazil had only reported M. leprae infec-
tion in armadillos of the species D. novemcinctus (Mey-

Clinically, most of the animals exhibited nodule-
like lesions indicative of leprosy or other degenerative
diseases, as demonstrated by a clinical exam conducted
by an expert veterinarian. However, no histopathologi-
cal study was conducted, as the tissue samples were not
formalin-fixed for anatomopathological examination.

Additionally, no blood samples were taken from the
animals. Therefore, we cannot conclude that the PCR-
positive armadillos had clinical leprosy.

The genome sequencing of the Tamil Nadu M. lep-
rae strain and other strains was conducted with a typing
system based on single-nucleotide polymorphism (SNP)
differences allowing continent distribution of the leprosy
bacilli, which were classified as SNP types 1 to 4 (Monot
et al. 2005). In addition, a correlation was observed be-
tween a mutation in the gyrA gene and the SNP types,
which were clustered in gyrAC and gyrAT populations;
the T SNP represents SNP type 3, while the C nucleotide
represents the other three types. The results from the se-
quecing data demonstrated that the analysed samples
from the armadillos in CE belong to the gyrAT (SNP type
3) population, which was also identified in samples from
wild armadillos in Louisiana (Monot et al. 2005) and in
humans in Brazil (Fontes et al. 2009). Although this is not
yet definitive evidence that armadillos act as a source of

<table>
<thead>
<tr>
<th>Biopsy source</th>
<th>Liver</th>
<th>Spleen</th>
<th>Ear</th>
<th>Nose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dasypus novemcinctus (n = 5)</td>
<td>4</td>
<td>2</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Euphractus sexcinctus (n = 1)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
infection in humans, this contributes to the evidence supporting the hypothesis of zoonotic transmission, as suggested by other authors (Job et al. 1986, Walsh et al. 1988, Cardona-Castro et al. 2009, Truman & Fine 2010).

Because M. leprae cannot be cultivated in vitro, its detection is based on the histopathological demonstration of the bacilli in dermal nerves, mouse footpad cultivation and PCR assays of the selective amplification of M. leprae DNA (Truman & Fine 2010). This study used the RLEP repetitive element sequence of M. leprae, which is reported to be specific for M. leprae and is not present in other mycobacterial or bacterial species. In addition, the use of the repetitive sequence as the PCR target DNA provides the advantage of higher sensitivity over other targets because it is present at multiple sites in the genomic DNA (Donoghue et al. 2001, Truman et al. 2008). It has been suggested that many homologous sequences of the RLEP may be present in other environmental Mycobacterium species that have not been thoroughly investigated, which might generate false-positive results. Despite this fact, Martinez et al. (2011) found that the RLEP PCR assay can be used as a more specific and sensitive diagnostic test to detect M. leprae infection compared to the ones based on gene targets Ag 85B, sodA and 16S rRNA. Because we used several strategies to minimise false-positive amplifications and contamination, we are confident that these are real infections.

In conclusion, the presence of M. leprae DNA in wild armadillos (D. novemcinctus and E. sexcinctus) in a leprosy transmission area in Brazil provides additional evidence supporting the hypothesis that armadillos can play a role as an environmental reservoir for the bacillus in this area. Moreover, the finding supports the idea that intensive contact with these animals may increase the risk of infection in CE. The current global control strategy depends on treating all human cases, but a definitive identification of an animal reservoir, as suggested by Truman et al. (2011), could challenge this strategy and may partially explain the continuing and growing presence of leprosy in the studied area. More detailed molecular studies will be useful in monitoring and confirming the transmission of M. leprae between wild armadillos and humans and in guiding new strategies for prevention.

REFERENCES


Armadillo Online! 2012. [homepage on the Internet]. Minneapolis.


Schmitt JV, Dechandt IT, Dophe G, Ribas ML, Cerri FB, Viesi JMZ, Marchioro HZ, Zunino MMB, Miot HA 2010. pArmadillo meat intake was not associated with leprosy in a case control study, Curitiba (Brazil). *Mem Inst Oswaldo Cruz* 105: 857-862.


