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Longitudinal study on the detection of canine *Leishmania* infections by conjunctival swab analysis and correlation with entomological parameters

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ABSTRACT

A longitudinal study was carried out on kennelled stray dogs in a canine leishmaniasis (CanL) endemic area, to evaluate early and late diagnostic performance of a non-invasive conjunctival swab (CS) nested (n)-PCR analysis for *Leishmania* detection in 2 cohorts of dogs, respectively. (A) Sixty-five IFAT- and CS n-PCR-negative dogs exposed to, and followed up once or twice a month during a full sand fly season (July–November 2008). In parallel, a sand fly survey was performed on site using standard sticky traps set twice a month, for a cumulative surface of 63 m^2. (B) Seventeen IFAT- and CS n-PCR-negative dogs found positive in July 2008 at the peripheral blood buffy-coat (BC) n-PCR. These dogs were examined again by BC n-PCR in September and November 2008, and before the subsequent transmission season (May 2009) along with CS n-PCR and IFAT. None of the cohort (A) dogs converted to positive CS n-PCR during the transmission season. Although ~2500 phlebotomine specimens were collected with peaks of 100–147 specimens/m^2 sticky trap, the cumulative density of the only proven CanL vector in the area (*Phlebotomus perniciosus*) was found to be very low (0.5/m^2). All cohort (B) dogs remained substantially seronegative; BC n-PCR showed an intermittent positive trend during the period surveyed, resulting in 82% conversions to negative by the end of the study, in contrast with 71% conversions to positive at the CS n-PCR analysis. In conclusion, while CS n-PCR was not found effective for the early detection of *Leishmania* contacts in dogs exposed to a low pressure of vectorial transmission, this assay showed to slowly convert to positive in a high rate of dogs, in the absence of seroconversion. CS n-PCR technique can be a suitable marker for assessing *Leishmania* exposure in dogs as a non-invasive alternative to current serological and molecular tools.

1. Introduction

Zoonotic visceral leishmaniasis, caused by the protozoan parasite *Leishmania infantum* (=*L. chagasi*) is a sand fly-borne disease endemic in the Mediterranean area, Asia and Latin America (Gramiccia and Gradoni, 2005). In most of this range, the domestic dog is the main reservoir host. Canine leishmaniasis (CanL) is a major veterinary and public health problem in endemic areas, but also in non-endemic ones where individual clinical cases or outbreaks of disease are occasionally reported, such as in northern Europe, the USA and Canada (Slappendel and Teske, 1999;

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Schantz et al., 2005; Shaw et al., 2009). Dogs may suffer from a severe disease characterized by chronic evolution of viscero-cutaneous signs; however diseased cases represent only a small subset of the total infected population. Numerous cross-sectional surveys in endemic areas have documented a large pool of dogs without clinical signs of the disease, which however are seropositive, or seronegative but PCR-positive (reviewed in Baneth et al., 2008). Longitudinal studies have demonstrated that, after exposure to infection, a progression to disease may rapidly occur or, more frequently, a latent (sub-patent) condition may persist for years either resulting in apparent spontaneous recovery, or in abrupt progression to overt CanL preceded by high-titer seroconversion and parasite demonstration (Oliva et al., 2006). Importantly, both seropositive without clinical signs and clinically patent animals are similarly infectious to phlebotomine vectors (Molina et al., 1994).

The diagnosis of *Leishmania* infection in dogs exposed to risk of transmission may require frequent samplings owing to the late appearance of specific antibodies and detection of parasites in tissues. Sensitive PCR diagnosis requires invasive aspirate sampling of bone marrow or lymph nodes. A non-invasive conjunctival swab (CS) sampling coupled with a sensitive and specific PCR analysis, was proposed for the diagnosis of CanL in clinical cases or experimentally infected dogs in Israel (Strauss-Ayali et al., 2004) and Brazil (de Almeida Ferreira et al., 2008), and found later to be effective also in untreated or drug-treated animals without clinical signs (Di Muccio et al., 2008). These findings prompted us to evaluate the diagnostic performance of CS nested (n)-PCR for both the early and the late detection of *Leishmania* contacts in dogs exposed to risk of transmission, in comparison with usual serological and molecular techniques.

2. Materials and methods

2.1. Place and dogs

The study was carried out in a public kennel for stray dogs, which collects animals from a wide area around the commune of Santa Maria Capua Vetere (Campania region, Southern Italy). This area is a well-known focus of both human visceral leishmaniasis and CanL (Gradoni et al., 1996), with *Phlebotomus perniciosus* acting as the sole *L. infantum* vector (Maroli et al., 1994). The kennel is suited to host around 250 stray dogs, with elevated turn-over due to new stray dog collections and in-kennel births on one hand, and adoptions and deaths on the other hand. While aggressive or sick animals are confined in cages, most of the dogs live free in packs in wide fenced areas. Despite the repeated introduction of *Leishmania*-seropositive dogs, as shown by compulsory IFAT analysis at entry, occurrence of infection transmission within the kennel has never been clearly established.

2.2. Study design

A longitudinal study was designed to evaluate the early and late diagnostic performance of CS n-PCR for *Leishmania* detection, respectively in: (A) a cohort of IFAT- and CS n-PCR-negative dogs exposed to and periodically followed up during a full sand fly season, and (B) a cohort of IFAT- and CS n-PCR-negative dogs detected as positive at the peripheral blood buffy-coat (BC) n-PCR analysis, followed up during 1 year. To meet the first objective (A), a conservative dog screening was performed prior the 2008 transmission season as follows: all dogs found in the kennel in February 2008 were examined clinically for suspected CanL signs; those found without clinical signs were submitted to IFAT serology, and animals detected as seronegative were subsequently submitted to CS n-PCR; finally, dogs found negative also by this assay were enrolled for the investigation, consisting in serial CS n-PCR examinations performed during the sand fly season. In parallel, entomological surveys were performed in the kennel and neighbouring areas to investigate on sand fly species prevalence, density and seasonal trend.

To meet the second objective (B), a subset of CS n-PCR-negative dogs selected as above and found BC n-PCR-positive in July 2008, was examined again by this technique in September and November 2008, and before the subsequent transmission season (May 2009) along with CS n-PCR and IFAT.

2.3. Canine samples

Peripheral blood (5 ml) was obtained from jugular vein and equally distributed in empty and EDTA-coated tubes for serum and BC sample collection, respectively. The latter was performed within 24 h at room temperature, and the obtained BC samples (350 μl) were stored at −20 °C pending DNA extraction. Conjunctivas of both eyes were separately sampled using sterile CS manufactured for bacteriological assays. The swabs were rubbed against the surface of lower eyelid, immerged into 1.8 ml sterile saline in 20 ml plastic tubes and kept at 4 °C for 24 h. After manual stirring of swabs, the saline containing eluted exfoliating cells was transferred into 2-ml sterile vials pending DNA extraction. Samples from right and left eyelid conjunctivas were processed separately to provide a replicate diagnosis for each animal.

2.4. Phlebotomine collection

Standard 20 cm × 20 cm castor-oil soaked papers were set twice a month from May to November 2008 in various sites inside the kennel and in wall holes along side roads within 1 km from the kennel. Collection of live specimens was performed by CDC light traps set overnight, and through hand aspirators in suitable resting sites. Sand fly specimens were identified by their morphological characteristics to species level according to Theodor (1958) and Léger et al. (1983). Presence of *Leishmania* promastigote infections was determined microscopically on live female specimens.

2.5. IFAT

The in-house IFAT antigen consisted of cultured promastigotes of the WHO reference strain for *L. infantum* zymodeme MON-1 (MHOM/TN/80/IPT-1). The assay pro-