Coinfection of and *Leishmania* spp. in Synanthropic Reservoirs (*Canis familiaris*) in an Endemic Area of The State of Querétaro, Use of FeSODe as an Antigenic Tool

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**Abstract**

The importance of synanthropic reservoirs in the epidemiology of metaxenic diseases plays an important role in field studies. Dogs, being the domestic animals that commonly cohabit with the human being in both rural and urban areas, play a fundamental role as reservoirs of blood and tissue protozoa. The migration of the human and its invasion to areas inhabited mainly by natural reservoirs and vectors of Trypanosomatids like *Leishmania* and *Trypanosoma* has increased the risk of contracting blood or tissue infection. The present study reveals firstly the seroprevalence of both parasites in canids that coexist openly with the population and in turn are part of the natural fauna of the place (synanthropic reservoir) and secondly the existing co-infection between *Leishmania* sp in the studied dogs.

Were collected 100 blood samples from canids close to the human and were tested for anti *T. cruzi* antibodies and anti-*Leishmania* first with two standard tests, ELISA and Indirect Hemagglutination (HAI) for *T. cruzi* and Immunochromatographic Test for *Leishmania* (SD bioline *Leishmania* Ab), whose results were not convincing. The enzyme superoxydodismutase (SODE) excreted by *T. cruzi* parasites and *Leishmania* species present in the endemic region (*L. mexicana mexicana*, *L. infantum chagasi* and *L. braziliensis braziliensis*).

The results obtained reveal a seroprevalence of 20% for *T. cruzi*, 15% for *L. mexicana mexicana*, 2% for *L. infantum chagasi* and 11% for *L. braziliensis braziliensis* and a coinfection between *L. mexicana mexicana*/*L. braziliensis braziliensis* and *L. mexicana mexicana*/*T. cruzi* and less frequent, coinfection among *L. braziliensis braziliensis*/*T. cruzi* with percentage values of 4%, 4% and 2% respectively.

**Keywords:** FeSODe; Synanthropic reservoirs; Co-infection in trypanosomatids; Antigenic tool

**Abbreviations**

HAI: Hemagglutination indirect; ELISA: Enzyme linked immunosorbent assay, SODE: Superoxide diismutase of iron

**Introduction**

Trypanosomatids are one of the major causes of infections affecting man and animals. Its effects not only cause high rates of morbidity and mortality, but also lead to the production of large economic losses that not only compromise the sick man, but also the family environment and the community in which he is inserted. In some cases they limit social and economic development, as is often the case in many Third World countries [1].

They are protozoan parasites of blood and tissues that affect a wide range of vertebrate hosts. The two most important genera that infect the human and mammals of the world are *Leishmania* and *Trypanosoma* that come to produce pathologies of diverse consideration.

*Leishmania* is a histoparasitosis, produced by protozoans of the genus *Leishmania*, of intracellular localization (macrophages) that is characterized by presenting cutaneous, mucous or visceral lesions, according to one of the 20 existing pathogenic species and is transmitted through the bite of diptera insects *The genres Phlebotomus and Lutzomyia*, where
there are domestic and wild reservoirs, and it is considered a
zoonosis [1]. Distributed in 98 countries around the world, it is
considered a cosmopolitan infection. It locates it within the TDR
program and indicates as a priority in the research and control
worldwide and a total of 350 million at risk of
infection. In recent years, it has been considered as an emerging disease
[4,5], due to climatic changes, population migrations, vector resistance and the difficulty of eliminating animal reservoirs as
well as their opportunistic relationship with individuals Immuno
compromised (AIDS) [6,7].

With regard to the American Trypanosomiasis better known
as Chagas disease, it is a zoonosis of wide complexity that
presents endemic only in the Western Hemisphere, from the
United States to Argentina. It is produced by the protozoan
heteroteric Trypanosoma cruzi, a parasite described by Carlos RJ
Das Chagas in 1909, who characterized it and established its life
cycle by presenting different mechanisms of transmission
among which are contaminated feces of triatomine insects of
various species (Rhodnius, Panstrongylus, Triatoma and others),
blood transfusions, vertical transmission (mother-child), organ
transplantation and oral transmission [8].

WHO considers it one of the 9 neglected diseases in the world
[3] because it is one of the most complicated parasitic diseases
in humans, both because of its unrealistic prevalence and
because of its serious clinical picture [9]. In the last 5 years, the
investigations revealed that the vectorial transmission reaches
80% of the cases detected and 20% are transmitted by
congenital route [10]. It is estimated that 12 million people are
infected in 21 Latin American countries [11-13], with 14,000
deaths year [14,15] more than 25 million people are at risk of
contracting the disease [13]. Prevalence rates vary widely depending on the geographical area; only in 2008 there were
more than 10,000 deaths from Chagas [15].

The impact of the vertical transmission of this disease is
estimated for all Latin America in more than 15,000 cases year
[16].

Reservoir animals, especially those in close contact with man,
play a key role in the transmission of Leishmania and
Trypanosoma. The discovery in Brazil in 1913 of the first dog
infected by Leishmania [17] raised the possibility of the
important role they play in the transmission of the disease.
Today, many cases of Leishmaniasis in canines have been
described in many countries [18-23], Leishmania mexicana
infection was reported for the first time in dogs from the
mexican states of campeche, quintana roo and oaxaca [24]
which is one of the main links in the peri-urban and urban
population due to its sinantropism. The prevalence of
Leishmania spp. in dogs from Central and South America varies
according to the region and the diagnostic method used with
rates from 25% to 75% [25]. In Mexico, the prevalence of the
disease reaches 58% [26]. Most dogs in rural areas may present
cutaneous and/or mucocutaneous lesions, playing a relevant
role in the transmission of the domestic cycle of the disease
[27,28].

Natural reservoirs in Chagas disease are domestic and wild
mammals that play an important role in maintaining the
domestic, peri-domestic and wild cycles. Among them, it has
been demonstrated that the dog is the fundamental key in the
domestic cycle, being considered the overcrowding as a risk
factor for the acquisition of the infection in the human beings.
This represents a close connection between the parasite’s
domestic and jungle cycle [29,30]. T. cruzi infection in dogs has
been described throughout Latin America, from southern Texas
to northern Argentina [31,32].

The diagnosis of both trypanosomatids is very complicated
and is usually accompanied by several tests necessary for
confirmation. Serological procedures for the detection of
antibodies in Leishmania infections are generally based on
Indirect Immunofluorescence (IFI) or Immune Enzymatic Assay
(ELISA) techniques. For cutaneous Leishmaniasis of the Old World
seem to have little diagnostic value, instead they are more
useful in cutaneous diseases of the New World. The above tests
can detect the infection but do not allow identification of the
species.

In general, the sensitivity of the tests for skin disease is low.
For visceral Leishmaniasis, the direct agglutination test and
more recently the development of an rk39 antigen-based
indicator test, which together with the PCR, may be specific for
genus and species, but are not yet available for application in
humans or in animals. On the other hand, the stage of the
amastigote can be difficult to detect in the imprints or in the
biopsy material and the isolation of the parasite directly or by
culture is difficult. In the diagnosis of Trypanosoma cruzi, 3
conventional immunological tests (ELISA, HAI or IFI) are
required, two of which must be reactive, so that the confirmed
infection is considered, this is considered to be costly, in addition
to cross reactions (Leishmania,Toxoplasma, Plasmodium) and
obtain a “false positive” result.

The use of detoxifying enzymes as an antigen in the diagnosis
of both infections has been very promising in previous studies
[33-37]. Other rapid diagnostic techniques such as
chromatography and gelatin particles bound to T. cruzi antigen
(Serodia) have given results of lower sensitivity.

The study of mixed infections by both trypanosomatids using
test with high sensitivity molecular antigens can provide us with
data of great interest in the epidemiology of both genera in
synanthropic reservoirs.

Materials and Methods

Biological sample

We studied 100 domestic dogs from an endemic area of T.
cruzi located in the municipality of Colón in the State of
Querétaro. The owners of the animals were asked to sign the
consent form, to make the blood sample. At several weekend
visits, 100 blood samples of canids were collected by puncture
of the central paw vein, in addition to scraping of skin and
mucosa (Figure 1).
The extracellular isoform of SOD is considered the most important since it plays a very relevant role in the pathogenesis of many pulmonary, neural and cardiovascular diseases [40,41].

In 2006 Marin et al. purified Fe-SOD excreted by Phytomonas showing that it had a pl of about 3.6 and a molecular weight of around 22kDa [39,42].

Subsequently, Mateo et al. purified and characterized FeSOD excreted by T.cruzi, showing that it presented characteristics similar to those found by Marin in 2006, pl of 3.8 and molecular weight in 25kDa [37].

**Obtaining FeSODs**

**Cell culture**

For the study and characterization of excreted FeSOD, promastigote forms of Leishmania mexicana, Leishmania infantum chagasi and Leishmania braziliensis, isolated from dogs and T. cruzi epimastigotes were isolated from an infected patient and cultured in MTL medium (Medium Trypanosomes Liquid) (Gibco*), supplemented with 10% (V/V) fetal bovine serum (SBF, PAAA*), heating to inactivate it at 56°C for 30 minutes.

The growth parasite density (1 × 10^7 cells/ml) was estimated by counting in a Neubauer® chamber and the cells were harvested in the exponential growth phase through centrifugation at 1500 g for 10 minutes at room temperature. The obtained cellular package was resuspended with 25 ml of MTL, without SBF and incubated for 24 hours or overnight at 28°C.

**Preparation of fraction H**

The promastigotes (Leishmania) and epimastigotes (Trypanosomes), obtained in their exponential phase of growth described in the previous step, undergo a process of lysis or cellular rupture. They are washed twice with phosphate solution pH 7, removing the residues from the culture medium. The pellet is resuspended in 3 ml STE-Buffer 1 lysis buffer (250 nm sucrose, 25 mm Tris-HCl and 1 mm EDTA, pH 7.8), sonicated cold in three cycles of 60 V for 30 seconds (at intervals of 1 min between cycles). The cell lysate was centrifuged (2500 g/10 min/4°C), discarding membrane debris, the supernatant obtained a new supernatant three times with buffer 1 until it had a 9 ml total volume fraction. It was centrifuged the last time in the same previous conditions, obtaining a new supernatant (Fraction H).

**Obtaining and purification of excreted FeSOD**

The pellet of cells already obtained was resuspended with 25 ml of MTL medium without SBF and incubated 24 hours at 28°C. It is then centrifuged at 1500 xg for 10 minutes, the pellet is discarded and the supernatant is filtered on nitrocellulose membrane (Minisart®). The supernatant is precipitated with ammonium sulfate between 35% and 85%. Again a concentration is made with 35% saline and centrifugation of 9000 g for 20 minutes. The second supernatant is precipitated a second time with ammonium sulfate to a total concentration of
85%. Dissolved the salt is allowed to stand in cold for 20 minutes. Again centrifuged at 9000 xg/20 minutes at 4°C.

The pellet obtained is resuspended in 2.5 ml of distilled water and desalted to 3.5 ml via a Sephadex G-25 (GE Healthcare Life Sciences®, PD 10 column) chromatography column, equilibrated in advance with 25 ml of distilled water (Fraction P85 or FeSODE-np). The last step is to add 25 μl of antiprotease (Protease Inhibitor Complete Mini, Roche®) thus minimizing the action of the proteases present in the medium.

The P85 fraction of both parasites were purified independently by column chromatography, first by ion exchange, and then by molecular weight filtration.

Once the P85 fraction was concentrated to a volume of 2 ml by lyophilization (lyoQuest, Telstar®), it was passed through an ion exchange column, QAE-Sephadex A-50 (Sigma-Aldrich®), previously equilibrated with the potassium phosphate buffer (20 mm, pH 7.4, 1 mm EDTA), and the elution of the absorbed proteins in the matrix was done through the application of a linear gradient of KCl (0-0.6 M) collecting fractions of 2.5 ml.

The protein concentration of the H P85 and Q1e and Fe-SODe fractions were quantified using the Bradford technique (Sigma Bradford test), using bovine albumin serum as the standard curve.

The obtained FeSODE is used as antigen and the ELISA test is applied for the search for anti-Leishmania and anti Trypanosoma antibodies. [36].

Indirect ELISA Test

With the excreted FeSOD fraction diluted with carbonate-bicarbonate buffer (at a final concentration of 1.5 μg and 5 μg), the polystyrene plates are sensitized. 100 μl per well is used. Incubate 2 hours at 37°C or leave overnight in a humid chamber.

To remove unfixed antigens, wash 3 times with 200 μl Wash Buffer solution (Tampon 4; PBS+Twen 20° 0.05%) by overturning the plate on filter paper to dry. Adsorption sites that have been left free because the antigen has not been bound are blocke with buffer 5 (Twen 20°, 0.2% BSA in PBS) and incubated for 2 hours at room temperature and under stirring to prevent binding Not specific between plaque and serum. Wash 3 times again; the plate is incubated for 45 minutes with 100 μl of canine serum diluted 1:80 to be studied.

At the end of the time, the plate is washed 3 times (Buffer 4) and incubated for 30 minutes at 37°C at 37°C and 100 μl of the immunoconjugate (Anti-IgG/anti-dog peroxidase-Sigma-Aldrich) is added at a 1: 1000. Wash again (Buffer 4) and add 100 μl to each substrate well of the immunoconjugate, orthophenylenediamine dihydrochloride (OPD-Sigma-Aldrich®) in Buffer 7; 12.5 ml distilled H2O2, 12.5 ml of 0.1M citrate-phosphate Buffer, pH 5, Buffer 6 and 10 μl H2O 30%. The plate is incubated in the dark for 20 minutes and 50 μl of reaction stop solution (3 N HCl) is measured at the end of this time by measuring the absorbance at 492 nm in the ELISA reader (Sunrise TM, TECAN). The mean and standard deviation (SD) of the optical density of the negative controls was used for the calculation of the cutoff value (mean + 3x SD).

Results

To evaluate the efficacy of FeSODE from L. mexicana, L.infantum chagassi, L. braziliensis and Trypanosoma cruzi, we tested 100 sera from dogs from the endemic zone of the State of Querétaro, all at a dilution of 1/80 by the technique of ELISA, only 20 were positive to T. cruzi (20%), 15 to Leishmania mexicana mexicana (15%), 2 to Leishmania infantum chagasi (2%) and 11 to Leishmania braziliensis braziliensis (11%) (Figure 2).

Figure 2: Prevalence of anti T. cruzi and anti Leishmania antibodies in feral canids of the community of Gallo, municipality of Colón in the State of Queretaro. Mexico.

The percentage of coinfection among the Trypanosomatid species were, L. mexicana / L. infantum (1%), L. mexicana/L. braziliensis (4%), L. mexicana/L. infantum/L. braziliensis (1%), L. mexicana/T. cruzi (4%), L. braziliensis/T. cruzi (2%) and for L. mexicana/ L. infantum/T. cruzi (1%) (Figure 3).
WHO promotes the ELISA test due to its high sensitivity in the diagnosis of Trypanosomiasis, which is why the enzyme, have been proposed as promising diagnostic tools. In 2002, WHO promotes the ELISA test due to its high sensitivity and low cost, being nowadays one of the main conventional tests.

The problem of using a single test is the presence of cross-reactions to parasites belonging to the same or different families, which is why the institution recommends the use of at least two positive conventional serological tests to confirm diagnosis. Different groups of researchers work to improve the quality of these tests based mainly on obtaining antigenic fractions exclusive of the parasite obtained via lysate or excreted by the same to the culture medium. These antigens excreted by parasites of the Trypanosomatid family, such as the FeSOD enzyme, have been proposed as promising diagnostic tools against infection by this group of parasites, examples tested as T. cruzi [33], Phytomonas [35] and Leishmania spp. [36] Superoxide dismutases are an important group of metalloenzymes that play an important role in the defense of superoxide radicals that protect cells from infection [38,40].

These enzymes are considered to be virulence factors that protect the parasite from attacks by the host cells; their activity was detected in the main Trypanosomatid species [41], in particular the excreted iron superoxydismutase is the one that has been shown Sensitive in trials with this group of parasites for the T. cruzi species, this enzyme was shown to be highly sensitive and of high diagnostic value in works.

In the present study we show for the first time in a rural community endemic to Trypanosoma cruzi, the importance of domestic canids as reservoirs for the infection of both genera of Trypanosomata, the role of these animals as synanthropic reservoirs to Chagas disease and Leishmaniasis is of relevance in this work due to the high prevalence observed for both Leishmania spp and which means that these animals play an important role in the epidemiology of both diseases in the study area.

In 1993, it detected for the first time the presence of leishmaniasis infection in wild dogs in several states of the Mexican Republic [24], mainly in the Yucatán Peninsula, an area endemic for the Leishmania. The relationship between man and dog so close, both rural and urban, make these animals the first epidemiological link as synanthropic reservoirs [43]. The risk to human infection is greater in terms of the relationship between dogs and their environment as well as the presence of vectors in human dwellings. Different studies support the theory that dogs would be the most important reservoir for Leishmania [18,19,24].

It is therefore necessary to control and supervise such animals thus avoiding a public health problem. The percentage obtained in our study referring to the canine population of Leishmania spp places it among the Latin American countries studied. Our results show the highest frequency of infection in dogs to Leishmania mexicana followed by Leishmania braziliensis and Leishmania infantum chagasi with populations of 15%, 11% and 2%, respectively. The serological prevalence of in these animals was 20%. These data, which are relevant for the first time in the State of Querétaro, demonstrate the importance of these Trypanosomatids in the epidemiology of the respective diseases.

Co-infections observed at both Leishmania spp and T. cruzi were more relevant for Leishmania mexicana and braziliensis and Leishmania mexicana and with Coinfection rates of 4%.

The presence of three species of Leishmania in these reservoirs in the State of Querétaro emphasizes the importance of this endemic until the moment unknown. Regarding, 20% of these animals were seropositive in an area where the level of human infection was 13% [19].

Chagas’ disease in Mexico is underestimated according to official data, a prevalence of 1.5% in the Mexican Republic in the only study carried out so far, may lead us to think of a low endemicity for this protozoan, thus the true prevalence This disease is far below reality [44].
It is contradictory that a country with the highest number of vectors counted has so low levels of official endemicity, only in the State of Querétaro the serological prevalence of was 8% in studies carried out by our group [34].

In countries where more than one species of *Leishmania* is endemic, the differential diagnosis between species becomes indispensable [15]. The two main forms of *leishmaniasis* found in Mexico are *L. mexicana* (cutaneous), *L. braziliensis* (mucocutaneous) and *L. infantum-chagasi* (visceral) [39].

The antigenic fractions of all *Leishmania* species have been prepared under the same technical conditions; therefore the low prevalence values of *L. infantum-chagasi* suggest the absence of cross-reaction between different species of *Leishmania*. The absence of a cross-reaction with Chagas disease is very important because the endemic areas of both diseases are overlapping, which leads to the diagnosis of false positives with consequent aggravating effects on the health of the presumed patient [31].

The results presented seem to demonstrate that: the diagnosis performed by the ELISA technique with the antigenic fraction FeSODe is more reliable than by conventional serological methods such as IIF and HAI and that the FeSOD excreted by the 4 parasites did not present cross-reaction between them and other Trypanosomatids such as the case of *T. cruzi*, thus being able to discriminate between *Leishmaniasis* and Chagas’ disease (Figure 4).

It is for this reason that the results offered in the present work contribute unique findings so far not shown on the importance of the reservoirs synanthropic canines in the epidemiology of *leishmaniasis* and *Trypanosomiasis*, with species of *Leishmania* not described in our area of study.

**Conclusion**

*L. braziliensis*, *L. infantum chagasi*, *L. mexicana* and excrete a FeSODe that presents immunogenic characteristics that make it a good molecular marker for serodiagnosis. In conclusion, an indicative ELISA capable of detecting specific antibodies by different species of *Leishmania* and *Trypanosoma* has been developed, demonstrating that FeSOD excreted by *Leishmania* spp. and is an ideal antigen because of its incredible sensitivity and high specificity for the routine diagnosis of these diseases.

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