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Serological and molecular detection of infection with *Mycobacterium leprae* in Brazilian six banded armadillos (*Euphractus sexcinctus*)

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ABSTRACT

Leprosy was recognized as a zoonotic disease, associated with nine-banded armadillos (*Dasypus novemcinctus*) in the Southern United States of America in 2011. In addition, there is growing evidence to support a role for armadillos in zoonotic leprosy in South America. The current study evaluated twenty specimens of the six-banded armadillo (*Euphractus sexcinctus*), collected from rural locations in the state of Rio Grande do Norte (RN), Brazil for evidence of infection with *Mycobacterium leprae*. Serum was examined using two "in-house" enzyme-linked immunosorbent assays (ELISAs) and via two commercially available (ML flow and NDO-LID®) immunochromatographic lateral flow (LF) tests, for detection of the PGL-I and/or LID-1 antigens of the bacterium. The presence of *M. leprae* DNA in liver tissue was examined using the multi-copy, *M. leprae*-specific repetitive element (RLEP), as target in conventional and nested PCR assays. Molecular and anti-PGL-I-ELISA data indicated that 20/20 (100 %) of the armadillos were infected with *M. leprae*. The corresponding detection levels recorded with the LF tests were 17/20 (85 %) and 16/20 (85 %), for the NDO-LID® and ML flow tests, respectively. Our results indicate that, in common with *D. novemcinctus*, six banded armadillos (a species hunted and reared as a food-source in some regions of Brazil, including RN), represent a potential reservoir of *M. leprae* and as such, their role in a possible zoonotic cycle of leprosy within Brazil warrants further investigation.

1. Introduction

Leprosy, caused principally by infection with the bacterium *Mycobacterium leprae*, is a chronic, disfiguring and disabling disease of humans that was recognized in many ancient societies [1]. The elimination of leprosy as a public health problem was announced by the World Health Organization (WHO) in 2000; a result obtained mainly through the application of free and effective multidrug antibiotic therapy [1].

Mycobacterium leprae was identified as the etiological agent of leprosy in 1873. Yet, despite almost 150 years of accumulated knowledge, leprosy still holds some surprises. In this context, in some regions of Mexico and the Caribbean leprosy is caused by *M.* lepromatosis, disproving the theory of *M. leprae* as the sole etiological agent [2]. In addition, a possible role for animals including armadillos, squirrels and non-human primates in the ecology of *M. leprae* and *M. lepromatosis* has emerged [3–7]. Indeed, nine-banded armadillos (*Dasypus novemcinctus*) were unequivocally shown to be involved in both the maintenance and

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transmission of human pathogenic strains of *M. leprae* in the United States of America, where leprosy is currently classified as a zoonotic disease [7].

The discovery of naturally infected populations of nine-banded armadillos in the USA in the 1970s, was initially viewed as advantageous for leprosy research, insofar that it provided researchers with a model system for the culture of the organism [8]. The infection of armadillos with *M. leprae* is believed to have been a relatively recent (last 500 years) event, that occurred through the colonization of the Americas by Europeans and the forced introduction of African slaves, who each brought their infectious diseases with them [5]. The recent confirmation of zoonotic leprosy involving *D. novemcinctus* in the southern USA [7], provides an elegant example of how invasive anthropogenic activities almost inevitably result in unforeseen consequences for future generations of humans.

Brazil has the second-largest number of leprosy cases worldwide and demonstrates the highest incidence of the disease in the Americas [1,9]. The state of Rio Grande do Norte (RN) shows one of the lowest coefficients of detection in the country but the municipality of Mossoró, located in the east of that state, is hyperendemic for the disease [10,11]. Intriguingly, this region maintains a substantial population of the six-banded armadillo (*Euphractus sexcinctus*) and the practices of hunting and consumption of those armadillos is commonplace in both rural and urban populations [12,13]. Integrated approaches based upon serological, molecular and clinical data supported the epidemiological investigations that uncovered the participation of armadillos in zoonotic leprosy in the Southern USA [6,7] and there is a limited, but growing, body of molecular, histopathological and epidemiological data that points to the same role for nine banded armadillos in Brazil [12–14] and Colombia [15]. Nevertheless, other workers contend that in Brazil armadillos are unlikely to pose a significant public health concern [16–18].

The detection of serum antibodies against phenolic glycolipid-1 (PGL-I; most commonly in the form of the natural disaccharide-octyl of PGL-I = ND-O), or towards LID-1, a fusion of the ML0405 and ML2331 proteins [19], has been used in ELISA and in rapid, immuno-chromogenic lateral-flow (LF) test formats to confirm exposure to *M. leprae* in humans [20,21]. Moreover, studies conducted in the USA and Brazil identified the potential of serology for the detection of non-human sources of *M. leprae* [6,7,14,22]. This was possible because of cross reactivity between armadillo immunoglobulins and the anti-human IgM or protein G conjugates employed in the tests [21]. Two LF tests are currently used in Brazil; the ML flow test is a single antigen (NT-P = trisaccharide-octil of PGL-I), system that detects human anti-PGL-I IgM antibodies [23,24], while the NDO-LID® system [21], combines the NDO antigen in conjugation to the LID-1 fusion protein. In contrast to the ML flow system, the NDO-LID® test has not previously been used to evaluate the infection status of armadillos.

Molecular detection of *M. leprae* in clinical and environmental samples has been reported using a variety of target sequences using both conventional and real time-PCR formats [25]. However, it has been established that assays targeting the multicopy, *M. leprae*-specific repetitive element (RLEP), represent the most specific and are among the most sensitive molecular tests available for detection of the bacterium in a variety of clinical samples of both human and animal origin [26,27].

The aim of this study was to examine the potential of six banded armadillos, a species found in the north, north-east, central-west, southeast and south of Brazil, as well as in Bolivia, Paraguay, Uruguay and northern Argentina, to act as a reservoir for *M. leprae*. A previous investigation, performed in the mid-western region of the state of São Paulo, found no-evidence of infection using conventional RLEP-PCR, in a sample of 22 specimens of *E. sexcinctus* [17], while a subsequent study detected the RLEP target in a one out of two specimens sampled in the northeastern state of Ceará [12]. The serological and RLEP-PCR data obtained herein, demonstrated that in common with *D. novemcinctus*,

six banded armadillos (a species hunted and reared as a food-source in some regions of Brazil including RN), represent a potential reservoir of *M. leprae* and as such, their role in a possible zoonotic cycle of leprosy warrants further investigation.

2. Material and methods

2.1. Setting

Mossoró is the second most populous city in the State of Rio Grande do Norte (RN), Brazil, with an urban population that increased from 192.267 in the 1991 census to 291.937 inhabitants in the most recent one, conducted in 2016. The city of Mossoró covers an area of 150 km² with the municipality, including rural areas, showing an extension of 2,110.207 km². Rio Grande do Norte is a small Brazilian state that recorded a rate of 8.21 new cases of leprosy per 100,000 inhabitants, a value significantly lower than its neighboring Northeastern states. However, the incidence of new cases in Mossoró recorded in 2013 was 45.4 cases/100,000 people/year, which represented almost half of the new cases in RN, a finding that classified Mossoró as hyperendemic in accordance with Brazilian Ministry of Health criteria [11].

2.2. Ethical approval

The project was approved by the Ethics Committee of The Federal Rural University of Rio de Janeiro (Protocol number 8068280716) and was licensed by The Brazilian Institute for the Environment and Natural Resources (IBAMA) for the capture and collection of wildlife material (Protocol number 50564-2).

2.3. Capture and processing of six-banded armadillos

A total of twenty (20) specimens of *E. sexcinctus* were obtained live by wildlife veterinarians from twenty distinct locations, within five rural municipalities (Pendências, Afonso Bezerra, Macau, Pedro Avelino e Guamaré). The collection of the first ten animals took place in May of 2016 and that of the remaining specimens in June of the same year. Specific details of which animals were collected at each location are provided in Supplementary data Table S1 and are depicted in the map presented in Supplementary Figure S2. The sites were selected based on previous knowledge of the practices of hunting and post-capture fattening, prior to consumption, of armadillos in those municipalities. As informed by the site owners, animals had been recently captured by hunters and were being maintained in earth-filled tanks. Individual animals were identified by tagging on site and then transported to a holding facility located within the wildlife animal hospital at The Federal Rural University do Semi-Árido (UFERSA), located in Mossoró, where they spent 48 h of acclimatization to minimize post-capture stress. Thereafter, animals were weighed and lightly anesthetized by the intramuscular administration of tiletamine-zolazepam (Zoletil® 50, Virbac, Brazil), at a dose of 4 mg/Kg to permit visual inspection of the entire body for the presence of lesions indicative of infection with *M. leprae* as recommended by Sharma et al. [8].

At the same time, blood was collected in an untreated 10 mL volume syringe by cardiac puncture using a 22 G 1 1/4-gauge needle. A portion of the blood was transferred to a 13×75 mm, 3.0 mL volume BD Vacutainer® tube containing 3.4 mg of spray-coated K_2EDTA (Becton Dickinson) and was transported to the clinical laboratory at the Veterinary Hospital of UFERSA for hematological analyses not related to this study. The remainder of each sample was passed to a 16×100 mm, 10 mL volume, plain/no additive, BD vacutainer tube (Becton Dickinson) and allowed to clot for 1 h at ambient temperature (25 °C), followed by centrifugation (1500 x g for 15 min at 4 °C), to produce fully separated serum. Serum samples were aliquoted in 100 µl volumes in Eppendorf centrifuge tubes (1.5 mL volume) and frozen on dry ice for transport to The Laboratory of Cellular Microbiology (LCM),

Oswaldo Cruz Institute, FIOCRUZ, Rio de Janeiro. Upon arrival, tubes were transferred to a freezer ($-20\,°C$) and stored until used in the immunological analyses as described below.

Euthanasia, performed by the administration of potassium chloride via the femoral vein at a dose of 2.56 mEq / Kg (1 mL / kg of a 19.1 % solution), was initiated upon confirmation of loss of the corneal reflex in the previously anesthetized animals. Samples of liver were collected for molecular analyses during necropsy, placed in 15 mL volume falcon tubes, containing 2 mL of RNA*later*® (Ambion Inc, USA) with short-term (24 h) storage at 4 °C, before freezing on dry ice for transport to FIOCRUZ for molecular analyses.

2.4. Immunochromatographic lateral flow tests

The NDO-LID® rapid test (Orange Life, Rio de Janeiro, Brazil) was developed in 2014, by impregnating nitrocellulose membranes with ND-O-LID-1, a conjugate of the ND-O antigen and the LID-1 protein [21]. This test is described as a semi-quantitative IgM/IgG method and was introduced to address concerns that tests based only on detection of anti-PGL-I IgM antibodies, were missing paucibacillary forms of the disease resulting in delayed diagnosis [21]. As recommended by the manufacturer and as reported recently [20], the kit uses a 10 μL volume of either whole blood or serum with a positive result based upon the chromogenic detection of IgM antibodies to PGL-I and IgG antibodies specific to LID-1.

The ML Flow test (acquired from Dr. Samira Bührer-Sékula - Royal Tropical Institute, KIT Biomedical Research, Amsterdam, the Netherlands) is based upon the detection of anti-PGL-I (NT-P-Bovine Serum Albumin; BSA) IgM antibodies and was first described in 2003 [28]. The ML flow system has been and continues to be widely used as a rapid, first line screening method for leprosy diagnosis in humans [29,30] and animals [24]. In common with the NDO-LID® rapid test, a 10 μL volume of either whole blood or serum is applied and provides a definitive result within 10 min. In the current study, the tests were used according to the recommended protocols, employing 10 u L volumes of undiluted armadillo serum.

2.5. Serum samples employed in a pilot study of the NDO-LID® rapid test

The ML flow test was previously evaluated with sera collected from nine-banded armadillos in Brazil [24], while the NDO-LID® rapid test has never been used with armadillo sera. Thus, to establish the suitability of the NDO-LID®test for the planned purposes of our research, a pilot study was conducted at the National Hansen's Disease Program, Baton Rouge, Louisiana, USA. A total of 78 sera, collected from nine-banded armadillos in the Southeastern U.S.A and previously characterized as PGL-I reactive (n = 35), LID-1 reactive (n = 22) or unreactive for both antigens (n = 21) using ELISA [6,22] were selected. The sera (10 μL) were examined in the NDO-LID® rapid test using the manufacturers recommended protocol.

2.6. Measurement of levels of IgM anti-PGL-I and IgG anti-LID-1 by ELISA

Levels of armadillo IgM against PGL-I and IgG against LID-I were measured by ELISA. The development of the assays was based upon a slightly modified version of previously reported protocols [22]; briefly, 96-well microplates (Nunc) were coated with 50 μl of the antigens NDO-BSA (2 ng/μL; donated by the Biodefense and Emerging Infections Research Resources Repository listed at https://www.beiresources.org/Catalog/antigen/NR-19329.aspx or LID-1 (1 ng/μL; provided by The Infectious Disease Research Institute, Seattle, WA, USA) in 100mMcarbonate/bicarbonate buffer (pH 9.6) at 4 °C for 16 h. Coated plates were blocked with phosphate-buffered saline (PBS; pH 7.4,supplemented with 5 % w/v BSA) for 1 h at room temperature (25 °C). Serum samples (50 μL),at the pre-determined dilution of 1:35 prepared in PBS-Tween (PBS/T = 10 mM phosphate buffer pH 7.4, 150 mM NaCl,

0.05 %),were tested in triplicate, with incubation at room temperature for 1 h,after which time individual wells were washed three times with 200 μl of PBS/T. Horse radish peroxidase (HRP)-conjugated secondary antibodies (Anti-Human IgM (μ-chain specific)-produced in goat (Sigma-Aldrich St. Louis, USA), diluted 1:5000 in PBS/T supplemented with 1 % w/v BSA were used in the PGL-I assays. In the case of the LID-I assays, HRP-conjugated recombinant protein G, Pierce Appleton, Wisconsin, EUA) diluted 1:2500 in PBS/T supplemented with 1 % w/v BSA was used. In both assays, plates were incubated for 1 h at 25 °C, to allow binding of the detection conjugates. Plates were washed as described above and the reaction was developed by the addition of 3,3′,5,5′-Tetramethylbenzidine (TMB – Sigma-Aldrich, St. Luis, MO, EUA), and protected from light using aluminum foil. Based on initial optimization results, the reaction was stopped after 15 min by the addition of 2.5 M H_2SO_4 (50 μL per well). Absorbance was measured at 450 nm on an Eon microplate spectrophotometer (Biotek, Inc. Winooski Vermont, EUA). Positive responses were defined when optical density (OD) values of > 0.101 (ELISA anti-PGL1) and > OD 0.5 (ELISA anti-LID1) were recorded. The cut-off values for each ELISA were determined using a total of seven serum samples, classified as negative for both PGL-I and LID-1, that had been collected from *D. novemcinctus* in the USA and shipped to FIOCRUZ. Sera samples absorbed into Nobuto paper strips (absorbing area contained the equivalent of 0.1 mL of serum) were resuspended in PBS/T with incubation on a rocking shaker at 4 °C during overnight to obtain the desired dilution (1:35).

The results of the ELISAs were expressed as the mean OD of triplicates. Two positive (for PGL-I and LID-1) human sera samples were diluted (1:200) and included in triplicate as assay performance controls to evaluate inter-assay reproducibility.

2.7. Nucleic acid extraction and molecular detection of M. leprae DNA from six banded armadillo liver

Nucleic acids contained in samples of armadillo liver (25 mg) preserved in RNA*later*® (Ambion Inc, USA) were extracted using the DNeasy Blood & Tissue kit (Qiagen), following the manufacturer's guidelines. Extractions were performed on two separate occasions to confirm reproducibility. In addition to the armadillo samples, the extraction procedures included duplicate, extraction negative controls composed of 25 mg of bovine liver. In all cases, DNA was eluted from the silica capture filters using 100 μL of AE buffer (10 mM Tris-Cl. 0.5 mM EDTA; pH 9.0). The DNAs extracted (2 μL), were submitted to molecular analysis for amplification of *M. leprae* specific repetitive element (RLEP) using conventional and nested-PCR, as described by Frota et al. [12]. Blank tubes (2), containing water in place of DNA were included for contamination control during each assay. All assays were repeated at least twice. Purified *M. leprae* DNA was included in all assays at final concentrations of 50 and 5 fg (fg), in order to confirm interassay reproducibility. The DNA was produced from bacilli of *M. leprae* (strain Thai-53), recovered from nude mouse footpads as reported previously [31] and quantified using the Qubit™ dsDNA HS Assay Kit (Invitrogen). Samples negative in the first-round PCR´s (using 40 cycles), were subsequently examined by nested PCR (using 25 cycles) together with the negative controls from the same assay (water and bovine liver), wherein 1 μL of the first reaction was used as template for 24 μL of nested master-mix [12]. Post amplification identification of PCR products was performed by restriction fragment length polymorphism (RFLP) analysis, using the enzymes *Dde*I (New England Biolabs) and *Msp*I (Promega), in separate reactions, to digest the 282-base pair (bp) product of the first-round assay and/or the 238 bp nested product. Digestions were made by combining 5 μL of amplicon, 1.2 μL of the appropriate reaction buffer, 1.2 units of restriction endonuclease, 1.2 μL of bovine serum albumin (0.1 mg/mL) and molecular biology grade water to a final volume of 12 μL. Incubation was at 37 °C for 3 h, followed by electrophoresis in agarose gels (2.5 %) at a constant voltage of 5 V/cm for 90 min. Gels were stained with ethidium bromide,

observed and digitally photographed under ultra-violet illumination. Banding patterns were compared between samples and via comparison to a DNA molecular weight marker (Gene-Ruler 50 bp DNA Ladder; Thermo Scientific, Brazil). The resulting images were analyzed using the free software Gel-Analyzer, available at http://www.gelanalyzer. com, to accurately determine the size of individual restriction fragments.

2.8. Statistical analysis

The agreement between the serological tests was determined through the Kappa index and the results analyzed using the program BioEstat version 5.3

3. Results

3.1. Description and clinical status of the test population

The test population comprised 20 animals (nine females and eleven males), with an average weight of 1.645 Kg (Standard deviation ± 0.491). Clinical examination detected the presence of skin lesions in six animals (30 %), splenomegaly in four (20 %) and lymphadenopathy in seven (35 %). These symptoms have previously been suggested to be correlated with *M. leprae* infection [6,14]. One animal (T03) died during the acclimatization period. Although the exact time of death could not be determined, it was possible to collect blood and liver tissue from that specimen.

3.2. Assessment of suitability of the NDO-LID test using sera from North American nine-banded armadillos

The results in relation to the evaluation of the NDO-LID rapid test with North American serum samples are provided in Tables 1a and 1b. Therein, positivity or negativity recorded in the LF system, was compared to the previously established classifications of the 78 samples of armadillo sera that had been made using anti-PGL-I ELISA and anti-LID-1 ELISA. The classifications allocated to the Kappa values were based

Table 1a

Pilot evaluation of the NOD-LID-1 rapid test employing sera previously recovered from nine-banded armadillos (*Dasypus novemcinctus*) in the Southeastern USA and characterized as positive or negative in Enzyme Linked Immunosorbent Assays (ELISA); using PGL-I (A) or LID-1 (B) as antigen.

(A)

NDO-LID1 TEST	ELISA-anti PGL = I		TOTAL
	POSITIVE[1]	NEGATIVE[2]	
POSITIVE	34	2	36
NEGATIVE	1	19	20
TOTAL	35	21	56

P < 0.0001.
 Kappa: 0.80 (very good concordance; Rosner B. 2011).
 [1]A total of 35 samples previously classified as positive in PGL-I ELISA.
 [2]A total of 21 samples previously classified as negative in PGL-I ELISA.

(B)

NDO-LID1 TEST	ELISA-anti LID1		TOTAL
	POSITIVE[3]	NEGATIVE[2]	
POSITIVE	14	2	16
NEGATIVE	8	19	27
TOTAL	22	21	43

P = 0.0001.
 Kappa: 0.54 (good concordance; Rosner B. 2011).
 [1]A total of 22 samples previously classified as positive in LID-1 ELISA.
 [2]A total of 21 samples previously classified as negative in LID-1 ELISA.

Table 1b

Performance characteristics of the NOD-LID-1 test for serological detection of *M. leprae* in *Dasypus novemcinctus* in comparison to Enzyme Linked Immunosorbent Assays (ELISA); using PGL-I (A) or LID-1 (B) as antigen.

(A)

NDO-LID-1 test x ELISA anti-PGL-I	%	IC (95 %)
Sensitivity	97.1	91.6 → 102.7
Specificity	90.5	77.9 → 103.0
pre-test probability	62.5	49.8 → 75.2
Positive Predictive Value	94.4	87.0 → 101.9
Negative Predictive Value	95.0	85.4 → 104.6
Accuracy	94.6	88.7 → 100.5

(B)

NDO-LID-1 test x ELISA anti-LID1	%	IC (95 %)
Sensitivity	63.6	43.6 → 83.7
Specificity	90.5	77.9 → 103.0
Pre-test probability	51.2	36.2 → 66.1
Positive Predictive Value	87.5	71.3 → 103.7
Negative Predictive Value	70.4	63.1 → 87.6
Accuracy	76.7	64.1 → 89.4

upon the criteria established by Rosner [32]. The agreement between the NDO-LID test and the anti-PGL-I ELISA was very good (Kappa: 0.80; p < 0.0001), with high levels of sensitivity (97.1 %) and specificity (90.5 %). Good agreement (Kappa: 0.54, p = 0.0001) was observed with the anti-LID-1 ELISA, with reasonable sensitivity (63 %) and satisfactory specificity (90.5 %).

3.3. Serological analysis of six banded armadillos

The results obtained for the twenty samples analyzed in each of the four serological tests are presented in Table 2. Positive results were recorded in 20/20 (100 %) of samples examined using the anti-PGL-I ELISA, while only the sample from armadillo T08 (1/20; 5 %) was considered positive in the anti-LID-1 ELISA. A total of 17/20 (85 %) of sera examined by the NDO-LID rapid test were scored as positive while 16/20 (80 %) positives were registered for the ML Flow test. These results were confirmed upon re-testing all samples. The NDO-LID test tended to produce sharper and more readily interpretable bands than ML flow system.

As observed in the preliminary evaluation performed with the *D. novemcinctus* sera, the LF tests showed some discordance with the anti-PGL-I ELISA data recorded for *E. sexcinctus*, specifically regarding false negatives. Only 2/20 (10 %) of the samples, specifically T03 and T16, were negative for both LF tests. In case of animal T03, the amplification of RLEP by first-round PCR strongly supported infection with *M. leprae* and the OD value of 0.286 in the anti-PGL-I ELISA (Table 2), indicated clear seroconversion. The sample T16 presented the lowest OD value (0.19), which was close to the cut-off used in the anti-PGL-I ELISA and molecular examination of DNA extracted from liver revealed positivity only in the nested-PCR (Table 2). In this context, nested-PCR data were reproducible for the DNA extracted from animal T16 on two separate occasions. Importantly, DNA from the bovine liver samples co-extracted and processed together with the 20 armadillo samples was consistently negative for the presence of RLEP amplicons.

Additional discrepancies between the LF and the ELISA included data obtained with samples T02 and T14, that were recorded as positive in the NOD-LID® test and negative for the ML flow test (Table 2), even after repeating the test twice. The OD value recorded for sample T14 was 0.426, which was higher than the values recorded for 5 samples (T09, T11, T12, T13 and T17), which each produced clearly positive responses in both LF tests (Table 2). Sample T14 was also positive for the amplification of RLEP in the first-round PCR. Sample T02, presented a lower OD value (0.222) but was clearly positive in the NDO-LID® test

Table 2
Serological, molecular and anatomopathological data recorded for twenty specimens of wild caught *E. sexcinctus* armadillos collected from five distinct Municipalities, in the State of Rio Grande do Norte (RN), Brazil.

Sample	Value (OD) ELISA anti-PGL1[a]	Value (O.D) ELISA anti-LID1[b]	ML flow test	N-OD-LID test	Amplification RLEP-PCR	Anatomopathological analysis
T01	0.821	0.08	+	+	+	skin lesions
T02	0.222	0.01	−	+	+	hemodilution
T03	0.286	− 0.03	−	−	+	animal died
T04	0.867	0.02	+	+	+	splenomegaly
T05	0.632	0.04	+	+	+	lymphadenopathy
T06	0.640	0.02	+	+	+	NA
T07	0.255	0	+	−	+	NA
T08	0.593	0.9	+	+	+	NA
T09	0.367	− 0.06	+	+	+	NA
T10	0.531	− 0.01	+	+	+	splenomegaly
T11	0.341	0.01	+	+	+	Lymphadenopathy and skin lesions
T12	0.341	− 0.02	+	+	+	skinlesions
T13	0.360	− 0.01	+	+	+	Lymphadenopathy and skin lesions
T14	0.426	0.08	−	+	+	splenomegaly
T15	0.302	− 0.03	+	+	+	lymphadenopathy
T16	0.190	− 0.02	−	−	+[c]	NA
T17	0.370	− 0.02	+	+	+	splenomegaly, skin lesions and steatosis
T18	0.464	0.1	+	+	+	Lymphadenopathy and skin lesions
T19	0.479	− 0.06	+	+	+[d]	lymphadenopathy
T20	0.268	− 0.01	+	+	+	lymphadenopathy

[a] Reactivity of anti-PGL1 IgM antibodies (cut-off value OD 0.101).
[b] Reactivity ofanti-LID1 IgG antibodies (cut off value OD 0.500) recorded for six banded armadillo sera, collected from 20 specimens originating from five distinct municipalities in the state of Rio Grande do Norte (RN), Brazil.
[c] positivity recorded only when examined by Nested PCR.
[d] positivity recorded in first round PCR following dilution of template. NA: no anatomopathological alterations observed.

and presented evident amplification in first-round PCR. Consequently, the molecular data coupled to the readily interpretable nature of the OL test, supported the positivity recorded for T02 in the anti-PGL-I ELISA.

In contrast to samples T02 and T14, the serum from animal T07 was positive by ELISA (OD 0.255) and in the ML flow system but classified as negative by the NOD-LID® test. Yet, in common with T02 and T14, amplification the 282 bp first-round PCR product indicated that T07 was indeed infected with *M. leprae* and that the reproducibly, false negative result in the NOD-LID® test most likely reflected an undetermined inhibitory mechanism.

3.4. Molecular analyses of E. sexcinctus armadillos

The sensitivity of the PCR assays was determined using the control *M. leprae* DNA. The first-round PCR produced a clear band for the 50 fg positive control DNA and a weak band for the 5 fg sample. Nested PCR of the first-round product of the 5 fg samples produced a clear band. As shown in Table 2, 18/20 samples tested positive for the presence of the 282bp RLEP amplicon in first-round PCR and they each generated a 238 bp amplicon in the nested assay. Importantly, all water and bovine liver controls were negative in both the first-round and the nested PCR´s. The two samples that failed to produced amplicon in the conventional PCR were T16, which as reported above was only positive by nested PCR and the sample T19. The inability to amplify T19 was observed for DNA extracted from two distinct liver fragments. Serial, ten-fold dilutions (1:10 and 1:100) of those samples were prepared in duplicate in AE buffer, to assess the possibility of inhibition. DNAs extracted from both liver fragments that were diluted 1:10, generated amplicon in all four of the first-round PCR reactions. In contrast, samples diluted 1:100 produced amplicon in only 1 of 4 first-round reactions but were positive in 3 of 4 nested reactions.

Digestion of the 282 bp amplicon generated from positive control DNA (50 fg), with *Dde*I generated bands of 214 bp and 68 bp, while digestion with *Msp*I produced fragments of 208, 30 and 44 bp. The same banding patterns were observed for amplicons from the nineteen, first-round positive liver samples. The banding patterns generated for the positive control, nested amplicon (238bp), were 170 and 68 bp for *Dde*I and 164, 30 and 44bp for *Msp*I. Digestion of amplicon generated from

T16 and the other nineteen samples generated identical patterns, confirming the presence of *M. leprae* DNA in all 20 samples.

4. Discussion

The discovery that nine-banded armadillos play an undisputable role in zoonotic leprosy in the USA, has resulted in a paradigm shift in how the disease is viewed and has stimulated research on zoonotic leprosy in other regions of the Americas including Brazil. However, it is evident that unraveling the dynamics of possible zoonotic leprosy in some regions of Brazil, including Rio Grande do Norte (RN), may be more complicated than was the case in the Southern USA. Firstly, because of the extensive heterogeneity in relation to the endemicity of the disease among humans [11,12] and secondly because Brazil presents a greater diversity of armadillo species than in North America, with 17 recognized species including *D. novemcinctus* and *E. sexcinctus* widely distributed within Brazilian territories [17].

Prior surveys employing RLEP-PCR and to a lesser degree serology, to examine populations of armadillos (principally *D. novemcinctus*), for the presence of *M. leprae* in various regions of Brazil and in Colombia, generated contradictory findings [12,13,15–17,24]. The inclusion of epidemiological evaluation of human/armadillo interactions (hunting, handling carcasses or consumption of armadillo meat, including viscera), using data collected via questionnaires, provided a tantalizing indication that at least in some regions of Brazil, the nine banded armadillo may be implicated in zoonotic transmission of *M. leprae* and as such, could contribute to the persistence of leprosy in those regions and possibly others where similar interactions are common place [13,14]. In contrast, other authors found no epidemiological evidence to link the consumption of armadillo meat with leprosy in their investigation conducted in the Southern Brazilian city of Curitiba [18].

Previous research performed in the Brazilian states of Espírito Santo and Pará, presented evidence for the infection of *D. novemcinctus* with *M. leprae* using the ML flow lateral flow system [24], or via molecular and/or histopathological methods [13]. In addition, infections of *D. novemcinctus* and *E. sexcinctus* with *M. leprae* were reported in the state of Ceará using the same nested RLEP-PCR assay as employed herein [12]. However, the earlier observation that *M. leprae* DNA was not

detected in any of the twenty-two *E. sexcinctus* collected in mid-western, São Paulo by Pedrini and colleagues [17], albeit using an alternative RLEP-PCR assay, indicated that six banded armadillos were unlikely to be an important reservoir for the pathogen.

Clinical inspection of the 20 specimens of *E. sexcinctus* examined herein, provided some evidence for non-definitive clinical signs (splenomegaly, skin lesions and lymphadenopathy) of the kind reported in other species of armadillo infected with *M. leprae* [8]. However, serological data clearly confirmed the ability of this species of armadillo to produce a robust humoral immune response to the PGL-I antigen of the pathogen, as demonstrated in three distinct detection platforms. The levels of 100 %, 80 % and 85 % positivity that were reproducibly recorded for the anti-PGL-I ELISA, ML flow test and NDO-LID® system, respectively, exceeded those reported to date using serological methods to examine specimens of *D. novemcinctus* captured in Brazil [14,24] or the USA [6]. Interestingly, data in relation to the anti-LID-1 ELISA showed that only a single animal had produced detectable antibodies towards the recombinant LID-1 antigen. A detailed evaluation, conducted by North American researchers of the dynamics of immune responses to a variety of antigens including NDO and LID-1 in experimentally infected, nine banded armadillos demonstrated that seroconversion patterns were highly individual and essentially unpredictable [22]. However, in contrast to our findings a substantial number of the animals responded to the LID-1 antigen [22]. The limited sero-reactivity observed herein, indicated that the LID-1 antigen was poorly antigenic in naturally infected *E. sexcinctus*.

Confidence in the validity of the serological findings was provided by the observation that 100 % of the liver samples were reproducibly positive for the presence of *M. leprae* DNA, with 19/20 detected in first-round PCR assays, confirming the ability of *M. leprae* to infect *E. sexcinctus* as reported in the neighboring state of Ceará [12]. Nevertheless, given the limited number of animals examined in each of the three studies performed to date with this species of armadillo, it would be imprudent to ascribe a greater significance to our positive data than to the negative findings of Pedrini et al. [17]. Indeed, it is pertinent to note that highly variable levels of infection have also been reported within Brazilian populations of *D. novemcinctus* (62 % in Pará; 18.5 % in Ceará, 0 % in Amazonas and 0 % in mid-western São Paulo and Mato Grosso) [12,13,16,17] and in the United States as reviewed by Balamayooran et al. [5]. The possibility that inter-study methodological differences may have contributed, at least in part, to the observed variability of detection should not be discounted. In this context, the study of Pedrini et al. [17] used a PCR assay of undetermined sensitivity, based upon the primers reported by Woods and Cole [33] and included no tests for the presence of inhibitory substances in their DNA preparations, which were produced using a phenol-chlorofom extraction/purification procedure. All DNA samples dervided from *E. sexcinctus* in that study were negative, while a positive control in the form of liver from an experimentally infected (presumable with an elevated bacterial load), specimen of *D. novemcinctus* produced amplicon of the expected size. The study of Frota et al. [12] also used phenol-chloroform extraction but employed a highly sensitive nested PCR and confirmed that inhibition was not responsible for the lack of amplification in samples of either species of armadillo recorded as negative for *M. leprae* DNA. Inhibition of amplification of one sample (T19), was recorded in our study using DNA extracted from duplicate tissue fragments but was resolved by dilution of template, a solution also employed by Frota et al. [12]. The level of 62 % positivity, recorded by da Silva et al. [13], was obtained using DNA extracted and purified using a commercial kit, similar to that used in our study. However, those authors used only first-round PCR to detect the RLEP biomarker and employed an adapted amplification protocol based on the widely employed primers reported by Donoghue et al. [34]. Interestingly, the use of nested PCR in our study resulted in the detection of an additional infected animal, demonstrating the increased sensitivity offered by that method. Clearly, it would be of value to perform a comparative evaluation of different extraction and

amplification protocols in an attempt to develop a standardized method that would permit more meaningful comparisons of data between future studies on this topic.

Performing surveys of wild animal populations for zoonotic agents is not straightforward. In the case of armadillos, difficulties begin with the capture of the animals but extend to the need to respect environmental legislation that limits the invasive and lethal sampling methods that can be employed. Our results indicate that both the ML flow, as reported previously [24] and the NDO-LID® test formats could be used for the convenient, rapid and reliable evaluation of populations of Brazilian armadillos, specifically six and most likely nine-banded armadillos, as a reservoir of *M. leprae*. In the absence of a precedent, it was considered essential to perform an evaluation of the NDO-LID® test using known positive and negative armadillo (*D. novemcinctus*) sera. On the one hand, the correlation recorded between the anti-PGL-I ELISA and the NDO-LID® test was shown to be very high, with only a single possible false negative and two apparent false positives. On the other hand, the agreement with the anti-LID-ELISA was less convincing, specifically the level of possible false negatives (8/22) was considered unacceptably high. In the case of both assays, revision of the OD values showed the possible false positive samples to be borderline (in relation to the cut-off values of the two ELISAs). Notwithstanding, the performance of the NDO-LID® test with sera of nine-banded armadillos possessing anti-PGL-I IgM was highly satisfactory, justifying its inclusion in the subsequent comparative evaluation performed with *E. sexcinctus*.

The disagreements recorded between the LF tests and the anti-PGL-I ELISA, when applied to the *E. sexcinctus* sera, were indicative of false negative results for both LF tests. This hypothesis was supported by detection of the RLEP biomarker in all LF negative samples. In most cases, the false negative samples showed lower reactivity in the anti-PGL-I ELISA relative to samples that were unequivocally positive in the LF tests (Table 2). It may be the case, that LF negative animals were in the early stages of infection with *M. leprae*, resulting in levels of seroconversion below the limit of detection of the LF tests. In the specific case of sample T03, negativity might be linked to the fact that this animal died during the acclimatization period and that the delay in processing, relative to the others, may have led to post-mortem degradation of serum immunoglobulins as reported for foxes in Scandinavia [35]. In practical terms, false positives would result in unnecessary costs associated with the transport of material to centralized laboratories and the execution of ELISA. However, the existence of false negatives would be more problematic since such samples would not be identified for further analysis. Thus, at least in theory, infected animals would be left in circulation which could result in a potential risk for zoonotic transmission.

As confirmed in this study, ELISA techniques should be considered the gold standard immuno-diagnostic method as suggested by other authors [22]. However, the use of such methods requires transport, under conditions capable of maintaining sample integrity, to specialist laboratories which may be impractical in many instances. Our data showed that LF tests may be used in situations that go beyond those previously described and support their use for studies investigating the role of armadillos in zoonotic leprosy in Brazil. The reasons for this are numerous but can be summarized as follows; simplicity of use, the requirement for only minimal training, ease of interpretation (particularly in the case of the NOD-LID® test), the ability to undertake non-lethal in-field analysis, proven reproducibility and a high level of concordance with the gold standard immuno-diagnostic method (anti-PGL-I ELISA) and with molecular biology-based (RLEP) detection.

Although global leprosy research is still focused on humans, the adoption of a One Health approach, encompassing aspects of human, animal and environmental health, could be highly beneficial for the development of more effective strategies for management and control of this important infectious disease [36]. The various negative impacts of *M. leprae* infection upon development, reproduction and longevity in populations of nine-banded armadillos in the southern USA were

recently described [37] and clearly demonstrated that more attention must be given to understanding the dynamics of *M. leprae* infections in armadillos in other regions of the Americas.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cimid.2019.101397.

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