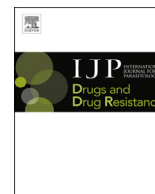


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Drugs and Drug Resistancejournal homepage: www.elsevier.com/locate/ijppawDecreased antimony uptake and overexpression of genes of thiol metabolism are associated with drug resistance in a canine isolate of *Leishmania infantum*Verónica Gómez Pérez ^a, Raquel García-Hernandez ^a, Victoriano Corpas-López ^b, Ana M. Tomás ^c, Joaquina Martín-Sánchez ^b, Santiago Castany ^{a,1}, Francisco Gamarro ^{a,*}, ¹^a Instituto de Parasitología y Biomedicina “López-Neyra”, CSIC, (IPBLN-CSIC), Parque Tecnológico de Ciencias de la Salud, Granada, Spain^b Departamento de Parasitología, Facultad de Farmacia, Universidad de Granada, Granada, Spain^c IBMC – Institute for Molecular and Cell Biology, Porto, Portugal

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ABSTRACT

Visceral leishmaniasis (VL) caused by the protozoan parasite *Leishmania infantum*, is one of the most important zoonotic diseases affecting dogs and humans in the Mediterranean area. The presence of infected dogs as the main reservoir host of *L. infantum* is regarded as the most significant risk for potential human infection. We have studied the susceptibility profile to antimony and other anti-leishmania drugs (amphotericin B, miltefosine, paromomycin) in *Leishmania infantum* isolates extracted from a dog before and after two therapeutic interventions with meglumine antimoniate (subcutaneous Glucantime[®], 100 mg/kg/day for 28 days). After the therapeutic intervention, these parasites were significantly less susceptible to antimony than pretreatment isolate, presenting a resistance index of 6-fold to Sb^{III} for promastigotes and >3-fold to Sb^{III} and 3-fold to Sb^V for intracellular amastigotes. The susceptibility profile of this resistant *L. infantum* line is related to a decreased antimony uptake due to lower aquaglyceroporin-1 expression levels. Additionally, other mechanisms including an increase in thiols and overexpression of enzymes involved in thiol metabolism, such as ornithine decarboxylase, trypanothione reductase, mitochondrial trypanothione and mitochondrial trypanothione peroxidase, could contribute to the resistance as antimony detoxification mechanisms. 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.

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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 of *Leishmania infantum*, and a risk factor for anthroponotic human visceral infection. Canine leishmaniasis (CanL) is endemic 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., 2011). 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).

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Anti-leishmania 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-García 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 (Croft 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 (Gramiccia 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-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich (St. Louis, USA). Miltefosine was purchased from Aeterna 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 \geq 1280) and by detection of *L. infantum* parasites with qPCR (1.06 parasites/ μ L) using specific primers for kDNA 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 μ L) 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 μ g/mL streptomycin. 3×10^4 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^V 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%

Triton X-100 in PBS for 30 min. 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 (1×10^8 per mL) were incubated with 100 μM Sb^{III} 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 Sb^{III} 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* ratios 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'-ACGATGGCGTCCAAGTACAG) and (5'-GAGTATGCGAGGTGCAAGTC) for *aquaglyceroporin-1* (*AQP1*), (5'-GCTGTACATGCAAGCTCTC) and (5'-ACGTACTCGTGCTCCACTTC) for *MRPA*, (5'-CTTCAACTGCATCCTCTTCG) and (5'-GCAGTCCATCGAATCGCAGG) for ornithine decarboxylase (ODC), (5'-CCTCGTGTGCCGCTATCTAG) and (5'-GCATGCGCTGCACAAGGTCA) for γ -glutamylcysteine synthetase (γ -GCS), and (5'-GAAGTACACGGTGGAGGCTG) and (5'-CGCTGATCAGACCTTCTTC) 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 against *GAPDH* was measured as an internal control.

The full-length coding sequence of *AQP1* from *L. infantum* (GeneDB *L. infantum*, accession code LinJ.31.0030) was isolated from the genomic DNA of the clinical isolates by PCR using sense and antisense primers (5'-CAACAGCACGCGCACTTC) and (5'-CAGTCAGCA CGAGCTGTG). A single-band 945 bp PCR product was obtained, cloned in the pGEM[®]-T Vector System (Promega) and sequenced.

2.8. Determination of intracellular levels of non-protein thiols

Intracellular non-protein thiol levels were measured by flow cytometry using CellTracker, as described previously (García-Hernández et al., 2012). Promastigotes (1×10^7 per mL) were washed twice with PBS, incubated with 2 μM CellTracker for 15 min at 37 °C, washed again with PBS and analyzed by flow cytometry using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA) equipped with an argon laser operating at 488 nm. Fluorescence emission between 515 and 545 nm was quantified using Cell Quest software.

2.9. Western blotting

For preparation of total protein lysate, *L. infantum* promastigotes (3×10^7 per mL) were harvested, washed in PBS, lysed by the addition of lysis buffer (2% DDM, 150 mM NaCl, 50 mM Tris-HCl pH 7.4 plus a protease inhibitor cocktail from Sigma) and incubated at 4 °C for 1 h. The clarified lysate was mixed with 2 \times Laemmli sample buffer and resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The samples (25 μg protein per lane) were 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 cytosolic trypanredoxin (cTXN), mitochondrial trypanredoxin (mTXN), cytosolic trypanredoxin peroxidase (cTXNPx), mitochondrial trypanredoxin 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 Sb^{III} of the different isolates of *L. infantum* was analyzed in promastigotes and intracellular amastigotes (Table 1). The EC_{50} 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 Sb^{III} 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

Table 1
Drug resistance profile in *L. infantum* lines.

Drug	<i>L. infantum</i> EC ₅₀ (μM) ± SD (RI)			
	Promastigotes		Amastigotes	
	576-1	576-3	576-1	576-3
Sb ^{III}	17.59 ± 1.75	109.01 ± 13.63 (6.20)*	20.21 ± 2.06	>60 (>3)*
Glucantime [®]	–	–	47.80 ± 6.36	159.33 ± 24.37 (3.30)*
AmB	0.07 ± 0.02	0.04 ± 0.02 (0.57)	0.10 ± 0.01	0.06 ± 0.01 (0.60)
MLF	12.61 ± 1.50	14.72 ± 0.16 (1.16)	3.81 ± 0.30	2.03 ± 0.43 (0.53)
PMM	84.46 ± 9.08	116.05 ± 10.63 (1.37)	34.36 ± 0.76	96.13 ± 10.43 (2.79)*

Promastigotes and intracellular amastigotes of lines 576-1 and 576-3 were grown for 72 h at 28 °C or 37 °C in the presence of increasing drug concentrations: Sb^{III}, Glucantime[®], amphotericin B (AmB), miltefosine (MLF) and paromomycin (PMM). Resistance indexes (RI) were calculated by dividing the EC₅₀ for line 576-3 by that line 576-1. Data are the means ± SD of three independent experiments. Significant differences were determined using the Student's *t*-test (*, *p* < 0.005). –, not determined.

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-leishmania 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 more appropriate approach for susceptibility studies, as 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 Sb^{III} 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 Sb^{III} for 1 h. Sb^{III} accumulation was found to be significantly lower (90%) in the resistant line 576-3 compared to line 576-1 (Fig. 1A). The lower Sb^{III} 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 Sb^{III} was found to be time-dependent and similar in both

Leishmania lines (Fig. 1B), thus confirming that the lower Sb^{III} accumulation was not due to increased efflux activity, but rather a decrease in drug uptake.

In *Leishmania*, antimony uptake is mediated by the transporter AQP1 (Gourbal et al., 2004). Previous studies indicated AQP1 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 AQP1 transcript correlated well with the accumulation of Sb^{III} and resistance levels in *Leishmania* parasites (Marquis et al., 2005). AQP1 gene expression in lines 576-1 and 576-3 was analyzed by RT-PCR to ascertain whether there was a correlation between AQP1 gene expression and antimony susceptibility and accumulation. The results showed that AQP1 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 AQP1 have been reported in antimony-resistant *Leishmania* parasites (Monte-Neto et al., 2015). The full-length coding sequence of the *L. infantum* AQP1 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 AQP1 activity.

Our data therefore support the suggestion that down regulation of AQP1 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.

3.4. Upregulation of thiol metabolic pathway in antimony resistance

An increase in thiol levels is considered to be one detoxification mechanism described in antimony-resistant *L. donovani* isolates (Mandal et al., 2007; Mittal et al., 2007; Mukhopadhyay et al., 2011;

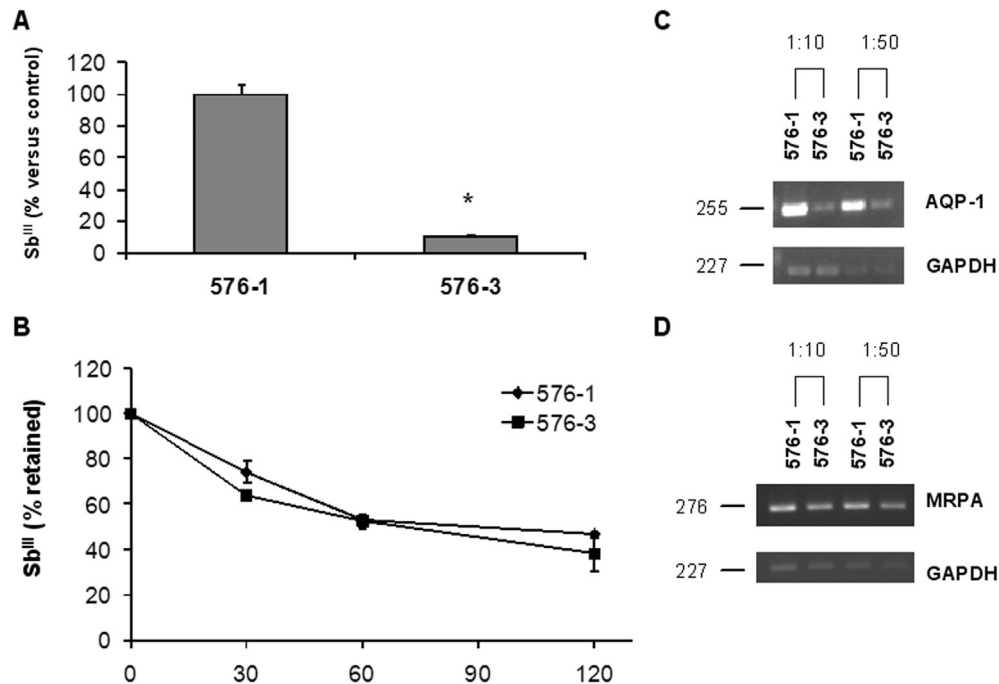


Fig. 1. Accumulation and efflux of Sb^{III} in *L. infantum* lines. (A) Promastigotes (1×10^8 /mL) of lines 576-1 (antimony susceptible) and 576-3 (antimony resistant) were incubated with 100 μ M Sb^{III} for 1 h. Antimony accumulation was measured by ICP-MS. The efflux assay (B) was performed for 1 h after incubation of lines with compensated concentrations of Sb^{III} to ensure similar labeling. The parasites were then washed and resuspended in PBS buffer without Sb^{III} and pelleted at different times. The data are the means \pm 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.

Rai et al., 2013). In order to study the involvement of thiols in the antimony resistant line, we first analyzed the total non-protein thiols using CellTracker. The results, quantified in terms of relative fluorescence units (RFU), demonstrate that the resistant line 576-3 showed 1.5-fold higher intracellular non-protein thiol levels (239.17 ± 38.43 RFU) than the susceptible line 576-1 (152.57 ± 23.38 RFU), and the difference showed statistical significance ($p < 0.005$).

Trypanothione (T[SH]₂), a conjugate of glutathione (GSH) and spermidine, is one of the major reduced thiols in *Leishmania* parasites (Fairlamb and Cerami, 1992). Increased levels of T[SH]₂ are achieved through overexpression of γ -GCS, the rate limiting enzyme of GSH biosynthesis, and ODC, an enzyme involved in spermidine biosynthesis (Grondin et al., 1997; Légaré et al., 1997; Haimeur et al., 1999; Guimond et al., 2003). Mechanisms of antimony resistance based on T[SH]₂ overproduction through overexpression of γ -GCS/ODC have previously been described in both experimental *Leishmania* antimony-resistant lines and in some clinical isolates of antimony-resistant *L. donovani* (Haimeur et al., 1999; Decuyper et al., 2005; Carter et al., 2006; Mukherjee et al., 2007; Goyeneche-Patino et al., 2008; Mandal et al., 2010; Wyllie et al., 2010). This led us to probe the possible relationship between intracellular thiol content and the gene expression status of γ -GCS, ODC and T[SH]₂ synthetase (TryS). The results show that ODC exhibited increased RNA levels (around 2-fold) in the antimony-resistant line 576-3 compared to the susceptible line 576-1 (Fig. 2A), whereas γ -GCS and TryS expression levels were unaffected (Fig. 2). Therefore, overexpression of ODC may contribute to increased levels of T[SH]₂ in the 576-3 antimony-resistant parasites. The above results concur with previous reports that revealed an increased expression of ODC in antimony-resistant isolates of *L. donovani* (Mukherjee et al., 2007).

In trypanosomatids, T[SH]₂ is involved in the maintenance of the intracellular reducing environment. TR is a flavoenzyme that catalyzes the NADPH-dependent reduction of trypanothione disulfide (TS₂) to T[SH]₂. Previous reports have described the amplification of the TR gene in antimony-resistant *L. donovani* field isolates (Mittal et al., 2007). Using Western blot analysis we observed a 4-fold increase in the levels of TR protein in the resistant line versus the susceptible one (Fig. 2B). Increased levels of TR will help to maintain increased levels of T[SH]₂, which is required for conjugation with antimony and to overcome the inhibitory effect of Sb^{III}.

In *Leishmania* the trypanredoxin/trypanredoxin peroxidase (TXN/TXNPx) system is crucial to defend parasites against the oxidative stress that can be found in different compartments (Flohé 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 (Matrangola 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 Sb^{III} (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 Sb^{III}, thus

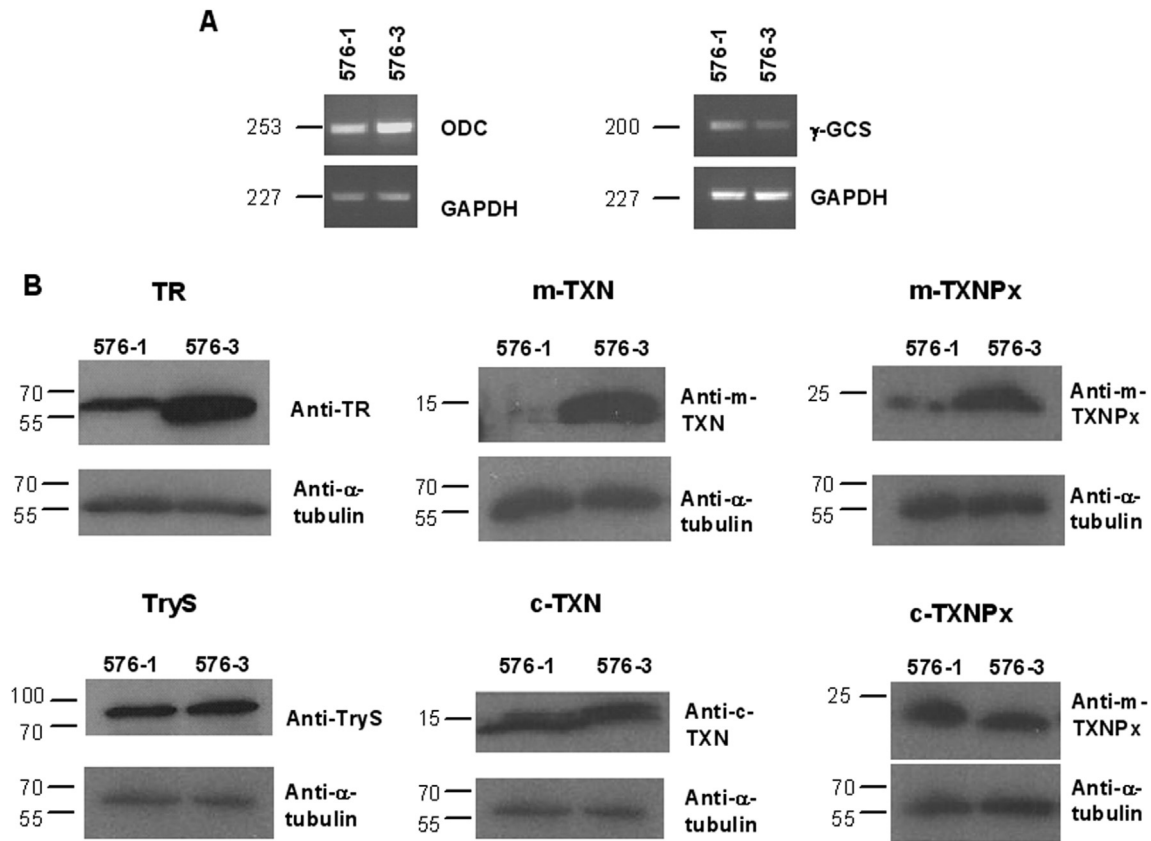


Fig. 2. Expression of genes related to thiol metabolism. (A) Gene expression analysis of ODC and γ -GCS in *L. infantum* lines. Total RNA was extracted from lines 576-1 (antimony susceptible) and 576-3 (antimony resistant). Upper panels: gene expression by RT-PCR as indicated by the amplified 253-bp ODC and 200-bp γ -GCS fragments. Lower panels: gene expression of GAPDH as internal loading control showing the amplified 227-bp GAPDH fragment. RT-PCR assay shown is representative of at least three independent experiments. The positions of molecular markers (bp) are indicated on the left. (B) Western blot analysis of thiols metabolizing enzymes TR, TryS, m-TXN, c-TXN, m-TXNPx, c-TXNPx in *L. infantum* lines. Anti- α -tubulin antibodies were used for loading control. Western blots shown are representative of at least three independent experiments. The positions of molecular markers (kDa) are indicated on the left.

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 Sb^{III} by significantly decreasing expression of the AQP1 transporter which leads to a reduction in intracellular accumulation of Sb^{III}. Other factors including an increase in thiols levels and overexpression of enzymes involved in thiol metabolism could also contribute to the Sb^{III} 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. However, the use of naturally infected dogs has limitations mainly due to the economic costs and the requirements and controls to prevent new reinfections and follow-up analyses for drug efficacy.

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