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Purification of a Fe-SOD excreted by *Leishmania braziliensis* for specific antibodies detection in Mexican human sera: Cutting-edge the knowledge

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ABSTRACT

Clinical diagnosis of leishmaniasis is highly complex, presenting a wide range of clinical manifestations, sometimes non-specific, and thus the epidemiological study and diagnostic need specific molecular markers for each *Leishmania* species. *Leishmania* spp. posses different Fe-SOD isoforms, one of which is excreted into the external milieu and, presenting immunogenic characteristics, is a very reliable molecular marker. Superoxide dismutases (SODs) are antioxidant metal-enzymes responsible for the dismutation of superoxide ion into hydrogen peroxide and molecular oxygen, and it is considered an important virulence factor. In this manuscript we have purified the iron(Fe)-SOD excreted by *Leishmania braziliensis* using ion-exchange and molecular-sieve chromatography and we have studied it as an antigen in serodiagnostic analyses in ELISA and Western blot techniques, testing 213 human sera from Mexico. Indeed, *L. braziliensis* Fe-SODe has been purified 123.26 times with a specific activity of about 893.66 U/mg of protein. Applying the purified enzymes in serological tests we found 17.84% sera positive. We have demonstrated that the purified enzyme is more sensitive than the non-purified ones and we also demonstrated, for the first time, the presence of antibodies against *L. braziliensis*, not the main species in the country, in human population from Hidalgo and Nuevo Leon States.

1. Introduction

According to WHO's official reports, leishmaniasis is endemic in 98 countries or territories, 12 million people are infected, 350 million are at risk, and 2 million new cases are reported every year, thus the infection represents a major public-health problem (WHO, 2015; WHO, 2010). Leishmaniasis is the "third world" typical disease, characterized by rural transmission, localized in remote areas in the urban hoods, where socio-economical and sanitarian conditions are poor and the disease is not diagnosed due

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to lack of medical tools (WHO, 2015). The epidemiology of leishmaniasis depends on the species, the ecological characteristics of the transmission areas, the level of population exposition to the parasite, and, particularly important, the human behaviour (Parham et al., 2015). Depending on the *Leishmania* species infecting the vertebrate host it can present three different clinical manifestations: cutaneous, mucocutaneous or visceral; all of them starting with the sandfly, the invertebrate vector, bite in where the metacyclic promastigotes are injected (Cáceres and Montoya, 2002). Clinical diagnosis of this disease is very complicated; they present a wide range of clinical manifestations, sometimes not-specific, such as *Leishmania peruviana* and *Leishmania braziliensis*; both start with a small cutaneous ulcers, but meanwhile for the first species it is a self-healing sore (WHO, 2010), in case of *L. braziliensis* infection, if not treated, it will develop to mucocutaneous leishmaniasis with a consequently very painful and facial disfiguration of the patient (de Vries et al., 2015; WHO, 2010). Indeed depending on each species a different kind of treatment is needed.

In 1912 Seidelin reported the first clinical case in Mexico of cutaneous leishmaniasis, called "gum picker's ulcer"(Seidelin, 1912) Since then, at least three clinical forms have been reported in this country: localized cutaneous, diffuse cutaneous and visceral leishmaniasis (Velasco-Castrejón, 1987). Previously, only three main endemic areas were known in Mexico: in the south-east area from the south of the State of Veracruz to the States of Quitana Roo and Chiapas; in the north, in the States of Tamaulipas, Nuevo Leon, Coahuilla, and San Luis de Potosi; and the third area, in the States of Puebla, Morelos, Oaxaca, and Michoacan, where visceral leishmaniasis was endemic (Velasco-Castrejón, 1987). Currently, leishmaniasis has been reported in 22 states, including Hidalgo and Nuevo Leon (Velasco-Castrejón et al., 2009; Monroy-Ostria and Sanchez-Tejeda, 2002). The main species of human leishmaniasis throughout Mexico is *Leishmania mexicana* but different species have also been found in the country, including *L. braziliensis* (Monroy-Ostria and Sanchez-Tejeda, 2002; Sanchez-Tejeda et al., 2001) and *Leishmania infantum* (syn. *chagasi*) (WHO, 2010). There is no study about prevalence of *L. braziliensis* infection in human in Mexico and it is considered as minor species, nonetheless 12 PCR positive patients have been reported in 2001 from the State of Nayarit in the north-west of the country (Sanchez-Tejeda et al., 2001). In the south of Mexico, Canto-Lara et al. (1999) identified, using specific polyclonal antibodies, isoenzyme electrophoresis and molecular techniques the presence of a *L. braziliensis* infection in one human from the State of Campeche. Furthermore, dogs and cats have been found to present antibodies against this species of *Leishmania* (Longoni et al., 2012; Longoni et al., 2011). The recognition of the proper species of *Leishmania* is crucially important; on one hand, some cutaneous leishmaniasis are self-healing and the treatment is not necessary, thus the correct pathogen identification makes possible to reduce costs and avoid chemotherapy with serious side effects, while on the other hand timely treatment of mucocutaneous leishmaniasis can save a patient from a very painful and disfiguring course of the disease. Unfortunately, differential diagnosis of leishmaniasis is still a major challenge, as current commercial kits are unable to distinguish among different *Leishmania* species, which is an important goal in areas endemic for more than one species. Different molecules have been proposed as diagnostic markers, but none of them proved successful. An iron superoxide dismutase excreted (Fe-SODe) by trypanosomatids has been used in ELISA technique with good results in *Phytomonas* spp. (Marín et al., 2006) and *Trypanosoma cruzi* (Mateo et al., 2010).

Fe-SOD excreted by different *Leishmania* species has been used to diagnose cutaneous, mucocutaneous, and visceral leishmaniasis in humans, dogs, and cats (Longoni et al., 2012; Longoni et al., 2011; Marín et al., 2009; Marín et al., 2007), showing high sensitivity and specificity while lacking cross reactions with other trypanosomatids like *T. cruzi* (Marín et al., 2009).

In the present work, we have purified the Fe-SOD excreted by *L. braziliensis* (mucocutaneous leishmaniasis) and used it for human leishmaniasis diagnostic. We tested 213 human sera from Mexican States of Hidalgo and Nuevo Leon. The main aim of this work was to assess the sensitivity of this purified antigen in comparison respect the same protein not purified eliminating all interference due by antigen sample preparation impurities. In addition, we report the presence of *L. braziliensis* Fe-SODe antibodies in patients from areas where *L. braziliensis* infections in humans have never been found before.

2. Material & methods

2.1. Parasite culture

Promastigotes of *L. braiziliensis* (MHOM/BR/75/M2904) were grown in tissue-culture flasks and an axenic medium trypanosomes liquid (MTL) medium (Hank's Balanced Salt Solution—HBSS (Gibco®), CO_3HNa, Lacto-albumin, yeast extract, bovine haemoglobin and antibiotics), supplemented with 10% heat-inactivated foetal bovine serum (FBS) at 26 °C until reaching a population of approximately 1×10^7 parasites/mL. Cells were harvested at the logarithmic growth phase by centrifugation (1500 $\times g$ for 10 min at room temperature). The pellet of cells (0.5–0.6 g wet weight/mL) was suspended in 25 mL of MTL medium that had not been enriched with foetal bovine serum (FBS) and cultured at 26 °C for 24 h.

2.2. Antigen preparation: extraction and purification of Fe-SODe

After 24 h, the promastigote culture was centrifuged (1500 $\times g$ for 10 min) and the supernatant was filtered (Minisart®,Φ 20 μm). The filtered supernatant was subjected to ice-cold ammonium sulphate precipitation at 35% salt concentration. Following centrifugation, the resultant supernatant was then treated with 85% ice-cold ammonium sulphate and the second precipitate collected. The resulting precipitate was finally dissolved in 2.5 mL of distilled water and desalted by chromatography in a Sephadex G-25 column (GE Healthcare Life Sciences®, PD 10 column), previously equilibrated with 25 mL of distilled water, bringing it to a final volume of 3.5 mL (Fraction P85e or SODe-np-Lb). Degradation due to the activity of protease present in the sample was minimized by the adding 25 μL of protease inhibitor to the fraction P85e (CompleteMini, Roche®). Fraction P85e was introduced to a