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Experimental and field investigations on the role of birds as hosts of *Leishmania infantum*, with emphasis on the domestic chicken

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ABSTRACT

In this study, 19 chickens were experimentally infected by *Leishmania infantum* and tissue samples, collected at different times, were cultured and subjected to conventional PCR and/or real time PCR (qPCR) to assess their susceptibility to infection. In addition, 121 serum samples from rural chickens ($n = 73$) and backyard birds ($n = 48$) were tested for anti-*L. infantum* antibodies by indirect immunofluorescence test. All the 19 animals showed to be molecularly positive at least at one tissue sample. In particular, 26 tissue samples from the experimentally infected chickens were positive on conventional PCR and/or qPCR but no clinical signs or seroconversion were detected and all tissue cultures were negative. Accordingly, all serum samples from rural chickens were negative whereas four (8.4%) from game birds (three *Anser anser* and one *Phasianus colchicus*) were positive. These results indicate that chickens are not suitable hosts for *L. infantum* under experimental condition. The occurrence of anti-*L. infantum* antibodies in domestic gooses (*A. anser*) and in a pheasant (*P. colchicus*) points out their possible role in the epidemiology of visceral leishmaniasis.

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1. Introduction

The leishmaniases are diseases of great zoonotic concern caused by protozoa of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae), which are transmitted by phlebotomine sand flies (Diptera: Psychodidae). *Leishmania infantum* is the most widespread causative agent of human and canine visceral leishmaniasis in China, Mediterranean countries and the Americas (Otranto et al., 2009b). In areas where visceral leishmaniasis is endemic, dogs are the most important reservoir hosts of *L. infantum* acting as a readily available source of infection for the vectors (Ashford, 1996; Dantas-Torres, 2007). However, natural hosts of *L. infantum* also include a number of sylvatic and synantropic animals (e.g., foxes, marsupials, and rodents) which are thought to play a role in the zoonotic transmission cycle of this parasite (Ashford, 1996). The actual role of some animals in the epidemiology of visceral leishmaniasis has not been fully understood, as it is the case of the domestic chicken (Alexander et al., 2002). The proximity of chicken houses is acknowledged as a possible environmental risk factor for *L. infantum* infection (Caldas et al., 2002; Moreira et al., 2003; Dantas-Torres et al., 2006). For instance, in Brazil, it has been reported that dwellings of persons affected by visceral leishmaniasis were about four times more likely to have chicken houses in their yards when compared to people living in the same area but not affected by the disease (Rodrigues et al., 1999). This could be explained by the fact that chickens constitute a “natural lure” for phlebotomine sand flies, including *Lutzomyia longipalpis*, the main vector of *L. infantum* in Brazil (Dantas-Torres and Brandão-Filho, 2006).

To our knowledge, no studies have assessed the susceptibility of chickens to experimental infection by *L. infantum*. In the same way, no studies have investigated the occurrence of anti-*L. infantum* antibodies in chickens and other backyard birds from areas where visceral leishmaniasis is endemic. Hence, the aim of the present work was to evaluate the susceptibility of chickens to experimental infection by *L. infantum*. Additionally, an indirect immunofluorescence antibody test (IFAT) was standardized to detect anti-*Leishmania* antibodies in chickens and other backyard birds in southern Italy where *L. infantum* infection is highly endemic.

2. Materials and methods

2.1. Parasites and inoculum preparation

A strain typed as *L. infantum* zymodeme MON-1 at the Istituto Superiore di Sanità (Rome, Italy) was cultured on Evan’s modi-

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fied Tobie's medium (EMTM) supplemented L-proline, 5% fetal calf serum and antibiotics (gentamicin 250 μg/ml and 5-fluorocytosine 500 μg/ml) at 24 °C, as described elsewhere (Brandonisio et al., 1992). Stationary phase promastigotes were collected and centrifuged (2000 × g for 10 min). The pellet containing the promastigotes was washed three times in sterile saline solution (NaCl 0.9%) by centrifugation (350 × g for 10 min) and then counted after immobilization by three drops of 70% ethanol. A suspension of stationary phase promastigotes of *L. infantum* at a concentration of 1×10^7 cells/ml in sterile saline solution was prepared as inoculum. This concentration was chosen by doubling the challenge dose used for resistant C57BL/6 mice (Goto et al., 2007).

2.2. Chickens and experimental infection

Twenty Hy-line 100-day-old chickens, weighting between 1170 and 1225 g, were housed in single cages (density 750 cm^2 per bird), according the Council Directive 1999/74/EC, at the Department of Veterinary Public Health of the University of Bari, Italy. The birds were kept under environmental controlled conditions (i.e. light/dark cycles of 10 h/14 h and temperature of 20–25 °C) and received commercial food (Specialmangimi Galtieri, Bari, Italy) and water ad lib.

At day 0 (T0), 19 chickens were injected subcutaneously (left side of the distal region of the neck) with 0.1 ml of the inoculum (see above). One chicken was left as a control and inoculated with 0.1 ml of sterile saline solution. At day 14 (T1), three chickens were euthanized whereas the other 16 birds were re-inoculated with the inoculum. Re-inoculated chickens were euthanized at 4, 8, 12 and 16 weeks (T2, T3, T4, and T5) post-inoculation (p.i.). Chickens were euthanized by endovenous inoculation of embutramide (200 mg/ml), mebenzonium iodure (50 mg/ml) and tetracaine cloridrate 5 mg/ml (Tanax, Intervet Italia, Milan, Italy) solution, and subjected to postmortem examination. From T1 to T5, tissue samples (i.e., skin, spleen, bone marrow, and liver) were collected from each animal and individually cultured. Part of these samples was stored at −20 °C until DNA extraction and PCR amplification. In addition, blood samples were weekly collected from chickens available from T1 to T5 (i.e., according with the euthanasia schedule) and tested for the presence of anti-*L. infantum* antibodies by an in-house IFAT, as described below. All experimentally infected chickens were daily examined for the presence of any clinical alteration (e.g., dermatitis, skin ulcers, feather loss, and weight loss) and body temperature was also recorded. All experimental procedures described above were approved by the Animal Ethics Committee of the University of Bari.

2.3. Indirect immunofluorescence test (IFAT)

An in-house IFAT for the detection of anti-*L. infantum* antibodies in chickens was performed as follows, using *L. infantum* promastigotes as antigen. Stationary phase promastigotes were centrifuged and washed three times in sterile phosphate-buffered saline (PBS). Wells of the IFAT slides (bioMérieux, Marcy l'Etoile, France) were each coated with 5 μl of the antigen, air-dried at room temperature overnight and fixed with cold acetone (−20 °C) for 10 min. Dried slides were stored at −20 °C until used. Then, 15 μl of sera diluted at 1:30 in PBS were added to the wells of IFAT slides and incubated at 37 °C for 30 min. After washing with PBS, each well was incubated with 15 μl of fluoresceinated rabbit anti-chicken IgG (Sigma–Aldrich, Steinheim, Germany) diluted at 1:50 in PBS, at 37 °C for 30 min. Slides were observed under a fluorescence microscope (Leica DMLS, Wetzlar, Germany) at 100× and 250× magnifications and samples were scored positive when they produced a clear promastigote cytoplasmatic or membrane fluorescence. In order to have a serum positive control, one chicken was inoculated three times (once a week) by intramuscular injection with 1.5 ml of a promastigote suspension (5×10^7 cells/ml) emulsified with mineral oil (Montanide ISA 740, Seppic, Paris, France) at a 2:1 ratio. Serum samples from this chicken were collected weekly until the 28th week p.i. A *L. infantum*-negative serum was obtained from the control animal inoculated with 0.1 ml of sterile saline solution. The cut-off dilution of 1:30 was adopted after testing both positive and negative controls using different conjugate and serum dilutions (data not shown).

2.4. Tissue culture

An amount of about 0.2 g of tissue samples (i.e., skin from the inoculation site, spleen, bone marrow, and liver) were collected from experimentally inoculated animals as previously reported. Individual samples were homogenized in a 1.5 ml Eppendorf tube using a pestle and immediately transferred to and cultured on EMTM. The remaining part of the tissue samples was stored at −20 °C until genomic extraction and PCR analysis (see below). Cultures were examined once a week for promastigote growth for one month. Contaminated cultures were discarded.

2.5. Molecular procedures

Total DNA was extracted from skin (inoculation site), spleen, bone marrow and liver samples using a commercial kit (Genomic DNA Purification Kit, Gentra Systems, Minneapolis, MN, USA), following the manufacturer instructions. A *L. infantum* kinetoplastid minicircle DNA was amplified using MC1 5′GTTAGCCGATGGTGGTCTTG3′ and MC2 5′CACCCATTTTTCCGATTTTG3′ primers (Cortes et al., 2004). DNA sample (4 μl) was added to the PCR reaction mixture (46 μl) containing 2.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 250 mM of each dNTP, 50 pmol of each primer and 1.25 U of AmpliTaq Gold (Applied Biosystems, Weiterstadt, Germany). Optimal conditions for PCR amplification were standardized as follows: initial denaturation at 94 °C for 12 min, 30 cycles consisting of denaturation at 94 °C for 30 s, annealing at 60 °C for 20 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. A positive control containing 5 pg of *L. infantum* genomic DNA and a negative control from each tissue sample collected from the control animal at T5 were included in the assays.

Amplification products (∼447 bp) were visualized on 2% (w/v) agarose gel, stained with ethidium bromide (10 mg/ml) using a 100 bp DNA ladder as a marker (Fermentas, Hanover, MD, USA) and then photographed using the Gel Doc 2000 gel documentation system (BioRad Laboratories, Hemel Hempstead, UK).

In addition, the same tissue samples were processed by real-time PCR (qPCR) as described elsewhere (Francino et al., 2006). Briefly, genomic DNA (3 μl) was added to a final volume of 25 μl containing 10 μl of RealMasterMix Probe (Eppendorf, Hamburg, Germany), 20 pmol of each primer and 5 pmol of FAM-labeled Taqman-MGB probe (Applied Biosystems, Foster City, CA, USA). All samples were run in duplicate and amplification, data acquisition and data analysis were carried out in a Mastercycler ep realplex S (Eppendorf, Hamburg, Germany). For each samples, a cycle threshold (C_T) was calculated based on the baseline cycles and the threshold value which is 10 times the mean standard deviation of fluorescence in all wells over baseline cycles.

2.6. Serological survey

From December 2005 to March 2006, 121 serum samples from backyard birds were collected, during the avian influenza control campaign, in different farms located in Apulia and Basilicata regions, where canine leishmaniasis is endemic (Otranto et al.,

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