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Leishmania infantum isolates exhibit high infectivity and reduced susceptibility to amphotericin B†

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Leishmaniasis is a neglected disease caused by a protozoan parasite of the *Leishmania* species in over 98 countries in five continents. Visceral leishmaniasis is one of the main forms of the disease and is mainly caused by *Leishmania infantum*, whose main vector is the dipteran *Lutzomyia longipalpis*. The presence of the vector in Uruguay was recorded for the first time in 2010 and an autochthonous outbreak of canine visceral leishmaniasis occurred in the northern locality of the country in 2015. We report the isolation in blood-free FBS-supplemented defined media of five isolates responsible for the referred outbreak, and characterize them in terms of their growth as promastigotes, infectivity and replication in human derived monocytes and drug resistance. Results indicate similar promastigote growth among the strains, enhanced infectivity and replication for the five strains isolated from the Uruguayan outbreak when compared with reference strains from South America, equivalent drug susceptibility for miltefosine and nifurtimox and a significant difference in IC_{50} values for amphotericin B between the Uruguayan strains, 3–4 fold higher than the reference strain.

1. Introduction

Leishmaniasis constitutes a group of diseases caused by over 35 different protozoan parasites of the genus *Leishmania* that are transmitted to humans and other wild or domestic animals by the bite of phlebotomine sandflies.[1] The disease is spread worldwide affecting 98 countries in five continents,[2] and is categorized as one of the "most neglected tropical diseases", strongly associated with poverty, affecting some of the poorest people on earth.[1,3] Its spread is tightly linked to environmental changes such as deforestation, building of dams and urbanization.[1]

There are three main forms of the disease: cutaneous, mucocutaneous and visceral leishmaniasis (VL), the latter being lethal in 95% of untreated cases and is characterized by irregular fever episodes, weight loss, anemia, hepatomegaly and splenomegaly. In the American continent, VL is caused by *Leishmania infantum*, whose main vector is the dipteran *Lutzomyia longipalpis*.[4] Mammals can get infected and stay infected without presenting symptomatology for long periods of time. During this subclinical forms of the disease, mammals are chronically infected and parasites can be transmitted to other mammals through competent vectors.[5] Infected dogs are the main urban reservoir for zoonotic visceral leishmaniasis mostly due to the high rate of canine infection in endemic areas and intense parasitism in the skin,[6] and are the most significant risk factor predisposing humans to infection.[7]

Treatment is only indicated when the disease is confirmed, and treatment options are highly dependent on several factors such as clinical manifestation, coinfections, parasite species and geographical localization.[8] This complexity is given in part because parasite species have different susceptibility to drugs in different geographical areas of the globe. Chemotherapy has proven to be the only

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Table 1 *Leishmania infantum* isolate nomenclature

Dog	Tissue	Isolate name	Isolate complete name
1	Ganglion	gPL8	MCAN_UY_2015_gPL8
1	Spleen	bPL7	MCAN_UY_2015_bPL7
2	Ganglion	gCH2	MCAN_UY_2015_gCH2
2	Spleen	bCH11	MCAN_UY_2015_bCH11
3	Medulla	mCO2	MCAN_UY_2015_mCO8

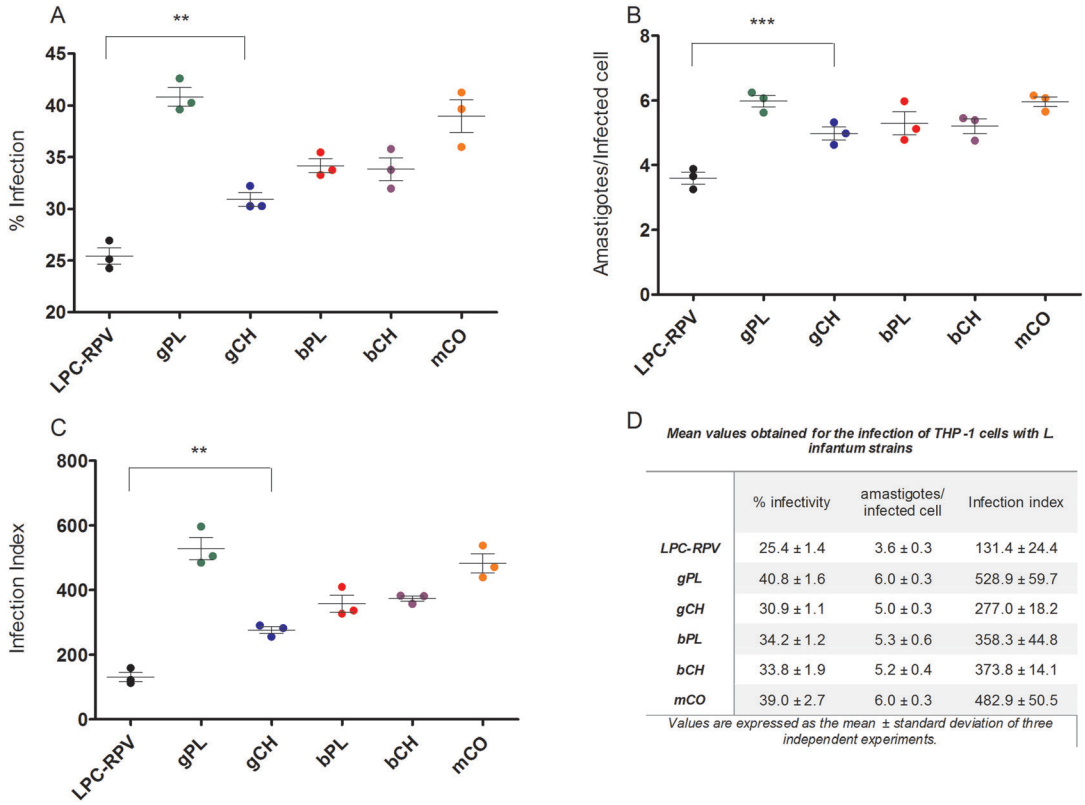

Fig. 1 Invasion, amastigote replication and infection indexes in THP-1 cells calculated at 48 hours post infection with the LPC-RPV strain and the five Uruguayan isolates. A. Percentage of the infected cells with respect to the total cells. B. Number of parasites per infected cell. C. Infection index. D. Summary table. Statistical differences were determined using the unpaired *t*-test, p-value < 0.05.

Table 2 IC_{50} values (μM) determined for the reference strain (LPC-RPV) and the five isolates

	LPC-RPV	gPL8	gCH2	bPL7	bCH11	mCO2
Nifurtimox	6.3 ± 0.1	6.0 ± 0.1	6.7 ± 0.2	6.5 ± 0.1	6.5 ± 0.1	6.7 ± 0.2
Miltefosine	5.3 ± 0.1	4.1 ± 0.1	4.1 ± 0.1	3.6 ± 0.1	3.8 ± 0.1	3.4 ± 0.1
Glucantime	50.1 ± 0.1	50.1 ± 0.1	50.2 ± 0.1	50.4 ± 0.1	50.5 ± 0.1	51.0 ± 0.2
Amphotericin B	6.5 ± 0.1	24.4 ± 0.1	24.8 ± 0.9	24.9 ± 0.1	24.6 ± 0.1	12.7 ± 0.1
Mevinolin	12.9 ± 0.2	25.4 ± 0.3	25.3 ± 0.2	25.3 ± 0.2	25.3 ± 0.2	25.4 ± 0.2

Values are expressed as the mean ± standard deviation of three independent experiments.

effective way of controlling infections and treatment often comprises antimony-containing drugs, such as sodium stibogluconate. Amphotericin B (AmB) is used when resistance to antimonials or pharmacological toxicity emerge, and also when antimonials are contraindicated for clinical conditions of the patient such as coinfections and parasite reactivation.[8,9] AmB has a better clinical efficacy and is better tolerated, but its high cost sometimes limits its availability.[10]

The presence of the vector *L. longipalpis* was recorded for the first time in 2010 in northern Uruguay, on the border with Argentina.[11] In 2015, the first cases of canine leishmaniasis caused by *L. infantum* were reported in the same region, which constituted the southernmost case of the disease.[12] In 2017, the first human case of VL was diagnosed in our country,[13] and few months later a new case was reported which ended with the patient's death because of comorbidity causes.[14] In the 2015 outbreak, our group detected the presence of *L. infantum* DNA in dog samples and in sandflies captured in the area, demonstrating the presence of an autochthonous transmission cycle.[12] Although new molecular methods for diagnosis tend to become independent of isolation, the culture of *Leishmania* spp. still remains essential for research and characterization purposes, antigen production for adequate diagnosis, and determination of biological characteristics and sensitivity to antiparasitic drugs. In the present work we describe the isolation in blood-free defined medium of five strains of *Leishmania infantum* that were responsible for the 2015 outbreak in Uruguay, and the characterization in terms of their growth, infectivity in human monocytes and drug susceptibility patterns.

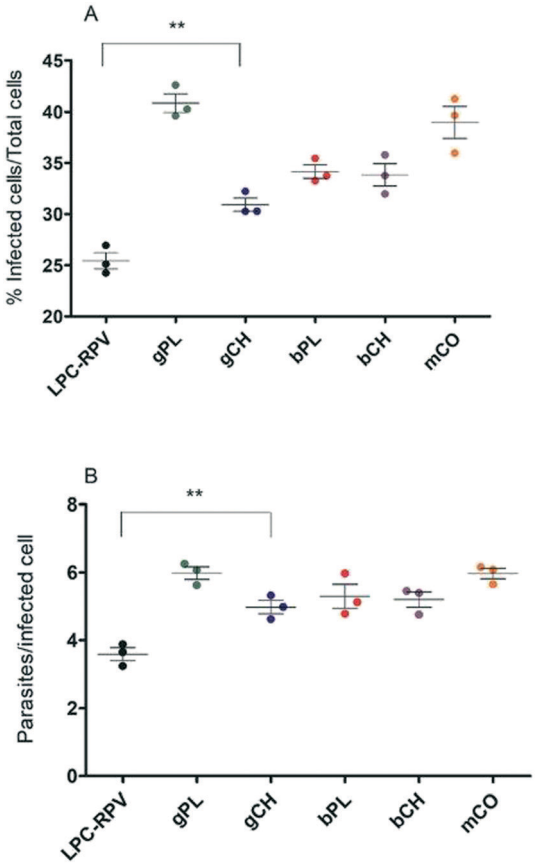

Fig. 2 Invasion and infection percentages in THP-1 cells at 48 hours post infection of the LPC-RPV strain and the isolates. A. Percentage of the infected cells with respect to the total cells. B. Number of parasites per infected cell. Statistical differences were determined using the unpaired *t*-test (**p-value < 0.05).

2. Material and methods

2.1. Parasites and cells

The *L.* (*L.*) *infantum* strain (MHOM/BR/2002/LPC-RPV) and *L.* (*L.*) *donovani* strain (MHOM/ET/1967/HU3) used as references were purchased from the *Leishmania* Collection of the Oswaldo Cruz Foundation (CLIOC) and gradually adapted to the RPMI medium described in the next section. All parasites were grown under axenic conditions at 28 °C. THP-1 monocytes (ATCC® TIB-202™) were cultured as recommended in RPMI-1640 (Cat No. 30-2001 Thermo Fisher Scientific, USA) supplemented to a final concentration of 0.05 mM β-mercaptoethanol and 10% fetal bovine serum (FBS, Gibco, USA).

2.2. Parasite isolation and culture

Samples were taken from symptomatic seropositive dogs, through bone marrow or lymph node aspiration, and spleen biopsies, which were aseptically collected and stored in cold PBS + penicillin/streptomycin until processing in the lab.

Samples were minced by scraping and cutting in a Petri-dish and then filtered through a 100 μm nitrocellulose filter. The cell suspension was seeded into a multiwell plate containing RPMI medium supplemented with 0.7% glucose, 0.1% ornitin, 0.4% fructose, 0.6% malate, 0.05% fumarate and 0.06% succinate, 20% FBS and vitamins and amino acid solution (Gibco, USA). Briefly, all solids (for 1 L) including RPMI were dissolved in 600 mL of distilled water and afterwards amino acid and vitamin solutions were added, pH was adjusted to 7.2 with NaOH. Finally, water is added to 800 ml final volume, sterilized by filtration with a 0.22 μm pore filter and stored at 4 °C until use. Aspirates were inoculated directly into the RPMI medium described above supplemented with FBS 20% v/v.

Plates were monitored every day and when promastigotes were observed, they were cryopreserved and stored in liquid nitrogen as passage 0 (P0) and passed to an F25 cm^2 flask into a 1 : 5 dilution of the same medium, when a stable culture was established, isolates were cryopreserved and considered P1. Experiments were made with parasites from P0 or P1 and never exceeded P5.

2.3. DNA extraction, amplification and species typing

DNA extraction from promastigotes in the log phase of growth was performed with a Quick-DNA Universal kit (Zymo Research, California, USA). PCR and restriction of the hsp70 fragment were performed as described previously in order to confirm the species level.[15]

2.4. Growth curve and IC_{50} determination

For the construction of growth curves, parasites were seeded at 1×10^6 cells per mL and at each time point counted at least 5 times. For IC_{50} experiments parasites were seeded at 3×10^6 cells per mL and incubated with serial dilutions of compounds starting from: 100 μM AmB, 50 μM nifurtimox, 25 μM miltefosine and 200 μM for mevinolin. Control conditions of parasites without drug (100% growth) and medium without parasites were included. After 72 h at 28 °C parasite viability was determined by the resazurin method as described by Rolón *et al.*[16] Results are expressed as the mean of three different and independent experiments.

2.5. Macrophage infectivity

THP-1 monocytes (ATCC®TIB-202™) were grown following ATCC recommendations and seeded at 30 000 cells per well onto 18 mm round glass coverslips in 12 wells. For stimulation, the cells were incubated with 100 nM PMA for 48 h, PMA was washed and the cells left in growth media for 24 hours more after incubation with parasites. 30 000 parasites per well were added and left to interact. After 48 hours, coverslips were washed with PBS, fixed with 95% (v/v) ethanol and stained with Fluoroshield™ with DAPI (sigma). Infectivity was assessed considering invasion and replication capacity counting infected cells and parasites per infected cell, respectively, using fluorescence microscopy. For each replicate, at least 500 macrophages were counted in total. The infection index was calculated as:

$$\text{Infection index} = \frac{\text{number of amastigotes} \times \text{number of infected macrophages}}{\text{number of total macrophages}}$$

Results are expressed in graphs as the mean of three different and independent experiments.

2.6. Statistical analysis

The statistical analysis of promastigote and amastigote results was performed using the unpaired *t*-test in Graph Pad Prism software. The level of significance of tests (*p*-value) was set as 0.05.

3. Results and discussion

From biological samples obtained from positive dogs of the canine leishmaniasis outbreak of 2015,[12] we were able to obtain five different isolates from three different dogs (Table 1).

After species typing with hsp70-RFLP (Fig. S1), growth curves were determined including the reference strain LPC-RPV, and the five isolates. When seeded at 5 × 106 parasites per mL, all strains showed equivalent exponential growth until approximately 140 hours of culture (1 × 108 p mL−1) when entering the lag phase of growth (Fig. S2). These parameters of exponential growth were used for further IC50 experiments. Five drugs were tested in vitro: nifurtimox, miltefosine, glucantime, AmB and mevinoline. For each drug the IC50 was determined and the values are shown in Table 2, and the dose response curves in Fig. S2. Behavior in the drug response curves and IC50 values for nifurtimox, miltefosine and glucantime are very similar, and no significant differences are found among the strains, values are in the range of 6, 4 and 50 μM, respectively.

On the other hand, when drugs such as AmB and mevinolin (Lovastatin) targeting the ergosterol pathway were used, significant differences among the values obtained for the reference strain and the isolates were observed. AmB increases membrane permeability by inducing pore formation in ergosterol-containing membranes, and mevinolin directly acts as an inhibitor of HMG-CoA reductase, a key enzyme of the sterol synthesis pathway. Results confirm that the isolates are less susceptible to these drugs than the reference strain: for AmB an IC_{50} value of 6.5 μM is found, and for the isolates at least twice this value is needed to kill 50% of parasites, 12.7 μM for the mCO2 isolate and more than four times for the rest of the isolates (24 μM values). For mevinolin, something similar happens but the isolates are much more alike, the IC_{50} of the isolates is around 25 μM, and for the reference strain 12 μM. The results obtained for AmB and mevinolin, both drugs targeting the ergosterol metabolism, suggest that a slight difference among these pathways may exist among the Uruguayan isolates and the reference strain; further experiments including the effect of these drugs in amastigote replication are needed in order to evaluate if this *in vitro* differential susceptibility is translated *in vivo* and to go deeper into the cause.

Differential susceptibility and drug resistance in *Leishmania* isolates are frequent and explanations align behind increased expression of enzymes involved in the tripanothion pathway, antioxidant defense, ABC transporters[17,18] and loss of expression sterol biosynthesis related enzymes.[19] Nifurtimox is a nitroheterocyclic drug used for the treatment of Chagas disease, but there are also reports of *Leishmania* promastigotes being susceptible[20] and identification of nitroreductases capable of activating the prodrug has been made.[21,22] Although the mode of action of nifurtimox is pleiotropic, oxidative stress is involved, as suggested by the observation that parasites lacking iron-dependent superoxide dismutase *SodB2* expression are more sensitive to the drug.[23] The results obtained for nifurtimox against the Uruguayan isolates and the reference strain do not let us infer differential expression of detoxification enzymes among them.

Current treatment protocols for canine leishmaniasis include meglumine antimoniate, miltefosine and allopurinol,[24] and the WHO specifically discourages canine use of AmB to avoid the occurrence of resistant leishmanial strains affecting humans. On the other hand, there are reports of experimental success of AmB in canine leishmaniasis.[25,26] Since VL has been expanding its geographical distribution into previously free areas, from northeastern Argentina, Misiones, Corrientes and Entre Ríos,[27,28] it is not unlikely that the strains responsible for the first Uruguayan outbreak come from treated dogs, ignoring the recommendations of health authorities.

Our second focus was to test the infectivity and replication capability of the isolates in human derived cells. For this, we incubated human derived monocytes THP-1 with the isolates and reference strains. In order to compare invasion, replication and the infective capacity we obtained two parameters from infections, the % of infected cells (Fig. 1A) and parasites per infected cell (Fig. 1B). We also calculated the infection index (Fig. 1C) and significant differences were observed. The Uruguayan isolates showed increased invasion and replication capability when compared to the reference strain. Regarding the percentage of infection we observed heterogeneity in invasion since for the gPL8 isolate we observed 40% of cells infected against 25% for LPC-RPV, 30% for gCH2, 33% for bPL7 and bCH11 and 34% for mCO2. For replication inside the macrophages, we observed 3.6 amastigotes per infected cell for LPC-RV and 5.5 as the mean of the rest of the isolates. This is a slight difference between replication of the strains but still represents a statistically significant difference. Both parameters are summarised in

the infectivity index where the mean value of the isolates is around 400, almost three times lower than the reference strain (131). All values are summarized in Fig. 1D.

Altogether, our results describe an isolation method in blood-free media of circulating *Leishmania infantum* from different organ sources. We characterized the isolated strains in terms of their growth under axenic conditions, drug susceptibility and human macrophages infectivity and replication. Highlights in terms of results are: the differential susceptibility to drugs that affect sterol biosynthesis to which Uruguayan isolates show differential resistance, and even though the molecular causes behind this phenotype should be explored, we cannot rule out AmB treatment at some point of the geographical expansion of the disease that may have contributed to the resistant phenotype.

Conflicts of interest

The authors declare no conflict of interest.

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