

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

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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 occur 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 10⁶ 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, Amezcua et al. 1984, Stallknecht et al. 1987, Zumarraga 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

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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.

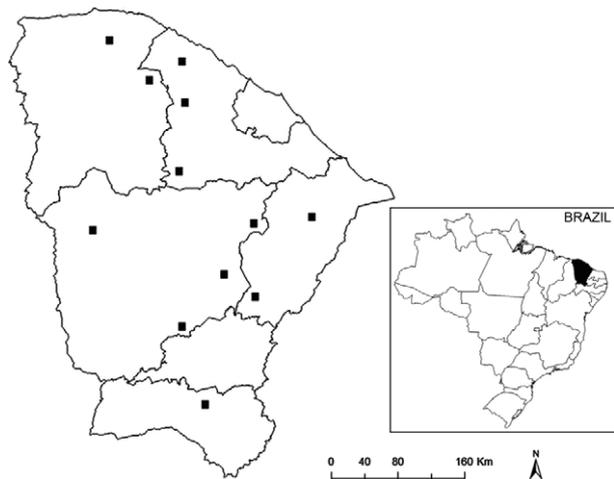
Euthanasia - Animal captures were authorised by the Brazilian Institute of Environmental and Renewable Natural Resources. Before euthanasia, armadillos were anaesthetised with tiletamine and zolazepam (5.0 mg/kg/1.M) (Virbac, Brazil). Euthanasia was conducted in the place of capture and the animals were 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 (RLEP) PCR was amplified in a nested PCR reaction. The primers for RLEP2-1 (5'-ATATCGATGCAGGCGT-GAG-3') and RLEP2-2 (5'-GGATCATCGATGCACT-GTTC-3') amplified a 282-bp sequence of the RLEP element. The second set of inner primers, RLEP2-3 (5'-GGGTAGGGGCGTTTTAGTGT-3') and RLEP2-2, 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 MgCl_2 , 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'-CCCGGACCGTAGCCACGCTAAGTC-3') and *gyrAR* (5'-CATCGCTGCCGGTGGGTCATTA-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



Distribution map of the 12 places where armadillos were collected in the state of Ceará, Brazil.

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 accession 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 identified and kept in separate boxes. A simple DNA extraction protocol was established to minimise the risk of contamination. The extraction method used one purification 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 Federal 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 species *E. sexcinctus* (sample 9) (Table I). *M. leprae* DNA was amplified in the ear biopsy samples of all six animals, 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 amplicon compared to the Donoghue primers that amplified a 99-bp product. In addition, the sequencing of the *gyrA* region (coding sequence location from 7515 to 11451 kb) was confirmatory for the presence of *M. leprae* DNA in the armadillos analysed. The detection limit of the nested PCR for *M. leprae* was 1 pg/μL (data not shown). All negative samples were amplified after adding 1 pg of purified *M. leprae* DNA. The exception was for one sample that was only amplified after a 1000-fold dilution, therefore confirming the presence of substances that were inhibitory to the PCR. The assay was repeated for the few isolates for which there were discrepancies between the researchers.

DISCUSSION

This is the first study to identify *M. leprae* in two species of wild armadillos (*D. novemcinctus* and *E. sexcinctus*) in Brazil. The tested armadillos came from an endemic leprosy area where the prevalence rate was reported 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* infection in armadillos of the species *D. novemcinctus* (Meyers et al. 1977, Truman et al. 1990, Deps et al. 2002).

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 histopathological 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. leprae* 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 between 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 sequencing 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 I
Polymerase chain reaction (PCR) results for the 29 wild armadillos analyzed according to the armadillo species in the state of Ceará, Brazil

Armadillo species	Total (n)	RLEP PCR positive n (%)
<i>Dasyus novemcinctus</i>	27	5 (19)
<i>Euphractus sexcinctus</i>	2	1 (50)
Total	29	6 (21)

RLEP: *Mycobacterium leprae*-specific repetitive element.

TABLE II

Distribution of positive polymerase chain reaction (PCR) results using the *Mycobacterium leprae*-specific repetitive element repetitive sequence primer pairs in different biopsy tissues from wild armadillos in the state of Ceará, Brazil

Armadillo species	Biopsy source			
	Liver	Spleen	Ear	Nose
<i>Dasyus novemcinctus</i> (n = 5)	4	2	5	4
<i>Euphractus sexcinctus</i> (n = 1)	1	1	1	1

infection for 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.

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