Research regarding anti-PGL-I antibodies by ELISA in wild armadillos from Brazil

Pesquisa de anticorpos anti PGL-I através de ELISA em tatus selvagens do Brasil

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ABSTRACT

Armadillos have been involved in leprosy transmission and are considered a source of *Mycobacterium leprae* in numerous reports. Clinicians from certain areas of the USA consider contact with armadillos a risk factor for leprosy. However, there is a challenge associated with the role of wild armadillos perpetuating human leprosy in the American Continent. The presence of anti-PGL-I antibodies was investigated in wild nine-banded armadillos from leprosy-endemic areas in State of Espirito Santo, Brazil, by ELISA performed on serum samples from 47 armadillos. Positive ELISA was obtained from 5 (10.6%) armadillos. Infected armadillos may play some role in leprosy transmission, disseminating bacilli in the environment, perhaps making it more difficult to interrupt transmission and reduce the number of new leprosy cases. ELISA is an efficient tool for seroepidemiological investigations of *Mycobacterium leprae* in armadillos.

Key-words: Mycobacterium leprae. Leprosy. Armadillos. ELISA. PGL-I.

RESUMO

Tatus têm sido envolvidos na transmissão da hanseníase e considerados como fonte de *Mycobacterium leprae* em muitas publicações. Médicos de partes dos EUA consideram o contato com tatus um fator de risco para hanseníase. Entretanto, há um desafio associado ao papel do tatu na perpetuação da hanseníase no Continente Americano. Foi pesquisada a presença de anticorpos anti-PGL-I em tatus selvagens de áreas endêmicas em hanseníase do Estado do Espírito Santo, Brasil, através de ELISA realizado em amostras de soro de 47 animais. ELISA positivo foi encontrado em 5 (10.6%) tatus. Tatus infectados podem ter algum papel na transmissão da hanseníase disseminando bacilos no meio ambiente, talvez tornando mais difícil a interrupção da cadeia de transmissão e redução do número de casos novos de hanseníase. A técnica de ELISA é um eficiente método para investigação soroepidemiológica da presença do *Mycobacterium leprae* em tatus.

Palavras-chaves: Mycobacterium leprae. Hasnseníase. Tatu. ELISA. PGL-I.

Although leprosy has declined in all endemic countries, in Brazil the number of new cases annually has remained almost the same for the last five years[29]. The State of Espírito Santo is located in the south-eastern region of Brazil and is classified as high prevalence for leprosy[18]. Leprosy transmission and the sources of *Mycobacterium leprae* have been discussed in numerous reports, but transmission from an untreated multibacillary (MB) patient to a susceptible individual is considered by most leprologists to be the only way to acquire leprosy.

Wild armadillos in the south central United States (USA) harbor a natural infection with *Mycobacterium leprae*[25] and in this region, clinicians consider contact with armadillos a risk factor for leprosy[17]. However, there is a challenge associated with the role of wild armadillos in helping to perpetuate human leprosy in this hemisphere. Besides the USA[26 27 28], *Mycobacterium leprae* infection in wild armadillos has been reported in Mexico[1], Argentina[15] and Brazil[5 7]. Controversy remains whether armadillos are reservoirs of *Mycobacterium leprae* and contribute to leprosy transmission in Brazil[5 6 7 8].

Currently, armadillos are considered a very important animal model for *Mycobacterium leprae* infection and as the principal source of leprosy bacilli for research and diagnostic purposes[24]. In experimentally infected armadillos, *Mycobacterium leprae* infection can be detected by PCR[5 12], autopsy[12 28], serology using ELISA[23], the ML Flow test[7] and histopathology[12 28].

The phenolic glycolipid 1 (PGL-I) is a highly specific antigen of *Mycobacterium leprae*[10] and is known to predominantly elicit

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IgM antibodies against its terminal trisaccharide. Multibacillary armadillos show a strong antibody response to *Mycobacterium leprae*. In experimentally infected animals, the timing of antibody appearance is highly correlated with the bacterial load in the animal's tissues and they persist over the course of the disease[20][23]. Most experimentally infected animals develop heavy infections with approximately 10^{12} recoverable bacilli in their liver and spleen within 18-24 months[13][24].

This report discusses the first survey conducted in a rural area of Brazil, where many wild nine-banded armadillos (known locally as *tatu galinha* or *tatu peba*) have been captured, slaughtered and eaten.

MATERIAL AND METHODS

Forty seven wild nine-banded armadillos, *Dasypus novemcinctus* species, were investigated for natural infection by *Mycobacterium leprae*. The animals were captured by a biotechnician in the rural area of the State of Espírito Santo, located in south-eastern region of Brazil from 2001 to 2002. The armadillos were transported from the wild to captivity at the Medical School of Santa Casa de Misericordia in Vitoria. They were cared for over a 3-4 day period and then submitted to intramuscular anesthesia with a mixture of tiletamine hydrochloride and zolazepam hydrochloride (Ketamine® and Zoletil®). Sex and weight were recorded and a complete physical examination of the skin, nose, ears and footpads was performed, searching for lesions, and of palpable lymph nodes, looking for lymphadenopathy. Blood was collected by intracardiac or femoral punction and after centrifugation, the serum was separated and stored at -20ºC for serological tests.

ELISA was performed to detect IgM antibodies against PGL-I of *Mycobacterium leprae* essentially as previously described[3], using NT-P-BSA as the semisynthetic analogue of PGL-I. NT-P-BSA (0.0023µg of sugar/ml) was diluted in a volatile ammonium acetate carbonate buffer (pH 8.2). 0.1µg/ml bovine serum albumin (BSA) was used as control. Briefly, microtiter plates were blocked for 60min with 100µl of a 1% (w/v) BSA in PBS. Before use, the plates were washed 4 times with PBST and 100µl per well of blocking solution PBS 1% (w/v) BSA was added. After incubation for 1h at 37ºC, 50µl of samples diluted to 1:100 in PBST containing 10% (V/V) normal goat serum (NGS) were added per well. In every plate, a standard serum and positive and negative control sera were used; all samples were tested in duplicate. After incubation for 1h, the wells were washed 4 times. Peroxidase-conjugated anti-human IgM (Cappel/Organon Teknika®, Turnhout, Belgium) diluted to 1:10 000 in PBST 10% NGS was added (50µl per well). After incubation for 1 h at 37ºC, the washing procedure was repeated and 50µl of the Sigma 3,3',5,5'- tetramethyl-benzidine (TMB) liquid substrate system was added to each well. After incubation at room temperature for about 15 min in the dark, the color reactions of the entire plate were stopped with 50µl of 0.5M sulfuric acid and optical density was read at 450nm when the standard serum reached a value of 0.6. The cutoff value for positivity was an OD of 0.200.

Ethics aspects

This study was approved by the Federal University of the São Paulo (Brazil) Research Ethic Committee and specific permission from the Brazilian Environmental Organ (IBAMA), under license number 018/2001, was provided.

RESULTS

Armadillos were captured from the rural area of six different cities of the State of Espirito Santo: Cariacica (Pedro Fontes Leprosy Colony), Guarapari, Muniz Freire, Serra (Private Enviromental Reserve-CST) and Vila-Velha.

Although 66 armadillos were captured, blood samples were collected only from 47 armadillos, which were included for serological analysis. The armadillos weighed between 350 and 5200 g and 24 were male (51%). Nonspecific clinical findings revealed the presence of nodules and ulcers in 17 (36%) armadillos.

ELISA was performed in serum samples from 47 armadillos and anti-PGL-I antibodies were detected in 5 (10.6%) by the ELISA method. The mean absorbance values for sera for each ELISA technique are presented in **Figure 1**. Among the 5 armadillos positive by ELISA, 4 presented ulcers and/or nodules (**Table 1**). Thorns were observed in the ears, nose and footpads of 42 armadillos. Inguinal lymph nodes were enlarged on both sides of one armadillo.
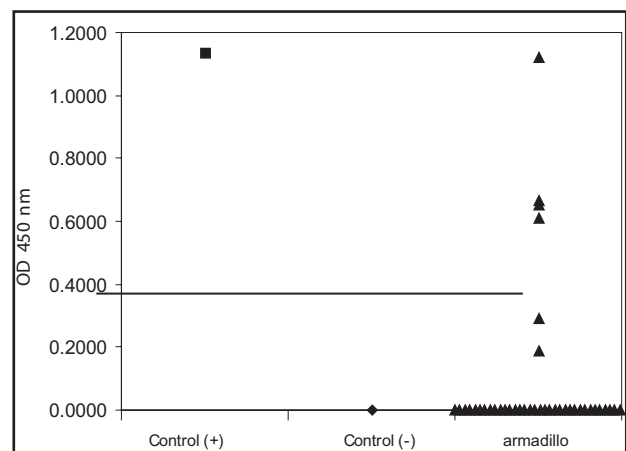

FIGURE 1

Distribution of the levels of PGL-I antibodies by ELISA technique in armadillos and negative and positive human controls.

TABLE 1

Positive armadillos animals, and clinical correlations with IgM antibodies to PGL-I levels/ELISA.

Animal (nº)	OD	Clinical lesions	Lymph nodes enlarged
F002	0.611	Ulcers	No
F005	1.121	Ulcers	Yes (inguinal)
F007	0.667	Ulcers	No
M017	0.650	Ulcers	No
M020	0.289	No	No

F: female, M: male, OD: optical density.

DISCUSSION

Clinical findings of *Mycobacterium leprae* infection in armadillos are not frequent. Leprosy in the armadillo shows manifestations similar to lepromatous leprosy in human beings. However, approximately 5% of the naturally infected armadillos develop clinical findings of leprosy, such as enlargement of lymph nodes, cutaneous nodules or tumors upon external examination or internal lymph node enlargement and hepatosplenomegaly during necropsy. Bacilli were obtained from the viscera of most of the armadillos[28].

In this study, the only armadillo that presented palpable lymph nodes also showed the highest level of anti-PGL-I IgM antibodies (animal F005), while the other three positive armadillos (F002, F007, M017) presented clinical lesions (ulcers).

The frequency of antibody positivity in this study was 10.6%, which was less than when armadillos from the same area were tested by ML Flow (29.7%)[7], and less than armadillos from Louisiana and Texas (16%)[22]. However, in the present study, some antigenic destruction could have occurred during the transportation of the frozen samples from Vitoria to Sao Paulo.

Years after the first demonstration that nine-banded armadillos could be experimentally infected with *Mycobacterium leprae*[14], observation showed that wild armadillos in the southern part of the USA also carried a natural infection. Cross-reactivity between armadillo and human IgM and other armadillo serum proteins were not detected by anti-human IgM peroxidase conjugate[20][21] and serodiagnostic testing has been used mainly for detecting experimental infection in armadillos used in research at laboratories. However, serology could be a helpful tool for investigating the epidemiology of natural leprosy infections in the armadillo[7][9][22].

PGL-I antigen is highly specific to *Mycobacterium leprae*[2] and false-positive results caused by armadillos nonspecific antibody responses to atypical mycobacteria have not been reported[24].

Detection of IgM anti-PGL-I by ELISA in MB patients ranges from 85% to 100% and in paucibacillary (PB) patients from 5% to 34%[4][11][16]. This difference between these two polar forms occurs because the lepromatous form (MB) presents deterioration of the cellular immunity, a high antigenic load and high antibody levels, while the tuberculoid form (PB) presents intact parameters of cellular immunity, few bacilli and minimal elevation or no increase in antibody levels. This particular aspect of the polar disease means serology is mainly designated for MB diagnosis[19].

PB leprosy in the armadillo means subclinical infection, since few animals develop clinical signs of leprosy even when they are considered to present the lepromatous form (MB). Positive and negative ELISA interpretations are based on absorption studies, achieving sensitivity of 89% and apparent specificity of 100% for detecting *Mycobacterium leprae* infection either in human beings or armadillos[21].

Leprosy in wild armadillos is considered a zoonotic transmission and the relative risks to humans would depend on a variety of host factors and the likelihood of susceptible individuals having some significant contact with infected armadillos[22]. In addition, armadillo species other than *Dasypus novemcinctus* were not analyzed in this work, although other species have been described in the State of Espirito Santo.

More studies should be designed aimed at studying the relationship between leprosy and wild armadillos in Brazil, especially in leprosy-endemic states. Armadillos infected by *Mycobacterium leprae* can spread bacilli in the environment, making it more difficult to interrupt transmission and achieve the goal of leprosy elimination in Brazil.

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