

Coinfection of *Leishmania Mexicana* and *Leishmania Infantum Chagasi* in The Pediatric Population of the State of Querétaro's (Mexico), Using Fesode as an Antigen

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Summary

Leishmaniasis is a disease caused by protozoa of the genus *Leishmania* of which there are currently about 20 pathogenic species to man. The WHO in 2010 recognizes this disease as one of the great neglected pathologies and estimates that around 98 countries in the majority of developing countries have endemicity. According to estimates by WHO at the present time we would have 12 million people infected with 350 million people living in areas at risk of acquiring the infection, registering 2 million new cases annually. In spite of this, these data are not real since in only 33 countries the disease is declared. At the present time, it is considered an emerging disease, fundamentally due to climate change, migrations, resistance to insecticides by its vectors and the difficulty of its elimination from natural reservoirs. The diagnosis is complicated both at the clinical and laboratory level, which is why effective markers of species and *leishmania* strain are needed to avoid recognized cross-reactions between species and genera of the same family. Superoxide Dismutases (SODs) are the most interesting group responsible for eliminating the Superoxide Ion (O_2^-) from the middle by its dismutation in Hydrogen Peroxide (H_2O_2) and molecular oxygen. All the protozoan parasites studied have shown iron-bound SOD activity, which is considered a virulence factor since it allows the parasites to survive the offensive cellular oxidant. The present work aims to show the prevalence of *Leishmania* in pediatric populations in the State of Querétaro by using these metalloenzymes that have been shown to be highly effective in the serological agnoses of different genera and species of the trypanosomatid family in the Mexican Republic and particularly in the State of Querétaro, there is a shortage of epidemiological data on species distribution.

Keywords: Free radicals; *Leishmania infantum chagasi*; *Leishmania mexicana*; *Lutzomyia sp*; Queretaro's State; Superoxidodismutase

Introduction

Leishmaniasis is one of the major poverty-related parasitic diseases, representing an increasing burden on affected populations. More than 20 species are pathogenic for humans classified into three main groups: Visceral Leishmaniasis (VL), Cutaneous

Leishmaniasis (CL) and Mucocutaneous Leishmaniasis (MCL), with different target cells harboring the parasite mainly from the mononuclear phagocytic system. WHO lists them as one of the 17 most important neglected diseases included in their TDR program, which are considered as priority research [1,2].

It is a cosmopolitan parasite, presenting wide geographic distributions except in Antarctica. The disease has been reported in about 98 countries [2]. An estimated 12 million people are infected worldwide, with 350 million living in areas at risk of contracting

the disease with an annual incidence of 2 million people mostly caused by cutaneous leishmaniasis. Unfortunately, most of the cases are not declared, only 33 countries are obliged to do so. Due to the economic, social and psychological damages caused, it is considered a disease with great impact on the global public health being one of the most important neglected diseases. Recently it has been considered an emerging disease [3,4], but due to the climate change observed in recent years and mainly to the migratory problem as well as its opportunistic relationship with AIDS patients [5,6].

This metaxenic disease, transmitted by different diptera of the Psychodidae family distributed fundamentally in two genera, *Phlebotomus* and *Lutzomyia* belonging to the Old and New World [3]. In the Mexican Republic, the problem of leishmaniasis is not really recognized since in only 7 States its endemicity is generally recognized, belonging to tropical areas (Campeche, Chiapas, Yucatan, Quintana Roo, Oaxaca, Tabasco and Veracruz [7]. In the rest of the states, reliable data are lacking, as is the study in question. In Mexico, only 3 species of leishmania, *L. braziliensis*, *L. mexicana* and *L. infantum chagasi* [2] have been recorded to date. It is for this reason that the study of leishmaniasis in pediatric populations in states far from tropical areas could clarify their true epidemiological dimension.

Material and methods.

Place of Study

Two communities from the rural area of the State of Querétaro, El Gallo, municipality of Colón and San Vicente Ferrer, municipality of El Marqués, within the State of Querétaro, were studied. The ages of participants in the study ranged from 6 to 17 years, with a sample size of 185 participants. The community of El Gallo has 1,525 inhabitants, 10% are peasants and the rest work in the nearby communities and municipal head. It only has an elementary school, a telesecondary and a kindergarten. The Health Center is attended by an undergraduate medical student, drainage is poor and potable water is scarce, the streets are stone and generally dirty. The town of San Vicente Ferrer is located in the Municipality of El Marqués 40 minutes from the city of Querétaro. The nearest town is Chichimequillas. The indigenous language is the Otomí and the activities they carry out are the production of leather or leather handicrafts, shoes, jackets, belts, shoes, etc. It has 1,839 inhabitants and is at 1,950 meters of altitude.

The socio-sanitary characteristics are similar to those found in the community of El Gallo. Epidemiological surveys were applied to parents of each school with authorization signature. This protocol was approved by the Bioethics and Research Committee of the University of Granada. The sample was obtained by puncture of the ulnar vein using the vacuum technique. The serum was separated in 500 µL aliquots into Eppendorf tubes by freezing at -30 ° C until processing. The excreted superoxydodismutase metalloenzyme (FeSODE) was used as antigen, applying ELISA

and Western Blot as working tests at a 1:80 dilutions. Obtaining FeSODE. Parasitic cultures for the study and characterization of excreted and homogenated FeSOD, promastigote forms of *Leishmania mexicana* (MHOM / BZ / 82 / Bel21), isolated from an infected patient from the State of Yucatán, were grown in MTL medium (Medium Trypanosomes Liquid) (Gibco®), supplemented with 10% (V / V) fetal bovine serum (SBF, PAAA®), heating to inactivate at 56 ° C for 30 minutes and *Leishmania infantum chagasi* (MCAN / ES / 2001 / UCM -10) isolated from dog of the province of Madrid. Spain cultivated in the same environment. Growth density (1x10⁷ cells / ml) was estimated by counting on a Neubauer® hemocytometer and 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. Extraction and purification of excreted FeSOD. The pellet of cells already obtained was resuspended with 25 ml of MTL medium without SBF and incubated for 24 hours at 28 ° C. It is then centrifuged at 1500 g 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 9,000 g for 20 minutes. The second supernatant is precipitated a second time with ammonium sulfate to its total concentration of 85%. Dissolved the salt is allowed to stand in cold for 20 minutes.

Again centrifuge at speed and time prior to 4 ° C. The obtained pellet is resuspended in 2.5 ml of distilled water and desalinated to reach a Volume of 3.5 ml via a Sephadex G-25 (GE Healthcare Life Sciences®, PD 10 column) chromatographic column, equilibrated in advance with 25 ml of distilled water (Fraction P85 or Fe-SODEnp). 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 the Mexican *Leishmania* species was purified 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. Proteins were quantified by the Bradford technique (Sigma Immunochemicals, St. Louis) using bovine albumin serum as standard. The obtained FeSODE is used as antigen and is applied ELISA and Western blot tests for anti-leishmania antibodies [8].

ELISA Test

With the FeSODE 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 (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 blocked with blocking buffer (Twen 20®, 0.2% BSA in PBS) and incubated for 2 hours at room temperature and under agitation to prevent them from occurring Non-specific connections between plaque and serum. Wash again 3 times, the plate is incubated for 45 minutes with 100 µl of serum diluted 1: 200 of the individuals to be studied. At the end of the time the plate is washed 3 times and 100 µl of the immunoconjugate (antiglobulin, anti-human, in Peroxidase-Sigma-Aldrich®) is added at a 1: 1000 dilution by incubating for 30 minutes at 37 ° C. Finally, the developing solution is applied as the substrate ortho-phenylenediamine dihydrochloride (OPD-Sigma-Aldrich®) in citrate-phosphate buffer. Incubate in the dark for 20 minutes and apply 50 µl of reaction stop solution (3 N HCl) 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).

Western blot test (Immunoblot)

It is an excellent molecular tool used due to the facility of specifically detecting an immune response against certain proteins [9,10]. For Western blot titration the fraction was concentrated to 1.5 µg of *L. mexicana* and *L. infantum* protein and applied to an IEF 3-9. The different proteins contained in the gel were then subjected to a membrane transfer process of nitrocellulose for 30 minutes, using the Phast System technique, to proceed with their transfer. Finished the process after 30 minutes is stained with red ponceau visualizing the proteins in the surface of the membrane, the residues of dye were eliminated by washing in water. The membrane is then blocked by incubation with Twen 20®, 0.2% buffer and 0.4% gelatin in PBS. Wash 3 times with 0.1% Twen 20® in PBS and incubate for 2 hours at room temperature with human serum at a 1: 100 dilutions. Before washing, the membrane was incubated for two hours at room temperature with a second antibody called, anti-human, Immunoglobulin G (Fc-specific), peroxidase conjugate (Sigma-immunochemical, 1: 1000 dilution). After washing, the substrate diaminobenzidine (0.5 mg / mL) in 0.1 M tris / HCl buffer, pH 7.4, containing 1/5000 H₂O₂ (10 V / V) was added and the reaction was stopped by washing several times with distilled water.

Results

The results observed in the present study show for the first

time the presence of leishmanial infection for two species, namely *L. mexicana* and *L. infantum chagasi* with variable percentages in the two communities studied (Figures 1-4). Prevalence by sex varies depending on the community and the species involved (Figures 5,7,9 and 11). The prevalence of infection by age groups is shown in (Figures 6, 8, 10,12).

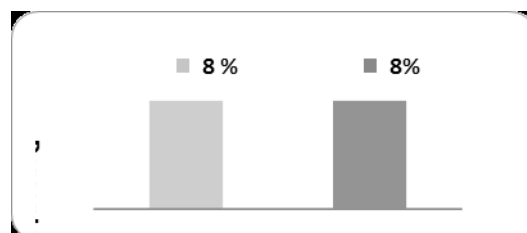


Figure 1: Pediatric prevalence to *Leishmania mexicana* in the El Gallo community Querétaro's State.

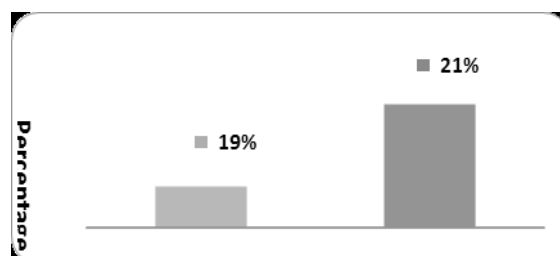


Figure 2: Pediatric prevalence to *Leishmania infantum chagasi* in the El Gallo community. Querétaro's State.

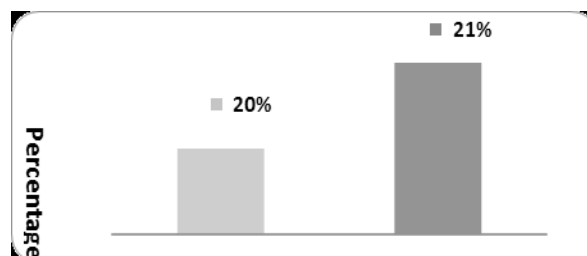


Figure 3: Pediatric prevalence to *Leishmania mexicana* in the San Vicente Ferrer community Querétaro's State.

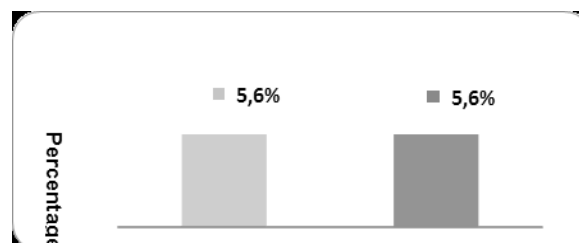


Figure 4: Pediatric prevalence to *Leishmania infantum chagasi* in the San Vicente Ferrer community. Querétaro's State.

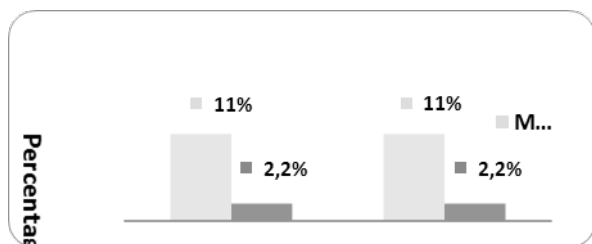


Figure 5: Pediatric prevalence to *L. mexicana* in the El Gallo community. Querétaro's State. (Forsex).

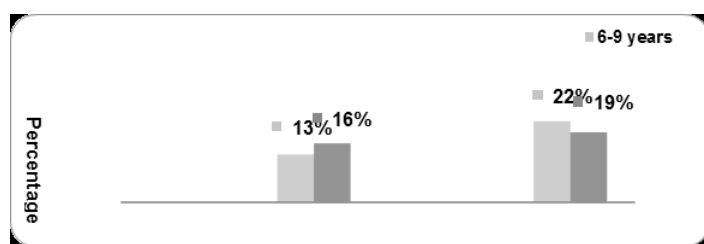


Figure 10: Pediatric prevalence to *L. mexicana* in the San Vicente Ferrer community. Querétaro's State. (for etarian groups).

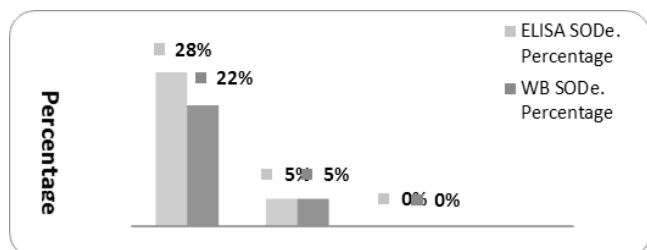


Figure 6: Pediatric prevalence to *L. mexicana* in the El Gallo community. Querétaro's State. (for etarian groups).

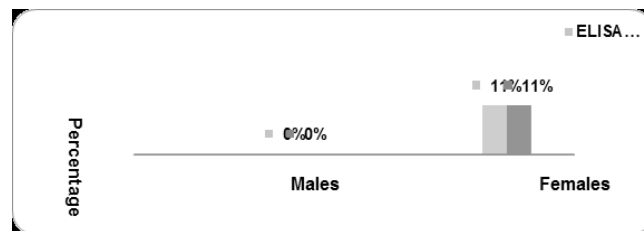


Figure 11: Prevalence pediatric to *L. infantum chagasi* in the San Vicente Ferrer community. Querétaro's State (For sex).

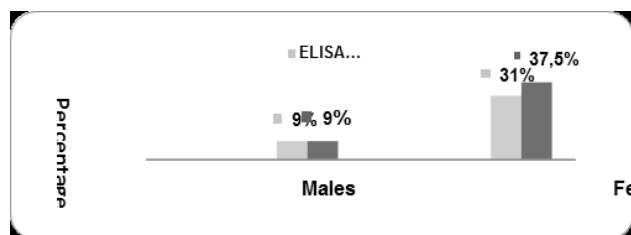


Figure 7: Pediatric prevalence to *L. infantum chagasi* in the El Gallo community. Querétaro's State. (for sex).

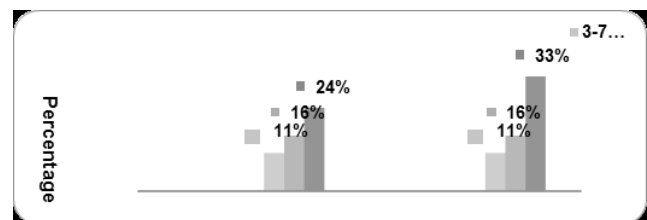


Figure 8: Pediatric prevalence to *L. infantum chagasi* in the El Gallo community. Querétaro's State. (for etarian groups).

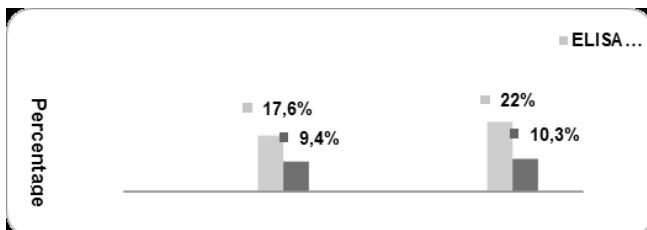


Figure 9: Pediatric prevalence to *L. mexicana* in the San Vicente Ferrer community. Querétaro's State. (for sex).

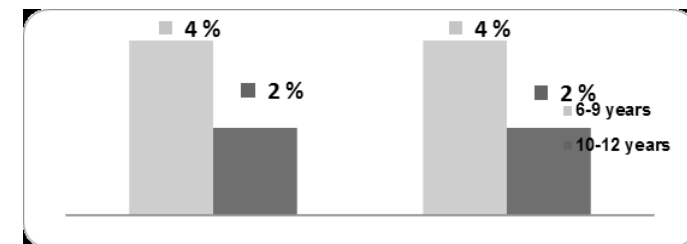


Figure 12: Pediatric prevalence to *L. infantum chagasi* in the San Vicente Ferrer community. Querétaro's State. (For etarian groups).

Discussion

The presence of *L. mexicana* and *L. infantum chagasi* in the two communities studied within their pediatric populations makes relevant the importance of these studies to know the epidemiological reality of this very important nosology in the Mexican Republic [11].

As can be observed in our study, the global data reflect infection rates ranging from 8% to 21% for *L. mexicana* in the communities of El Gallo and San Vicente Ferrer respectively. In the case of *L. infantum chagasi*, the observed rates ranged from 21% to 5.6% in both communities. In relation to the distribution of infection by sex in the community of Gallo and San Vicente Ferrer, the percentages observed were 11% in men and 2% in women for *L. mexicana*. The distribution was very different for *L. infantum chagasi* with Percentages ranging from 9% in men to 37.5% in women. If we relate the prevalence of infection to the different age groups studied, we found a higher percentage for *L. mexicana* in the community of El Gallo in the groups of 3 to 7 years, whereas

in the community of San Vicente Ferrer, groups Most affected in terms of infection levels with respect to the immunological test assayed. For *L. infantum chagasi* its distribution with respect to the age groups studied show different levels of infection being the ages between 13 and 17 years the most affected in the community of El Gallo not so in the community of San Vicente Ferrer, where the Ages between 6 and 9 years had the highest levels of infection. These data obtained using the techniques of ELISA and Western blot show prevalences of leishmaniasis in the population studied above the mean values found by other authors. The species that presented in coinfection presented variable prevalences depending on the test tested and the species of leishmania, in the community of El Gallo the highest percentage Was for *L. infantum chagasi*, superior to that presented for *L. mexicana*, in the case of the community of San Vicente Ferrer, the data are the reverse, with *L. mexicana* having the highest percentage of infection. The use of this new antigen that discriminates different species of leishmania has allowed us to know the presence of these two species in both communities being coinfection one of the data observed in the present study, there being no cross reactions when the antigenic target used is The excreted iron superoxydodismutase (FeSODe) metalloenzyme. [12]. The data presented in this study show the epidemiological importance of leishmaniasis in rural communities in the State of Querétaro, which is endemic for Chagas' disease. These results are the first obtained for this group of population within the State in which the endemy for this parasite until the moment was unknown.

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