Detection of *Mycobacterium leprae* DNA in nine-banded armadillos (*Dasypus novemcinctus*) from the Andean region of Colombia

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Summary

Objective To use DNA detection methodologies to test for *M. leprae* in nine-banded armadillos inhabiting forested regions located around the cities and towns where leprosy patients have been identified.

Design Ear lobe biopsies of 22 nine-banded armadillos were studied during a 2 year period. The biopsies were processed for DNA extraction and amplification by nested polymerase chain reaction (N-PCR) of a fragment of the high copy DNA locus of *M. leprae* known as R-LEP.

Results Nine of the 22 (40.9%) armadillos evaluated showed positive signals for *M. leprae*. Sequencing confirmed that PCR products were identical to the corresponding region of *M. leprae* DNA.

Conclusions In Colombia, South America, the consumption of and contact with the nine-banded armadillo (*Dasypus novemcinctus*) are common, ignoring the fact that this animal can host and be a possible zoonotic reservoir of *Mycobacterium leprae*, the causal agent of leprosy. This is the first study demonstrating that *M. leprae* is present in nine-banded armadillos in a region of Colombia using specific DNA detection. The possibility of leprosy transmission due to contact and consumption of armadillo meat or use of blood for therapeutic purposes should be further investigated.

Introduction

Leprosy has long been considered exclusively a human disease. However, over the course of the past 30 years, naturally-acquired and experimental leprosy has been observed in...
chimpanzees, sooty mangabey monkeys,1,2 and the nine-banded armadillo (Dasypus novemcinctus).3–5 It has been hypothesised that infected animals may serve as an environmental reservoir and source of transmission of leprosy; it is also suspected that some of the natural infections in non-human primates and armadillos were acquired from their handlers.6

Transmission of leprosy continues despite efforts to eradicate the disease, and new cases continue to be reported. In Colombia, based on WHO’s definition, leprosy has been eliminated as a public health threat;6 however, each year 400–500 new cases of leprosy are documented pointing to continued transmission of M. leprae in communities.7,8

In 1971, the armadillo was identified as a research model for experimental infection with M. leprae because it displayed clinical symptoms and pathologies similar to human disease supported by the isolation of large numbers of the bacilli.9 In 1975, wild nine-banded armadillos in Louisiana were noted naturally infected and suffering from leprosy that was identical to the human disease.4,10

Although a definitive mode of transmission has not been identified, earlier studies have reported an association between contact with the nine-banded armadillo and the development of leprosy in regions where armadillos have their natural habitat;11–15 however, studies by Filice et al.16 did not find any association between contact with armadillos and the development of leprosy. More recently a case-control study of 28 leprosy patients determined an association between armadillo exposure through hunting, cleaning, and ingestion of meat and the development of leprosy.17

Cultural practices in Colombia bring armadillos and humans into close physical contact: armadillos are used as a source of meat; their carapace is used to make musical instruments, containers, vases and bowls. Traditional medicinal practices in rural communities use armadillo derivates for multiple purposes. It is believed that discomfort during pregnancy can be relieved by ingesting a hot water infusion of pulverized toasted armadillo carapace and tail. Relief of inflammation, ear ache, and varicose veins is thought to be gained by applying armadillo fat emulsions to the offending site and, according to popular belief, ingestion of fresh blood of the armadillo can cure asthma.18 Considering these practices and the close proximity of humans and armadillos in Colombian regions where leprosy is prevalent, and that transmission is occurring, we set out to determine whether infection with M. leprae can be documented in these animals using specific DNA detection.

Several techniques are used for identifying M. leprae infection in animals. The most universally applicable, the most sensitive and specific technique is PCR, followed by ELISA serology that is dependent on species specific immune conjugates, necropsy and histopathology.19 Truman and others showed a positive cross-reaction between the IgM antibodies of humans and armadillos using ELISA, and detected the antigen PGL-I (phenolic glycolipid-I), which is specific for M. leprae.20 Recently, an immunochromatographic flow test (ML Flow test) was tested for detecting anti-PGL-I IgM in armadillo M. leprae infection.21

However, ELISA and ML flow tests were designed for the detection of human specific antibodies. Efforts have been underway to identify and purify Dasypus novemcinctus immunoglobulins with the aim of preparing specific antisera to evaluate the immune response in these animals;22 however, these specific antisera are not available commercially.

Based on these previous reports, we used specific M. leprae DNA detection by PCR. Specific DNA detection is an alternative to detect M. leprae in armadillo tissues and
several different *M. leprae* DNA targets have been published with different applications for clinical and research purposes.\textsuperscript{23,24} Donoghue *et al.*\textsuperscript{25} proposed a very sensitive PCR test for the detection of low bacterial loads using primers that amplify a family of dispersed repeats (RLEP). Thirty seven copies of RLEP exist in the *M. leprae* chromosome; all but one contains an invariant 488-bp core flanked in some cases by additional segments ranging from 44 to 100 bps (accession number AL583921).\textsuperscript{26,27} This specific *M. leprae* DNA amplification has high sensitivity and specificity and is used for clinical and research purposes.\textsuperscript{28,29}

**Materials and Methods**

**STUDY POPULATION**

During a period of 2 years, 22 armadillos were captured at their dens using net pouches attached at the exits\textsuperscript{30} in the rural area of the Barbosa municipality, a forested region near Medellín city (6°N, 75°W, 1300 metres above sea level, average temperature 23 °C). Figure 1 shows the geographical location where the armadillos were captured.

The armadillos were classified according to morphologic characteristics, age, weighed, sexed and the clinical appearance of skin lesions documented.

![Map of the study area](image_url)
BIOPSY SAMPLES

Armadillos were lightly sedated through an injection of 0.1 to 0.3 mg/kg acepromazine. They received local anesthesia through an injection of 2% xylocaine. Subsequently a 0.5 cm ear biopsy was taken with a sterile scalpel and the biopsy stored in 70% ethanol. The site of the biopsy was cleaned, followed by topical application of oxytetracycline/polimixin B, and Methyl Violet-Diazinon® (antiseptic larvicide). The armadillos continued to be observed until they recovered their activity and were then returned to the forest.

PCR TESTS

*M. leprae* DNA from biopsy samples was extracted using DNeasy kit (Qiagen, USA), according to the producer’s recommendations. Every sample was handled individually for DNA extraction and PCR on different days to avoid cross contamination between samples. All precautions to avoid contamination were followed up. All work surfaces, pipettes and plastic racks were cleaned using 70% ethanol and exposed to UV light for 30 minutes before the samples were manipulated. One room was used to develop the DNA extraction procedure and another, separated room, was used to make the PCR reaction. Both procedures were carried out inside a class II laminar flow hood which had undergone previous disinfection and UV sterilisation. Barrier pipette tips were used for each step. Ultra pure DNase/RNase free distilled water was used for DNA extraction purposes and as a negative and positive DNA control for PCR reaction. A post PCR room was also used to perform the gel electrophoresis for PCR products visualisation in 2.5% agarose gel run for 2 hours at 80 volts.

The first PCR was performed using the LP1 and PL2 primers, and then a nested PCR was carried out using the primers LP3 and LP4. Following the protocol for amplification described by Donoghue et al., an amplification band of 99 pb was expected. Samples were tested in duplicate. Sequences of the primers used are:

- LP1 5'-TGCATGTGTCATGCCCTTTGAGG-3'
- LP2 5'-CACCGATACCAGCGGCAGAA-3'
- LP3 5'-TGAGGTGTCCGCGTGTCGTC-3'
- LP4 5'-CAGAAATGTTGCAAGGGA-3'

*M. leprae* strain NHDP-63 (human origin) propagated in armadillos at the National Hansen’s Disease Program (NHDP); Baton Rouge, LA was used as the positive control. The infected tissue was processed and *M. leprae* DNA was extracted at Colorado State University (CSU) in Fort Collins, CO (NIH- NIAID Leprosy Research Support NO1-AI-25469). The negative control included: PCR master mix, primers, Taq polymerase, and water instead DNA template. Visualisation of the products was done by agarose gel electrophoresis, ethidium bromide staining and UV transillumination. Positive DNA bands from the biopsy samples were submitted for sequencing with the forward and reward primers (LP3 and LP4).

ETHICAL CONSIDERATIONS

This research was performed following the rules and principles stipulated by the International guiding principles for biomedical research involving animals, which are based on the animal rights (Universal Declaration of the Animal Rights 1977). The armadillos in the study
were handled according to article 2, minimising stress and manipulation. Relocation of
wild armadillos was mandatory according to Law 17 of Colombia from January 22, 1981,
for the conservation of fauna and flora.

Results

MORPHOLOGIC CHARACTERISTICS OF ARMADILLOS

Twenty two adult armadillos classified as nine-banded by morphologic characteristics were
studied: 14 male and 8 female; mean weight 4·2 kg, (± 0·66); average length of the carapace
was 30·7 cm (± 5·25); total length from nose to the end of the tail was 67 cm (± 7). The mean
body temperature was 31°C ± 2·5, the mean respiratory frequency was 29·4 ± 11·3 breaths
per minute and the cardiac pulse rate was 106 ± 35·7 beats per minute. Superficial skin
scars were reported in eight armadillos. None of the armadillos showed chronic ulcers or
visible lepromas.

PCR TESTS

Nine of the 22 armadillos (41%) showed positive results for *M. leprae* DNA by nested PCR,
three of them females and six males (P < 0·01). Figure 2 shows the nested PCR results.

Nine armadillos (A) samples A3, 4, 5, 6, 10, 11, 13, 14, and 20 showed positive DNA
amplification; of these nine, three armadillos had superficial skin scars (A6, 11, and 14).

Of the 13 PCR negative animals, five armadillos had skin scars (A9, 15, 16, 21, and 22);
no significant statistical associations were found between skin scars and positive nested PCR
results (P > 0·05).

Five PCR products from three armadillos with skin lesions (A6, 11, and 14) and two
without lesions (A3, and 5) were sequenced and aligned using the Basic Local Alignment
Search Tool. All sequences were 100% identical to *M. leprae*.

Discussion

The presence of *M. leprae* DNA in nine-banded armadillos from a region not previously
explored, and where leprosy cases are found is a new finding. This interpretation is based on
the identification of a small fragment of DNA repeated 37 times in the *M. leprae*

![Figure 2. Nested PCR for M. leprae using LP3-LP4 primers.](image-url)
chromosome. However, the possibility that the DNA products detected belong to an unknown armadillo specific relative of *M. leprae* is not impossible, although difficult to prove.

Leprosy transmission in Colombia is evident since new cases are reported every year, although the prevalence of the country is considered by WHO to have achieved leprosy elimination. The nine-banded armadillo could be a source of infection in this region since the behaviour of the population with respect to the animals is rooted in the culture, as was mentioned previously.

The current study is a screening based on the detection of *M. leprae* DNA in these animals, although the animals studied did not show external lesions or lepromas that would have allowed the taking of biopsy samples to confirm the leprosy diagnosis. Sequence of the amplicons confirmed the origin of the DNA; however association of the PCR results with histopathology to show involvement of *M. leprae* in dermal nerves would be useful to demonstrate disease.

Another study that used PCR in armadillos found *M. leprae* DNA in 52.8% of the animals they sampled, similar to the percentage found in our study (41%). However, the very same animals were analysed using other techniques that provided quite different prevalence values: histopathology ~3%, serology ~7%, and necropsy ~13%.19

Humans were considered to be the only reservoir of *M. leprae*; however leprosy in wild armadillos of the species *Dasypus novemcinctus* (nine-banded armadillo) was reported in 1975, as was natural transmission among armadillos in southern parts of the United States.34 Reports from the USA, Mexico, Argentina, and Brazil indicate that nine-banded armadillos (*Dasypus novemcinctus*) are natural hosts of *M. leprae*.20,21,23,35–38 The nine-banded armadillo builds burrows in moist soil near creeks, streams, and dry creek bed arroyos putting it in direct contact with potentially pathogenic environmental microorganisms, such as *M. leprae*,34,39,40 *Paracoccidioides brasiliensis*,41,42 *Sarcocystis* sp,43,44 *Trypanosoma cruzi*45 and *Coccidiodes imminitis*,46 that may be transmitted to humans.

This is the first study in Colombia to investigate whether *M. leprae* is present in armadillos from leprosy endemic regions and where contact between humans and armadillos is common, since these animals are part of the wild fauna and play an important role in the cultural practices of the local population.

While none of the PCR-positive animals showed clinically visible lesions indicative of leprosy, this finding is not surprising since according to Truman,47 *M. leprae* infection in armadillos produces few gross symptoms, and it is often not possible to distinguish *M. leprae*-infected from uninfected animals.47 To conclusively establish the disease of leprosy in the animals, studied necropsy to confirm organ and tissue involvement and to isolate *M. leprae*, is required. This was not possible in our study protocol because of the absence of clinical guidance. Therefore, we cannot conclude that the tested PCR positive armadillos have leprosy.

Available serologic tests lack specificity and sensitivity for leprosy diagnosis in humans.48 When applied to armadillos, positive cross-reaction between IgM antibodies was shown.47 However, since these tests are not conclusive for human diagnosis, they are also of limited value to document leprosy in armadillos.

Since human/armadillo contact has a cultural basis in several regions where leprosy is endemic, or where leprosy cases had been associated with armadillo exposure,11–15,17 further studies about the relationship between leprosy patients and armadillos in Colombia could provide more information about the environmental reservoir and local transmission of
leprosy, allowing to conduct educational activities for the population at risk to get infected by the close relation and uses that the population gives to this animal.

A standardised protocol for gaining a real understanding of the ecology of leprosy is suggested, since the reports on this topic come from various laboratories, all generated using different techniques and therefore not comparable to estimate leprosy prevalence in armadillos.

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