

Research article

***In vitro* susceptibility to pentavalent antimony in *Leishmania infantum* strains is not modified during *in vitro* or *in vivo* passages but is modified after host treatment with meglumine antimoniate**

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Abstract

Background: Leishmaniasis is a common parasitic disease in Southern Europe, caused by *Leishmania infantum*. The failures of current treatment with pentavalent antimonials are partially attributable to the emergence of antimony-resistant *Leishmania* strains. This study analyses the *in vitro* susceptibility to pentavalent antimony of intracellular amastigotes from a range of *L. infantum* strains, derived from the same infected animal, during *in vitro* and *in vivo* passages and after host treatment with meglumine antimoniate.

Results: Sb^V-IC₅₀ values for strains from two distinct isolates from the same host and one stock after two years of culture in NNN medium and posterior passage to hamster were similar (5.0 ± 0.2 ; 4.9 ± 0.2 and 4.4 ± 0.1 mgSb^V/L, respectively). In contrast, a significant difference ($P < 0.01$, t test) was observed between the mean Sb^V-IC₅₀ values in the stocks obtained before and after treatment of hosts with meglumine antimoniate (4.7 ± 0.4 mgSb^V/L vs. 7.7 ± 1.5 mgSb^V/L). Drug-resistance after drug pressure in experimentally infected dogs increased over repeated drug administration (6.4 ± 0.5 mgSb^V/L after first treatment vs. 8.6 ± 1.4 mgSb^V/L after the second) ($P < 0.01$, t test).

Conclusions: These results confirm previous observations on strains from *Leishmania*/HIV co-infected patients and indicate the effect of the increasing use of antimony derivatives for treatment of canine leishmaniasis in endemic areas on the emergence of *Leishmania* antimony-resistant strains.

Background

Leishmaniasis, a protozoan parasitic disease, is endemic in 88 countries, with an estimated yearly incidence of 1–1.5 million cases of cutaneous leishmaniasis and 500,000 cases of visceral leishmaniasis. Three hundred and fifty million people are estimated to be at risk, and there is an overall prevalence of 12 million cases [1]. Zoonotic visceral leishmaniasis occurs mainly in Latin America, the Med-

iterranean Basin and Asia. The parasite responsible for this disease is *Leishmania infantum* and the dog is the main reservoir. Leishmaniasis has re-emerged in recent years because of the increase in risk factors, some of which are related to HIV infection and intravenous drug use. In Western Europe the cumulative number of cases of *Leishmania*/HIV co-infections, reported to the WHO up to December 1999 was 1627 [2]. Canine leishmaniasis in

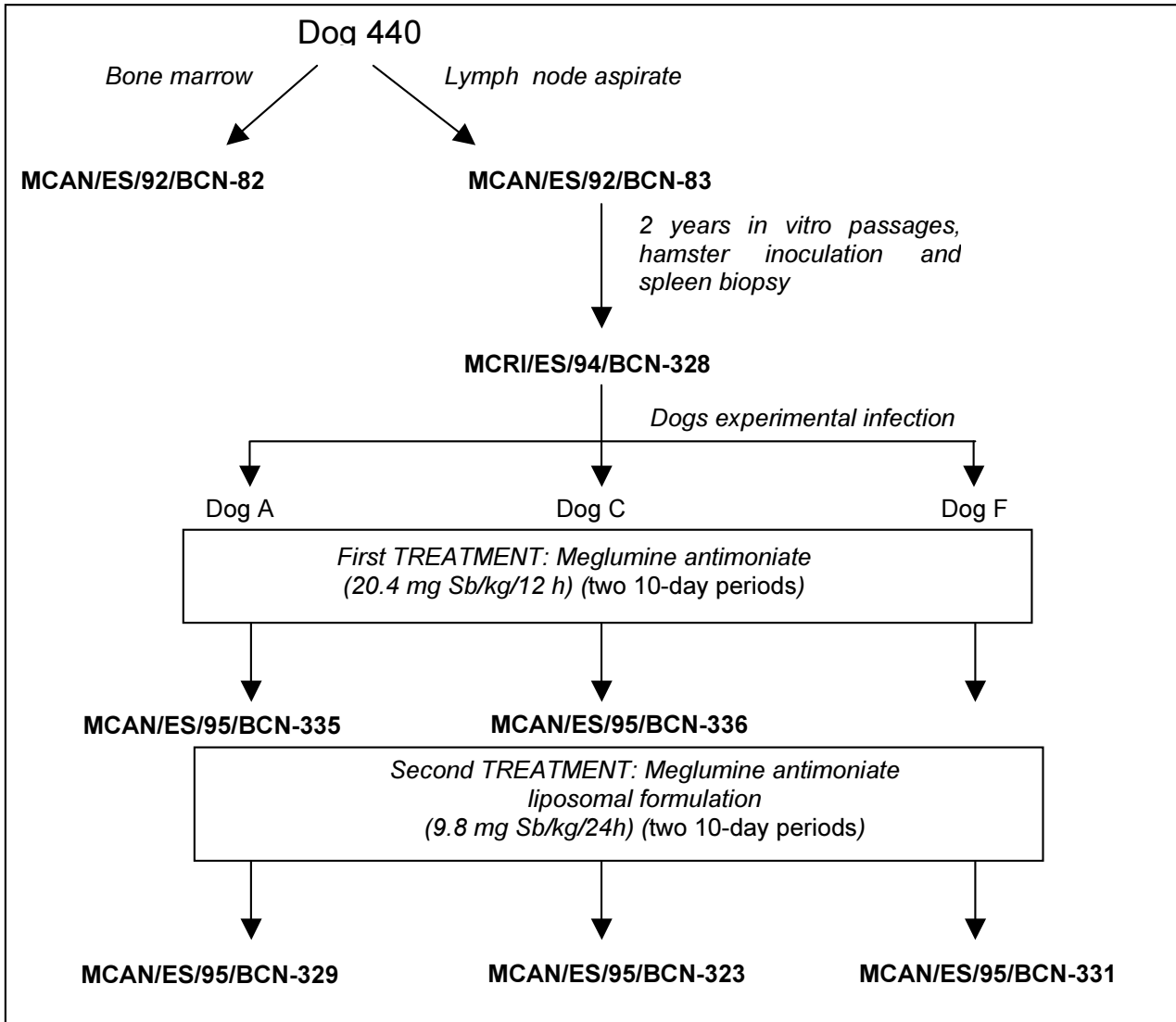


Figure 1

Flow chart for the origin of *L. infantum* stocks, derived from the same infected dog, after several *in vitro* and *in vivo* passages.

endemic areas is a serious veterinary and public health problem. The dog is one of the main hosts responsible for the spread of visceral leishmaniasis, which was initially only in rural areas, but is now extending to suburbs. Significant foci are located on the periphery of cities where small gardens encourage the presence of sandfly vectors [2]. The main treatment for dogs is through pentavalent antimonials. Nevertheless these drugs usually produces only temporary remission of clinical signs, and relapses are frequent [3]. Other alternatives such as amphotericin B [4], aminosidine [5] and allopurinol [6] have also

shown variable results. The ineffectiveness of treatment for cutaneous and visceral leishmaniasis is attributable to host physiology, the compound and its preparation and the susceptibility of the parasite strain to the drugs [7-11].

Grogl et al. [12] induced promastigote resistance to Pentostam *in vitro* in *Leishmania* strains from American cutaneous leishmaniasis, by discontinuous exposure of promastigotes to the drug. These authors observed that after repeated *in vitro* passages and *in vivo* infection, resistance was stable in the absence of drug pressure. The

decrease in Sb^V susceptibility *in vitro* of intracellular amastigotes of *Leishmania* strains obtained during a second episode of anthroponotic [10] or zoonotic human visceral leishmaniasis [9,13] has also been observed. In addition, Gramiccia et al [3] observed that the *in vivo* activity of Sb^V in mice infected with *L. infantum* strains from dogs treated with meglumine antimoniate was lower than in mice infected with strains obtained before the dogs were treated.

Here we evaluated the *in vitro* susceptibility to Sb^V of intracellular amastigotes of *L. infantum* strains, derived from one infected animal. These strains were obtained in a number of studies that involved repeated *in vitro* cultivation, hamster and canine experimental infections and two courses of treatment with meglumine antimoniate. This study aims to determine the stability of *in vitro* Sb^V susceptibility of *L. infantum* stocks during successive *in vitro* and *in vivo* passages and the role of host treatment in this susceptibility.

Results

The *in vitro* susceptibility to Sb^V of two *L. infantum* strains (MCAN/ES/92/BCN-82 and MCAN/ES/92/BCN-83), simultaneously isolated from bone marrow and lymph node aspirate from the same dog were almost identical (IC₅₀: 5.0 ± 0.2 and 4.9 ± 0.2 mgSb^V/L). Also, the Sb^V-IC₅₀ for MCRI/ES/94/BCN-328 (4.4 ± 0.1 mg/L) was very similar to that of the original strain (BCN-83) (Figure 1), despite repeated *in vitro* passages and hamster inoculation.

The mean IC₅₀ in these strains from untreated hosts (BCN-82, BCN-83 and BCN-328) (4.7 ± 0.4 mgSb^V/L) was lower ($P < 0.01$, t test) than in five strains (BCN-335, BCN-336, BCN-323, BCN-329 and BCN-331) isolated after dogs had been treated with meglumine antimoniate (7.7 ± 1.5 mgSb^V/L). The IC₅₀ was also lower in strains obtained after one treatment course with meglumine antimoniate (BCN-335 and BCN-336) (6.4 ± 0.5 mgSb^V/L) than in strains obtained after a second course with this drug (BCN-323, BCN-329 and BCN-331) (8.6 ± 1.4 mgSb^V/L) (Table 1).

The promastigote growing capacity of strains BCN-82, BCN-83 and BCN-328 was higher than in strains from dogs treated with meglumine antimoniate (Table 1).

Discussion and Conclusions

Leishmania isolates from an infected host have a multi-clonal composition with an heterogeneous expression of different phenotypes such as drug resistance [12], virulence [14] or growth capacity *in vitro*. The variations in clonal composition difficult comparison and interpretation of results from studies performed with different strains and, even, with the same strain after repeated pas-

Table 1: Growth rate of promastigotes and Sb^V susceptibility of intracellular amastigotes, of *L. infantum* strains, after repeated *in vitro* and *in vivo* passages and dogs treatment with meglumine antimoniate.

Strain Code	Growth ^a	IC 50 (mg Sb ^V /L) ^b	IC 50 Mean values
MCAN/ES/92/BCN 82	61	5.0 ± 0.2	Untreated animals
MCAN/ES/92/BCN 83	69	4.9 ± 0.2	4.7 ± 0.4
MCRI/ES/94/BCN 328	57	4.4 ± 0.1	
MCAN/ES/95/BCN 335	24	6.7 ± 0.5	
MCAN/ES/95/BCN 336	22	6.0 ± 0.2	Treated animals
MCAN/ES/98/BCN 323	29	9.7 ± 0.3	7.7 ± 1.5
MCAN/ES/98/BCN 329	31	9.1 ± 0.6	
MCAN/ES/98/BCN 331	28	6.9 ± 0.5	

a: promastigotes × 10⁶/mL Schneider's medium + 20% HIFCS, after three days of culture b: values are the means ± the standard deviation of three counts in two separate assays (n = 6)

sages *in vitro* or *in vivo*. Differences in the expression of certain phenotypes, such as "virulence" or "growth rate *in vitro*" may modify the clonal composition of the strain during successive passages. This, in turn, may modify the expression of other phenotypes like drug resistance.

In our study no differences were observed in Sb^V susceptibility of intracellular amastigotes of *L. infantum* strains isolated from untreated animals, despite repeated *in vitro* passages and hamster infection. In contrast, Sb^V susceptibility was lower in strains isolated after treatment with meglumine antimoniate of experimentally infected dogs. It is worth emphasising that the Priorat, where the strain was first isolated, is a rural region, with a high prevalence of canine leishmaniasis [15] and where dogs were rarely treated at the time when the first isolation was performed. We can thus assume that the drug pressure received by strains BCN 82 and BCN-83 was very low.

The *in vitro* growth capacity of *Leishmania* varies from one isolate to another and isolates that are initially difficult to grow adapt to the culture after repeated passages, probably because of the selection of phenotypes with higher growing rate. The promastigote growth rate in NNN and Schenider's culture media of all strains isolated after treatment was lower than in the original strain (BCN-83), without modification in the course of successive passages. This suggests that the selected clones more resistant to Sb^V also had a different promastigote growth rate phenotype

Our results correlates with previous studies performed with other designs, methodologies and *Leishmania* species

[3,9,10,12,13]. The study reinforces that the Sb^V susceptibility of *Leishmania* strains is stable over *in vitro* and *in vivo* passages, in the absence of drug pressure. Moreover, Sb^V susceptibility decreases after host treatment with meglumine antimoniate. We conclude that the increasing use of antimony derivatives in veterinary practice in Southern Europe will lead to a rapid overspread of *L. infantum* Sb^V-resistant strains.

Material and Methods

Parasites and drug

L. infantum strains BCN-82 and BCN-83 were isolated from bone marrow and lymph node aspirate from an asymptomatic dog, from the Priorat region (Spain), a highly endemic area for canine leishmaniasis [15]. BCN-83 was maintained for almost two years by repeated passages in NNN medium, it was then inoculated by intraperitoneal injection into one hamster. After three months the parasite was isolated again from the hamster spleen in NNN medium (coded as BCN-328) and promastigotes were used to experimentally infect dogs [16] for subsequent pharmacokinetic studies [17,18].

Dogs were treated with meglumine antimoniate (Glucantime, Rhône Mérieux) (20.4 mg of Sb/kg/12 h), for two 10-day periods, each separated by an interval of 10 days [17].

After a temporary remission of the symptoms, all dogs relapsed and two isolates were obtained from dogs A (BCN-335) and C (BCN-336). All dogs were treated again using a meglumine antimoniate liposomal formulation at a dose equivalent to 9.8 mgSb/kg/24 h for two 10-day periods, separated by an interval of 10 days [18]. Positive cultures were obtained again from dogs A (BCN-329), C (BCN-323) and F (BCN-331) at a range of times after treatment. (Figure 1)

Parasite isolation and *in vitro* maintenance was done through passages in NNN medium. Promastigotes from NNN were cultured in Schneider's insect medium at 26°C with 20% heat inactivated foetal calf serum (HIFCS). Their growth rate was then calculated, to establish the optimal conditions for *in vitro* susceptibility tests [19].

The pentavalent antimony used in *in vitro* assays was a solution of Sb^V in 8% hydrochloric acid at a concentration of 1 mg/mL (Varian Associates, Inc. Palo Alto, CA). Further working dilutions were performed in culture media. Previous assays demonstrated that the distinct working dilutions of HCl have no effect on the growing capacity of cultures [19].

Susceptibility test

The *in vitro* susceptibility tests to Sb^V were performed on intracellular amastigotes, cultured in the murine monocyte-macrophage cell line RAW 264.7 (American Type Culture Collection) in RPMI-1640 medium (Bio-Whittaker 12-1115; Boehringer-Ingelheim, Verriers, Belgium) with 10% of HIFCS in a 8 LabTeck Chamber Slide System (Nalge Nunc, Hamburg, Germany) as described previously [19]. Cells exposed to serial dilutions of the drug were cultured for 2 days at 37°C in a 5% CO₂ atmosphere. Drug activity was evaluated by calculating the percentage of infected cells. Counting was performed at three places in the well and each assay was performed twice.

The concentration of Sb^V that produced a 50% reduction in infected cells (IC50) was determined from least-squares linear regression of growth rate or percentage *vs.* log antimony concentration.

Student's unpaired test was used to determine the statistical significance of the values obtained.

Authors contributions

Author JC carried out the studies on susceptibility, performed the statistical analysis and together with MP conceived the study and its design.

Both authors read and approved the final manuscript.

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