

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. possess 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-SOD 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.

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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 Quintana 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. braziliensis* (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®), CO₂HNa, 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 ×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 ×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

QAE-Sephadex A-50 column (Sigma Immunochemicals®), 30 × 2 cm Φ, equilibrated with buffer 1 (20 mM potassium phosphate, pH 7.4, containing 1 mM EDTA). The adsorbed proteins were eluted with a linear gradient of KCl (0–0.6 M). Fractions with a total volume of 2.5 mL were recollected. Fractions exhibiting specific SOD activity were pooled (peak Q1e) and concentrated by ultrafiltration in Microcon® filter tubes (Amicon®) at 11,200 ×g for 30 min. The peak Q1e was introduced to a Sephadex G-100 (Sigma Immunochemicals®) molecular sieve chromatography column (75 × 1.6 cm Φ) equilibrated with Buffer 1 and eluted with 200 mL of the same buffer. The eluted fractions (4.5 mL each fraction) which showed SOD activity were again collected (peak SODe-Lb), concentrated (to 2 mg/mL) and used for the assays as described below.

2.3. Protein determination

The protein content of the fractions P85e and the peaks Q1 and SODe-Lb, and all the fractions resulting from the elution of the two columns were quantified using the Sigma Bradford test, which uses BSA as a standard (no traceability was certified for the BSA standard) (Bradford, 1976).

2.4. Spectrophotometric enzyme assays

SOD activity was measured on the fraction P85e, the peaks Q1 and SODe-Lb and all the fractions eluted by spectrophotometric measurement of NBT-UV light oxidation as previously described by Beyer and Fridovich (1987).

2.5. Determination of the molecular weight

Apparent molecular weight was calculated using the equation ($y = -0.952 \times + 118.51$) obtained from the linear trend line generated from the graph of chromatography standards (Bovine Serum Albumine, 67 kDa; Albumin from chicken egg white, 45 kDa; carbonic anhydrase, 30 kDa; and cytochrome C, 12.4 kDa; all from Sigma Immunochemicals, St. Louis) (Microsoft Excel®, 2007). Molecular weight was confirmed by testing the SODe-Lb fractions with native gel electrophoresis in PhastGel Homogeneous 12.5% as described in the Phast System manual (GE Healthcare Life Sciences®). Molecular weights of standard proteins were: phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa), all from LMW (GE Healthcare Life Sciences®). The gels were stained for protein with high-sensitivity 0.5% silver nitrate staining according to the Phast System manual (GE Healthcare Life Sciences®).

2.6. Isoelectric point determination

Isoelectro focusing (IEF) in polyacrylamide Phast Gel 3–9 (GE Healthcare Life Sciences®) enabled us to determine the isoelectric points of the purified enzymes, using trypsinogen (9.3), lentil-lectin acidic band (8.8), horse-heart myoglobin (6.8), soybean trypsin inhibitor (4.6) and amyloglucosidase from *Aspergillus niger* (3.6) as markers (Longoni et al., 2013). The gels were stained with the silver-nitrate solution for proteins and using the Beyer and Fridovich's (1987) method, in which enzyme activity appears as a chromatic band on a blue background.

2.7. Serological test (ELISA)

The antigenic fractions: SODe-np-Lb (corresponding to fraction P85), and the purified SODe-Lb, at a total concentration of 1.5 µg and, 0.5 µg and 0.05 µg, respectively, were used to sensitize polyethylene microtitre plates (Nunc, Denmark) in carbonate buffer (pH 8.2) for 2 h at 37 °C. Antigens not fixed in the plate were eliminated and washed 3 times with phosphate buffered saline (PBS), Tween 20® 0.05% (wash buffer). Free absorption sites where the antigen did not come together were blocked for 2 h (37 °C) using a blocking solution (PBS-Tween 20® at 0.2%, bovine serum albumin at 1%). After washing as before, the plate was incubated for 45 min in the presence of human serum at a 1:200 dilution. After a further washing it was again incubated for 30 min (37 °C) with immunoconjugate (anti-IgG with anti-human peroxidase, Sigma Immunochemicals®), at a dilution of 1:1000. The enzymatic, chromogenic reaction was performed for 20 min in darkness on an OPD substrate (Phenylenediamine dihydrochloride, Sigma Immunochemicals®) using 10 µL of 30% H₂O₂ for each 25 mL. The reaction was blocked by adding 50 µL of 3 N HCl. Absorbance was read at 492 nm using a microplate reader (Sunrise™, TECAN). All samples were analysed in triplicate in microtitre plates. The mean and standard deviation (SD) of the optical density of negative-control sera (4 healthy humans) were utilized to calculate the cut-off value (mean + 3 × SD).

2.8. Western blot analysis

The SODe-Lb antigen fractions (total concentrations of 0.5 µg of protein) were merged into IEF 3–9 gels and subsequently transferred to nitrocellulose for 30 min, as described in the Phast System manual. The membrane was blocked for 2 h at room temperature using 0.4% gelatine and 0.2% Tween 20 in PBS, followed by three washes in 0.1% Tween 20 in PBS (PBS-T) and incubated for 2 h at room temperature with human sera at a dilution of 1:200. After a second washing, the membrane was incubated for two more hours at room temperature with the second antibody, anti-human immunoglobulin G conjugate with peroxidase

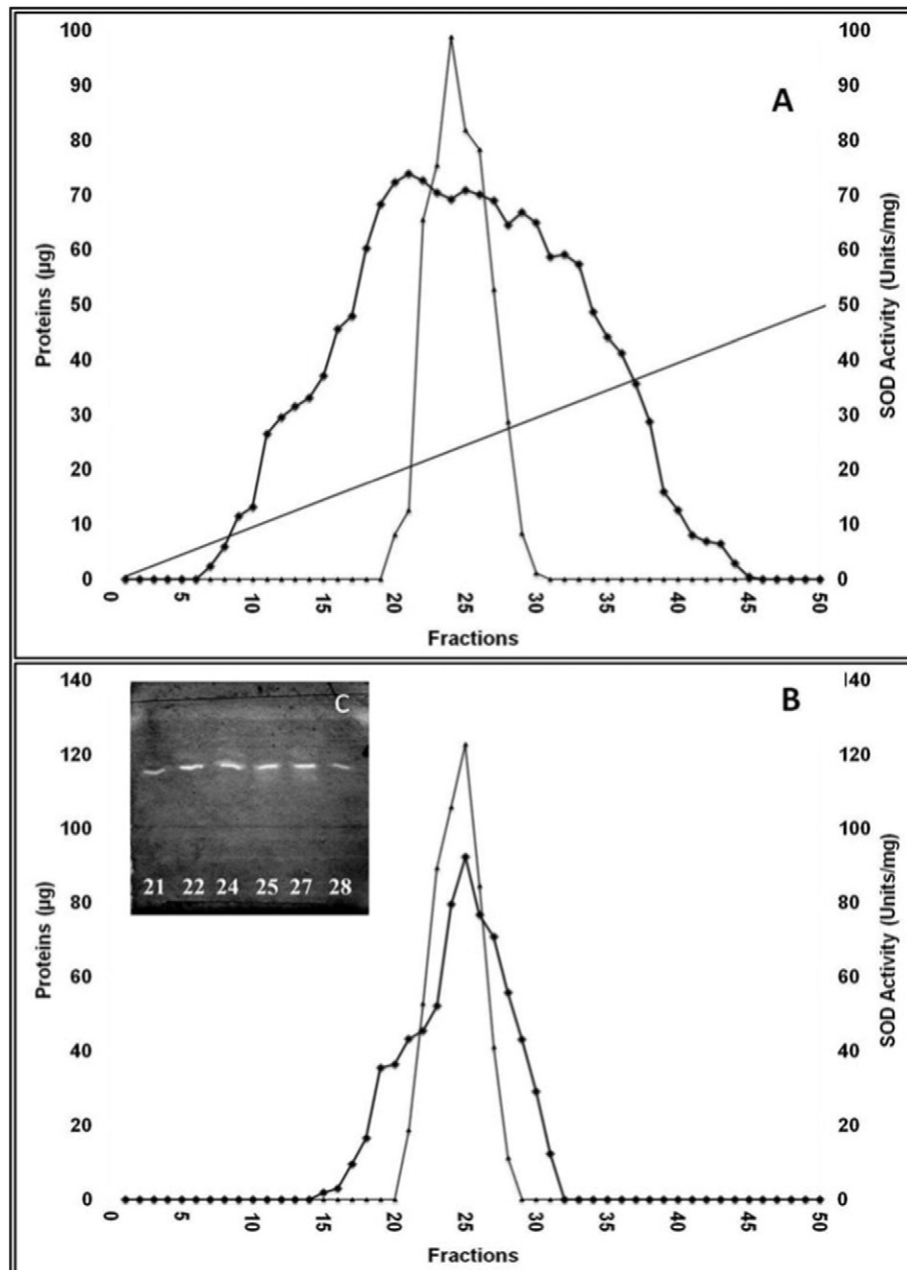


Fig. 1. Purification profiles of SOD excreted from *Leishmania braziliensis*. (A) QAE A-50 ion exchange chromatography. (—■—): protein concentration; (—▲—): SOD activity; (—): gradient 0–0.6 M KCl. (B) Sephadex G-100 sieve chromatography. (—■—): protein concentration; (—▲—): SOD activity. (C) Isoelectric focusing gel electrophoresis pl 3–9 of the fractions eluted by Sephadex G-100, the SOD activity was stained following the Beyer and Fridovich's (1987) protocol.

(Sigma Immunochemicals®; dilution 1:1000). The latter was washed with diaminobenzidine substrate (0.5 mg/mL in a buffer of Tris/HCL 0.1 M, pH 7.4 containing 1/5000 H_2O_2 [10 v/v]) and then added to the reaction, which was stopped with a series of washes with distilled water.

2.9. Human sera

The sera from a total of 213 patients were obtained between 2011 and 2012 from Hidalgo (81) and Nuevo Leon (132) States, Mexico. All 213 patients were not infected with any *Leishmania* sp. at the time of the blood taking. Complete history is not

Table 1*Leishmania braziliensis* excreted superoxide dismutase purification summary.

Fraction	Total protein (mg)	Total activity ^a (Units) ± SD	Specific activity ^a (U/mg ± SD)	Purification	Yield (%)
P85	34.71	663.31 ± 9.98 ^a	7.25 ± 4.41 ^a	1	100
Peak Q1	1.106	631.59 ± 5.05 ^a	322.49 ± 7.89 ^a	44.48	95
SODe-Lb	0.442	395.00 ± 0.00	893.66 ± 0.00	123.26	60

“SD” is the standard deviation of the mean of four determinations.

^a SOD activity determined by the technique of [Beyer and Fridovich, \(1987\)](#).

available for all the sera. The negative control sera (4, healthy individuals who have never travelled to Latin America) were obtained from human sera put down by the Blood Bank in Granada (Spain) and were not reactive to in the serological tests.

Informed consent was obtained individually from all participants before the collection of blood samples.

2.10. Ethics

This study was approved by the Ethics Committee (CEIH) of the University of Granada (Spain) in accordance with the Helsinki Declaration of 1975, revised in 1983.

3. Results

The supernatant recovered from the 24 h culture of 1×10^7 cells/mL in “FBS free MTL medium” was centrifuged, filtered and subjected to two ice-cold ammonium sulphate additions, to obtain a precipitate (SODe-np-Lb).

L. braziliensis SODe-np-Lb (total proteins amount 34.71 mg) was applied to a QAE-Sephadex A-50 ion exchange chromatography column. Protein concentration and Fe-SOD activity were quantified in all the fractions eluted using the Bradford and the Beyer and Fridovich methods, respectively, revealing only one active peak marked as Q1e (fractions 20–30) as shown in [Fig. 1-A](#). IEF 3–9 polyacrylamide gel electrophoresis of the fractions eluted from QAE-Sephadex A-50 columns was also performed to visualize the SOD activity (data not shown).

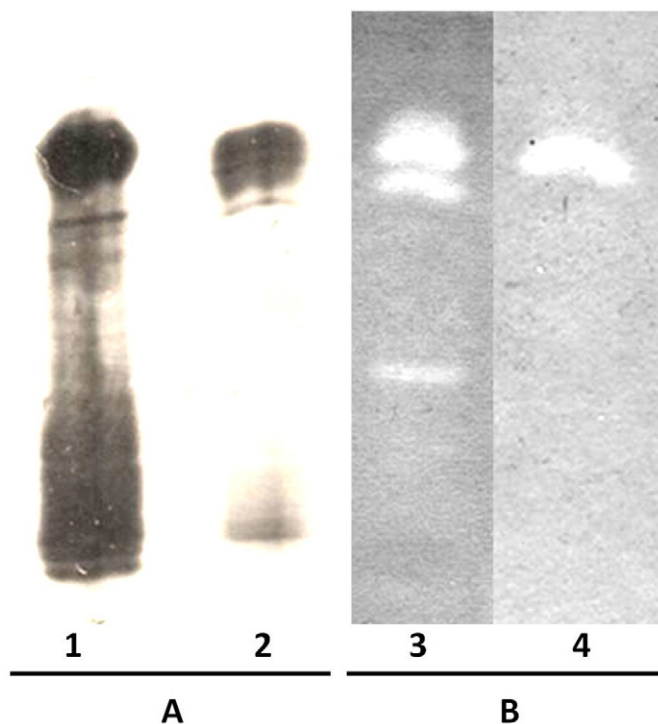


Fig. 2. Electrophoresis profile of fraction p85 (Lines 1 and 3) and purified protein SODe-Lb (Lines 2 and 4). A: native gel electrophoresis in PhasGel homogeneous 12.5% stained following the manufacturer silver nitrate protocol. B: Isoelectro focusing (IEF) in polyacrylamide Phast Gel 3–9 stained following the Beyer and Fridovich SOD activity protocol ([Beyer and Fridovich, 1987](#)).

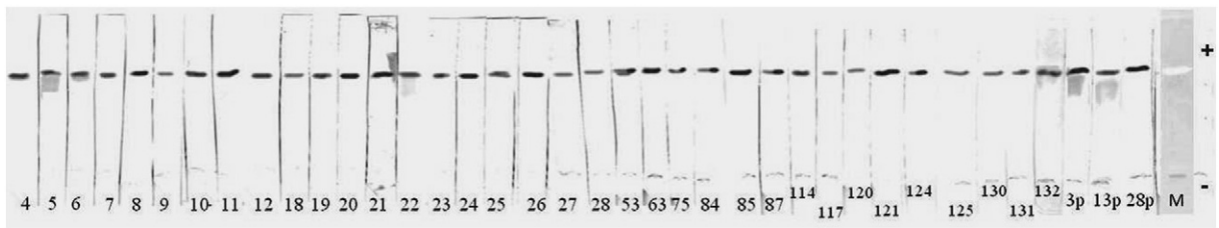


Fig. 3. Immunoblot of the ELISA positive serum samples at a dilution of 1/200 against SODe-Lb. Lines 4–132 correspond to sera from Monterrey (Nuevo Leon State) and Lines 3p, 13p and 28p correspond to sera from Pachuca (Hidalgo State). Line M: SODe activity in isoelectric focusing and staining following the technique of Beyer and Fridovich (1987).

The Q1e peak encompassing fractions 20 to 30 was concentrated to a final volume of 1 mL and a concentration of 1.106 mg protein and then applied to a Sephadex G-100 molecular sieve chromatography column. Protein concentration and SOD activity were quantified in all fractions eluted as shown in Fig. 1-B. We obtained only one protein peak with SOD activity (fractions 21–27). IEF 3–9 polyacrylamide gel electrophoresis of the fractions eluted from Sephadex G-100 column was also performed to visualize the SOD activity (Fig. 1-C).

The SODe-Lb peak obtained by molecular-sieve chromatography (fractions 21–27) was desalted and concentrated. Table 1 summarizes the purification, indicating that SODe-Lb was purified approximately 123 times to a specific activity of about 894 U/mg of protein (Table 1). Differences between purified and not purified proteins are shown in Fig. 2.

The SODe-Lb molecular weight (MW) was determined by native homogeneous 12.5% electrophoresis analysis, revealing only one band of about 25 kDa. In the same way, the SODe-Lb isoelectric point (pI) was determined using an IEF 3–9 polyacrylamide gel, showing a single activity band with a pI of about 3.75 (data not shown).

With the aim of demonstrating that the purified Fe-SOD excreted by *L. braziliensis* may be a suitable molecular tool for cutaneous human leishmaniasis diagnosis, 213 human sera from Mexico (81 from Pachuca city, Hidalgo State and 132 from Monterrey City, Nuevo Leon State) were assayed by ELISA and Western blot techniques at a dilution of 1/200, using as antigens the non-purified Fe-SODe (SODe-np-Lb) and purified Fe-SODe (SODe-Lb) (Fig. 3).

Using as antigen the SODe-np-Lb by ELISA technique, a total of 61 sera were positive (29.57%), 50 from Monterrey and 11 from Pachuca. When the SODe-Lb was used as an antigen, only 38 sera were positive (17.84%), 35 from Monterrey and 3 from Pachuca (Table 2).

Table 2

Relationship of positive sera from Monterrey (Nuevo Leon State) and Pachuca (Hidalgo State) to *L. braziliensis* by ELISA (ELISA) and Western blot (WB). Antigen fraction: iron superoxide dismutase excreted not purified (Np), and iron superoxide dismutase excreted purified (P).

Sera	ELISA			WB	Sera	ELISA			WB	Sera	ELISA			WB	Sera	ELISA			WB
	Np	P	0.05 ^a	P		Np	P	0.05 ^a	P		Np	P	0.05 ^a	P		Np	P	0.05 ^a	P
	1.5 ^a	0.5 ^a	0.05 ^a	0.5 ^a		1.5 ^a	0.5 ^a	0.05 ^a	0.5 ^a		1.5 ^a	0.5 ^a	0.05 ^a	0.5 ^a		1.5 ^a	0.5 ^a	0.05 ^a	0.5 ^a
<i>Monterrey (Nuevo Leon State)</i>																			
2	+	–	–	–	24	–	+	+	+	57	+	–	–	–	82	+	–	–	–
3	+	–	–	–	25	–	+	+	+	60	+	–	–	–	83	+	–	–	–
4	+	+	+	+	26	+	+	+	+	61	+	–	–	–	84	+	+	+	+
5	+	+	+	+	27	–	+	+	+	62	+	–	–	–	85	–	+	+	+
6	–	+	+	+	28	+	+	+	+	63	+	+	+	+	87	+	+	+	+
7	+	+	+	+	29	+	–	–	–	64	+	–	–	–	89	+	–	–	–
8	–	+	+	+	30	+	–	–	–	65	+	–	–	–	90	+	–	–	–
9	+	+	+	+	32	+	–	–	–	66	+	–	–	–	91	+	–	–	–
10	+	+	+	+	33	+	–	–	–	67	+	–	–	–	114	–	+	+	+
11	+	+	+	+	36	+	–	–	–	70	+	–	–	–	117	–	+	+	+
12	–	+	+	+	38	+	–	–	–	71	+	–	–	–	120	–	+	+	+
18	–	+	+	+	41	+	–	–	–	74	+	–	–	–	121	–	+	+	+
19	+	+	+	+	42	+	–	–	–	75	+	+	+	+	124	–	+	+	+
20	+	+	+	+	45	+	–	–	–	76	+	–	–	–	125	–	+	+	+
21	+	+	+	+	46	+	–	–	–	78	+	–	–	–	130	–	+	+	+
22	–	+	+	+	53	+	+	+	+	80	+	–	–	–	131	–	+	+	+
23	+	+	+	+	54	+	–	–	–	81	+	–	–	–	132	–	+	+	+
<i>Pachuca (Hidalgo State)</i>																			
2	+	–	–	–	15	+	–	–	–	28	–	+	+	+	45	+	–	–	–
3	+	+	+	+	16	+	–	–	–	35	+	–	–	–	64	+	–	–	–
13	+	+	+	+	26	+	–	–	–	41	+	–	–	–	65	+	–	–	–

^a µg/well used of each antigen fraction.

As shown in Table 2, using both final concentrations of SODE-Lb 0.5 µg and 0.05 µg, we observed the same results by ELISA technique. When we used the purified protein (SODE-Lb) as antigen the same results were revealed by testing the sera with the Western blot technique, giving a 100% correspondence between the two techniques (Table 2).

Western blot results (Fig. 3) showed single band for positive sera, confirming a specific reaction. On the other hand ELISA negative sera showed no visible band in Western blot analysis.

4. Discussion

SOD has been identified in a great variety of organisms and it has been subject of multiple studies, as a candidate for a chemotherapeutic target, in particular in those pathogens such as protist parasites which possess an Fe-SOD not present in human and/or animals (Wilson et al., 1994; Meshnick and Eaton, 1981). Fe-SOD excreted by different species of *Leishmania* has been already studied as well. Sera from patients affected by *L. peruviana*, *Leishmania amazonensis* and *L. braziliensis* (with proven identification of the agents) had been tested with the Fe-SOD excreted by these *Leishmania* species and then the results had been compared for specificity evaluation of the antigen (Marín et al., 2009). Recently the Fe-SOD excreted by *L. infantum* has been purified and tested as antigens in the diagnosis of visceral canine leishmaniasis. Purified enzyme proved to be more sensitive than the non-purified one, eliminating all false positive or negative results, using samples from positive, negative and unknown-status dogs (Longoni et al., 2013). The Fe-SOD excreted by *L. amazonensis* and *L. peruviana* has also been purified and used as antigens in human leishmaniasis diagnosis (Longoni et al., 2014), confirming its high specificity eliminating all kind of cross reaction that the not purified antigen was producing (Longoni et al., 2014).

Many studies have been conducted to discover a specific antigen for *Leishmania* with the aim to increase the specificity of the serodiagnostic. Most of them focus on visceral leishmaniasis; only few antigens have been studied for cutaneous leishmaniasis. Currently, only the rk39 antigen for *L. infantum* is accepted and recommended by the WHO (2010). Nowadays there is no serological assay available to detect an infection due by *L. braziliensis*. Okumura et al. (2014) reported a serological identification of *L. braziliensis*, among others species of *Leishmania* at the same time, using a not purified pool of antigens from promastigotes of different *Leishmania* spp.; it has already well demonstrated that the not purified pool of antigens from whole promastigotes are not species specific inducing false results, both positive or negative, indeed it cannot be considered as a reliable antigen (Marín et al., 2009). In the present study, using the purified Fe-SOD excreted by *L. braziliensis* as an antigen, we tested 213 human sera from Mexico in ELISA and Western blot techniques. The serum samples used in this study were from patients who tested negative in parasitological examinations and had no dermal lesions at the time of the blood taking. Thus, these patients were not having an active infection but they could have had an infection in the past, which would be revealed in a positive serological test.

In the present study using non-purified Fe-SODE as the antigen, we found around 10% false outcome compared to the results obtained with the purified protein. Using the purified Fe-SODE as antigen, we found 17.84% positive and complete correspondence between the two techniques, ELISA and Western blot, as observed previously using *L. infantum* Fe-SODE purified (Longoni et al., 2013). As it has been demonstrated by our previous works using positive control sera, the purified protein, excreted by *L. infantum* (Longoni et al., 2013) and by *L. peruviana* and *L. amazonensis* (Longoni et al., 2014), is much more sensitive than the non-purified one. Based on these results we are confident with the results here obtained and the difference between purified and non-purified proteins is explained by the elimination of all impurities present in the non-purified antigen that generate false reaction, positive or negative, as WHO affirms (WHO, 2010). Although the enzyme is reliable, the results were somewhat surprising, thus a further confirmation of those results is given when the sera were separated according to the State from where they were. In Nuevo Leon State 26.51% of the sera were positive to the Fe-SODE-Lb meanwhile only 3.70% of sera from Hidalgo State were positive. If we focus our attention on the geography of these Mexican States we can observe that Nuevo Leon is bordering Texas where some cases of weird non-self healing and very resistant to treatment lesions in patients have been reported (Maloney et al., 2002; McHugh et al., 1996) which brought clinicians to suspect a *L. braziliensis* infection (Petersen, 2009), whereas the State of Hidalgo is far from those areas where *L. braziliensis* has been reported. Mexico-US border is one of the biggest borders worldwide. In those areas where they share vertebrate and invertebrate vectors, where the migration is massive and where the health policies are not the same (Esteve-Gassent et al., 2014) it should not be surprising that there are reported cases of possible *L. braziliensis* infection from US and not from the Mexican areas, despite the wake-up call to Mexican health authorities in 2001 by Sanchez-Tejeda et al. (2001).

Summarizing, in the present work we made an important forward step in *Leishmania* diagnostic. We have confirmed the high sensitivity of the purified Fe-SODE, demonstrating for the first time that it is possible to use 200-fold less amount (0.05 µg/well) compared with the amount used when applying the complete pool of proteins obtained from promastigote culture (Marín et al., 2009) which is still currently used for serodiagnosis (Okumura et al., 2014) and even 13-fold less amount of purified protein compared with the results previously published by our group (Longoni et al., 2013; 2014). Despite the technique used to purify the protein is quite expensive this paper would open the door to massive generation of a recombinant antigen which will decrease considerably the cost of the assay. Moreover the use of the purified protein shows also 100% correspondence between the two techniques used: ELISA and Western blot, being the second more sensitive but also more expensive and usually not affordable in endemic countries. Second but not less important, we have surprisingly described for the first time the presence of *L. braziliensis* antibodies in human populations from the central and northern Mexico that could reveal a possible focus of infection for *L. braziliensis* in that area in future.

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Conflict of interests

None of the authors has conflict of interests.

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References

- Beyer, W.F., Fridovich, I., 1987. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Anal. Biochem.* 161, 559–566.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* 72, 248–254.
- Cáceres, O., Montoya, Y., 2002. Diseño y evaluación de tres oligonucleótidos para la detección de *Leishmania* por PCR. *Rev. Peru. Med. Exp. Salud Pública* 19, 7–11.
- Canto-Lara, S.B., Van Wynsberghe, N.R., Vargas-González, A., Ojeda-Farfán, F.F., Andrade-Narváez, F.J., 1999. Use of monoclonal antibodies for the identification of *Leishmania* spp. isolated from humans and wild rodents in the State of Campeche, Mexico. *Mem. Inst. Oswaldo Cruz* 94, 305–309.
- de Vries, H.J.C., Reedijk, S.H., Schallig, H.D.F.H., 2015. Cutaneous leishmaniasis: recent developments in diagnosis and management. *Am. J. Clin. Dermatol.* 16, 99–109.
- Esteve-Gassent, M.D., Pérez de León, A., Romero-Salas, D., Feria-Arroyo, T.P., Patino, R., Castro-Arellano, I., et al., 2014. Pathogenic landscape of transboundary zoonotic diseases in the Mexico–US border along the Rio Grande. *Front. Public Heal.* 2, 177.
- Longoni, S.S., Marín, C., Sauri-Arceo, C.H., López-Cespedes, A., Rodríguez-Vivas, R.I., Villegas, N., et al., 2011. An iron-superoxide dismutase antigen-based serological screening of dogs indicates their potential role in the transmission of cutaneous leishmaniasis and trypanosomiasis in Yucatan, Mexico. *Vector Borne Zoonotic Dis.* 11, 815–821.
- Longoni, S.S., López-Cespedes, A., Sánchez-Moreno, M., Bolio-Gonzalez, M.E., Sauri-Arceo, C.H., Rodríguez-Vivas, R.I., et al., 2012. Detection of different *Leishmania* spp. and *Trypanosoma cruzi* antibodies in cats from the Yucatan Peninsula (Mexico) using an iron superoxide dismutase excreted as antigen. *Comp. Immunol. Microbiol. Infect. Dis.* 35, 469–476.
- Longoni, S.S., Sánchez-Moreno, M., López-Rivera, J.E., Marín, C., 2013. *Leishmania infantum* secreted iron superoxide dismutase purification and its application to the diagnosis of canine leishmaniasis. *Comp. Immunol. Microbiol. Infect. Dis.* 36, 499–506.
- Longoni, S.S., Marín, C., Sánchez-Moreno, M., 2014. Excreted *Leishmania peruviana* and *Leishmania amazonensis* iron-superoxide dismutase purification: specific antibody detection in Colombian patients with cutaneous leishmaniasis. *Free Radic. Biol. Med.* 69, 26–34.
- Maloney, D.M., Maloney, J.E., Dotson, D., Popov, V.L., Sanchez, R.L., 2002. Cutaneous leishmaniasis: Texas case diagnosed by electron microscopy. *J. Am. Acad. Dermatol.* 47, 614–616.
- Marín, C., Rodríguez-González, I., Sánchez-Moreno, M., 2006. Identification of excreted iron superoxide dismutase for the diagnosis of *Phytomonas*. *Mem. Inst. Oswaldo Cruz* 101, 649–654.
- Marín, C., Longoni, S.S., Mateo, H., de Diego, J.A., Alunda, J.M., Minaya, G., et al., 2007. The use of an excreted superoxide dismutase in an ELISA and Western blotting for the diagnosis of *Leishmania (Leishmania) infantum* naturally infected dogs. *Parasitol. Res.* 101, 801–808.
- Marín, C., Longoni, S.S., Urbano, J., Minaya, G., Mateo, H., de Diego, J.A., et al., 2009. Enzyme-linked immunosorbent assay for superoxide dismutase-excreted antigen in diagnosis of sylvatic and Andean cutaneous leishmaniasis of Peru. *Am. J. Trop. Med. Hyg.* 80, 55–60.
- Mateo, H., Sanchez-Moreno, M., Marín, C., 2010. Enzyme-linked immunosorbent assay with purified *Trypanosoma cruzi* excreted superoxide dismutase. *Clin. Biochem.* 43, 1257–1264.
- McHugh, C.P., Melby, P.C., LaFon, S.G., 1996. Leishmaniasis in Texas: epidemiology and clinical aspects of human cases. *Am. J. Trop. Med. Hyg.* 55, 547–555.
- Meshnick, S.R., Eaton, J.W., 1981. Leishmanial superoxide dismutase: a possible target for chemotherapy. *Biochem. Biophys. Res. Commun.* 102, 970–976.
- Monroy-Ostria, A., Sanchez-Tejeda, G., 2002. Molecular probes and the polymerase chain reaction for detection and typing of *Leishmania* species in Mexico. *Trans. R. Soc. Trop. Med. Hyg.* 96 (1), S101–S104.
- Okumura, Y., Yamauchi, A., Nagano, I., Itoh, M., Hagiwara, K., Takahashi, K., Uezato, H., Maeda, M., Seishima, M., 2014. A case of mucocutaneous leishmaniasis diagnosed by serology. *J. Dermatol.* 41, 739–742.
- Parham, P.E., Waldo, J., Christophides, G.K., Michael, E., 2015. Climate change and vector-borne diseases of humans. *Philos. Trans. R. Soc. B* 370, 20140377. <http://dx.doi.org/10.1098/rstb.2014.0377>.
- Petersen, C.A., 2009. Leishmaniasis, an emerging disease found in companion animals in the United States. *Top. Companion Anim. Med.* 24, 182–188.
- Sanchez-Tejeda, G., Rodríguez, N., Parra, C.I., Hernandez-Montes, O., Barker, D.C., Monroy-Ostria, A., 2001. Cutaneous leishmaniasis caused by members of *Leishmania braziliensis* complex in Nayarit, State of Mexico. *Mem. Inst. Oswaldo Cruz* 96, 15–19.
- Seidelin, H., 1912. Leishmaniasis and babesiasis in Yucatan. *Ann. Trop. Med. Parasitol.* 6, 295–299.
- Velasco-Castrejón, O., 1987. Leishmaniasis in Mexico. *Rev. Latinoam. Microbiol.* 29, 119–126.
- Velasco-Castrejón, O., Rivas Sánchez, B., Mungía Saldaña, A., Hobart, O., 2009. Leishmaniasis cutánea de perros en México. *Enferm. Infecc. Microbiol.* 29, 135–140.
- WHO, 2010. World Health Organization, control of the leishmaniases. World Health Organ. Tech. Rep. Ser. pp. 22–26
- WHO, 2015. World Health Organization, leishmaniasis. Fact Sheet no 375.
- Wilson, M.E., Andersen, K., Britigan, B.E., 1994. Response of *Leishmania chagasi* promastigotes to oxidant stress. *Infect. Immun.* 62, 5133–5141.