Decreased antimony uptake and overexpression of genes of thiol metabolism are associated with drug resistance in a canine isolate of *Leishmania infantum*

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1. Introduction

Leishmaniasis caused by the intracellular protozoan *Leishmania* is the second most important parasitic disease after Malaria, affecting humans and animals in wide areas of the Americas, Asia, Africa and Europe. Domestic dogs are considered the main reservoir host of *L. infantum* and affects millions of dogs in Asia, Europe, North Africa and South America, and is currently an emergent disease in North America. It has been estimated that at least 2.5 million dogs are infected in southwestern Europe (Moreno and Alvar, 2002; Athanasiou et al., 2012). The infection is spreading to non-endemic areas and cases of CanL are reported with increasing frequency in Northern European countries and in the United Kingdom, even in dogs that have never visited endemic zones (Shaw et al., 2009). The introduction and spread of the disease to regions where infections had not previously been observed may create new epidemiological scenarios, further complicating the zoonotic potential and control of the disease.

In the absence of effective human and canine vaccines, the only feasible way to treat and control leishmaniasis is through the use of affordable chemotherapy. Meglumine antimoniate is one of the most common drugs used in Europe to treat CanL (Solano-Gallego et al., 2008). The combination of meglumine antimoniate with allopurinol is considered to be the most effective therapy and constitutes the first line protocol against CanL (Miró et al., 2008).
Anti-leishmanial therapy can usually decrease the parasite load, although it is extremely difficult to achieve a parasitological cure in dogs. For this reason, the majority of short-term therapeutic interventions are usually followed by a relapse within 1 year of discontinuing treatment (Baneth and Shaw, 2002; Ikeda-Garcia et al., 2007; Manna et al., 2008). As infected dogs never achieve parasitological cure and are frequently treated several times with the same compounds, selection and transmission of antimony-resistant parasites could appear (Campino and Maia, 2012; Sereno et al., 2012).

Therapeutic failure and relapse in leishmaniasis is known to have a multifactorial origin, involving features related to the host (immunity, genetic and nutritional factors, among others), the drug (quality, pharmacokinetics) and the parasite (drug resistance, increased infectivity, coinfection with other pathogens) (Vanaerschot et al., 2014). While the occurrence of Leishmania resistance to pentavalent antimonials is well known in human medicine (Crocket et al., 2006; Vanaerschot et al., 2013), only limited information is available for dogs. L. infantum isolates taken from dogs which had received several treatment courses have demonstrated contradictory results, with no differences in susceptibility in isolates from treated and untreated dogs (Carrió and Portús, 2002), or a decreased susceptibility to antimony after several therapeutic interventions (Gramatica et al., 1992).

In the present study we investigate if a L. infantum isolate from a dog with CanL relapse after a therapeutic intervention has developed drug resistance as a factor responsible of therapeutic failure, and the mechanism of resistance developed. The singularity of this study was based on the use of Leishmania isolates at the time of diagnosis and after therapeutic interventions, facilitating the comparative studies. It is important to detect resistant parasites in dogs with therapeutic failure in order to avoid and reduce the emergence and spread of resistant parasites throughout the canine populations; a factor that requires special consideration when dogs are treated with the same anti-leishmania drugs that are available for human visceral leishmaniasis.

2. Materials and methods

2.1. Chemicals

Trivalent antimony (Sb(III)), amphotericin B (AmB), paromomycin, Triton X-100, paraformaldehyde, 4',6-diamidino-2-phenylindole dilactate (DAPI), n-dodecyl-β-D-maltoside (DDM), 3-(4,5-dimethithiazol-2-yl)-2,5-diphenyltetrazium bromide (MTT) and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich (St. Louis, USA). Miltefosine was purchased from Älterna Zentaris (Frankfurt, Germany). Glucantime® was purchased from Sanofi-Aventis (Paris, France). L-glutamine and penicillin/streptomycin were obtained from Gibco. All chemicals were of the highest quality available.

2.2. Clinical case, parasite isolation and culture

A five-years-old male boxer dog was positively diagnosed for leishmaniasis through detection of anti-leishmania specific antibodies by indirect immunofluorescence (IgG titer > 1280) and by detection of L. infantum parasites with qPCR as described (Corpas-López et al., 2015). The animal had many clinical signs compatible with CanL, including onychogryphosis, adenopathy, skin lesions and uveitis, among others. The clinical status was quantified by determining a Clinical Score (CS). Briefly, each symptom was assigned a value according to the severity of it (0 = absence; 1 = mild; 2 = moderate; 3 = severe) and the CS was the sum of the values for every symptom. The initial clinical scores (CS = 18) rises after the first treatment series (CS = 24) and declined to the initial CS value after the second one (CS = 18). In the follow-up period without drug pressure, the dog showed a higher CS value (CS = 20), due to the aggravation of eye symptoms (uveitis, keratitis) besides increased creatinine.

After the trial, the dog was returned to its owner with full information on its clinical and parasitological state, who decided to treat the animal with other drugs.

The dog was housed in the facilities of an animal shelter, with access to water and food ad libitum. It was fitted with a Scalibor® deltamethrin collar at the time of the assay. The building where the dog was housed was protected with adequate mosquito nets to prevent sandflies from entering. This building was also sprayed with insecticide (Fenitrothion) on a monthly basis.

Animal experiments were approved by the Ethics Committee for Animal Experimentation of the University of Granada (Protocol: 450-26113) and all procedures were carried out according to the international guidelines and the European Union Directive (86/609/EEC). The dog was treated with meglumine antimoniate (Glucantime®) at 100 mg/kg/day administered subcutaneously for two 28-day periods, separated by a 30-day interval. The parasites were isolated from bone marrow and ganglion aspirates in Evans’ Modified Tobie’s Medium (EMTM). After their isolation, parasites were cultured at 28 °C in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (hiFBS, Invitrogen). After 6 passages (2 months), parasite strains were labeled, mixed with DMSO and stored in liquid nitrogen.

2.3. Drug-susceptibility analysis in Leishmania promastigotes

To determine parasite susceptibility to Sb(III) and other anti-leishmania drugs such as AmB, paromomycin and miltefosine, 2 × 10^5 promastigotes were incubated in 96-well plates (100 μL) at 28 °C for 72 h in the presence of increasing drug concentrations before determining cell proliferation by the MTT colorimetric assay as described previously (Gómez-Pérez et al., 2015). The 50% effective concentration (EC50) was defined as the drug concentration required for half maximal inhibition of cellular growth rate. The EC50 for each line was calculated by nonlinear regression analysis using SigmaPlot 2000 software for Windows (SPSS Inc., Chicago, IL, USA). Resistance index (RI) was calculated by dividing the EC50 obtained for the resistant line by the EC50 obtained for the parental susceptible line.

2.4. Human myelomonocytic cell line (THP-1) culture

THP-1 cells were grown at 37 °C and 5% CO2 in RPMI-1640 supplemented with 10% hiFBS, 2 mM glutamate, 100 U/mL penicillin and 100 μg/mL streptomycin. 3 × 10^6 cells/well, in 96-well plates, were differentiated to macrophages with 20 ng/mL of PMA treatment for 48 h followed by 24 h of culture in fresh medium (Gómez-Pérez et al., 2014).

2.5. Susceptibility analysis in intracellular Leishmania amastigotes

To determine the susceptibility of intracellular Leishmania amastigotes to Sb(III), Sb(IV) in the form of meglumine antimoniate (Glucantime®), AmB, paromomycin and miltefosine, late stationary-phase promastigotes were used to infect macrophage differentiated-THP-1 cells at a macrophage/parasite ratio of 1:10. Infected cell cultures were then incubated at different compound concentrations for 72 h, as described previously (Gómez-Pérez et al., 2014). Samples were then fixed for 30 min at 4 °C with 2% paraformaldehyde in PBS, followed by permeabilization with 0.1%
Intracellular parasites were detected by nuclear staining with DAPI (Invitrogen). The percentage of infection and the mean number of amastigotes from infected macrophages were determined in 200 macrophages/well.

2.6. Antimony accumulation and efflux by ICP-MS

Promastigotes (1 x 10^4 per mL) were incubated with 100 μM SbIII for 60 min at 28°C, then centrifuged and the resultant pellet stored at -80°C until antimony accumulation was measured as described previously (Manzano et al., 2013). To determine antimony efflux, the different isolates were incubated with compensated SbIII concentrations (50 μM for line 576-1 and 500 μM for line 576-3) in culture medium at 28°C for 1 h to allow a similar labeling in the Leishmania lines. The parasites were then washed with PBS, resuspended in culture medium at 28°C and pelleted at different times (0, 30, 60 and 120 min). The cell pellet was dissolved in 200 μL of concentrated nitric acid at room temperature for 24 h. The sample was diluted to 3 mL with distilled water and then injected into an inductively coupled plasma mass spectrometer (ICP-MS; PerkinElmer) for quantitation. Antimony was measured at its m/z ratio of 121 and 123 with rhodium as an internal standard. All chemicals used for sample pre-treatment were of at least analytical grade.

2.7. Nucleic acid isolation and gene expression

Genomic DNA and total RNA were extracted from the Leishmania isolates using the RNeasy Plus Mini Kit (Qiagen) and DNeasy Blood and Tissue Kit (Qiagen), respectively. Isolated RNA was transcribed into cDNA using the qScript cDNA Synthesis Kit (Quanta Biosciences, Inc) following the manufacturer’s instructions. The synthesized cDNAs were diluted 1:10 and 1:50 and specific fragments amplified with sense and antisense primers (5'-ACGATGCCGTCACAGTACAG) and (5'-GATTATGGGAGGTCGGACTG) for aquaglyceroporin-1 (AQP1), (5'-GCTTGGATCATGAAGAGGCACATC) and (5'-ACCTACTGGTCGCTCAGACTTCTC) for MRPA, (5'-CTTAACTGTGATCTTCTC) and (5'-GATGTTCTGATGCAATTCGACAGG) for ornithine decarboxylase (ODC), (5'-CTCTATTGGCCGCTCAGACTTCTC) and (5'-GACATGGTTGAGGTCGAGGACT) for γ-glutamylcysteine synthetase (γ-GCS), and (5'-GATTACGGGAGGTCGAGGCT) and (5'-GGTCATGACCACTTCTC) for GAPDH using 35 amplification cycles at an annealing temperature of 54°C. PCR products were electrophoresed on 1% agarose gel, stained with ethidium bromide, viewed under a UV illuminator and the relative intensity resolved by 10% SDS-PAGE and transferred to PVDF membranes. Membranes were subsequently blocked at room temperature for 1 h in 5% skimmed milk in PBS with 0.05% Tween 20 (PBS-T) and washed three times with PBS-T. Immunodetection was performed by incubating the blocked membranes at room temperature for 1 h in the presence of antibodies against cystosolic tryptaredoxin (cTXN), mitochondrial tryptaredoxin peroxidase (cTXNPx), mitochondrial tryptaredoxin peroxidase (mTXNPx), trypanothione synthetase (TryS) and trypanothione reductase (TR): anti-cTXN (dilution 1:3000) (Castro et al., 2004), anti-mTXN (1:3000) (Castro et al., 2004), anti-cTXNPx (1:3000) (Castro et al., 2002), anti-mTXNPx (1:3000) (Castro et al., 2002), anti-TryS (1:1000) (Sousa et al., 2014) or anti-TR (1:1000) (H. Castro and A. Tomás, personal communication). The membranes were washed and incubated with HRP anti-mouse IgG (1:5000) or HRP anti-rabbit IgG (1:5000) (Promega). Immunoreactive proteins were viewed using a chemiluminescence detection kit (Pierce ECL Western Blotting Substrate) according to the manufacturer’s instructions. The results were normalized using anti-α-tubulin antibodies (Sigma).

2.10. Statistical analysis

Statistical comparisons between groups were performed using Student’s t-test. Differences were considered significant at a level of p < 0.005.

3. Results and discussion

3.1. Isolation of L. infantum lines from a dog with naturally acquired leishmaniasis

In this study, we have analyzed parasites isolated from ganglion aspirate at the time of diagnosis (MCAN/ES/2014/DP576-1, abbreviated as line 576-1) and 30 days after two therapeutic interventions with Glucantime® (100 mg/kg/day, subcutaneously for 28 days), separated by a 30-days interval (MCAN/ES/2014/DP576-3, abbreviated as line 576-3).

Several factors could have contributed to the absence of a therapeutic response to Glucantime® in this dog, including factors related to the parasite, the drug or the host. We evaluated whether line 576-3 presents modifications in drug susceptibility that could be associated with the development of resistance and, in positive case, we analyzed the mechanism of resistance.

3.2. Drug susceptibility profiles of L. infantum isolates

The susceptibility profile to ShIII of the different isolates of L. infantum was analyzed in promastigotes and intracellular amastigotes (Table 1). The EC50 values for line 576-3 were significantly higher than for line 576-1, in both promastigotes and intracellular amastigotes (Table 1). These data suggest that parasites developed resistance to ShIII after the second therapeutic intervention, presenting a RI of around 6 for promastigotes and greater than 3 for intracellular amastigotes (Table 1). Considering Glucantime® was the drug used to treat this infected dog, we analyzed the
susceptibility profile to this drug in intracellular amastigotes from line 576-3, obtaining an EC50 of 159.3 μM, 3.3-fold higher than line 576-1 (Table 1). Our data clearly substantiate that relapse after the second therapeutic intervention was due to the acquisition of drug resistance in line 576-3.

We also studied the cross-resistance profile to other anti-leishmanial drugs, including AmB, miltefosine and paromomycin (Table 1). The results showed that intracellular amastigotes from line 576-3 present a significant cross-resistance to paromomycin (RI around 2.8). However, we did not observe any cross-resistance to AmB and miltefosine in promastigotes and intracellular amastigotes. As previously described, promastigotes of *L. donovani* resistant to antimony present a significant cross-resistance to paromomycin and not to AmB and miltefosine (García-Hernández et al., 2012); however, other studies using antimony resistant and sensitive field *L. donovani* isolates shown equal susceptibility to paromomycin (Kulshrestha et al., 2011). These results support the variability in the response of *Leishmania* to drugs. Additionally, drug pressure could induce genomic changes in parasites that could be responsible of the resistance to paromomycin in intracellular amastigotes.

Furthermore, lack of correlation between the promastigote and intracellular amastigote susceptibilities to paromomycin observed in the present study, supports the intracellular amastigote model as a more appropriate approach for susceptibility studies, as it has been previously reported (Vermeersch et al., 2009; Kulshrestha et al., 2011).

We then tried to elucidate the mechanism of antimony resistance in line 576-3 by analyzing antimony uptake and the expression profiles of known genes involved in transport and thiol based redox metabolism.

### 3.3. Antimony accumulation and efflux in *L. infantum* lines

A decrease in drug concentration within the parasite, either by reducing drug uptake or by increasing efflux/sequestration of the drug, represents the primary mechanism of antimonial resistance in *Leishmania* (Brochu et al., 2003). To determine whether a reduction in SbIII uptake was one of the mechanisms of resistance developed in line 576-3, the intracellular accumulation of this metal ion was measured by ICP-MS after incubation with 100 μM SbIII for 1 h. SbIII accumulation was found to be significantly lower (90%) in the resistant line 576-3 compared to line 576-1 (Fig. 1A). The lower SbIII accumulation in line 576-3 could explain the resistance to antimonials. To determine whether the reduced level of accumulation was due to an increase in antimony efflux, both *L. infantum* lines were loaded under conditions that yielded similar amounts of intracellular antimony and the portion of antimony retained in the parasites was measured at different times. The efflux of SbIII was found to be time-dependent and similar in both *Leishmania* lines (Fig. 1B), thus confirming that the lower SbIII accumulation was not due to increased efflux activity, but rather a decrease in drug uptake.

In *Leishmania*, antimony uptake is mediated by the transporter AQPI (Gourbal et al., 2004). Previous studies indicated AQPI RNA levels were down-regulated in several *Leishmania* promastigote species (*L. tarentolae*, *L. major* and *L. infantum*) that were experimentally resistant to antimonials (Marquis et al., 2005), as well as in different antimony-resistant *Leishmania donovani* clinical isolates (Decuyper et al., 2005; Mandal et al., 2010; Mukhopadhyay et al., 2011).

In general, the level of AQPI1 transcript correlated well with the accumulation of SbIII and resistance levels in *Leishmania* parasites (Marquis et al., 2005). AQPI1 gene expression in lines 576-1 and 576-3 was analyzed by RT-PCR to ascertain whether there was a correlation between AQPI1 gene expression and antimony susceptibility and accumulation. The results showed that AQPI1 expression in the resistant line 576-3 was around 5-fold lower than the expression in the susceptible line 576-1 (Fig. 1C).

Gene deletion and point mutations in AQPI have been reported in antimony-resistant *Leishmania* parasites (Monte-Neto et al., 2015). The full-length coding sequence of the *L. infantum* AQPI gene was cloned and sequenced from the 576-1 and 576-3 isolates. Only one of five clones sequenced presented a point mutation that lead to a premature stop codon, while the rest of the clones did not show any point mutations or deletions that could be associated with a reduction in AQPI1 activity.

Our data therefore support the suggestion that down regulation of AQPI is the main resistance mechanism in *L. infantum* isolate from a case of CanL with therapeutic failure.

ABC transporter MDR-related proteins (MRPA/PgpA) are known to be involved in antimonial-resistance in *Leishmania* through sequestration of the metal-thiol conjugates in an intracellular organelle located close to the flagellar pocket (Légaré et al., 2001). The role of MRPA in conferring antimony resistance by sequestration of metal-thiol conjugates has also been reported in clinical isolates of *Leishmania* (Mukherjee et al., 2007).

MRPA gene expression analysis in antimony susceptible and resistant *L. infantum* lines was determined by RT-PCR using specific primers. The results showed that there were no differences in expression levels between susceptible and resistant lines (Fig. 1D), suggesting that MRPA is probably not responsible for the defect in antimonial accumulation in resistant *L. infantum* line 576-3.
in independent experiments. Significance was determined using the Student’s t-test (*; p < 0.001). (C) Gene expression analysis of AQP1 and (D) ABC transporter MRPA. Total RNA was extracted from lines 576-1 and 576-3. Upper panels: gene expression of AQP1 or MRPA by RT-PCR using cDNA dilutions of 1:10 and 1:50, respectively, as indicated by absorbance intensities (A) Promastigotes (1 × 10⁷/mL) of lines 576-1 (antimony susceptible) and 576-3 (antimony resistant) were incubated with 100 µM SbIII for 1 h. Antimony accumulation was measured by ICP-MS. The efflux assay (B) was performed for 1 h after incubation of lines with compensated concentrations of SbIII to ensure similar labeling. The parasites were then washed and resuspended in PBS buffer without SbIII and pelleted at different times. The data are the means ± SD of three independent experiments. Significant differences were determined using the Student’s t-test (*, p < 0.001). (C) Gene expression analysis of AQP1 and (D) ABC transporter MRPA. Total RNA was extracted from lines 576-1 and 576-3. Upper panels: gene expression of AQP1 or MRPA by RT-PCR using cDNA dilutions of 1:10 and 1:50, respectively, as indicated by the amplified 255-bp AQP1 or 276-bp MRPA fragment. Lower panels: gene expression of GAPDH as internal loading control showing the amplified 227-bp GAPDH fragment. The RT-PCR assay shown is representative of at least three independent experiments.

In Leishmania the trypanothione/trypanothione peroxidase (TXN/TXNPx) system is crucial to defend parasites against the oxidative stress that can be found in different compartments (Fromholt et al., 1999). Increased levels of both proteins have been detected in antimony-resistant L. donovani field isolates (Wyllie et al., 2010). Leishmania have two TXNPx, one localized in the cytoplasm (cTXNPx) and the other in the mitochondrion (mTXNPx), which protect the cell from peroxide-induced damage (Castro et al., 2002). Proteomic analysis of experimental antimony-resistant lines from L. braziliensis and L. infantum showed an overexpression of cTXNPx (Matrangolo et al., 2013). Additionally, overexpression of cTXNPx confers resistance to antimony in L. braziliensis and L. donovani; however, overexpression of cTXNPx in L. infantum does not seem to be directly associated with resistance to SbIII (Lyer et al., 2008; Andrade and Murta, 2014). Therefore, we studied whether TXN/TXNPx proteins were upregulated in the antimony-resistant line 576-3. The results showed an overexpression of mTXN (by around 12-fold) and mTXNPx (around 4-fold), while no differences were observed in the expression of cTXN and cTXNPx (Fig. 2B). The overexpression of the mitochondrial pathway could induce an increase in T[SH]2 levels which forms a complex with SbIII, thus...
inactivating the toxic effects of antimony and enhancing the parasite’s antioxidant defense.

4. Conclusions

The present study has characterized the mechanisms of antimony resistance for the first time in a clinical isolate of L. infantum from a dog with naturally acquired CanL. We confirmed that L. infantum line 576-3 confers resistance against SbIII by significantly decreasing expression of the AQP1 transporter which leads to a reduction in intracellular accumulation of SbIII. Other factors including an increase in thiols levels and overexpression of enzymes involved in thiol metabolism could also contribute to the SbIII detoxification mechanism.

A major contribution of this study in a canine L. infantum isolate is to find an antimony-resistant mechanism similar to that previously described in other human clinical isolates. Therefore, more studies are required in L. infantum isolates from dogs that relapse after treatment to better understand the therapeutic failure in CanL. The use of naturally infected dogs and a controlled therapeutic intervention represent an excellent experimental strategy to study therapeutic failure/resistance and search for more effective therapeutic strategies following relapse, to avoid the spread of drug-resistant lines in endemic areas in attempts to control the disease.

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is related to the expression of host and parasite gamma-glutamylcysteine synthetase. Antimicrob. Agents Chemother. 50, 88–95.


