

Transmission Potential of Antimony-Resistant *Leishmania* Field Isolates

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We studied the development of antimony-resistant *Leishmania infantum* in natural vectors *Lutzomyia longipalpis* and *Phlebotomus perniciosus* to ascertain the risk of parasite transmission by sand flies. All three resistant strains produced fully mature late-stage infections in sand flies; moreover, the resistant phenotype was maintained after the passage through the vector. These results highlight the risk of circulation of resistant *Leishmania* strains and question the use of human drugs for treatment of dogs as *Leishmania* reservoirs.

Drug-resistant microorganisms are currently a major global health threat. They are the main cause of nonresponse to treatment, prolonged illness, and higher risk of death. Furthermore, they seriously reduce treatment options. For visceral leishmaniasis, a vector-borne and widespread disease with potentially fatal outcomes, treatment alternatives are limited and largely dictated by economic conditions (1). The worldwide emergence of *Leishmania* resistance to the most widely used antileishmanial agent, antimonials, represents a threatening problem (2, 3, 4, 5). In leishmaniasis, various environmental factors can contribute to the selection of antimony (Sb)-resistant strains (6, 7, 8). Several field studies suggest a probability of natural cyclical transmission of resistant strains to explain the observed epidemiological patterns in different regions of the world (9, 10). In Algeria, where antimony drug pressure is sought to be low, antimony-resistant *Leishmania infantum* strains have been repeatedly isolated from dogs (11), which serve as a main reservoir of visceral leishmaniasis. Previously, it was shown in *in vitro* experiments that selection for drug resistance affects the amastigote-promastigote differentiation process of *Leishmania* (12) and is associated with competitive cost (13). In addition, a higher fitness of Sb-resistant *Leishmania donovani* strains corresponding to an increased number of metacyclic stages has been described (14). However, many *Leishmania* strains developed efficiently in the culture but did not produce late-stage infection in the sand fly vector (15). During passage through the sand fly gut, *Leishmania* must overcome several barriers which are critical for successful completion of the parasite life cycle (16).

Strains of *L. infantum* isolated from dogs that have undergone several courses of antimonial therapy were shown to grow in *Phlebotomus perniciosus* sand flies used for xenodiagnosis in these dogs (17), and this finding raised questions about circulation of these strains in nature and their transmissibility to humans (18). While these studies dealt with resistant strains under strong drug pressure, there is no study performed on antimony-resistant strains isolated in areas where antimony is not the primary pressure source, like the ones that have been isolated in Algeria and South America (11, 19).

Thus, we investigated in detail the development of antimony-resistant *L. infantum* parasites in *Lutzomyia longipalpis* and *Phlebotomus perniciosus*, two natural sand fly vectors of visceral leishmaniasis (20). Three antimony-resistant (LEM5285-R, LEM5696-R, and LEM5695-R) and three antimony-susceptible (LEM5680-S, LEM5684-S, and LEM5688-S) *L. infantum* strains, isolated from naturally infected dogs originating from various localities in the Algiers region (Algeria), where leishmaniasis is endemic (11), were used; two of them belong to the MON-1 zymodeme and four to the MON-281 zymodeme (Table 1) (21). We classified these strains as resistant ones because they express 50% inhibitory concentrations (IC₅₀s) toward trivalent derivatives of Sb (SbIII) and pentavalent derivatives of Sb (SbV) that are 2- to 3-fold higher than the IC₅₀s determined for 50 *L. infantum* strains isolated in France and Algeria (11, 22). Before passage through the sand fly, susceptibilities of promastigotes and amastigotes were measured twice within a 6-month interval to confirm that the resistant phenotype is stable.

The susceptibility of *Leishmania* promastigote forms to SbIII was determined by cell counting using a flow cytometer as previously described (11). Briefly, parasites were incubated with increasing concentrations of SbIII, ranging from 3.12 to 50 µg/ml, for 3 days. Dead cells were discriminated from the whole population by propidium iodide staining and counted on a flow cytometer (FACSCalibur; Becton Dickinson). As summarized in Table 1, the IC₅₀s of promastigotes to SbIII vary between 7.0 and 10.6 µg SbIII/ml for susceptible strains, compared to IC₅₀s of 15.2 to 28.3 µg SbIII/ml for resistant strains.

To confirm these results, we also ascertained the susceptibility

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TABLE 1 Antimony susceptibility of *L. infantum* before and after experimental infections of *P. perniciosus*^a

<i>L. infantum</i> isolate	WHO code	Zymodeme	Mean IC ₅₀ ± SD (µg/ml)			
			SbIII		SbV	
			Pro(In)	Pro(Pern)	Ama(In)	Ama(Pern)
LEM5680-S	MCAN/DZ/2008/ENV34	MON-1	7.0 ± 0.5	ND	30.1 ± 5.0	ND
LEM5684-S	MCAN/DZ/2008/ENV38	MON-1	9.0 ± 0.5	10.5 ± 2.9	35.0 ± 4.0	29.3 ± 3.2
LEM5688-S	MCAN/DZ/2008/ENV41	MON-281	10.6 ± 1.0	8.5 ± 1.0	25.7 ± 9.0	34 ± 6.0
LEM5285-R	MCAN/DZ/2006/ENV6	MON-281	25 ± 1.0	23.4 ± 1.0	>100	>100
LEM5695-R	MCAN/DZ/2008/ENV48	MON-281	15.2 ± 3.4	ND	60 ± 3.1	ND
LEM5696-R	MCAN/DZ/2008/ENV49	MON-281	28.3 ± 4.6	ND	>100	ND

^a The susceptibility of promastigotes to SbIII before [Pro(In)] and after [Pro(Pern)] development in *P. perniciosus* and susceptibility of intramacrophagic amastigotes to SbV before *P. perniciosus* infection [Ama(In)] and amastigotes derived from promastigotes after *P. perniciosus* infection [Ama(Pern)]. Results are mean values from three independent experiments. Shading indicates results obtained in experiments with the three Sb-resistant *L. infantum* strains. ND, not determined.

of intracellular amastigotes (the clinically relevant stage of *Leishmania*) to SbV. Briefly, THP-1 monocytes were differentiated into macrophages in a 16-well chamber (LABTECKII; Nunc) by adding 20 ng/ml of phorbol myristate acetate (Sigma) for 48 h. Macrophages were washed two times with RPMI medium (Life Technology) and infected for 4 h at a parasites/host cell ratio of 10:1. Then, 12.5, 25.0, 50.0, or 100.0 µg SbV/ml was added. Drugs were renewed after 48 h, and after 120 h macrophages were fixed with methanol and stained with Giemsa to evaluate the susceptibility to SbV antimony. As shown in Table 1, three isolates belonging to the MON-281 zymodeme were characterized as antimony (SbV) resistant, with IC₅₀s greater than 100 µg SbV/ml for LEM5285-R and LEM5696-R and an IC₅₀ of 60.0 ± 3.1 µg SbV/ml for LEM5695-R. Susceptible isolates expressed IC₅₀s for SbV between 25.7 and 35.0 µg SbV/ml (Table 1).

Then, we compared the intravectorial development of resistant and susceptible strains. Three Sb-resistant and three Sb-susceptible *L. infantum* strains were used for experimental infection of *Lutzomyia longipalpis* females. In each experiment, approximately 150 sand fly females (5 to 7 days old) were fed through a chick skin membrane on heat-inactivated rabbit blood containing 10⁶ promastigotes/ml. Engorged females were separated, maintained at 26°C under conditions described previously (23), and dissected at days 2, 8, and 9 after blood meal (pbm). Localization and intensity of *Leishmania* infection in sand fly midguts were estimated *in situ* under a light microscope, by scoring the proportions of sand flies with weak (<100 parasites/gut), moderate (100 to 1,000 to parasites/gut), and heavy (>1,000 parasites/gut) infections (24). All experiments were repeated twice. The χ² test was used for comparison of infection rates and intensities using the STATISTICA 6.0 program (StatSoft, Inc.). On day 2, no differences were observed in the development of Sb-resistant and Sb-susceptible *L. infantum* strains ($P = 0.46$), with both phenotypes showing high infection rates (>80%). Most sand fly females produced heavy or moderate infection in their abdominal midgut, with parasites enclosed within the peritrophic matrix. In late-stage infection, days 8 and 9 pbm, parasites migrated anteriorly to the thoracic midgut (infection rates above 70%) and colonized the stomodeal valve in 70 to 90% of infected females. All tested strains produced mature late-stage infection with metacyclic promastigotes, and no significant differences in infection intensities were observed between Sb-resistant and Sb-susceptible strains ($P = 0.27$) (Fig. 1).

To test the stability of the Sb resistance phenotype after development in sand flies, we isolated one resistant strain

(LEM5285-R) and two susceptible strains (LEM5688-S and LEM5684-S) from the guts of *P. perniciosus* females with mature infections (on day 9 pbm). *P. perniciosus* females were infected using the same methods described above for *Lutzomyia longipalpis*. Sand fly-derived promastigotes were transferred into SDM79 medium supplemented with 10% fetal calf serum (FCS) and antibiotics (amikacin, penicillin, and fluorocytosine) in order to prevent bacterial and fungal growth (25). Seven to 10 days after isolation from the sand fly gut, their susceptibilities to SbIII and SbV were tested as described above. We also checked the SbV resistance of intramacrophagic amastigotes derived from gut-isolated promastigotes. As shown in Table 1, the cyclical passage of the LEM5384-R *Leishmania* strain through sand fly did not alter its SbIII or SbV antimony resistance profiles. As suspected, the cyclical passages of susceptible strains (LEM5688-S and LEM5684-S) did not change their antimony susceptibility.

The worldwide increase in drug resistance frequency among the *Leishmania* population, including areas where antimonial pressure is low (6), and the description of fitness advantages in Nepalese Sb-resistant *L. donovani* strains (26) raise concerns about the risk of spreading of such strains. Here, we confirmed previous finding by Gradoni et al. (17) that Sb-resistant *L. infantum* strains grow in sand flies. Importantly, we studied in detail the development of *L. infantum* originating from an area with a low drug pressure, and for the first time we showed that resistant strains are comparable to the resistant ones in all studied aspects of the vectorial part of the life cycle. For the first time, we demonstrated that the Sb resistance phenotype was maintained after passage through the natural sand fly under conditions without any drug pressure. The most important prerequisite for successful transmission, a high number of parasites colonizing the stomodeal valve and the thoracic part of the sand fly midgut, was achieved in all the strains tested. However, this aspect should be confirmed in the future using additional field-isolated antimony-resistant strains.

Nevertheless, our results highlight the risk of circulation of resistant *Leishmania* strains and also question the use of human drugs for treatment of dogs as *Leishmania* reservoirs. Treatment of canine and human leishmaniasis, both based on antimonial compounds, provide favorable conditions for selection and long-lasting circulation of resistant strains, particularly in areas where leishmaniasis is endemic and where no cost-effective therapeutic alternatives are available. As mentioned by Ait-Oudhia et al. (27), further work should be done in order to verify the epidemiological

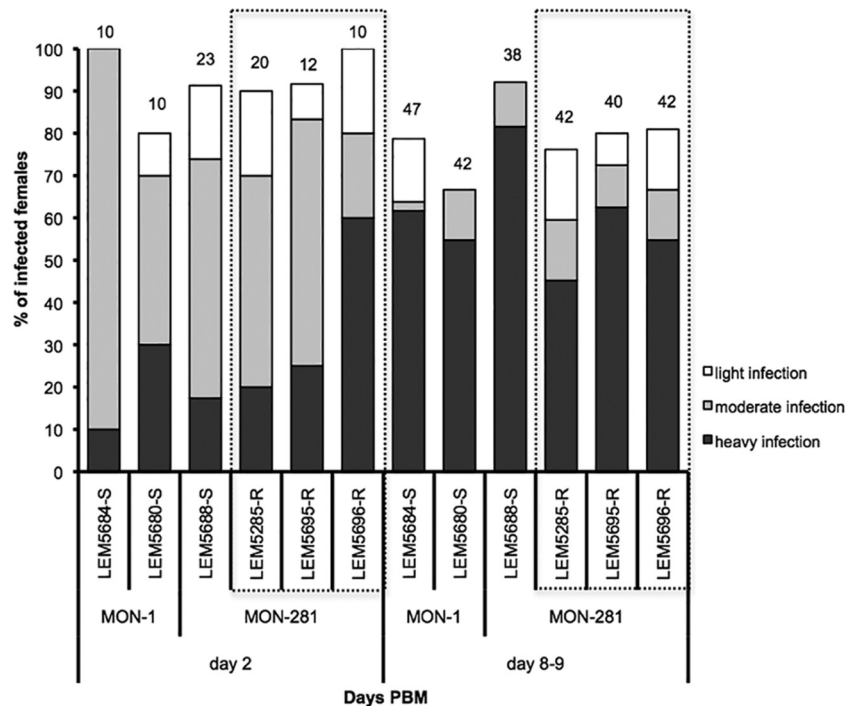


FIG 1 Development of antimony-resistant (box with dashed lines) and antimony-susceptible *L. infantum* strains in *Lutzomyia longipalpis*. Infection rates and intensities of infection by Sb-susceptible (LEM5684-S, LEM5680-S, LEM5688-S) and Sb-resistant (LEM5285-R, LEM5695-R, LEM5696-R) *L. infantum* strains in *Lutzomyia longipalpis* determined at day 2 and days 8 and 9 post blood meal (PBM). Numbers above the bars indicate the number of dissected females.

consequences of the natural occurrence of *L. infantum* antimony resistance in field conditions and their impact on the emergence and spreading of chemoresistance and consequently on control of the disease.

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REFERENCES

- Vanlerberghe V, Diap G, Guerin JP, Meheus F, Gerstl S, Van der Stuyft P, Boelaert M. 2007. Drug policy for visceral leishmaniasis: a cost-effectiveness analysis. *Trop. Med. Int. Health* 12:274–283. <http://dx.doi.org/10.1111/j.1365-3156.2006.01782.x>.
- Abdo MG, Elamin WM, Khalil EAG, Mukhtar MM. 2003. Antimony-resistant *Leishmania donovani* in eastern Sudan: incidence and *in vitro* correlation. *East Mediterr. Health J.* 9:837–843.
- Hadighi R, Mohebbi M, Boucher P, Hajjarian H, Khamesipour A, Ouellette M. 2006. Unresponsiveness to glucantime treatment in Iranian cutaneous leishmaniasis due to drug-resistant *Leishmania tropica* parasites. *PLoS Med.* 3:e162. <http://dx.doi.org/10.1371/journal.pmed.0030162>.
- Mittal MK, Rai S, Ashutosh Ravinder Gupta S, Sundar S, Goyal NN. 2007. Characterization of natural antimony resistance in *Leishmania donovani* isolates. *Am. J. Trop. Med. Hyg.* 76:681–688.
- Rojas R, Valderrama L, Valderrama M, Varona MX, Ouellette M, Saravia NG. 2006. Resistance to antimony and treatment failure in human *Leishmania (Viannia)* infection. *J. Infect. Dis.* 193:1375–1383. <http://dx.doi.org/10.1086/503371>.
- Sereno D, Maia C, Ait-Oudhia K. 2012. *Leishmania* antimony resistance and environment: elusive links to explore. *Int. J. Parasitol.* 2:200–203. <http://dx.doi.org/10.1016/j.ijpdr.2012.07.003>.
- Ait-Oudhia K, Gazanion E, Vergnes B, Oury B, Sereno D. 2011. *Leishmania* antimony resistance: what we know what we can learn from the field. *Parasitol. Res.* 109:1225–1232. <http://dx.doi.org/10.1007/s00436-011-2555-5>.
- Perry MR, Wyllie S, Raab A, Feldmann J, Fairlamb AH. 2013. Chronic exposure to arsenic in drinking water can lead to resistance to antimonial drugs in a mouse model of visceral leishmaniasis. *Proc. Natl. Acad. Sci. U. S. A.* 110:19932–19937. <http://dx.doi.org/10.1073/pnas.1311535110>.
- Adaui V, Maes I, Huyse T, Van der Broeck F, Talledo M, Kuhls K, De Doncker S, Maes L, Llanos-Cuentas A, Schönian G, Arevalo J, Dujardin JC. 2011. Multilocus genotyping reveals a polyphyletic pattern among naturally antimony-resistant *Leishmania braziliensis* isolates from Peru. *Infect. Genet. Evol.* 11:1873–1880. <http://dx.doi.org/10.1016/j.meegid.2011.08.008>.
- Singh S, Kumar DV, Ramesh Negi NS, Singh S, Salotra P. 2006. Visceral leishmaniasis, or kala azar (KA): high incidence of refractoriness to antimony is contributed by anthroponotic transmission via post-KA dermal leishmaniasis. *J. Infect. Dis.* 194:302–306. <http://dx.doi.org/10.1086/505079>.
- Ait-Oudhia K, Gazanion E, Sereno D, Oury B, Dedet JP, Pratlong F, Lachaud L. 2012. *In vitro* susceptibility to antimonials and amphotericin B of *Leishmania infantum* strains isolated from dogs in a region lacking drug selection pressure. *Vet. Parasitol.* 187:386–393. <http://dx.doi.org/10.1016/j.vetpar.2012.01.034>.
- Sereno D, Guilvard E, Maquaire S, Cavaleyra M, Holzmüller P, Ouassii A, Lemesre JL. 2001. Experimental studies on the evolution of antimony-resistant phenotype during the *in vitro* life cycle of *Leishmania infantum*:

- implications for the spread of chemoresistance in endemic areas. *Acta Trop.* 80:195–205. [http://dx.doi.org/10.1016/S0001-706X\(01\)00154-1](http://dx.doi.org/10.1016/S0001-706X(01)00154-1).
13. Agnew P, Holzmüller P, Michalakis Y, Sereno D, Lemesre JL, Renaud F. 2001. *In vitro* growth of *Leishmania amazonensis* promastigotes resistant to pentamidine is dependent on interactions among strains. *Antimicrob. Agents Chemother.* 45:1928–1929. <http://dx.doi.org/10.1128/AAC.45.6.1928-1929.2001>.
 14. Ouakad M, Vanaerschot M, Rijal S, Sundar S, Speybroeck N, Kestens L, Boel L, De Doncker S, Maes I, Decuypere S, Dujardin JC. 2011. Increased metacyclogenesis of antimony-resistant *Leishmania donovani* clinical lines. *Parasitology* 138:1392–1399. <http://dx.doi.org/10.1017/S0031182011001120>.
 15. Cihakova J, Volf P. 1997. Development of different *Leishmania major* strains in the vector sandflies *Phlebotomus papatasi* and *Phlebotomus duboscqi*. *Ann. Trop. Med. Parasitol.* 91:267–279. <http://dx.doi.org/10.1080/00034989761120>.
 16. Dostalova A, Volf P. 2013. *Leishmania* development in sand flies: parasite-vector interactions overview. *Parasit. Vectors* 5:276. <http://dx.doi.org/10.1186/1756-3305-5-276>.
 17. Gradoni L, Maroli M, Gramiccia M, Macianti F. 1987. *Leishmania infantum* infection rates in *Phlebotomus perniciosus* fed on naturally infected dogs under antimonial treatment. *Med. Vet. Entomol.* 4:339–342.
 18. Gramiccia M, Gradoni L, Orsini S. 1992. Decreased sensitivity to meglumine antimoniate (Glucantime) of *Leishmania infantum* isolated from dogs after several courses of drug treatment. *Ann. Trop. Med. Parasitol.* 86:613–620.
 19. Robledo SM, Valencia AZ, Saravia NG. 1999. Sensitivity to Glucantime of *Leishmania viannia* isolated from patients prior to treatment. *J. Parasitol.* 85:360–366. <http://dx.doi.org/10.2307/3285647>.
 20. Maroli M, Feliciangeli M, Bichaud L, Charrel R, Gradoni L. 2013. Phlebotomine sand flies and the spreading of leishmaniasis and other diseases of public health concern. *Med. Vet. Entomol.* 27:123–147. <http://dx.doi.org/10.1111/j.1365-2915.2012.01034.x>.
 21. Ait-Oudhia K, Lami P, Lesceu S, Harrat Z, Hamrioui B, Dedet JP, Pratlong F. 2009. Increase in the prevalence of canine leishmaniasis in urban Algiers (Algeria) following the 2003 earthquake. *Ann. Trop. Med. Parasitol.* 103:679–692. <http://dx.doi.org/10.1179/000349809X12554106963591>.
 22. Oury B, Diabate M, Gazanion E, Barnabe C, Vergnes B, Garcia D, Pratlong F, Bastien P, Dedet JP, Sereno D. 2008. Chemosensitivity to antimony of *Leishmania infantum* in the region of Montpellier (South of France), p 37–42. *In Dupoy-Camet J, Dei-Cas E (ed), X European Multicolloquium of Parasitology*. Medimond, Paris, France.
 23. Volf P, Volfova V. 2011. Establishment and maintenance of sand fly colonies. *J. Vector Ecol.* 36:S1–S9. <http://dx.doi.org/10.1111/j.1948-7134.2011.00106.x>.
 24. Myskova J, Votypka J, Volf P. 2008. *Leishmania* in sand flies: comparison of quantitative polymerase chain reaction with other techniques to determine the intensity of infection. *J. Med. Entomol.* 45:133–138. [http://dx.doi.org/10.1603/0022-2585\(2008\)45\[133:LISFCO\]2.0.CO;2](http://dx.doi.org/10.1603/0022-2585(2008)45[133:LISFCO]2.0.CO;2).
 25. Sadlova J, Yeo M, Seblova V, Lewis MD, Mauricio I, Volf P, Miles MA. 2011. Visualisation of *Leishmania donovani* fluorescent hybrids during early stage development in the sand fly vector. *PLoS One* 6:e19851. <http://dx.doi.org/10.1371/journal.pone.0019851>.
 26. Vanaerschot M, Decuypere S, Berg M, Roy S, Dujardin JC. 2013. Drug-resistant microorganisms with a higher fitness—can medicines boost pathogens? *Crit. Rev. Microbiol.* 39:384–394. <http://dx.doi.org/10.3109/1040841X.2012.716818>.
 27. Ait-Oudhia K, Gazanion E, Vergnes B, Oury B, Sereno D. 2011. The fitness of antimony-resistant *Leishmania* parasites: lessons from the field. *Trends Parasitol.* 27:141–142. <http://dx.doi.org/10.1016/j.pt.2010.12.003>.