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Molecular diagnosis and phylogeographic analysis of *Trypanosoma evansi* in dogs (*Canis lupus familiaris*) suggest an epidemiological importance of this species in Colombia

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ABSTRACT

Surra disease is a zoonosis caused by Trypanosoma (Trypanozoon) evansi, a salivary trypanosome, originally from Africa, which affects a wide range of mammalian worldwide. Dogs are highly susceptible to T. evansi infection and they often exhibit strong clinical signs than can lead to death, even within weeks in untreated acute cases. The present survey is the first report through clinical, parasitological and molecular approaches, of two fatal cases of T. evansi in Colombian dogs. After analysing two presumptive cases of infection with Trypanosoma spp., in dogs by parasitological methods, we confirmed by molecular techniques the presence of T. evansi, finding clinical signs such as anaemia, thrombocytopenia and hepatosplenomegaly, with fatal outcomes within a week even after the treatment. A phylogenetic and phylogeographic analysis of both isolates from T. evansi, suggest a complex evolutionary relationship with species of Trypanozoon subgenus. Moreover, the haplotype H2 was observed for the first time in Colombia, in common areas where human cases of T. evansi infection has been reported. These findings imply a relevant problem for animal health in the country, and highlight the importance of this infection in domestic animals and the possibility of human cases.

1. Introduction

Trypanosoma (Trypanozoon) evansi, the agent of "Surra", is a flagellate protozoan parasite that affects several mammalian species in Africa, Asia and Latin America. Nowadays, this disease is considered mandatory reporting to the World Organisation for Animal Health – OIE (Desquesnes et al., 2013; OIE, 2013). Evolutionary hypothesis suggest that *T. evansi* derived from *T. brucei* through the complete loss of the maxicircles of the kinetoplastic DNA, which are required to survival of the procyclic form in tsetse flies (Wen et al., 2016; Carnes et al., 2015; Sánchez et al., 2015; Lai et al., 2008). For this reason, this species is "trapped" into the host in its bloodstream form and it is transmitted mainly by biting insects (tabanids and stomoxes), iatrogenic or oral transmission. However in Brazil, vampire bats are also implicated in a unique type of biological transmission (Desquesnes et al., 2013; Schnaufer et al., 2002; OIE, 2013).

Since 2005, at least six human cases of *T. evansi* have been reported in Africa and Asia, highlighting the importance of domestic animals as a source of infection to human (Van Vinh Chau et al., 2016; Haridy et al., 2011; Kaur et al., 2007; Powar et al., 2006; Shegokar et al., 2006; Joshi et al., 2005). In South America, *T. evansi* was described for the first time in 1827 on the Marajo Island (Amazon estuary), and it has subsequently been found in Argentina, Paraguay, Brazil, Bolivia, Venezuela, Guyana, Colombia and Panama infecting domestic animals such as horses, cattle, buffaloes and dogs (Arias et al., 1997; Eberhardt et al., 2014; Franke et al., 1994; Reyna-Bello et al., 1998; Songa et al., 1990; Herrera et al., 2004), and wild reservoir like capybaras *(Hydrochoerus hydrochaeris)* and coatis (*Nasua nasua)* (Arias et al., 1997; Eberhardt et al., 2014; Nunes and Oshiro, 1990). However, human cases of *T. evansi* have not been reported in this continent.

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Dogs are highly susceptible to *T. evansi* infection and they often develop strong clinical signs like fever (39 °C–41 °C), edema, anaemia, paresis, weakness and lack of appetite leading to death, sometimes within a week and most often within a month especially in acute cases not treated and also sometimes even despite treatments (Desquesnes et al., 2013). Most of the cases are related to hunting dogs or dogs living around slaughter houses, which suggests peroral infection; although transmission by stomoxes is also possible if the dog lives in close contact with another infected animal (Franke et al., 1994; Desquesnes et al., 2013). The course of the disease and clinical picture varies from animal to animal thereby making the putative diagnosis more difficult (Franke et al., 1994; Rjeibi et al., 2015). Although parasitological and serological techniques are available for diagnosis of *Trypanosoma* spp ., in dogs, the intermittent parasitemia and cross reaction with other protozoa (*Leishmania* spp., *Ehrlichia* spp., and *Babesia* spp.) (Zanette et al., 2014), reduce the sensitivity and specificity of these tests. Therefore, molecular and genotyping techniques are considered the best strategies for study focus of infection with *Trypanosoma* spp., in this hosts.

Although some cases of *T. evansi* have been reported in dogs from Colombia (Correa et al., 2010), molecular confirmation has not been reported in this host, whereby the clinical and epidemiological characteristics of this infection are unknown. In this paper, we made the first clinical, parasitological and molecular report, of two fatal cases of Surra in dogs from two different regions of Colombia, showing common clinical sings and new genotypes of species in America that highlight the importance of this host as a possible source of infection to humans.

2. Materials and methods

Between July and August 2016, two cases of active trypanosomiasis in dogs from the municipalities of Caldas (Antioquia department) and Morelia (Caquetá department) Colombia, respectively, were referred to BCEI lab of the University of Antioquia, to determine the species and genotypes associated with the infection. Caldas is located in the northwest section of the country, at a latitude 6°05′19″N and longitude 75°38′10″W, at average altitude of 1750 m. The ecological zone consists of subtropical moist forests, with rainfall average 2125 mm/year and an average annual biotemperature of 19 °C (IDEAM, 2016). Morelia is located in the south of the country, at a latitude 1°29′12″N and longitude 75°43′31″W, at average altitude of 258 m. The ecological zone consists of tropical rainforest, with rainfall average 3840 mm/year and an average annual biotemperature of 25 °C (IDEAM, 2016). The main clinical features of each case are referred to below:

2.1. Description of cases

2.1.1. Case 1

In July 2016, a five-year-old male creole dog, from a farm of rural area of the municipality of Caldas, and used as pet, was admitted to the veterinary clinic of the Lasallista University Corporation with a dehydration of 9%, jaundiced mucous membranes, generalized lymphadenitis, distended abdomen and a corporal condition of 2.5/5 (Fig. 1A). In his history, the owner reported that the dog lived outside house with other domestic animals as cattle and horses. A blood smear stained with Giemsa showed numerous trypanosomes with morphology typical of monomorphic, slender, flagellated (with a parasitemia of approximately 154×10^6 trypanosomes/mL) and structures compatible with *Hepatozoon canis* (Fig. 1B). Blood samples were transferred to BCEI lab in Medellin (Antioquia-Colombia) for the diagnosis and molecular characterization.

2.1.2. Case 2

In August 2016, a sixteen-month-old male creole dog, from a farm of rural area of the municipality of Morelia, and used as hunting dog, was admitted to a veterinary clinic of this town with progressive weight loss, corporal condition of 2/5, lack of appetite and decay (Fig. 1C). In his history, the owner reported that in earlier days the dog suffered an open wound caused by the bite of a capybara. A blood smear examination indicated the presence of unknown flagellate (with a parasitemia of approximately 25.3×10^6 trypanosomes/ml) (Fig. 1D). Blood samples were transferred to BCEI lab in Medellin (Antioquia-Colombia) for the diagnosis and molecular characterization.

2.1.3. Clinical and laboratory analyses

To assess the clinical status of each patient, additional tests were performed between two to five days after initial clinic admission, in the reference laboratories of each clinic. A Complete Blood Count (CBC) along with a classic biochemical quantitative analysis with emphasis on Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), urea and creatinine, were performed as screening tests to determine the general health status of each animal. Additionally, a stool ova, parasites test and chest radiograph, were performed in the animal of Case 1.

2.2. Blood sample collection and DNA extraction

For each animal, a sample (5 ml) of blood was collected from radial vein, using EDTA.K3 and stored at 4 °C until use. Genomic DNA was extracted from 200 μl of blood with EDTA using the Genomic DNA Purification Kit (Invisorb® Spin Blood Mini Kit) according to the manufacturer's instructions. Total DNA was diluted with 100 μl elution buffer and stored at −20 °C until molecular diagnosis

2.3. Molecular diagnosis

Infection with *Trypanosoma* spp., was detected using a semi-nested PCR (sn-PCR) based on the small sub-unit ribosomal gene (18S) (Geysen et al., 2003), and the subgenus *Trypanozoon* using a PCR for the surface proteins (ESAG) (Holland et al., 2001). The oligonucleotide sequences used for n-PCR and PCR in the present study and their annealing temperatures (°C) are shown in (Table 1). All PCR assays were conducted in 25 μl reaction volumes containing 1X reaction buffer (100 mM Tris-HCl, 50 mM KCl, pH 8.8), 0.2 mM dNTP, 1–4 mM of $MgCl_2$, 0.4 μM of each primer, 0.625 U of Taq polymerase (Thermo Scientific®), and 5 μl (100 ng) of DNA samples, except in the sn-PCR, where 1 μl of the first PCR product was used. PCR products were analyzed by 2% agarose gel electrophoresis, stained with GelRed™ and visualized under UV light.

To determine the trypanosome species in the 18S positive samples, RFLP-based methods were used. For this, 5 μl of semi-nested PCR products were digested with Msp 1 and Eco571 enzymes in buffer Y + /Tango with S-adenosylmethionine according to the manufacturer's specifications (New England Biolabs®). The reaction was incubated 1 h at 37 °C, and 10 μl of the restriction product was analyzed by 2% agarose electrophoresis as described above. In this analysis, 18S semi-nested PCR products from *T. theileri* (strain C1587ANT, Colombia), *T. evansi* (Isolated Montevideo, Uruguay), *T. cruzi* (strain Gal61, Colombia) and *T. vivax* (strain LIEM176, Venezuela) were used as controls, to compare the pattern of bands of each sample.