Available online at www.sciencedirect.com
SCIENCE DIRECT
Veterinary Parasitology 138 (2006) 366–370
veterinary parasitology
www.elsevier.com/locate/vetpar

Short communication

Post mortem parasitological evaluation of dogs seroreactive for *Leishmania* from Rio de Janeiro, Brazil

Maria de Fátima Madeira [a,*], Armando de O. Schubach [b], Tânia M.P. Schubach [b], Sandro A. Pereira [b], Fabiano B. Figueiredo [b], Cibele Baptista [a], Cristianni A. Leal [a], Cíntia X. Melo [a], Eliame M. Confort [a], Mauro C.A. Marzochi [c]

[a] *Serviço de Parasitologia, Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz (FIOCRUZ), Av. Brasil 4365, 21045-900 Rio de Janeiro, RJ, Brazil*
[b] *Serviço de Zoonoses, Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz (FIOCRUZ), Av. Brasil 4365, 21045-900 Rio de Janeiro, RJ, Brazil*
[c] *Secretaria Municipal de Saúde da Cidade do Rio de Janeiro, Brazil*

Received 1 November 2005; received in revised form 20 January 2006; accepted 28 January 2006

Abstract

A parasitological study was conducted on 66 dogs seroreactive for *Leishmania* captured as a control measure of visceral leishmaniasis in the State of Rio de Janeiro, Brazil. Biological samples from different anatomical sites were collected during autopsy of the animals and cultured on biphasic medium (NNN/Schneider). The *Leishmania* isolates were characterized by isoenzyme electrophoresis. *Leishmania* was isolated from 80.3% of the animals: 12 animals with *Leishmania* (*Viannia*) *braziliensis* isolated exclusively from cutaneous lesions, 39 with *L.* (*L.*) *chagasi* isolated from different sites in the same animal, and 2 with simultaneous isolation of *L.* (*V.*) *braziliensis* from cutaneous lesions and *L.* (*L.*) *chagasi* from different sites. Isolation in culture revealed the absence of *Leishmania* parasites in 13 animals. The results obtained confirm the existence of mixed infections in dogs in Rio de Janeiro and indicate the need to complement the investigation of seroreactive dogs using methods for the parasitological diagnosis and identification of *Leishmania* species.

Keywords: *Leishmania*; Dog; Diagnosis; Co-infection

1. Introduction

Tegumentary (TL) and visceral (VL) leishmaniasis are relevant parasitic diseases in public health (WHO, 1990). In the State of Rio de Janeiro located on the southeast coast of Brazil, TL is caused by *Leishmania* (*Viannia*) *braziliensis*. Over the last few years, TL has

* Corresponding author. Tel.: +55 21 3865 9594; fax: +55 21 3865 9541.
E-mail address: fmadeira@ipec.fiocruz.br (M. de Fátima Madeira).

0304-4017/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2006.01.059

been expanding to the urban areas of different municipalities (Kawa and Sabroza, 2002; Serra et al., 2003). In the municipality of Rio de Janeiro, VL is caused by *Leishmania* (*Leishmania*) *chagasi*, with tens of canine cases being notified annually and with an average of 2.4 annual human cases having been reported over the last 5 years (Ministério da Saúde, 2003).

In the case of VL, control actions in Brazil include the culling of seroreactive dogs with indirect fluorescent antibody test (IFAT) titers of 1:40 or higher (Ministério da Saúde, 2003). Such indication for euthanasia does not exist in the case of TL (Ministério da Saúde, 2000). However, dogs with TL may be seroreactive (Madeira et al., 2005a,b), a fact making the differential diagnosis difficult. In areas with overlapping transmission of VL and TL, differentiated control measures for each disease should be based on parasitological diagnostic methods together with the identification of the *Leishmania* species involved.

The objective of the present study was to identify the *Leishmania* species isolated from different anatomical sites of seroreactive dogs collected for euthanasia in Rio de Janeiro.

2. Materials and methods

2.1. Animals and collection of biological samples

Sixty-six dogs of various breeds and ages from the State of Rio de Janeiro, Brazil, with serological titers for leishmaniasis ranging from 1:40 to 1:1280 determined by IFAT were carried out and analyzed at the Leishmaniasis Referral Center and Zoonosis Service (IPEC/FIOCRUZ/RJ), respectively. Venous blood was collected from each animal. After euthanasia, fragments were obtained from the following sites: cutaneous scars or lesions (when present); intact skin (scapular region or abdomen); cervical, popliteal and mesenteric lymph nodes; spleen; liver, and bone marrow aspirate. Tissue fragments were immersed in saline containing 50 μg 5′-fluorocytocine, 1000 IU penicillin and 200 μg streptomycin per milliliter and stored at 4 °C for 24 h. After this period, each fragment was transferred aseptically to a biphasic culture medium (NNN supplemented with Schneider's medium and 10% fetal bovine serum, FBS) and stored at 26–28 °C. Bone marrow obtained by puncture of the iliac region and blood (200–300 μL) were directly seeded onto the culture medium after collection. Fresh cultures were observed weekly for 30 days.

2.2. Characterization of the parasitic isolates

Multi-locus enzyme electrophoresis was used for characterization of the isolates according by Cupolillo et al. (1994). The following five enzymatic systems were analyzed by 1% agarose gel electrophoresis: nucleosidase (NH1 and NH2, E.C.3.2.2.1), glucose-6-phosphate dehydrogenase (G6PDH, E.C.1.1.1.49), glucose phosphate isomerase (GPI, E.C.5.3.1.9), and 6-phosphogluconate dehydrogenase (6PGDH, E.C.1.1.1.43). *Leishmania* (*V.*) *braziliensis* (MHOM/BR/75/M2903), *L.* (*L.*) *chagasi* (MHOM/BR/74/PP75) and *L.* (*L.*) *amazonensis* (IFLA/BR/67/PH8) were used as reference samples.

3. Results

3.1. Animals and parasitological test

Fifty-five of the 66 dogs studied were from various places in the municipality of Rio de Janeiro and 11 from neighboring municipalities: Angra dos Reis-Ilha Grande (4), Mangaratiba (3), Maricá (3), and Miguel Pereira (1). The serological titers showed the following frequency: 1:40 (9%), 1:80 (16.7%), 1:160 (16.7%), 1:320 (13.6%), 1:640 (27.3%), and 1:1280 (16.7%). Thirty dogs (45.4%) had cutaneous lesions located on the ears, muzzle, face, scrotal bag, limbs, and lip. *Leishmania* was isolated from one or more anatomical sites in 53 animals (80.3%) (Table 1).

3.2. Isoenzyme characterization

A maximum of seven samples from each animal were selected for electrophoretic analysis. All isolates obtained from fragments of cutaneous lesions and scars could be identified. No *Leishmania* parasites were detected in 13 animals, 1 with signs suggestive of

Table 1
Serological titers of 66 dogs obtained with an indirect fluorescent antibody test (IFAT) for leishmaniasis and results of isolation of *Leishmania* from different clinical samples cultured on biphasic medium (NNN/Schneider)

IFAT		*Leishmania* isolates in culture	Isoenzyme electrophoresis		
Titers	Number		*L.* (*L.*) *chagasi*[a]	*L.* (*V.*) *braziliensis*[b]	*L.* (*L.*) *chagasi* and *L.* (*V.*) *braziliensis*[c]
1:40	6	2	0	2	0
1:80	11	8	2	5	1
1:160	11	8	4	4	0
1:320	9	7	6	0	1
1:640	18	17	16	1	0
1:1280	11	11	11	0	0
Total number of dogs, *n*	66 (100)	53 (80.3)	39 (59.1)	12 (18.2)	2 (3)

Values in parentheses are in percent.
[a] VL, visceral leishmaniasis.
[b] TL, tegumentary leishmaniasis.
[c] Mixed infection.

VL, 8 with cutaneous lesions and 4 asymptomatic animals.

Leishmania (*L.*) *chagasi* was isolated from three or more sites in 39 (59.1%) dogs: cutaneous lesion (8/8), blood (13/39), intact skin (31/39), spleen (31/39), liver (27/39), popliteal (31/39), cervical (24/27) and mesenteric lymph nodes (17/27), and bone marrow (18/24). The serological titers were 1:80 (2), 1:160 (4), 1:320 (6), 1:640 (16), and 1:1280 (11) (Table 1). Thirty-six animals were from the municipality of Rio de Janeiro, two were from Ilha Grande and one was from Mangaratiba. Thirteen animals were considered to be asymptomatic and 26 were symptomatic for VL. *Leishmania* (*V.*) *braziliensis* was exclusively isolated from skin lesions located on the ear, muzzle, face, scrotum, hind limb, and lip of 12 (18.2%) animals in good general condition. The animals presented the following serological titers: 1:40 (2), 1:80 (5), 1:160 (4), and 1:640 (1). Co-infection was observed in two animals from the municipality of Rio de Janeiro (Jacarepaguá and Campo Grande neighborhoods). In one dog with an IFAT titer of 1:80, *L.* (*V.*) *braziliensis* was isolated from a scar on the ear and *L.* (*L.*) *chagasi* was isolated from blood, intact skin, spleen, liver, bone marrow, and popliteal, cervical, and mesenteric lymph nodes. In the other animal with an IFAT titer of 1:320, *L.* (*V.*) *braziliensis* was isolated from two cutaneous lesions (ear and muzzle) and *L.* (*L.*) *chagasi* was isolated from the spleen and popliteal lymph node.

4. Discussion

The method of isolation in culture on biphasic medium, regarded as the gold standard, was used for the parasitological diagnosis of leishmaniasis to analyze 66 seroreactive dogs from the State of Rio de Janeiro, Brazil. *Leishmania* sp. isolates were obtained from 80.3% dogs.

In areas of VL transmission such as the municipality of Rio de Janeiro, dogs with serological titers of 1:40 or higher are considered to be suspicious (Marzochi et al., 1985; Ministério da Saúde, 2003). Among the 55 seroreactive dogs from this municipality, *L.* (*L.*) *chagasi* was exclusively isolated from 36 animals, *L.* (*V.*) *braziliensis* from 5, and both species from 2; 12 animals were negative. These results suggest that the serological parameter should not be used as a single criterion to evaluate the prevalence of VL in dogs. The other hand, other parasites that might infect dogs in Rio de Janeiro as sporotrichosis (Schubach et al., 2006) could be confused with cutaneous lesion caused by *L.* (*V.*) *braziliensis*.

Although *Leishmania* infection cannot be ruled out in negative animals, the IFAT was able to identify 65.4% of animals with VL in the municipality of Rio de Janeiro and three cases, described for the first time, in the municipalities of Angra dos Reis and Mangaratiba.

Although serological parameters are important for the epidemiological surveillance of leishmaniasis,

previous studies had already showed that a low sensitivity of IFAT to canine diagnosis (Dye et al., 1993). The greatest obstacle to the interpretation of serological data is the absence of parasitological information. The latter employed in this study, permitted to identify two additional cases of mixed infection with *L.* (*V.*) *braziliensis* and *L.* (*L.*) *chagasi*, besides another recently reported case (Madeira et al., 2005b). Both co-infected dogs had cutaneous lesions and were asymptomatic for VL. In one dog, *L.* (*V.*) *braziliensis* was isolated from fragments obtained from a cutaneous scar, a fact confirming the persistence of *L.* (*V.*) *braziliensis* in healed lesions (Falqueto et al., 1986; Schubach et al., 2001; Mendonça et al., 2004). The fact of the IFAT positive to be criterion of inclusion of dogs in this study did not allow the comparison with epidemiological survey on canine population in other studies.

Although canine VL is characterized by a variety of signs and symptoms (Alvar et al., 2004), dermatological alterations are relevant signs (Silva et al., 2001), suggesting that other cases of co-infection remain unnoticed in endemic areas with overlapping TL and VL. In the present study 45.4% of the 66 seroreactive dogs had cutaneous lesions; of these, 8 were negative, *L.* (*L.*) *chagasi* was isolated from 8 animals and *L.* (*V.*) *braziliensis* from 14. In the latter cases, although different anatomical sites were investigated, *L.* (*V.*) *braziliensis* was only detected in cutaneous lesions. Previous studies have suggested that this species tends to be restricted to cutaneous lesions in naturally infected dogs (Madeira et al., 2005a). In contrast, only *L.* (*L.*) *chagasi* has been detected in healthy skin (Madeira et al., 2004) and internal organs. These findings indicate the need for the parasitological investigation of suspected dogs and add to the discussion regarding the importance of the domestic dog in the transmission cycle of TL (Reithinger and Davies, 1999).

The dissemination of *L.* (*L.*) *chagasi* to tissues seems to follow a sequence, with endothelial reticulum organs being parasitized before the skin (Tafuri et al., 2001), although this process is not well understood (Dos-Santos et al., 2004). In this study we found similar percentages of isolation of *L.* (*L.*) *chagasi* (79.4%) from skin, spleen and popliteal lymph nodes, irrespective of the serological titer or clinical condition of the animals. However, the chance of detection of *L.* (*L.*) *chagasi* was higher for cervical lymph nodes (88.8%) compared to the other sites investigated.

An interesting aspect of parasites of the genus *Leishmania* is the diversity of host tissue tropism (Noyes et al., 1977; Colmenares et al., 2002) and the present results suggest that *L.* (*V.*) *braziliensis* and *L.* (*L.*) *chagasi* behave differently in naturally infected dogs. Thus, it seems reasonable to suppose that domestic dogs play different roles in the transmission cycles of TL and VL and that the identification of the etiologic agents circulating in each region is necessary to implement differentiated control measures.

Acknowledgements

We thank the Paulo Dacorso Filho Center for Zoonosis Control and the Leishmaniasis Control Program of Rio de Janeiro for the identification and collection of the seroreactive animals. This study was partially financed by Fundação de Apoio a Pesquisa do Rio de Janeiro (FAPERJ). It was performed in accordance with Ethical Procedures (CEUA/FIOCRUZ/P195-03).

References

Alvar, J., Cañavate, C., Molina, R., Moreno, J., Nieto, J., 2004. Canine leishmaniasis. Adv. Parasitol. 57, 1–88.

Colmenares, M., Kar, S., Goldsmith-Pestana, K., McMahon-Pratt, D., 2002. Mechanisms of pathogenesis: differences amongst *Leishmania* species. Trans. R. Soc. Trop. Med. Hyg. 96, S1/3–S1/7.

Cupolillo, E., Grimaldi Jr., G., Momen, H., 1994. A general classification of new world *Leishmania* using numerical zymotaxinomy. Am. J. Trop. Med. Hyg. 50, 296–311.

Dos-Santos, W.L.C., David, J., Badaró, R., de-Freitas, L.A.R., 2004. Association between skin parasitism and a granulomatous inflammatory pattern in canine visceral leishmaniosis. Parasitol. Res. 92, 89–94.

Dye, C., Vidor, E., Dereure, J., 1993. Serological diagnosis of leishmaniasis: on detecting infection as well as disease. Epidemiol. Infect. 103, 647–658.

Falqueto, A., Coura, J.R., Barros, G.C., Grimaldi, G., Sessa, P.A., Carias, V.R.D., Jesus, A.C., Alencar, J.T.A., 1986. Participação do cão no ciclo de transmissão da leishmaniose tegumentar no município de Viana, estado do Espírito Santo, Brasil. Mem. Inst. Oswaldo Cruz 81, 155–163.

Kawa, H., Sabroza, P.C., 2002. Espacialização da leishmaniose tegumentar na cidade do Rio de Janeiro. Cad. Saúde Pública 18, 853–865.

Madeira, M.F., Schubach, A.O., Schubach, T.M.P., Leal, C.A., Marzochi, M.C.A., 2004. Identification of *Leishmania (Leishmania) chagasi* isolated from healthy skin symptomatic and asymptomatic dogs seropositive for Leishmaniasis in the Municipality of Rio de Janeiro, Brazil. Braz. J. Infect. Dis. 8, 444–448.

Madeira, M.F., Schubach, A.O., Schubach, T.M.P., Serra, C.M.B., Pereira, S.A., Figueiredo, F.B., Confort, E.M., Quintella, L.P., Marzochi, M.C.A., 2005a. Is *Leishmania* (*Viannia*) *braziliensis* preferentially restricted to the cutaneous lesions of naturally infected dogs? Parasitol. Res. 97, 73–76.

Madeira, M.F., Schubach, A., Schubach, T.M.P, Pacheco, R.S., Oliveira, F.S., Pereira, S.A., Figueiredo, F.B., Baptista, C., Marzochi, M.C.A., 2005b. Mixed infection with *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Leishmania*) *chagasi* in a naturally infected dog from Rio de Janeiro, Brazil. Trans. R. Soc. Trop. Med. Hyg., in press.

Marzochi, M.C.A., Coutinho, S.G., Souza, W.J.S., Toledo, L.M., Grimaldi Jr., G., Momen, H., Pacheco, R.S., Sabroza, P.C., Souza, M.A., Rangel, F.B., Tramontano, N.C., 1985. Canine visceral leishmaniasis in Rio de Janeiro, Brazil. Clinical, parasitological, therapeutical and epidemiological findings (1977–1983) Mem. Inst. Oswaldo Cruz 80, 349–357.

Mendonça, M.G., De Brito, M.E., Rodrigues, E.H., Bandeira, V., Jardim, M.L., Abath, F.G., 2004. Persistence of *Leishmania* parasites in scars after clinical cure of American cutaneous leishmaniasis: is there a sterile cure? J. Infect. Dis. 189, 1018–1023.

Ministério da Saúde, 2000. Manual de controle da leishmaniose tegumentar Americana. In: Ministério da Saúde, MS (Ed.), Brasília, 62 pp.

Ministério da Saúde, 2003. Manual de vigilância e controle da Leishmaniose visceral. In: Ministério da Saúde, MS (Ed.), Brasília, 122 pp.

Noyes, H., Chance, M., Ponce, C., Ponce, E., Maingon, R., 1977. *Leishmania chagasi*: genotypically similar parasites from Honduras cause both visceral and cutaneous Leishmaniasis in humans. Exp. Parasitol. 85, 264–273.

Reithinger, R., Davies, C.R., 1999. Is the domestic dog (*Canis familiaris*) a reservoir host of American cutaneous leishmaniasis? A critical review of the current evidence. Am. J. Trop. Med. Hyg. 61, 530–541.

Schubach, A., Cuzzi-Maya, T., Oliveira, A.V., Sartori, A., Oliveira-Neto, M.P., Mattos, M.S., Araujo, M.L., Souza, W.J.S., Haddad, F., Perez, M.A., Pacheco, R.S., Momen, H., Coutinho, S.G., Marzochi, M.C.A., Marzochi, K.B.F., Costa, S.C.G., 2001. Leishmanial antigens in the diagnosis of active lesions and ancient scars of American tegumentary leishmaniasis patients. Mem. Inst. Oswaldo Cruz 96, 987–996.

Schubach, T.M.P., Schubach, A., Okamoto, T., Barros, M.B.L., Figueiredo, F.F., Cuzzi, T., Pereira, S.A., Santos, I.B., Paes, R.A., Paes-Leme, L.R., Wanke, B., 2006. Canine sporotrichosis in Rio de Janeiro, Brazil: clinical presentation, laboratory diagnosis and therapeutic response in 44 cases (1998–2003). Med. Micol. 44, 87–92.

Serra, C.M.B., Leal, C.A., Figueiredo, F.B., Schubach, T.M.P., Duarte, R., Uchôa, C.M.A., Silva, R.M.M., Madeira, M.F., 2003. Canine tegumentary leishmaniasis in Morada das Águias (Serra da Tiririca), Maricá, Rio de Janeiro, Brazil. Cad. Saúde Pública 19, 1877–1880.

Silva, E.S., Gontijo, C.M.F., Pacheco, R.S., Fiuza, V.O.P., Brazil, R.P., 2001. Visceral leishmaniasis in the metropolitan region of Belo Horizonte, state of Minas Gerais, Brazil. Mem. Inst. Oswaldo Cruz 96, 285–291.

Tafuri, W.L., Oliveira, M.R., Melo, M.N., Tafuri, W.L., 2001. Canine visceral leishmaniosis: a remarkable histopathological picture of one case reported from Brazil. Vet. Parasitol. 96, 203–212.

World Health Organization, 1990. Control of Leishmaniases: Report of a WHO Expert Committee. World Health Organization, Geneva, pp. 1–158.