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Phenotype evaluation of human and canine isolates of *Leishmania infantum*

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ABSTRACT

Human visceral leishmaniasis (VL) and canine leishmaniasis (CanL) in countries of South and Central America are caused by *Leishmania infantum* and has been endemic in Brazil for several years. The parasite biodiversity as well as the pharmacologic properties of drugs and the host species, are involved in the efficacy or inefficacy of leishmaniasis treatments. Although there are substantial number of reports describing the genetic characterization of the clinical field isolates of *L. infantum*,the phenotypic parameters have been less studied. In this study isolates from human and canine leishmaniasis (Hum1 and Can1) obtained in Campinas, São Paulo state, Brazil were identified as *L. infantum*. The Hum1 and Can1 isolates exhibited typical promastigote growth pattern. Regarding morphological features Can1 isolate differed in cell size. The infectivity *in vitro* of both isolatesis lower compared to the reference strain of *L. infantum*. Moreover, the *in vivo* infectivity of the three parasites is similar in Balb/c mice. The Hum1 isolate is more sensitive to leishmanial drugs (amphotericin B, miltefosine and glucantime) than the Can1 isolate when inside human macrophages, but not when inside canine macrophages. These findings indicated that *L. infantum* isolates differs in some phenotypic characteristics.

1. Introduction

Human visceral leishmaniasis (VL) and canine leishmaniasis (CanL) in countries of South and Central America are caused by the intracellular parasite *Leishmania infantum* [1,2]. There is a spectrum of clinical signs of VL, but the most common ones are fever, anorexia, weight loss, abdominal distension, weakness, splenomegaly and hepatomegaly. Untreated symptomatic VL patients will progress to death [3]. The disease has been endemic in Brazil for several years, an average of 3500 cases recorded each year (2009–2018), and has also been reported from Mexico to Argentina [4,5]. CanL is characterized by a variable spectrum of clinical signs, but typical symptoms include fatigue, anorexia, weight loss, lymphadenopathy, alopecia, dermatitis, onychogryphosis and ocular involvement such as keratoconjunctivitis and uveits [2,6,7]. The presence of infected dogs has been identified as a risk factor for occurrence of VL, and CanL is endemic to millions of dogs affected in various countries such as China, Brazil and Spain [2,6].

In Brazil the Public Health System adopts the pentavalent antimonial compound N-methyl glucamine antimoniate as the drug of choice for VL and amphotericin B as the second choice treatment [8]. However, an emergence of *Leishmania* species resistant to pentavalent antimonials has been notified in various countries such India, Nepal and Sudan [9]. Moreover, *in vitro* resistance has been recently reported in clinical isolates from Brazilian patients with LV refractories to meglumine [10]. Until a few years ago the Ministry of Health and the Ministry of Agriculture prohibited the treatment of CanL [11]. Nowadays, the treatment of CanL is permitted, and in the veterinary practice miltefosine is the drug of choice for dogs with clinical leishmaniasis in Brazil. Although the protocols are effective in promoting clinical improvement, parasites are reduced but still detected in the skin and/or lymphoid tissues of dogs with CanL [12].

The variety of host and vector species involved in the natural transmission of *Leishmania* exert different selective pressures, and their circulation in various eco-epidemiological contexts can induce parasite biodiversity, *i.e.* the generation of structurally different parasite populations [13]. The parasite biodiversity as well as the pharmacologic

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properties of drugs and the host species, are involved in the efficacy or inefficacy of VL and CanL treatments [14,15]. Based on the fact that research of new anti-leishmanial compounds as well as diagnosis and vaccines are conducted with old and well adapted laboratory strains, which may differ genetically and phenotypically from field isolates, researchers have recommended adding clinical isolates to the secondary screening of anti-leishmanial compounds [16,17].

There are a substantial number of reports describing the genetic characterization of the clinical field isolates of *L. infantum*, while phenotypic parameters have been less studied [18,19]. This study was designed to identify two parasite isolates from VL and CanL obtained in Campinas, São Paulo state, Brazil, and to analyze their experimental infectivity in both human and dog macrophages and in a mouse model, as well as their morphology and susceptibility to drugs.

2. Materials and methods

2.1. Parasites

The reference strain of *L. infantum* (MHOM/BR/1972/LD) is from a Brazilian patient with LV. One clinical field isolate (Hum1) was obtained in 2014 at the Hospital das Clínicas, State Campinas University, Campinas, São Paulo, southeastern Brazil, from the bone marrow and lymph node samples of an 8-year-old female patient from Bahia state, northeast Brazil and residing in Campinas, with hepatosplenomegaly and pancytopenia. The hospital admission form was signed by the minor's guardian. The patient only started the treatment after parasite isolation and PCR analysis. The other clinical isolate (Can1) was obtained in 2014, from the puncture of bone marrow and lymph node of a dog collected by the Campinas Regional Service SR-05 (Sucen, São Paulo, Brazil), diagnosed with CanL by the ELISA serological test and euthanized. After isolation of the parasite, promastigotes were immediately stored at −80 °C and in liquid nitrogen. The *L infantum* promastigotes were all cultured in Schneider medium supplemented with 50 μg/mL gentamicin, 10% inactivated fetal bovine serum (FBS) and 5% filtrated human urine at 26 °C. *Leishmaniaamazonensis* (MHOM/BR/M2269) and *L. braziliensis* (MHOM/BR/BA/88) promastigotes were cultured in RPMI medium supplemented with 50 μg/mL gentamicin and 10% FBS at 26 °C.

2.2. Macrophages

The canine macrophage cell line (DH82) was obtained from the Rio de Janeiro Cell Bank, Rio de Janeiro, Brazil, and cultivated in a DMEM medium supplemented with 50 μg/mL gentamicin and 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2 [20]. The human cell line THP1 was obtained from the Rio de Janeiro Cell Bank and cultivated in a RPMI-1640 medium supplemented with 50 μg/mL gentamicin, 1 mM sodium piruvate and 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2. Before the experiments, the THP1 cells were differentiated into macrophages by exposure to 1 mg/mL phorbol-12- meristate-13-acetate for four days [21].

2.3. Animals

Six-week old female BALB/c mice (a total of 12 animals) were purchased from the Animal Center of Campinas State University. The protocols used were approved by the Animal Care Committee of Campinas State University under project license number 4710-1.

2.4. Polymerase chain reaction (PCR)

The samples (promastigotes) were subjected to DNA extraction by using QIAamp® DNA Mini and quantified in the Nanodrop Spectrophotometer 2000c (Thermo Scientific). A reaction mixture was made, containing 5 μL buffer (10X PCR buffer), 1 μL dNTPs (500 μM), 2 μL MgCl2 (25 μm), 1 μL primers (Forward 5′-CTTTTCTGGTCCCGCGGGTAGG and Reverse 3'CCACCTGGCCTATTTTACACCA-3 kDNA region size) to detect a 145-bp target sequence in the DNA of *L. infantum*[22], 1 μL of target DNA, 0.5 μL of taq DNA polymerase (1U/μL) and completed to 50 μL with deionized water. After an initial denaturation at 94 °C for 5 min, PCR was performed with 29 cycles of denaturation 94 °C (30 s), annealing (60 °C, 30 s) and polymerization (72 °C, 30 s). Gel electrophoresis was performed for the product's visualization, where 4 μL of PCR product were mixed with 1 μL loading dye and loaded on a 2% agarose gel. 5 μL of each sample were loaded in the wells and 2 μL of DNA ladder (100 bp) (Invitrogen) in a separate well. Gel tray was placed in a gel tank containing 1X TBE buffer. The gel was run for 25 min at 120 V and 500 A current. A solution of ethidium bromide (1.5 mg/mL) was used to stain the gel overnight and an UV transilluminator was used to visualize the amplified products [23,24].

2.5. Promastigote evaluation

For the determination of promastigotes growth, 2×10^5/mL were added to 25 cm^2 flasks containing Schneider medium and were kept at 26 °C. On a daily basis, an aliquot was removed and the number of promastigotes was determined using a Neubauer chamber. The experiments were carried out in triplicate and were repeated independently three times. For the morphometric analysis, promastigotes were transferred to glass slide, fixed with methanol and stained with Giemsa. The cell size (body and flagellum lengths) was measured for 100 parasites from each *Leishmania*[25]. The images were taken using the Axio-vision 4.3 software, in 1000x magnification of microscope Zeiss (Zeiss Primo star Axiocam, Germany) and analysed by the software Image J (Atlanta, GA, USA).

2.6. In vitro infection assays

The THP1 and DH82 macrophages (5×10^4/well) cultured in 24-well plates containing 13 mm diameter glass cover slips were infected with promastigotes in RPMI 1640 and DMEM media, respectively, at 10:1 or 20:1 ratios (promastigote: macrophage) and mantained at 37 °C in a humidified atmosphere with 5% CO_2. After 24 or 48 h, the cell cultures were washed to remove extracellular parasites and the coverslips washed with PBS, stained with Giemsa, and mounted on glass slides to evaluate the percentage of infected cells and the average of intracellular amastigotes [26]. The slides were examined microscopically, and at least 200 cells were counted. For the assay of antileishmanial drug susceptibility of clinical isolates, infected macrophages obtained as described above were washed with PBS and re-incubated for 48 h with amphotericin B (Sigma-Aldrich), miltefosine (hexadecylphosphocholine, Cayman Chemical Company) and glucantime (meglumine antimonate, Sanofi-Aventis) which were prepared as described in Barbosa et al. and Moraes et al. [26,27]. These were repeated at least three times, each performed in triplicate.

2.7. In vivo infection assays

Three experimental groups of BALB/c mice (3 animals per group) were intraperitoneally inoculated with 2×10^6 *L. infantum* promastigotes and one control group was not infected (3 animals). To estimate the parasite load at the end of the experimental period (two months after parasite inoculation), spleen and liver from euthanized mice were extracted, weighed, and homogenized using a 70 μm cell strainer to obtain cell suspension. Serial dilutions of the cell suspension were plated in a 96-well plates and maintained at 26 °C for 10 days. The wells were examined for viable promastigotes and the highest dilution positive for parasites was considered to calculate the parasite load [28]. The same limiting dilution procedure was used to calculate parasite load in the bone marrow. In addition, bone marrow suspensions were cultured in 6-well plates containing 5 mL of Schneider medium at 26 °C for up to 10

days. For histopatological preparation, tissue samples were fixed by immersion in 4% paraformaldehyde in 0.1 M PBS and processed for standard paraffin embedding [29]. Tissue sections were stained with haematoxylin and eosin (HE). The images were taken using the Axio-vision 4.3 software, in 400x and 1000x magnification of microscope Zeiss (Zeiss Primo star Axiocam, Germany).

2.8. Statistical analyses

The results were expressed as mean ± SD. Comparison between quantitative values were done by the ANOVA Turkey HSD multiple comparisons test, verifying the homogeneity of variances. Comparisons whose probability of equality is less than 5% ($p < 0.05$) were considered significant. All statistical estimates were made using the software R 3.6.0 and Graphpad Prism 7.

3. Results

Initially DNA extracted from Hum1 and Can1 promastigotes isolated from VL and CanL cases, respectively, were used as a template for PCR using primers for *L. infantum*. The correctly sized PCR product was amplified from DNA of Hum1 and Can1 isolates and reference strain of *L. infantum*, and did not appear in DNA of *L. braziliensis* promastigotes (Fig. 1), thus confirming the clinical isolates as *L. infantum*.

In order to avoid the impact of the long term *in vitro* culture on virulence and other phenotypic characteristics of parasites, the assays were performed with promastigotes from the first *in vitro* passages. The growth curves of parasite cultures from Hum1 and Can1 isolates peaked at day 5 ($\approx 1.7 \times 10^8$ parasites/mL) followed by a slow decline in the parasite number (Fig. 2). The reference strain of *L. infantum* promastigotes growth peaked at day 7 ($\approx 1.9 \times 10^8$ parasites/mL) followed by a slow decline in parasite number (Fig. 2). All promastigotes (isolates and reference strain) had typical and similar growth patterns.

The morphometric analyses of isolates and reference strain of *L. infantum* in addition to *L. amazonensis* promastigotes were performed. As observed in Fig. 3, by light microscopy all promastigotes were slender and spindle-shaped with a single and long flagellum. The values of the sizes of promastigotes are showed in the box graphics. The Can1 isolate cultures have the smallest promastigotes ($p < 0.001$) and reference strain of *L. infantum* and Hum1 isolate promastigotes are similar in cell size. *Leishmania amazonensis* promastigotes are larger than all *L. infantum* promastigotes ($p < 0.001$).

The infectivity of parasites was evaluated in this study. As shown in Fig. 4 the level of infection obtained for reference strain of *L. infantum* was around 40% of infected macrophages and 3.5–3.8 intracellular amastigotes for both human (THP1) and canine (DH82) macrophage cell lines. The Can1 and Hum1 isolates also infect the two macrophage cell lines. However, the infection levels were significantly different from reference strain of *L. infantum, i.e.* clinical isolates were less infective than the reference strain for both THP1 and DH82 macrophages ($p < 0.001$). Light microscopy demonstrated the ability of parasites to infect macrophages and to remain in vacuoles (Fig. 4C). The data of *in vivo* infectivity are shown in Fig. 5. There was a tendency towards higher

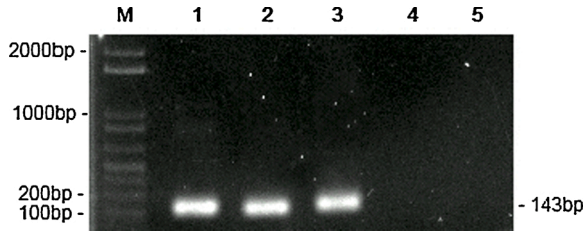

Fig. 1. Identification by PCR using RV1/RV2 primers for *L. infantum*. 148 bp markers (M), Hum1 isolate (1), Can1 isolate (2), reference strain of *L. infantum* (3), L. *braziliensis* (4) and negative control (5).

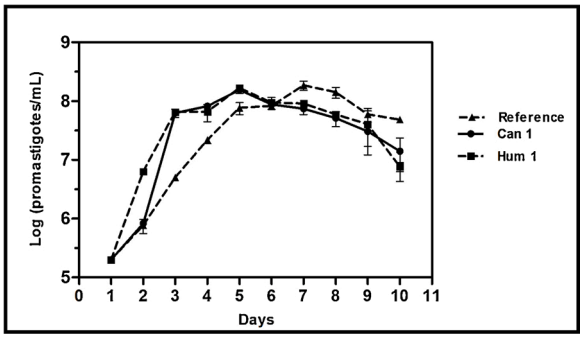

Fig. 2. Growth curve of *L. infantum* promastigotes. 2 × 105 cell/mL reference strain, Can1 and Hum1 promastigotes were incubated in Schneider medium at 26 °C. The results were obtained by the daily counting of parasites. Each value is expressed as the mean ± standard deviation of 3 independent experiments.

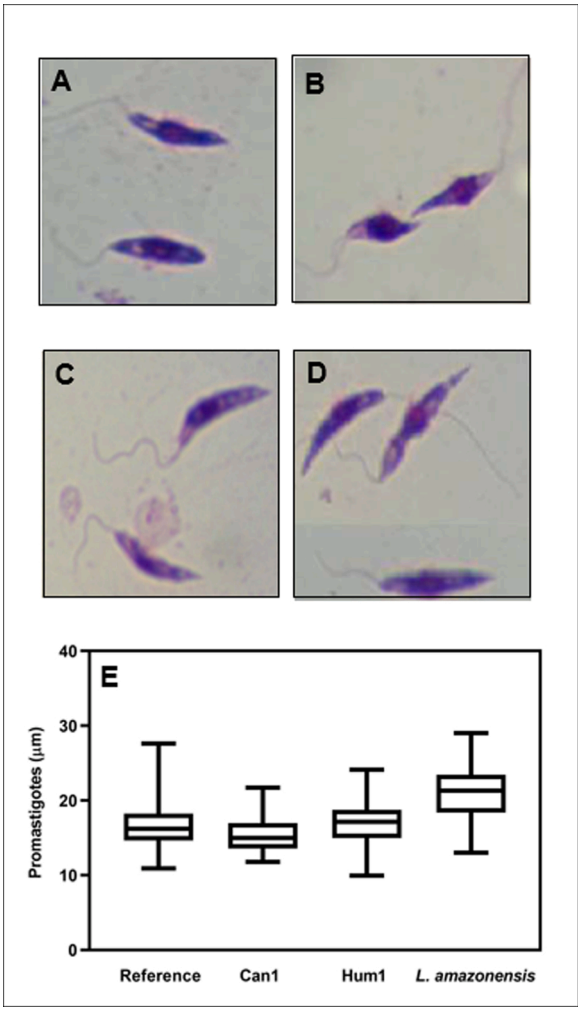

Fig. 3. Morphological analysis of *Leishmania* promastigotes. Light microscopy images of the reference strain of *L. infantum* (A), Can1 (B), Hum1 (C) and L. *amazonensis* (D) promastigotes stained with Giemsa and the cell size of parasites (E).

spleen parasite load of mice infected with reference strain of *L. infantum* compared with parasite load of mice infected with promastigotes clinical isolates, although the differences were not statistically significant (Fig. 5A). There were also no significant differences between the three experimental groups regarding parasite loads in the liver and bone marrow (Fig. 5). It should be noted that all spleen and bone marrow

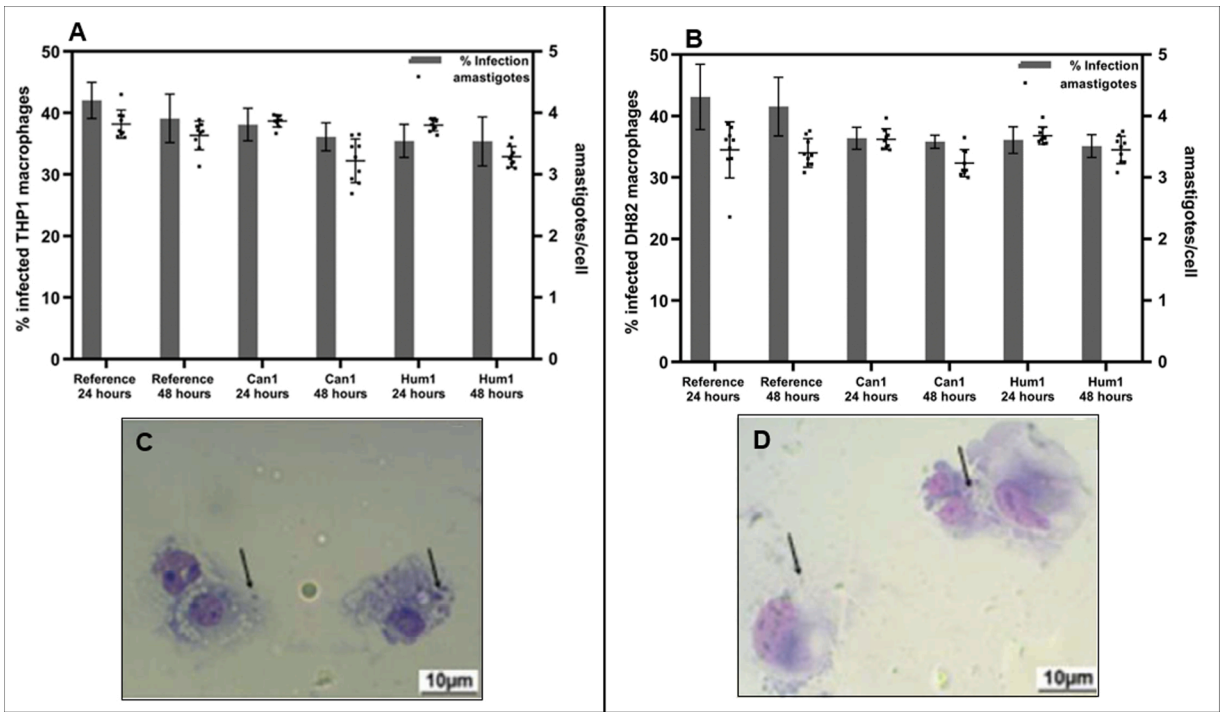

Fig. 4. *Leishmania infantum* promastigotes *in vitro* infectivity. The percentage of infected THP1 macrophages and the number of intracellular amastigotes per cell (A) and the percentage of infected DH82 macrophages and the number of intracellular amastigotes per cell (B). Light images of THP1 macrophages infected with Hum1 isolate (C) and DH82 macrophages infected with Can1 isolate (D) showing vacuolated cells containing amastigotes (arrows).

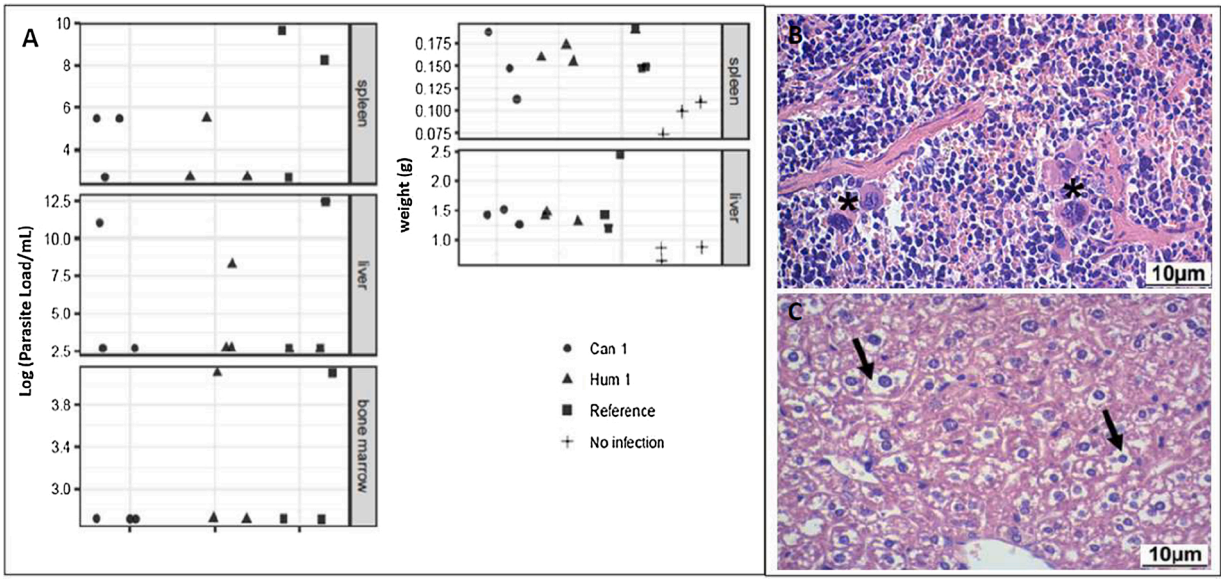

Fig. 5. *Leishmania infantum* promastigotes *in vivo* infectivity. Balb/c mice noninfected or infected with 2×10^6 reference strain, Hum1 or Can1 promastigotes, were sacrified after 2 months and evaluated for parasite loads and weights of organs (A). Each dot represents one mouse. The histological pattern from the spleen (B) and liver (C) of a mouse infected with the reference strain. Asterisks indicate multinucleated giant macrophages in B, and arrows indicate hypertrophic vacuolated hepatocytes in C.

cultures not serially diluted tested positives for promastigotes after ten days (data not shown). The data indicate that the clinical isolates as well as the reference strain were able to infect and visceralize. They are able to reach to the tissues and induce hepatosplenomegaly as also attested by the significant increase in the weight of these organs compared to their weight in normal animals (Fig. 5). In the spleens of mice infected with the reference strain and Hum1 and Can isolates, slight disorganization of white pulp, inflammatory cells infiltration, multinucleated giant macrophages scattered throught tissue including red pulp and near the splenic capsule, fibrous connective tissue, and vacuolization and hypertrophy of cells were observed (Fig. 5B). In the livers of these infected animals, the presence of inflammatory cell infiltrates close to blood vessels and spread in foci by the parenchyma and tissue disorganization with hypertrophic vacuolated cells were observed (Fig. 5C). It should be noted that these alterations did not led to severe disarrangement of spleen and liver of mice infected with the reference strain and

Hum1 and Can isolates for at least 2 months of infection.

The susceptibility of reference strain of *L. infantum* and Hum1 and Can1 isolates to the anti-leishmanial drugs was analyzed. Two different doses of each drug, ranging close to the IC50 of each of one of them [26, 27] were tested against THP1 and DH82 macrophages infected with the parasites (Fig. 6). As expected the susceptibility to amphotericin B, miltefosine, and glucantime (128 μg/mL) was observed in the infected cell cultures, and did not significantly vary between Hum1 and Can1 isolates infecting DH82 macrophages (Fig. 6). Differences could only be observed for amphotericin B, miltefosine and glucantime treatments between Hum1 and Can1 isolates infecting THP1 macrophages, i.e Hum1 isolate appear to be more sensitive to drugs (around 30% for amphotericin B, 25% for miltefosine and 35% glucantime; $p < 0.001$) when inside human macrophages.

4. Discussion

Phenotypic characterization of *Leishmania* is a prerequisite for identification of genetic loci and mechanisms involved in parasite virulence [13]. In the present report, two recent parasite isolates from VL and CanL obtained in Campinas, São Paulo state, Brazil and identified as *L. infantum* by PCR analyses. We cannot excluded that patient reported here represents an imported case for VL, since she is frm Bahia in northeastern Brazil, an endemic area for VL [4], and lived in Campinas, southeast region. The city of Campinas is a non-endemic area of leishmaniasis, although a canine outbreak was reported in recent years [30]. The analyses of promastigote growth pattern showed no differences among Hum1 and Can1 isolates and the reference strain *L. infantum*. The promastigotes from clinical isolates were from the first *in vitro* passages to avoid adaptation of parasites to culture conditions [25]. In any case, the possibility of the artificial axenic conditions of culture media favor the proliferation at similar rates of isolate promastigotes cannot be excluded. Other authors too did not find differences between promastigote clinical isolates of *L. infantum* cannot be excluded [13], *L. donovani*[31] and *L. braziliensis*[25]. However, when a morphometric parameter, cell size, was used, Can1 promastigotes at stationary phase were smaller than Hum1 and reference strain promastigotes. Can1 promastigotes are slender and splinter-shaped with a single flagellum suggesting no cytoskeleton impairment.

The comparison between promastigotes infectivity demonstrated that infection index of Hum1 and Can1 isolates were similar to each other, but lower than the infection index of reference strain for both type

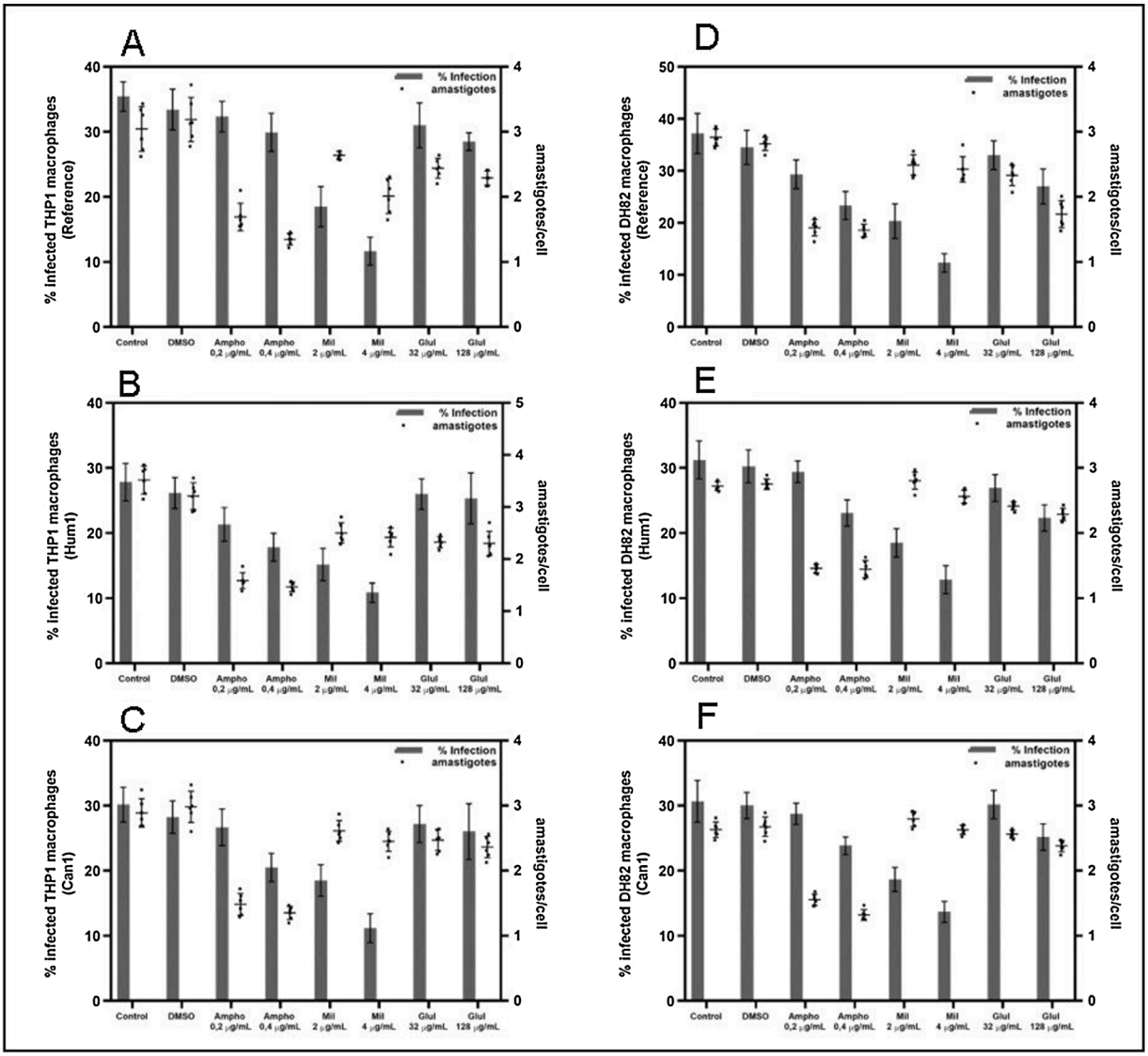

Fig. 6. Effect of antileishmanial drugs in *L. infantum* infected macrophages. The *in vitro* THP1 and DH82 macrophages infected with reference strain (A and D), Hum1 (B and E) and Can1 (C and F) promastigotes were treated with amphotericin B (Ampho), miltefosine (Mil) or glucantime (Glu) for 48 h, and the percentage of infected cells and intracellular amastigotes per cell were evaluated.

of macrophages. Similar analyses have been previously described for *L. infantum* isolates. For example, Maia and coworkers did not detect differences in the *in vitro* infectivity of twoisolates from human and dog [32], Campos-Ponce and coworkers studying viscerotropic strains which were expected to show higher infectivity than dermotropic strains of *L. infantum* from Central Americashowed that all had similar infection rates [33]. Moreover, Domingues-Bernal and coworkers, showed that three isolates from Spain had different *in vitro* infectivity [34]. We cannot rule out that genetic differences between the parasites are responsible for our findings, but we suggest that the adaptation of the reference strain of *L. infantum* in artificial axenic conditions of culture media for a long time explains the better efficiency of *in vitro* infection of macrophages. This is also supported by the results of the *in vivo* experiments. In contrast to *in vitro* experiments, a similarity between reference strain and Hum1 and Can1 isolates by *in vivo* infectivity was evidenced. The pattern of infection (spleen, liver and bone marrow parasite loads, weights of organs and histopathology) in Balb/c mice inoculated with any of three *L. infantum* promastigotes is the same, and similar to that described in the literature [25,35]. Some previous studies showed divergences between *in vitro* and *in vivo* activities of *L. infantum* isolates, Marques and coworkers showed that although a reference strain of *L. infantum* is less sensitive to reactive oxygen and nitrogen species, biomolecules with leishmanicidal effect, it had a limited capacity to infect mice [36]. Studies conducted by Cunha andcoworkers, with clinical isolates of *L. infantum* from two HIV + and two HIV- patients, indicated that *in vitro* infectivity is similar in three out of four isolates, while their *in vivo* infectivity differs in BALB/c mice; the isolates from HIV + patients are less infective *in vivo*[37]. The differences in the results of *in vitro* and *in vivo* infectivity of parasites can be attributed to the complex tissue environment (red blood cells, dendritic cells, neutrophils complement *etc.*) faced by promastigotes, compared to the *in vitro* situation (macrophage monocultures) [38]. Despite this, the results point to the importance of carrying out both *in vitro* and *in vivo* infectivity assays, and also suggest the adaptative capacity of *L. infantum* promastigotes.

Although the clinical isolates used in this work were obtained from untreated patient and dog, and apparently have not been under drug pressure, the Hum1 isolate appears to be more sensitive to drugs than the Can1 isolate only when infecting human macrophages. It should be noted that previous studies using different *in vitro* models (different parasite:cell ratios, macrophage lines, time of drug exposition, *etc.*) also indicated a tendency of low susceptibility of strains/isolates from dogs to the main leishmanial drugs [32,39]. In addition, the reasons for the differences in the drugs susceptibility of the isolates to be detected only in the assays with human THP1 macrophages are not known. Nevertheless, host cell dependent variation in drug susceptibility has been reported previously. For instance, Hendrick and coworkers found variations in antimonial, anphotericin B and miltefosine susceptibility in clinical human isolates of *L. infantum* infecting mouse primary macrophages and cell lines [16], as well as Seifert and coworkers for *L. donovani* strain and Terreros and coworkers for a *L. amazonensis* strain, both infecting macrophages of different types [40,41]. The authors attributed the results to the differences in macrophage differentiation rate, which can influence intracellular promastigote to amastigote transformation, surface-structures and metabolic responses of the various cell lines and primary macrophages. These results reinforce the use of more than one macrophage cell type, parasite strain and clinical isolate in the *in vitro* assays for the drug resistance monitoring in the field and for validation of active compounds [17].

In conclusion, isolates from human and canine leishmaniasis from Campinas, São Paulo state, Brazil were identified as *L. infantum*. The Hum1 and Can1 isolates exhibited typical promastigote growth pattern. Regarding morphological features Can1 differed in cell size. The infectivity *in vitro* of both isolates is lower compared to the reference strain of *L. infantum.* Moreover, the *in vivo* infectivity of the three parasites is similar in Balb/c mice. The Hum1 isolate is more sensitive to leishmanial drugs than the Can1 isolate when inside human macrophages. These results provide a starting point for the genetic and immunological understanding of these field isolates of *L. infantum*.

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Declaration of Competing Interest

None.

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