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Zoonotic parasites infecting free-living armadillos from Brazil.

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Abstract

Abstract: Armadillos are specialist diggers and their burrows are used to find food, seek shelter and protect their pups. These burrows can also be shared with dozens of vertebrate and invertebrate species and; consequently, their parasites including the zoonotics. The aim of this study was to diagnose the presence of zoonotic parasites in four wild-caught armadillo species from two different Brazilian ecosystems, the Cerrado (Brazilian savanna) and the Pantanal (wetland). The investigated parasites and their correspondent diseases were: Toxoplasma gondii (toxoplasmosis), Trypanosoma cruzi (Chagas disease), Leishmania spp., (leishmaniasis), Paracoccidioides spp. (Paracoccidioidomicosis) and Mycobacterium leprae (Hansen's disease). Forty-three free-living armadillos from Pantanal and seven road-killed armadillos from the Cerrado were sampled. Trypanosoma cruzi DTU TcIII were isolated from 2 out of 43 (4.65%) armadillos, including one of them also infected with Trypanosoma rangeli. Antibodies anti-T. gondii were detected in 13 out of 43 (30.2%) armadillos. All seven armadillos from Cerrado tested positive for Paracoccidioides brasiliensis DNA, in the lungs, spleen, liver and ear fragments. Also, by molecular analysis, all 43 individuals were negative for M. leprae and Leishmania spp. Armadillos were infected by T. cruzi, T. rangeli, P. brasiliensis, and presented seric antibodies to T. gondii, highlighting the importance of those armadillos could have in the epidemiology of zoonotic parasites. Key words: Cingulata, Trypanosoma cruzi, Toxoplasma gondii, Paracoccidioides brasiliensis, Mycobacterium leprae, Leishmania sp.

Introduction

A single family (Dasypodidae), autochthonous to South America, with twenty species and nine genera (Messias-Costa et al., 2001), composes the order Cingulata (armadillos). This order is part of the super-

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order Xenarthra that, together with the marsupials, are the oldest mammals in the history of South America (Wetzel, 1982). Armadillos belong to a basal mammalian group that represents 0.5% of the existing mammals. This primitive group varies widely, from the pink fairy armadillo (*Chlamyphorus truncatus,* Harlan, 1825) weighing just 90 grams, to the giant armadillo (*Priodontes maximus* Kerr 1792) weighing up to 50 kg (Emmons and Feer, 1997). Furthermore, armadillos have unusual physiologic characteristics, such as low body temperature and low basal metabolic rates, when compared to other placental mammals with similar body mass (McNab, 1985). Armadillos are specialist diggers and their burrows are used to hide their babies, protect from predators and for food.

Armadillos live and dig burrows in organic or inorganic matter, under a variety of biotic and abiotic conditions. Desbiez and Kluyber (2013) described that giant armadillos dig burrows that are used by more than 54 vertebrates, such as tamanduas (*Tamandua tetradactyla*), ocelots (*Leopardus pardalis*), tayras (*Eira barbara*), wild peccaries (*Tayassu pecari* and *Pecari tajacu*), rodents and marsupials. This intimate contact with different species may cause the armadillos to acquire infectious agents, including the zoonotics.

Humans have a direct relationship with armadillos causing negative impacts on populations, usually killing them for cultural beliefs, poaching for medicine, or hunting for handcrafted musical instrument, especially in Latin America (Rodrigues et al., 2019). In South America, armadillos are hunted for food due to their taste and high protein level, and are considered relevant species for public health (Deps et al., 2008, Richini-Pereira et al., 2009).

In 1912, Carlos Chagas identified the nine-banded armadillo (*Dasypus novemcinctus* Linnaeus, 1758) as one of the reservoirs for *Trypanosoma cruzi* , the parasite that causes Chagas Disease (Chagas, 1912). This armadillo species is also considered the main host of *Leishmania naiffi* (Lainson et al., 1981). *Mycobacterium leprae* , responsible to cause Hanseniasis in humans, is the most studied agent in armadillos, especially in *D . novemcinctus* and has been described infecting wild armadillos since the 70's (Walsh et al., 1975). Also in the 70's, Sogorb et al. (1977) highlighted that armadillos could act as intermediate host for *Toxoplasma gondii* , responsible for toxoplasmosis. The geophilic and thermo dimorphic fungus, *Paracoccidioides brasiliensis* , which causes systemic mycosis in humans, was found infecting armadillos in several endemic areas of Latin America, including Brazil (Bagagli et al., 2008). Finally, multiple studies also described armadillos as potential reservoirs of zoonotic pathogens (da Silva et al., 2008; Bagagli and Bosco, 2008; Truman et al., 2011).

As a part of the Xenarthra Health initiative of the Giant Armadillo Conservation Program, the present study was developed with the aim to diagnose five zoonotic parasites (*T. gondii, T. cruzi, Leishmania*spp*., Paracoccidioides* spp*., and M. leprae*) in four wild-caught armadillo species (*Cabassous unicinctus* Linnaeus, 1758, *Dasypus novemcinctus* , *Euphractus sexcinctus*Linnaeus, 1758 and *P. maximus Kerr 1792*) in the midwestern Brazil.

2. Material and methods

2.1 Study areas

The Mato Grosso do Sul State, located in Midwestern Brazil, is divided into two main regions: the floodplains, which encompass the Pantanal wetlands, and the plateau, which is dominated by the dry, tropical Cerrado savanna. Most of the Pantanal floodplains is still pristine and some areas harbor low impact traditional extensive livestock (Harris et al., 2005).

The plateau region is dominated by Cerrado vegetation, composed of dense grasslands with sparse shrubs, savanna and woodland areas. Nevertheless, large portions of the plateau have been modified for economical purposes (e.g. agriculture and intensive livestock) and native remnants have been fragmented into small patches (Reynolds et al., 2016). This study has taken place on both regions of Mato Grosso do Sul state.

2.1.1 Pantanal

The Pantanal has a high biodiversity and is one of the largest continuous freshwater wetlands on earth

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(160,000 km^2) (Franco et al., 2013). In this pristine region, the study area was located at a private ranch (Baía das Pedras) in the Nhecolândia sub region of the Pantanal (19°20' S, 55°43' W). **(Fig. 1)**

2.1.2 Cerrado

The Brazilian savanna vegetation is called "Cerrado" and covers 2 million km^2 of Central Brazil. In the more impacted region dominated by Cerrado, a different approach was used and three main state and federal highways (BR 262, 163 and 267) were surveyed. Agriculture (e.g. sugarcane, soybean, corn, *Eucalyptus*plantation), cattle ranching, charcoal production and road kill are the main threats for the fauna in the Cerrado region (Ascensão et al., 2017). (Fig.1)

2.2 Capture and chemical immobilization in the Pantanal

Free ranging armadillos from Pantanal were manually captured and anesthetized. Small species such as *D. novemcinctus* , *E. sexcinctus* and *C. unicinctus* were captured by hand or using long-handled dip nets. *P. maximus* were caught using cylindrical iron traps that were placed at the entrance of burrows (Desbiez et al., 2020). Anesthesia was induced using a combination of butorphanol tartrate (Zoetis Indústria de Produtos Veterinários Ltda, São Paulo/SP, Brazil; 0.1 mg/kg), detomidine hydrochloride (Agener-União, Apucarana/PR, Brazil; 0.1 mg/kg), and midazolam hydrochloride (Cristália, Itapira/SP, Brazil; 0.1 mg/kg), by intramuscular injection in the hind limbs (Kluyber et al., in press).

During anesthesia, animals were identified, sexed, weighed and had their age estimated based on Desbiez et al. (2019). Additional microchip tagging and morphometric measurements were performed. Animals were released in the same day at the location they were captured, immediately after complete anesthesia recovery.

2.3 Sampling

Captures and biological sample collection in the Pantanal occurred between June 2011 and January 2015 and included blood collection and ear tissue biopsy. Blood was collected through venipuncture of saphenous vein, using syringes and/or Vacuntainer® tubes with and without anticoagulant.

From blood samples collected in tubes containing EDTA, approximately 0.6–1.0 ml of blood was aseptically inoculated into two tubes containing Novy-Mc Neal-Nicole medium (NNN) with a liver infusion tryptose medium (LIT) overlay (hemoculture). Serum was obtained after blood centrifugation (10 min at 3,500 rpm) from tubes without anticoagulants and stored at -20C°. Ear tissue was biopsied (1.0 cm fragment) and immediately stored in plastic tubes containing 99% ethanol. Samples were stored in a portable cooler with ice and taken to a field laboratory, where they were preprocessed and stored, to be later sent to laboratory analyses.

In the Cerrado, biological samples were obtained from road-killed animals encountered between January and March of 2014, on three main state and federal highways (BR 262, 163 and 267). Highways were monitored every 15 days by driving a car slowly with two people looking for carcasses. For every animal encountered, a necropsy was performed, and biological samples were collected and associated with the GPS position of each of the road-killed animal.

Two tissue fragments of approximately 2 cm diameter from spleen, liver, lungs and one fragment (1 cm) of the ear were collected and stored in separated tubes with ethanol 99% and transported to the laboratories for molecular analysis. Only samples derived from carcasses there were not in apparent autolysis were collected.

2.4 Laboratorial analyses

2.4.1 *Trypanosoma* sp, : The presence of *T. cruzi*was evaluated by the hemoculture previously performed in the field. Cultures were analyzed every 15 days for three to five months and when at least one flagellate parasite was observed in optical microscopy, samples were classified as positive. From positive samples, parasites were grown in LIT medium, cryopreserved and deposited in the*Trypanosoma* collection from wild and domestic animals and vectors-COLTRYP (Oswaldo Cruz Foundation). Genomic DNA was extracted using the standard phenol-chloroform protocol (Sambrook et al., 1989). The obtained DNA was first characterized

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by multiplex PCR amplification of the mini-exon intergenic region (Fernandes et al., 2001); and secondly using the PCR-RFLP protocol targeting the histone 3 gene and digestion with AluI (Westenberger et al., 2005). Moreover, a Nested PCR targeting the 18S rRNA gene (600 bp) was performed as described by Noyes et al. (1999) and dos Santos et al. (2018). From this latter, amplified PCR products were purified using Illustra GFX PCR DNA and a gel band purification kit (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). The purified PCR products were sequenced with ABI 3730 BigDye Terminator (v3*1) Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystem DNA Analyzer on the PDTIS/FIOCRUZ sequencing platform. Nucleotide sequences were manually edited using the DNASTAR's Lasergene Sequence Analysis Software (Burland, 2000) and aligned using CLUSTALW. The obtained sequences were compared with nucleotide sequences deposited in GenBank using the NCBI BLAST (Basic Local Alignment Search Tool) algorithm to identify *Trypanosoma* species and/or *T. cruzi* DTUs.

2.4.2 *Toxoplasma gondii:* For the serodiagnosis of*T. gondii* , the Modified Agglutination Test (MAT) was applied with the use of tachyzoites inactivated by formalin as an antigen, as described by Dubey and Desmonts (1987). As a cut-off point, a 1:25 dilution (Dubey, 2010) was used, with concomitant tests being performed with positive and negative controls previously known to mice. Positive samples were diluted until the final reaction titer. The antigen for carrying out the MAT was provided by Dr. Jitender P. Dubey of the United States Department of Agriculture (USDA), Agriculture Research Service, Animal and Natural Resources Animal Parasitic Diseases Laboratory, Beltsville, Maryland (USA)

2.4.3 *Leishmania* sp. : Nucleic acids contained in samples of armadillo ear tissue were extracted using the DNeasy Blood & Tissue kit (Qiagen), according to manufacturers. Each extraction procedure included duplicate extraction controls containing 25mg of bovine liver. PCR targeting a conserved region of the kinetoplast DNA of*Leishmania sp.* was conducted using the pureTaq Ready-To-Go PCR beads (Amersham Biosciences, Buckinghamshire, UK) and primers forward 5'-GGGAGGGGCGTTCTGCGAA-3' and reverse 5'GGCCCACTATATTACACCAACCCC-3' (Cassia-Pires et al., 2014). The PCR products were visualized after electrophoresis on 8% polyacrylamide gel and silver staining using a specific kit (DNA Silver Staining, GE Healthcare). Positive and negative controls were derived from fragments of liver from noninfected and infected (*Leishmania braziliensis* – IOC-L2483) hamsters.

2.4.4 *Mycobacterium leprae* : The DNA samples extracted for *Leishmania* sp. analysis were also used to detect *M. leprae* . Specific sequences of 16 Variable Number Tandem Repeats (VNTRs) were applied for four separate multiplex PCR assays, as described by Kimura et al., (2009), with a volume of 2μL of DNA used in each multiplex reaction. The sensitivity of the multiplex assay, incorporating the AC8b, GTA9, GGT5, AT17, 6-3 VNTR targets, was determined using nude mouse-derived *M. leprae* cells as follows: a total of $2x10^5$ cells was combined with duplicate samples of 25mg of bovine liver and DNA extracted using the same protocol described for the armadillo samples, with elution performed in 100μL of AE.

The mouse-derived samples (denominated S0), were considered to contain a quantity of DNA equivalent to $2x10^3$ cells/μL (assuming 100% extraction efficiency). The S0 samples were submitted to 10-fold serial dilution in AE buffer to generate samples containing quantities of DNA ranging from equivalent to $2x10^3$ cells/μL to 2 cells/μL. The sensitivity of the multiplex assay was determined based on the last dilution that produced clearly amplified products, suitable for genotyping based on fluorescent fragment size analysis (Kimura et al., 2009).

The *M. leprae* specific repetitive element (RLEP) PCR was amplified in a nested PCR reaction. The primers for RLEP2-1 (5'-ATATCGATGCAGGCGTGAG-3') and RLEP2-2 (5'-GGATCATCGATGCACTGTTC-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 1μL aliquot of the isolated DNA was added to 24μL of PCR mix, which contained 15mM Tris-HCl (pH 8.0), 50mM KCl, 1.5mM MgCl2, 0.2mM dNTP, 5% DMSO, 1.25 units of Taq DNA Polymerase and 0.2μM of each primer.

The mixture was denatured at 94^oC for 4 min, followed by 35 PCR cycles (30s at 94^oC, 30s at 59.6^oC and 1min at 72^oC), with a final extension at 72^oC for 10min. Each run included negative and positive controls.

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For the nested PCR, 0.5µL of product was used as the DNA template. The amplification reactions were visualized on a 1.5% agarose gel. When the results for the same sample were different, a third PCR was performed for confirmation.

Different amounts of purified DNA from *M. leprae* 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 10fg to 1µg. Purified*M. leprae* DNA was also used as a positive control for the amplifications.

2.4.5 *Paracoccidioides* sp.: PCR analyses and sequencing were performed to detect the infection by *Paracoccidioides* sp. (Hrycyck et al., 2018) in distinct tissues. The DNA extraction from ear, lung, liver and spleen samples was performed using the commercial kit Macherey-Nagel (MN) and the DNA was quantified on NanoVue (GE Healthcare) and stored at -20°C before use. A Nested PCR technique was applied aiming to amplify the ITS1-5.8S-ITS2 region with primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCGTAGGTGAACCTGCGG-3'). Later, a second amplification using the specific primers PbITS-E (5'-GAGCTTTGACGTCTGAGACC-3') and PbITS-T (GTATCCCTACCTGATCCGAG) was applied. Both amplifications were performed by employing GoTaq MasterMix (Promega). Cycling conditions were stablished according to the Taq polymerase manufacturer requirement with annealing temperature of 55°C for ITS1/ITS4 and 62°C for PbITS-E/ PbITS-T primers. The PCR products were detected by 1.5% agarose gel electrophoresis.

2.5 Statistical Analysis

A Fisher's exact test was applied to evaluate the association between the presence of *T . gondii* antibodies and the species, sex and age class.

2.6 Ethics Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. Animals were captured and biological samples were collected under licenses numbers 27587-8 and 53798-7 from the Brazilian Federal Environmental Protection Agency (ICMBIO), which regulates and protects wildlife in the country. The study was approved by the Animal Care and Use Committee of Tropical Medicine Institute - CEUA - 2013/182.

Results

In the Pantanal, forty-three armadillos of four species were captured (*P. maximus* , *E. sexcinctus* , *C. unicinctus* and*D. novemcinctus*), while, in the Cerrado, seven individuals of two species were sampled (*E. sexcinctus* and *D. novemcinctus*). Individual age estimation, sex and average body mass for the species are presented in Table 1. None of the individuals from the Pantanal showed clinical signs of infection and were all considered to be in good health. Ten road-killed armadillos of two species (*E. sexcinctus*and *D. novemcinctus*) were recorded in the Cerrado and had biological samples collected. However, due to the advanced level of autolysis, only samples derived from seven of these were considered suitable for molecular analysis: six *E. sexcinctus* and one*D. novemcinctus* (Table 1). Blood samples were not obtained from those animals, and serological and blood analysis were not performed (*T. gondii* and *Trypanosoma sp*).

Hemocultures performed for *Trypanosoma sp* . detection revealed that two out of 43 armadillos (4.65%) from the Pantanal were positive. These cultures were from two *D. novemcinctus* , one captured on July 2013 (end of wet season), and the other captured on January 2014 (beginning of wet season). Both isolates were diagnosed as*Trypanosoma cruzi* and characterized as zymodeme 3 (Z3) in the multiplex PCR. Further characterization using PCR-RFLP of H3/AluI genotyped the isolates as belonging to DTU TcIII. The same isolates were also submitted to characterization through the sequence analysis of the PCR products obtained in the nested 18S PCR. One of them was confirmed as *Trypanosoma cruzi* DTU TcIII/TcV (100% of coverage and identity), because similarities in the sequences do not allow the differentiation between these two *T. cruzi* DTUs using this molecular target. The other isolate was characterized as *T. rangeli,* subpopulation A

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(100% of coverage and 99.5% of identity), demonstrating a probably mixed infection in this individual. Both isolates are cryopreserved in COLTRYP and the sequenced deposited in the GenBank, respectively Coltryp 566 (GenBank number MT253589 and Coltryp 542 (GenBank number MT253688).

Presence of anti-*T. gondii* antibodies were detected in 13 (30.2%) of the 43 individuals (Table 2), with presented titers ranging from 1:25 to 1:100. Detection of *T. gondii* antibodies presented no variation among species (p = 0.59), between sexes (p = 1) or age classes (p = 1, CI = 0.06 – 5.02, odds ratio (OR) = 0.73). Even within each species there were no differences between sexes (*Pm* – p = 1, *Es* – p = 1) or age class (*Pm* – p = 0.6, CI = 0.1 – 36.37, OR = 1.91; *Es* – p = 0.51, CI = 0 – 6.04, OR = 0).

All ear biopsy samples of the 43 individuals from the Pantanal and the seven from Cerrado tested negative for both *Leishmania* sp. and*M. leprae* . In contrast, all lungs, spleen, and liver samples from the Cerrado tested positive for *P. brasiliensis* (Table 2).

Discussion

Many wild mammal species can share armadillo burrows looking for food, thermal refuges, to escape from predators or to use the sand in front of burrows as a latrine or a rest place, as described for giant armadillos by Desbiez and Kluyber (2013). It has been hypothesized that the use of armadillos' burrows by several vertebrate species and the constant mild temperatures encountered inside these excavations, may favor survival and proliferation of parasites and vectors (Desbiez and Kluyber, 2013). Furthermore, armadillo burrows may act as shelters for living insects, such as kissing bugs and the sandflies, which act as vectors of protozoans such as *T. cruzi* and *Leishmania* sp. respectively; besides some parasites that can resist long periods in the environment, such as *M. leprae, P. brasiliensis* and *T. gondii* oocysts.

Armadillos are considered important species for public health, given that its meat is widely consumed in several regions, from the south of the United States throughout South America. (Cardona-Castro et al., 2009; Rodrigues et al., 2019). In Latin America, armadillos are hunted for cultural practices such as medicinal use, manufacturing of musical instruments but especially, for food (Capellão et al., 2008; Rodrigues et al., 2019).

Although the hunting of wildlife is not allowed in Brazil (Environmental Crime Law - 9.605/98), armadillos are still considered one of the favorite bush meats and a main protein source, in rural areas (Deps et al., 2008). Different leprosy studies in the United States and Brazil, suggest that meat intake and direct contact with contaminated armadillos may be considered an important source of infection (Kerr et al. 2015, Truman et al., 2011). In general, studies highlight that contact with contaminated environments and poor sanitary conditions are some of the most important factors that favor zoonotic parasites transmission (Sharma et al., 2015; Ker-Pontes et al., 2006).

Trypanosoma cruzi is a multihost wild parasite species transmitted by contaminated feces of triatomines bugs and between mammals through predation (Jansen et al., 2015). Curiously, it was in an armadillo (*D. novemcinctus*) that Carlos Chagas first observed*T. cruzi* infection in the wild (Chagas, 1912). After that, Yaeger et al. (1988) and Forrester et al. (1992) described significant prevalence of *T. cruzi* for *D. novemcinctus* in the United States. In Brazil, this armadillo species is also considered a potential reservoir of *T. cruzi* and several studies reported infected individuals (Barret and Naiff, 1990; Herrera et al., 2004; Roque et al., 2008). Few studies have been conducted focusing on *T. cruzi* in other wild armadillo species, but there have been records of individuals of *E. sexcinctus,* C. *villosus, C. vellerosus, T. matacus*and *C. unicinctus* exposed to *T. cruzi* (Yeo et al.,2005; Noireau et al., 2009; Telleria and Tibayrenc, 2010; Orozco et al., 2013; Cardona-Castro et al., 2009). The high number of studies focusing only on *D. novemcinctus* might be explained by its wide geographic distribution and the absence of health surveillance studies for other armadillo species.

According to Herrera et al. (2004), the burrowing habits of certain species, such as armadillos, in combination with the vector's ecology (i.e. living inside burrows), may influence the transmission of *T. cruzi* . In fact, underground burrows, as the ones used by armadillos, are usually associated with triatomines from the *Panstrongylus*genus (Jansen et al., 2017). Moreover, armadillo's habits of bringing grass and leaves to the inside

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of their burrows and to place plant debris on the burrow's entrance to cover it, (Loughry and McDonough, 2013), creates an ideal habitat for triatomines and; consequently, sets place for an opportunistic behavior. This vector's habitat use behavior was also described by de Lima et al. (2015), who recorded the presence of triatomine bugs in arboreal nests of Coatis (*Nasua nasua*) in the Pantanal wetlands of Brazil. Finally, D'Alessandro et al. (1984) indicated the insectivorous diet of armadillos as a possible cause of accidental ingestion of triatomines, and one of their main sources of infection by *T. cruzi.*

After the first biochemical characterizations of *T. cruzi*isolates, proposed by Miles et al. (1977), isolates derived from armadillos were usually associated with zymodeme Z3, which lead Yeo et al. (2005) to propose an association between armadillos and the *T. cruzi* subpopulations currently recognized as DTUs TcII to TcVI (Zingales et al., 2009). Nowadays, although the frequency of infections by *T. cruzi* DTUs TcIII and TcIV (formerly described as Z3 zymodeme) are higher than other *T. cruzi* DTUs, armadillos have also been found infected with DTUs TcI and TcII, demonstrating their importance to maintain distinct transmission cycles in nature (Jansen et al., 2017). It is likely that the scarcity of long-term studies, and health monitoring programs of different armadillo species, might be leading the field to an underestimation of the role of armadillos as hosts for *T. cruzi* .

In the present study, four different species of armadillos were studied, but *T. cruzi* could only be isolated from blood cultures of two of them, *D. novemcinctus* and *P. maximus* . Both isolates were characterized as *T. cruzi* TcIII based on PCR/RFLP (H3/AluI) corroborating previous studies that demonstrate that this DTU is the most common in armadillos (Barros et al., 2017; Jansen et al., 2018). The characterization of one of these isolates was also confirmed by the sequence analysis of the 18S rDNA PCR products. However, the other isolate was characterized as *T. rangeli,* subpopulation A. Because the Miniexon assay was positive only for *T. cruzi* Z3 (further confirmed by the H3 PCR) and did not detect *T. rangeli,* although it is able to Fernandes et al., 2001, two distinct results were considered as an indicative of mixed infection, a trait probably more common in nature than it is usually detected (Jansen et al., 2020). *T. rangeli* is also a multihost wild mammal parasite transmitted by kissing bugs, but unlike *T. cruzi,* the transmission takes place by the inoculative pathway and the only proven vectors are the triatomine bugs of the genus *Rhodnius* . The subpopulation A is commonly described in bats from distinct areas in South America, but has also been described in primates and carnivores (Maia da Silva et al., 2007; 2009; Dario et al., 2017). To our knowledge, this is the first description of *T. rangeli,* subpopulation A, in armadillos.

This is the first report of armadillo exposure to *T. gondii* in the Pantanal of Mato Grosso do Sul, and the first record of this infection for the species *C. unicinctus.* Previous studies have described the exposure to *T. gondii* for *D. novemcinctus, E. sexcinctus,* and *P. maximus* (Sogorb et al., 1977; da Silva et al., 2008a; da Silva et al., 2008b; Deem et al., 2009). In a study performed by da Silva et al., (2008) and Vitaliano et al., (2014), *T. gondii* could be isolated from *E. sexcinctus* and*D. novemcinctus.* Consumption of carrion by omnivore armadillo species, such as *E. sexcinctus* (Medri, 2008), can be an important source of infection.

The habit of armadillos to dig burrows, live in organic and inorganic matter and forage for food on the ground, in conjunction with the fact that *T. gondii* oocysts can resist long periods in the environment (Dubey, 2010), might favor the contact of armadillos with soil contaminated by *T. gondii* sporulated oocysts, eliminated by felids that share the same environment. Wild felids are the only definitive hosts of *T. gondii* in the wild (Wang, 2002). The ocelot (*Leopardus pardalis*) is one of the main species of feline that frequently uses giant armadillo burrows to rest or as a thermal refuge. Due to its size, *Puma concolor* is not able to explore inside burrows, but, like *L. pardalis* , have been using the sand in front of giant armadillo burrows, to rest or as a latrine (Desbiez and Kluyber, 2013). Furthermore, the infectiousness of *T. gondii* from *P. maximus* has been demonstrated through the isolation of *T. gondii*in a mouse that had been inoculated with lungs and brain fragments from an infected *P. maximus* (Sogorb et al., 1977). Hence, armadillos may act as intermediate hosts and should be considered relevant species for the maintenance of *T . gondii* cycle, which is directly connected to their role in ecological food-chains (Kin et al., 2014).

At least seven *Leishmania* species have been described infecting xenarthras, especially sloths and tamanduas (Roque and Jansen, 2014). However, few descriptions are available concerning *Leishmania*infection in arma-

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dillos and, infections had been detected only in*D. novemcinctus* (reviewed by Roque and Jansen, 2014). Two distinct species of *Leishmania* , *L. naiffi* and *L. guyanensis* were described in *D. novemcinctus.* Importantly,*D. novemcinctus* is the only non-human host in which *L. naiffi* has been isolated (Naiff et al., 1991, Roque and Jansen, 2014). Serology can indicate the exposure of a given mammal host to*Leishmania* infection, but the lack of available commercial reagents for armadillo impairs this kind of investigation (Jansen et al., 2015). In this sense, cultures or molecular diagnosis on punctures or fragments of hematopoietic tissues are the frequently chosen methods to identify *Leishmania* infections in armadillos (Roque and Jansen, 2014).

Molecular tests like polymerase chain reaction (PCR) targeting the kDNA of these parasites may provide sensitivity and specificity of almost 100%. Richini-Pereira et al. (2014) detected *Leishmania* sp. in two armadillo's species studied (1 *D. novemcinctus* and 1 *E. sexcinctus*) in Brazil. However, in the present study, all the samples analyzed using PCR analysis were negative for *Leishmania* sp. As described by Roque and Jansen, (2014), few *Leishmania* studies, and lack of species- specific standard tests, are still a challenge for wild mammal diagnosis.

In 1971, armadillos were experimentally infected with *M. leprae*for the first time by Kirchheimer et al. (1971). Later, Walsh et al. (1975) reported that wild armadillos could be naturally infected, demonstrating that *M. leprae* infection was not limited to man as previously thought. In the USA, Truman et al. (2011) described the potential role of armadillos in the zoonotic transmission of *M .leprae* to humans. *Mycobacterium leprae* infects two kinds of human body cells: skin macrophages and Schwann cells (Kaplan, 1986). The ideal body temperature for *M. leprae* is about 30°C and that is the reason the bacteria and its clinical signs are encountered in colder parts of the body such as the extremities. This characteristic explains, in part, the infection in armadillos, which present average body temperature between 30 and 35°C (Shepard, 1965). Kerr et al. (2015), proposed that ecological characteristics of armadillos, such as its burrowing habits, and *M. leprae* 's viability in the soil and water, could result in a possible infection route (inhalation) among armadillos. Transmission might occur through aerosol, direct contact, antagonist encounters or while they are copulating (Truman et al., 2011). Mohanty et al. (2016) also described that some species build their burrows close to rivers or in humid soil, possibility creating a new route of infection through the contact or exposure to different parasites, including *M. leprae* .

Studies performed by Truman et al. (2011) and Loughry et al. (2009) in the USA recorded wild *D. novemcinctus* infected with *M. leprae* , mainly in Texas and Louisiana. Moreover, according to Sharma et al. (2015) when *M. leprae* was investigated in 645 armadillos from 8 locations in the southern USA, 106 (16.4%) individuals were positive using serologic (anti-PGL-I antibody) and/or PCR (RLEP and 18 kDa) techniques. This indicates a continued spread of *M. leprae*infection in these animals as their range has expanded East and North throughout the USA.

In Brazil, recent studies have shown *D. novemcinctus* and *E. sexcinctus* individuals naturally infected by *M. leprae* in an hyperendemic area in the Ceará state, northeastern Brazil (Frota et al., 2012; Kerr et al., 2015). In the north, studies performed in Pará state by da Silva et al. (2018) found 62% (10/16) of *D. novemcinctus*positive for *M. leprae.* In the southeastern Brazil, Deps et al. (2002), studied 14 *D. novemcinctus* in a rural area of Espírito Santo state and none of the animals examined presented macroscopic lesions suggesting infection, but five of them were PCR positive for

M. leprae.

Although *D. novemcinctus* is frequently described as one of the main reservoirs for *M. leprae, E. sexcinctus* was also described naturally infected for the first time in an endemic area for human Hanseniasis, in Ceará state, northeast of Brazil (Frota et al., 2012). Regardless of the methods applied, *M. leprae* was not detected in any of the armadillo samples in this study. The results are in accordance with previous studies in the region (Pedrini et al., 2010). Using molecular analysis (PCR) the authors investigated 22 *E. sexcinctus* , and other three species of armadillo (*D. novemcinctus* , n = 18*; C. unicinctus* , n = 2*; C. tatouay* , n = 2) from two different regions, in the southeast and in the central states of Brazil, including Mato Grosso do Sul state, and all individuals tested negative. According to Truman et al. (2008), negative results might also be

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related to a non-endemic area for *M. leprae* , as is the case of the herein investigated areas.

Soil is the probable main habitat for *P. brasiliensis.* Humans were considered the only hosts of *P. brasiliensis* for a long time, until the description of naturally infected armadillos in endemic areas of Brazil (Bagagli et al., 2008). Then, the use of molecular analysis enabled the diagnosis of *P. brasiliensis* in other mammal species, like rodents (*Cavia aperea* and *Sphiggurus spinosus*) and carnivores (*Procyon cancrivorus* and*Gallictis vittata*), which are also considered as new hosts for this fungus (Costa et al., 1995; Richini-Pereira et al., 2008; Bagagli and Bosco, 2008).

There is little comprehension about the real meaning of *P. brasiliensis* infection in armadillos, both for the fungus eco-epidemiology and for the animal health, and its possible impacts in wildlife and conservation. The prevalence of *P. brasiliensis*infection in nine-banded armadillos is very high, especially in endemic regions for this parasite where the fungus was isolated in 75-100% of the studied armadillos (Bagagli and Bosco, 2008). Both armadillos and*Paracoccidioides* species have originated and evolved in South America over the last millions of years, with a high likelihood to stablish biological relationships that might be positive for the fungus dissemination while probably neutral for the armadillos. However, we cannot rule out the possibility that this association is actually more recent (e.g. in the last 500 years), as the result of human interference in nature, in a similar manner to the relationship suggested for*M. leprae* and the nine-banded armadillo (Bagagli et al., 2008; Truman et al., 2011).

As seen for for *M. leprae* , *D. novemcinctus* is the most studied species, but *P. brasiliensis* could also be isolated in*Cabassous centralis* and molecularly detected in *D. septemcinctus* and *D. hybridus* (Bagagli and Simões, 2005; Richini-Pereira et al., 2009; Corredor et al., 2005). Although studies performed by several authors presented similar prevalence rates (75-100%; Fernandes et al., 2004; Silva-Vergara et al., 2000; Bagagli et al., 2008; Richini-Pereira et al., 2009), this is the first study describing armadillos infected by *P. brasiliensis* in a non-endemic area and the species *E. sexcinctus* infected by this fungus in the Cerrado biome in Mato Grosso do Sul. These results also highlight the importance of the relationship between this fungus and armadillos even in non-endemic areas.

Frequent isolation of *P. brasiliensis* is usually observed in mesenteric lymph nodes and the fungus has also been molecularly detected in feces samples, suggesting that the armadillo infection might be occurring by alternative routes, like the gastrointestinal tract (Bagagli et al., 2008). Lungs, liver and spleen were the organs where*P. brasiliensis* was detected in this study. As described by Bagagli et al. (2008), after the fungus is inhaled by the respiratory tract, the mycelial form can be transformed in yeast in the lungs and be systemically disseminated in armadillos, in a very similar way to that described in humans.

This fungus can survive long periods in the soil, and can also be found inside armadillo burrows (Silva-Vergara et al., 2000; Bagagli and Simões, 2005). The constant digging activity inside burrows may further increase the exposure of armadillos to pathogen inhalation and, based on Silva-Vergara et al. (2000), armadillo infection may increase the long-term viability of the fungus.

5. Conclusion

Armadillo's ecological studies may also favor the obtaining of relevant information concerning the health status of a given environment. Long-term surveillance health programs involving armadillos should be encouraged, and the data obtained from these should help to better: 1) Understand the prevalence data or occurrence of diverse parasites, especially the zoonotic ones, in the ecosystems they occur; 2) Identify the role of armadillos as reservoir hosts of different parasite species; 3) Provide precise geographic locations and disease risk map analysis; 4) Develop actions and preventive disease control initiatives considering the parasites that can be transmitted by armadillos; and 5) Develop low-cost strategies for health studies considering wild animals, humans and their ecosystems.

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7. Conflict of interest declaration

The authors declare that the content of this manuscript have not been previously published and have no conflict of interest to the publication of this article.

8. Data Availability Statement

The data that support the findings of this study are openly available in [repository name e.g "figshare"] at http://doi.org/[doi], reference number [reference number].

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