Review

Meta-analysis and discussion on challenges to translate *Leishmania* drug resistance phenotyping into the clinic

Denis Sereno^{1,2*}, Zoubir Harrat³, Naouel Eddaikra³

¹ IRD, Univ Montpellier, MIVEGEC, Montpellier, France;

² IRD, Univ Montpellier, InterTryp, Montpellier, France; denis.sereno@ird.fr.

² Laboratory of Eco-epidemiology and Parasitic Population Genetics, Pasteur Institute of Algier, Algier, Algeria; zharrat@gmail.com, neddaikra@yahoo.fr.

* Correspondence: denis.sereno@ird.fr; Tel.: +33-046-741-6328

Abstract

Antimicrobial resistance (AMR) threatens the prevention and treatment of infections caused by a large range of microorganisms. *Leishmania* is not an exception and treatment failure due to drug-resistant organisms is increasingly reported. Currently, no molecular methods and marker are validated to track drug-resistant organism and antimicrobial susceptibility tests are roughly not amenable to a clinical setting. Taking these facts into account, it is essential to reflect on ways to translate basic knowledge into methodologies aimed to diagnose leishmania drug resistance. As a matter of fact, a meta-analysis of the literature discloses the reliability of the promastigotes antimicrobial susceptibility tests (AST) to predict intracellular amastigotes susceptibility status. Promastigote cultures that are easy to perform, typically inexpensive and amenable to standardization should represent a candidate to diagnose resistance. Using AST performed on promastigote, we propose a way to improve leishmania drug resistance diagnosis in the framework of guidance and guideline of the bacterial drug resistance diagnosis. In this review, we highlight challenges that remained and discuss the definition of clinical breakpoints, including the epidemiological cutoff (ECOFF), to track drug-resistant isolates. Our analysis paves the ways to standardize and analyze anti-leishmania susceptibility tests output in order to guide the characterization of drug-resistant isolates, the clinical decision during treatment and the search for new molecular markers.

Leishmaniasis are caused by obligate unicellular eukaryote parasites belonging to the genus *Leishmania* (Trypanosomatida: Trypanosomatidae). There are twenty-one *Leishmania* species with documented pathogenicity in humans (Akhoundi et al., 2016). These digenetic or heteroxenous parasites have a life cycle that involves two hosts, a vertebrate and an invertebrate (the sandfly). Infection is initiated when metacyclic promastigotes are regurgitated by sandflies having a blood meal on a vertebrate host. The clinical manifestations of leishmaniasis range from simple or diffuse cutaneous leishmaniasis (CL), mucocutaneous (MCL), mucosal (ML), post-Kala-azar dermal (PKDL) and visceral (VL) leishmaniasis (Akhoundi et al., 2017). These diseases rank after malaria in term of annual incidence and affect 98 countries and territories worldwide. Visceral leishmaniasis kill between 20 000 to 30 000 persons annually, 1 millions cutaneous leishmaniasis cases reported during the past 5 years and, over 1 billion peoples living in endemic area at risk of infection (Alvar et al., 2012; <http://www.who.int/leishmaniasis/en/>).

Heterogeneity in clinical presentation, disease progression, complications and the response to treatment with various etiological agents of patients with leishmaniasis, makes the choice of therapy sometimes problematic. Guidelines for the treatment of the different clinical forms are published and include only a few a drugs: meglumine antimoniate, sodium stibogluconate, amphotericin B, miltefosine, pentamidine and paromomycin (Aronson et al., 2016; Gradoni et al., 2017; Uliana et al., 2017; Alves et al., 2018). The broad antimicrobial activity of Sb-containing compounds was investigated at the same time as the discovery of their antileishmanial and antitrypanosomal activities (Camac 1911); these compounds are currently the first-line drugs in most parts of the world. In 1997, the first indication of therapeutic failure related to leishmania drug-resistant isolates was reported in the northern Bihar province of India are now problematic (Lira et al., 1999). Amphotericin B (AmB), initially described as an alternative to Sb for the treatment of MCL, has also been successfully used to treat severe VL and HIV/*Leishmania* coinfection (Uliana et al., 2017). Therapeutic failure or relapse after treatment is reported but currently rare (Purkait et al., 2012; Pandey et al., 2012). Currently, AmB (especially in its liposomal form) is now recommended by the World Health Organization and by the

main scientific societies as the first line drug to treat any form of VL worldwide, not only severe or HIV-associated conditions but also in children and immunocompetent adults. Miltefosine is the sole registered oral drug effective against leishmaniasis. Since its registration in India in 2002, a clinical decrease in its efficacy has been reported (Dorlo et al., 2012). Pentamidine is a second-line drug for the management of Sb-resistant VL cases, but its cure rate is inferior to that of amphotericin B (Mishra et al., 1992). Additionally, the toxicities associated with pentamidine (cardiac toxicity, diabetes mellitus, hypotension, and gastrointestinal side effects) limit its use. Paromomycin, an aminoglycoside antibiotic, was rediscovered as an antileishmanial agent for the treatment of VL in the 1980s (Jha et al., 1998), but its clinical use remains limited.

In recent decades, the rise of antibiotic-resistant bacteria and the extensive use of antibiotics have led to a global public health crisis that has prompted considerable efforts to standardize guidance related to antibiotic-resistant bacteria (<http://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance>). Unfortunately, neglected tropical infectious diseases, such as leishmaniasis, have not yet been included in these efforts, even if leishmania resistance are reported with increasing frequency and will certainly pose future challenges (Ponte-Sucre et al., 2017). Here, we performed a meta-analysis of information related to the susceptibility of leishmania parasites to the main molecules used to treat leishmaniasis and propose ways to translate drug resistance phenotyping into the clinical setting, within the framework of the well-defined guidelines of the bacterial antibiotic resistance.

2. *Leishmania* drug resistance diagnosis: Where are we?

Drug resistance and therapeutic failure are not synonymous. Therapeutic failure encompasses an ensemble of factors linked to the host (i.e Genetic, Immunologic...) to the infective agent (i.e Drug resistance...), to the drugs (i.e pharmacodynamics/pharmacokinetic...) and, to the chemotherapeutic protocol. Nevertheless, the first indication that informs a clinician on the therapeutic choice is the drug

resistance status of the isolate. A schematic representation of the protocols that can be used is given in figure 1.

To carry AST, isolates from needle aspirates, punch biopsies, or blood samples are usually subjected to initial cultivation in a diphasic agar medium, such as NNN medium (reviewed in Evans DA, 1987) (Figure 1). Once established in this medium, parasites must be transferred into a semi-defined culture medium (reviewed in Schuster and Sullivan, 2002). Considering the workflow described in Figure 1, 4 to 30 days after biopsy are required to isolate promastigotes in culture. If the *in vitro* test is performed on the promastigote stage of the parasite, then another 3 days is required. For tests performed on intracellular amastigotes, after the initial proliferation phase in biphasic medium, parasites are transferred into a semi-defined medium, to obtain homogeneous populations of metacyclic promastigotes that are used to infect host macrophages, at a determined host cell/parasite ratio (Inocêncio da Luz et al., 2009). Infected macrophages are then incubated for 5 days (Maes et al., 2013).

The use of molecular markers of resistance has the advantages of being faster and not requiring parasite isolation and cultivation (Figure 1). As a matter of fact, currently no molecular markers are available to diagnose drug resistance in clinical settings.

3.1. Molecular marker

An exhaustive review of molecular mechanisms and markers of resistance was recently published (Ponte-Sucre et al., 2017). Here, we will highlight some key points for translating basic research on mechanisms of leishmania drug-resistance into clinical applications. Most Indian Sb-resistant *L. donovani* isolates present molecular adaptations that were initially characterized in experimentally selected Sb-resistant strains. First, they present an intrachromosomal amplification of the 'H-Locus', which encodes MRPA, an ABC transporter that sequesters Sb within a vacuole after its conjugation with trypanothione (Leprohon et al., 2009). A second mechanism involves limiting the entry of Sb. In Leishmania, this metalloid enters via an AQP1 transporter. Modulation of AQP1 levels

prompted the emergence of resistant leishmania strains (Marquis et al., 2005). The level of AQP1 activity as an Sb transporter is abolished in isolates with a homozygous two-base pair insertion in the AQP1 sequence; this insertion is linked to some highly Sb-resistant field isolates (Marquis et al., 2010). Hybrid strains with intermediate Sb susceptibility are heterozygous for the AQP1 insertion (Imamura et al., 2016). Experimental selection for miltefosine resistance identified inactivating mutations or deletions in the MIL translocation machinery (LMT and/or LROs3) (Perez-Victoria et al., 2006). Mutations in LMT (2 sense mutations) are documented in *L. donovani* (Mittal et al., 2007; Cojean et al., 2012) and *L. infantum* (Mondelaers et al., 2016). Amphotericin B-resistant field isolates are scarce, mainly because of the high efficacy of this molecule in treating leishmania infection (Cojean et al., 2012). Experimental selection for amphotericin B resistance highlighted a change in the sterol composition of the membrane of drug-resistant organisms (Mbongo et al., 1998).

3.2. Antimicrobial susceptibility test (AST)

To perform AST (Box 1), living parasites must be isolated from biopsies to measure susceptibility of the isolates, then the diagnosis is empirical and relies on direct isolate-to-isolate comparisons (Mittal et al., 2007; Srivastava et al., 2017). A tentative plan for the standardization of antileishmanial testing processes was proposed (Maes et al., 2013; Hendrickx et al., 2016).

Intracellular amastigotes mimic the conditions encountered by the parasite in a human infection, they are considered as the ideal form to assay. The determination of drug activity model involves staining the slide and counting the mean number of intracellular amastigotes. The method is labor intensive and requires skilled individuals. The use of reporter gene technologies enable an easy and quick quantification of parasites within host cells, reduce the time to measure the intracellular burden of leishmania, and eliminate bias due to manual counting (Serenio et al., 2007; Dube et al., 2009; Lang et al., 2009; Singh et al., 2009; De La Llave et al., 2011; Michel et al., 2011; Pulido et al., 2012; Jaiswal et al., 2016). The ability of these technologies to ascertain the drug susceptibility of field isolates was probed (Hadighi et al., 2006; Lachaud et al., 2009). Indeed, the time required for the

selection of recombinant parasites limits its interest in the clinic. Progress in microscopy imaging techniques and methodologies for image analysis has allowed high spatial resolution allowing to simultaneously assesses a variety of host cell features in correlation with compound potency/toxicity. Such imaging methods make use of fluorescent dyes, allowing the visualization of DNA and/or cytoplasm to quantify drug-mediated effects on intracellular parasites (Forestier et al., 2015). The prohibitive cost and the technical skill required, render them impractical for an everyday use. Finally, the output of test on intramacrophagic amastigotes depends on factors related to the nature of the host cell (Berman et al., 1984; Gebre-Hiwot et al., 1992; Seifert et al., 2010) or to the inherent infective capacity of leishmania isolates (Inocência da Luz et al., 2009).

Leishmania can be grown in vitro as amastigotes under axenic conditions, i.e., without the requirement for a host cell (Serenio et al., 1997; Monte-Alegre et al., 2001; Guptan et al., 2001). These *in vitro*-cultured amastigotes have biochemical, morphological and immunological similarities to intracellularly cultured ones (Saar et al., 1998; Li et al., 2008; Pescher et al., 2011; Brotherton et al., 2012). Nevertheless, not all *Leishmania* species are amenable to culture as amastigotes in axenic condition, therefore no extensive studies have been performed on field isolates.

The usefulness of promastigotes in measuring the drug susceptibility of field isolates is debatable, but promastigote cultures of most of the 21 identified human pathogenic species can be achieved in large panels of culture media (Evans DA, 1987; Schuster and Sullivan, 2002) and drug susceptibility determinations can be achieved with various tests (Serenio et al., 2007; Kulshrestha et al., 2013). With this system, the determination of drug susceptibility is simple, rapid, cheap, and does not require host cells that simplify the standardization process.

3. What do we need to translate AST into the clinical setting?

Notably, the methodology used to ascertain the drug susceptibility of isolate must be 1) amenable to standardization, 2) inexpensive, 3) easy to handle, and 4) easy to interpret and normalize. In addition, to have direct patient benefit in terms of appropriate chemotherapy, the lag between

165 parasite isolation and drug susceptibility testing should be limited. All these requirements should be
166 potentially fulfilled by AST carried out on promastigotes, therefore the first question to be studied is:
167 does promastigotes AST reflect the susceptibility of intracellular amastigotes?

169 3.1. How to set AST for clinical setting: do promastigotes (AST) reflects amastigote susceptibility?

170 Taking the framework of recommendation of the PRISMA group (Moher et al., 2009), we
171 collected data from seven databases: Scopus, PubMed, Science Direct, Web of Science, MEDLINE,
172 EMBASE and Google Scholar, using the following keywords: *Leishmania* drug susceptibility,
173 *Leishmania* antimony, *Leishmania* amphotericin, *Leishmania* pentamidine, *Leishmania* paromomycin,
174 *Leishmania* miltefosine. Papers with a description of tests performed on promastigotes and/or
175 intramacrophagic amastigotes were selected. Paper where checked for: information on the culture
176 media for leishmania and of the host cell, the Host-cell nature, temperature of incubation, protocol to
177 ascertain promastigote viability and amastigote burden. In addition, we selected only papers whom
178 AST are performed at both parasitic stages on at least 5 isolates, belonging to the same species or not.
179 Only 11 publications where selected. The data were extracted and statistical analyses performed using
180 the linear regression function of GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA).
181 Statistical significance at $P < 0.05$ were calculated using Spearman's t-test.

182 It is thought that the susceptibility of leishmania promastigotes to trivalent-Sb (SbIII) does not
183 accurately reflect the sensitivity of intramacrophagic amastigotes to SbV formulations (Sundar 2001;
184 Yardley et al., 2006; Vermeersh et al., 2009). Our analysis revealed that promastigote susceptibility
185 reflects of the susceptibility of intracellular amastigote (Table 1). We recorded a highly significant
186 relationship ($P < 0.05$ in 3 of the 6 data series extracted from the literature) between susceptibility at the
187 two parasite stages (Table 1). Examples of correlation are given in figure 2A. For miltefosine,
188 promastigote susceptibility was previously shown to reflect susceptibility of intracellular amastigotes
189 (Kulshrestha et al., 2011; Kulshrestha et al., 2013). Our meta-analysis confirms this assertion with an
190 additional set of data extracted from the literature (Table 1 and figure 2B). For amphotericin B, on 3 of

the 6 currently published studies selected, a highly significant relationship at $P < 0.05$ is recorded, for the 3 other (Table 1 and figure 2C). No publications that satisfy our inclusion criteria were found for pentamidine. Only one publication was collected for paromomycin with no significant correlation recorded (Table 1). Altogether, with only one study performed on American Leishmania species, our meta analysis highlight the need of further studies to address such correlation in leishmania species responsible for cutaneous and mucocutaneous leishmaniasis in south and central America. Indeed, this meta analysis pinpoint that promastigote is of interest to perform AST in order to track Miltefosine, Amphotericin B as well as Antimony drug susceptibility status of new isolates. Further experiments are required to test this system on American Leishmania species and for pentamidine and paromomycin.

3.2. How to delineate drug resistant isolates

As the first set of breakpoints, the definition of ECOFF values is of major importance (Box 1). In contrast to clinical breakpoints, ECOFF does not define resistance based on the likelihood of treatment failure. The ecological concept of resistance that underlies the definition of the ECOFF states “a microorganism is defined as wild type for a species by the absence of acquired and mutational mechanisms of resistance to the agent” (Khalmeier 2014). The definition of the wild-type phenotype is obtained by the study of the susceptibility of several unrelated isolates, allowing for the establishment of the ECOFF, which is the upper limit of the normal susceptibility distribution for a given antimicrobial drug and a given species. Any isolate presenting susceptibility above this value is considered resistant, irrespective of whether the achieved level of resistance compromises therapy.

To compute the ECOFF, the susceptibility of at least 20 isolates must be ascertained. We therefore screened the literature previously gathered (Chapter 3.1) for papers in which the susceptibility toward antileishmanial drugs was performed on at least 20 isolates. Only 15 publications satisfy our criteria. Data were extracted and the ECOFF ascertained, using an online application, Cutoff Finder that use R version 2.15.0 (2012-03-30) (<http://molpath.charite.de/cutoff/>) (Budczies et al., 2012). Strikingly, the

multiplicity of AST protocols described in the literature reveal that guidance and SOPs previously published are not followed (Table 2). We further investigated if the amastigote ECOFF value can be deduced from AST performed on promastigote. Using the linear equation of the regression line (Figure 2), we calculated the IC_{50} of amastigote and then ascertained ECOFF (see Figure 2 and Table 3). The computed ECOFF reflects well those obtained on intramacrophagic amastigotes for Miltefosine and to a lesser extent for Antimony (Table 3). In conclusion AST performed on promastigotes reflect well the susceptibility status of intracellular amastigotes, giving the opportunity to compute the basal susceptibility level of the 21 human pathogenic leishmania species, but also to search for geographic variation in leishmania drug susceptibility and finally to be used as a parasite drug resistance diagnostic tool.

3.3. How to translate drug-resistance diagnosis into clinical setting

ECOFFs, pharmacokinetic (PK) and pharmacodynamic (PD) cutoffs and clinical cutoffs defined together clinical breakpoints (Box 1) that defines the clinical phenotypes: S (high likelihood of clinical success), I (uncertain clinical success) or R (high likelihood of therapeutic failure). Leishmania susceptibility is often defined by the IC_{50} and less frequently by the IC_{90} (concentrations of drug required to inhibit 50% or 90% of growth). Unfortunately, to compute these values, it is necessary to gather drug response information for at least 5 concentrations. This is time-consuming and do not provide information on the minimal critical concentration that kills or completely inhibits the growth of the parasite population, an important parameter for the clinician. From, the experience gained from medical bacteriology, methods that use agar or liquid dilution for the determination of the MIC (minimal inhibitory concentration) would be a faster and easier alternative to IC_{50} or IC_{90} calculation. In addition, the effort undertaken to standardize interpretation of AST results for the disc diffusion test (Kronvall and Smith, 2016) will be beneficial for leishmania drug resistance diagnosis, if amenable with this micro-organism.

4. Conclusions

To prevent and minimize the risk of the emergence of Leishmania drug resistance, there is an urgent need to reflect on the definitions and guidelines of drug resistance diagnosis. This will also help to track the underlying factors triggering the emergence of drug-resistant isolates and to characterize new molecular markers of resistance. In addition, This will assist practitioners in the future definition of species-specific treatment schemes and help to define the most effective treatment protocols and molecules. Altogether, determining Leishmania drug resistance in the light of **Clinical Breakpoints (CBPs)** offer a unique opportunity to include neglected tropical diseases in the well-defined framework of antimicrobial therapy.

Funding the authors received no specific funding for this work.

Competing interests: The authors have declared that no competing interests exist.

References

- Aït-Oudhia, K., Gazanion, E., Sereno, D., Oury, B., Dedet, J.P., 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, doi: [10.1016/j.vetpar.2012.01.034](https://doi.org/10.1016/j.vetpar.2012.01.034).
- Akhoundi, M., Downing, T., Votýpka, J., Kuhls, K., Lukeš, J., Cannet, A., Ravel, C., Marty, P., Delaunay, P., Kasbari, M., Granouillac, B., Gradoni, L., Sereno, D. 2017. Leishmania infections: Molecular targets and diagnosis. *Mol. Aspects. Med.* 57, 1-29. doi: [10.1016/j.mam.2016.11.012](https://doi.org/10.1016/j.mam.2016.11.012).
- Akhoundi, M., Kuhls, K., Cannet, A., Votýpka, J., Marty, P., Delaunay, P., Sereno D. 2016. A Historical Overview of the Classification, Evolution, and Dispersion of *Leishmania* Parasites and Sandflies. *PLoS. Negl. Trop. Dis* 10, e0004349. doi: [10.1371/journal.pntd.0004349](https://doi.org/10.1371/journal.pntd.0004349).
- Alvar, J., Vélez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., den Boer, M., WHO Leishmaniasis Control Team. 2012. Leishmaniasis worldwide and global estimates of its incidence. *PLoS. One.* 7, e35671, doi: [10.1371/journal.pone.0035671](https://doi.org/10.1371/journal.pone.0035671).
- Alves, F., Bilbe, G., Blesson, S., Goyal, V., Monnerat, S., Mowbray, C., Muthoni Ouattara, G., Pécou, B., Rijal, S., Rode, J., Solomos, A., Strub-Wourgaft, N., Wasunna, M., Wells, S., Zijlstra, E.E., Arana, B., Alvar, J. 2018. Recent development of visceral leishmaniasis treatments: successes, pitfalls, and perspectives. *Clin. Microbiol. Rev.* 31, e00048-18. PMID: [30158301](https://pubmed.ncbi.nlm.nih.gov/30158301/).
- Aronson, N., Herwaldt, B.L., Libman, M., Pearson, R., Lopez-Velez, R., Weina, P., Carvalho, E.M., Ephros, M., Jeronimo, S., Magill, A. 2016. Diagnosis and Treatment of Leishmaniasis: Clinical Practice Guidelines by the Infectious Diseases Society of America (IDSA) and the American Society of Tropical Medicine and Hygiene (ASTMH). *Clin. Infect. Dis.* 63, 1539-1557. PMID: [27941143](https://pubmed.ncbi.nlm.nih.gov/27941143/).

281

282 Berman, J.D. 1984. *Leishmania tropica*: quantitation of *in vitro* activity of antileishmanial agents by

283 Giemsa staining, viability, and 3H-formycin B incorporation. J. Parasitol. 70, 561–562, PMID: 6502359.

284

285 Bhandari, V., Kulshrestha, A., Deep, D.K., Stark, O., Prajapati, V.K., Ramesh, V., Sundar, S., Schonian,

286 G., Dujardin, J.C., Salotra, P. 2012. Drug susceptibility in *Leishmania* isolates following miltefosine

287 treatment in cases of visceral leishmaniasis and post kala-azar dermal leishmaniasis. PLoS. Negl. Trop.

288 Dis. 6, e1657, doi: 10.1371/journal.pntd.0001657.

289

290 Bilbao-Ramos, P., Dea-Ayuela, M.A., Cardenas-Alegría, O., Salamanca, E., Santalla-Vargas, J.A. 2017.

291 Leishmaniasis in the major endemic region of Plurinational State of Bolivia: Species identification,

292 phylogeography and drug susceptibility implications. Acta. Trop. 176, 150-161. doi:

293 10.1016/j.actatropica.2017.07.026.

294

295 Brotherton, M.C., Racine, G., Ouameur, A.A., Leprohon, P., Papadopoulou, B., Ouellette, M. 2012.

296 Analysis of membrane-enriched and high molecular weight proteins in *Leishmania infantum*

297 promastigotes and axenic amastigotes. J. Proteome. Res. 11, 3974-3985. doi: 10.1021/pr201248h.

298

299 Budczies, J., Klauschen, F., Sinn, B.V., Schmitt, W.D., Darb-Esfahani, S. 2012. Cutoff Finder: a

300 comprehensive and straightforward web application enabling rapid biomarker cutoff optimization.

301 PLoS. One. 7, 1–7. doi: 10.1371/journal.pone.0051862.

302

303 Camac C.N. 1911. Intramuscular and intravenous injections of antimony in trypanosomiasis. Br. Med.

304 J. 2, 104-105. PMID: 20765726.

305

Cojean, S., Houzé, S., Haouchine, D., Huteau, F., Lariven, S., Hubert, V., Michard, F., Bories, C.,
 Pratlong, F., Le Bras, J., Loiseau, P.M., Matheron, S. 2012. *Leishmania* resistance to miltefosine
 associated with genetic marker. *Emerg. Infect. Dis.* 18, 704-706. doi: [10.3201/eid1804.110841](https://doi.org/10.3201/eid1804.110841).

De La Llave, E., Lecoœur, H., Besse, A., Milon, G., Prina, E., Lang T. 2011. A combined luciferase
 imaging and reverse transcription polymerase chain reaction assay for the study of *Leishmania*
 amastigote burden and correlated mouse tissue transcript fluctuations. *Cell. Microbiol.* 13, 81-91, doi:
[10.1111/j.1462-5822.2010.01521.x](https://doi.org/10.1111/j.1462-5822.2010.01521.x).

Deep, D.K., Singh, R., Bhandari, V., Verma, A., Sharma, V. 2017. Increased miltefosine tolerance in
 clinical isolates of *Leishmania donovani* is associated with reduced drug accumulation, increased
 infectivity and resistance to oxidative stress. *PLoS. Negl. Trop. Dis.* 11, e0005641, doi:
[10.1371/journal.pntd.0005641](https://doi.org/10.1371/journal.pntd.0005641).

Dorlo, T.P., Balasegaram, M., Beijnen, J.H., de Vries, P.J. 2012. Miltefosine, a review of its
 pharmacology and therapeutic efficacy in the treatment of leishmaniasis. *J. Antimicrob. Chemother.*
 67, 2576-2597, doi: [10.1093/jac/dks275](https://doi.org/10.1093/jac/dks275).

Dube A, Gupta R, Singh N. Reporter genes facilitating discovery of drugs targeting protozoan
 parasites. *Trends Parasitol* 2009; 25: 432-439. doi: [10.1016/j.pt.2009.06.006](https://doi.org/10.1016/j.pt.2009.06.006).

Eddaikra, N., Aït-Oudhia, K., Kherrachi, I., Oury, B., Moulti-Mati, F., Benikhlef, R., Harrat, Z., Sereno,
 D. 2018a. Antimony susceptibility of *Leishmania* isolates collected over a 30-year period in Algeria.
PLoS. Negl. Trop. Dis. 1, e0006310, doi: [10.1371/journal.pntd.0006310](https://doi.org/10.1371/journal.pntd.0006310).

331 Eddaikra, N., Kherrachi, I., Bensegheir, S., Aït-Oudhia, K., Benikhlef, R., Oury, B., Harrat, Z., Sereno,
332 D. 2018b. Antimonial susceptibility and *in vivo* behaviour of *Leishmania major* isolates collected in
333 Algeria before and after treatment. Acta. Trop. 180, 7-11, doi: [10.1016/j.actatropica.2017.12.020](https://doi.org/10.1016/j.actatropica.2017.12.020).
334
335 Evans DA. Leishmania. In: *In Vitro Methods for Parasite Cultivation*, Taylor A.E. & Baker J.R., 1987;
336 eds. Academic Press, London, UK, 52-75
337
338 Forestier, C.L., Späth, G.F., Prina, E., Dasari S. 2015. Simultaneous multi-parametric analysis of
339 *Leishmania* and of its hosting mammal cells: A high content imaging-based method enabling sound
340 drug discovery process. Microb. Pathog. 88, 103-108. doi: [10.1016/j.micpath.2014.10.012](https://doi.org/10.1016/j.micpath.2014.10.012).
341
342 Gebre-Hiwot, A., Tadesse, G., Croft, S.L., Frommel, D. 1992. An *in vitro* model for screening
343 antileishmanial drugs: the human leukaemia monocyte cell line, THP-1. Acta. Trop. 51, 237-245,
344 PMID: [1359751](https://pubmed.ncbi.nlm.nih.gov/1359751/).
345
346 Gradoni, L., López-Vélez, R., Mokni, M. 2017. Manual on case management and surveillance of the
347 leishmaniasis in the WHO European Region. World Health Organization Regional Office for Europe,
348 Copenhagen.
349
350 Gupta, N., Goyal, N., Rastogi, A.K. 2001. *In vitro* cultivation and characterization of axenic
351 amastigotes of *Leishmania*. Trends. Parasitol. 17, 150-153. PMID: [11286801](https://pubmed.ncbi.nlm.nih.gov/11286801/).
352
353
354 Hadighi, R., Mohebbi, M., Boucher, P., Hajjarian, H., Khamesipour, A., Ouellette, M. 2006.
355 Unresponsiveness to Glucantime treatment in Iranian cutaneous leishmaniasis due to drug-resistant
356 *Leishmania tropica* parasites. PLoS .Med. 3, e162. doi: [10.1371/journal.pmed.0030162](https://doi.org/10.1371/journal.pmed.0030162).

357

358 Hendrickx, S., Guerin, P.J., Caljon, G., Croft, S.L., Maes, L. 2016. Evaluating drug resistance in visceral
359 leishmaniasis: the challenges. *Parasitology*. 21, 1-11. [PMID:27866478](#).

360

361 Imamura, H., Downing, T., Van den Broeck, F., Sanders, M.J., Rijal, S., Sundar, S., Mannaert, A.,
362 Vanaerschot, M., Berg, M., De Muylder, G., Dumetz, F., Cuypers, B., Maes, I., Domagalska, M.,
363 Decuypere, S., Rai, K., Uranw, S., Bhattarai, N.R., Khanal, B., Prajapati, V.K., Sharma, S., Stark, O.,
364 Schönlan, G., De Koning, H.P., Settimo, L., Vanhollebeke, B., Roy, S., Ostyn, B., Boelaert, M., Maes, L.,
365 Berriman, M., Dujardin, J.C., Cotton, J.A. 2016. Evolutionary genomics of epidemic visceral
366 leishmaniasis in the Indian subcontinent. *eLife*. 5, e12613. [doi: 10.7554/eLife.12613](#).

367

368 Inocêncio da Luz, R., Vermeersch, M, Dujardin, J.C., Cos P., Maes L. 2009. *In vitro* sensitivity testing of
369 *Leishmania* clinical field isolates: preconditioning of promastigotes enhances infectivity for
370 macrophage host cells. *Antimicrob. Agents. Chemother.* 53, 5197-5203. [doi: 10.1128/AAC.00866-09](#).

371

372 Jaiswal, A.K., Rao, K.B., Kushwaha, P., Rawat, K., Modukuri, R.K. 2016. Development of *Leishmania*
373 *donovani* stably expressing DsRed for flow cytometry-based drug screening using chalcone thiazolyl-
374 hydrazone as a new antileishmanial target. *Int. J. Antimicrob. Agent.* 48, 695-702. [doi:](#)
375 [10.1016/j.ijantimicag.2016.09.018](#).

376

377 Jha, T.K., Olliaro, P., Thakur, C.P., Kanyok, T.P., Singhania, B.L., Singh, I.J., Singh N.K., Akhoury, S.,
378 Jha, S. 1998. Randomised controlled trial of aminosidine (paromomycin) v sodium stibogluconate for
379 treating visceral leishmaniasis in North Bihar, India. *BMJ*. 316, 1200-1205. [PMID: 9583927](#).

380

381 Kahlmeter, G. 2014. Defining antibiotic resistance-towards international harmonization. *Ups. J. Med.*
382 *Sci.* 119, 78-86. [doi: 10.3109/03009734.2014.901446](#).

383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407

Kronvall, G., Smith, P. 2016. Normalized resistance interpretation, the NRI method: Review of NRI disc test applications and guide to calculations. APMIS. 124, 1023-1030. doi: [10.1111/apm.12624](https://doi.org/10.1111/apm.12624).

Kulshrestha, A., Bhandari, V., Mukhopadhyay, R., Ramesh, V., Sundar, S., Maes, L., Dujardin, J.C., Roy, S., Salotra, P. 2013. Validation of a simple resazurin-based promastigote assay for the routine monitoring of miltefosine susceptibility in clinical isolates of *Leishmania donovani*. Parasitol. Res. 112, 825-828. doi: [10.1007/s00436-012-3212-3](https://doi.org/10.1007/s00436-012-3212-3).

Kulshrestha, A., Singh, R., Kumar, D., Negi, N.S., Salotra, P. 2011. Antimony-resistant clinical isolates of *Leishmania donovani* are susceptible to paromomycin and sitamaquine. Antimicrob. Agents. Chemother. 55, 2916-2921, doi: [10.1128/AAC.00812-10](https://doi.org/10.1128/AAC.00812-10).

Kumar, D., Kulshrestha, A., Singh, R., Salotra, P. 2009. *In vitro* susceptibility of field isolates of *Leishmania donovani* to Miltefosine and amphotericin B: correlation with sodium antimony gluconate susceptibility and implications for treatment in areas of endemicity. Antimicrob. Agents. Chemother. 53, 835-838, doi: [10.1128/AAC.01233-08](https://doi.org/10.1128/AAC.01233-08).

Lachaud, L., Bourgeois, N., Plourde, M., Leprohon, P., Bastien, P., Ouellette, M. 2009. Parasite susceptibility to amphotericin B in failures of treatment for visceral leishmaniasis in patients coinfectd with HIV type 1 and *Leishmania infantum*. Clin. Infect. Dis. 48, e16-22. doi: [10.1086/595710](https://doi.org/10.1086/595710).

Lang, T., Lecoecur, H., Prina, E. 2009. Imaging *Leishmania* development in their host cells. Trends. Parasitol. 25, 464-473, doi: [10/1016/j.pt.2009.07.006](https://doi.org/10.1016/j.pt.2009.07.006).

Leprohon, P., Légaré, D., Raymond, F., Madore, E., Hardiman, G. 2009. Gene expression modulation is associated with gene amplification, supernumerary chromosomes and chromosome loss in antimony-resistant *Leishmania infantum*. Nucleic. Acid. Res. 37, 1387-1399. doi: 10.1093/nar/gkn1069.

Li, Q., Zhao, Y., Ni, B., Yao, C., Zhou, Y., Xu, W., Wang, Z., Qiao, Z. 2008. Comparison of the expression profiles of promastigotes and axenic amastigotes in *Leishmania donovani* using serial analysis of gene expression. Parasitol. Res. 103, 821-828. doi: 10.1007/s00436-008-1048-7.

Lira, R., Sundar, S., Makharia, A., Kenney, R., Gam, A. 1999. Evidence that the high incidence of treatment failures in Indian kala-azar is due to the emergence of antimony-resistant strains of *Leishmania donovani*. J. Infect. Dis. 180, 564-567, doi: 10.1086/314896.

Maes, L., Cos, P., Croft, S.L. The Relevance of Susceptibility Tests, Breakpoints, and Markers. In: Ponte-Sucre A., Diaz E., Padrón-Nieves M. (eds) Drug Resistance in Leishmania Parasites. 2013; Springer, Vienna.

Maia, C., Nunes, M., Marques, M., Henriques, S., Rolão, N., Campino, L. 2013. *In vitro* drug susceptibility of *Leishmania infantum* isolated from humans and dogs. Exp. Parasitol. 135, 36-41, doi: 10.1016/j.exppara.2013.05.015.

Marquis, N., Gourbal, B., Rosen, B.P., Mukhopadhyay, R., Ouellette, M. 2005. Modulation in aquaglyceroporin AQP1 gene transcript levels in drug-resistant *Leishmania*. Mol. Microbiol. 57, 1690-1699. doi: 10.1111/j.1365-2958.2005.04782.x.

432 Mbongo, N., Loiseau, P.M., Billion, M.A., Robert-Gero, M. 1998. Mechanism of amphotericin B
 433 resistance in *Leishmania donovani* promastigotes. Antimicrob. Agents. Chemother. 42, 352-357. PMID:
 434 9527785.

435 Michel, G., Ferrua, B., Lang, T., Maddugoda, M.P., Munro, P., Pomares, C., Lemichez, E., Marty, P.
 436 2011. Luciferase expressing *Leishmania infantum* allows the monitoring of amastigote population size,
 437 *in vivo*, *ex vivo* and *in vitro*. PLoS. Negl. Trop. Dis. 5, e1323. doi: 10.1371/journal.pntd.0001323.
 438

439 Mishra, M., Biswas, U.K., Jha, D.N., Khan, A.B. 1992. Amphotericin versus pentamidine in antimony-
 440 unresponsive kala-azar. Lancet. 340, 1256-1257, PMID: 1359322.

441

442 Mittal, M.K., Rai, S., Ashutosh-Ravinder, Gupta, S., Sundar, S., Goyal, N. 2007. Characterization of
 443 natural antimony resistance in *Leishmania donovani* isolates. Am. J. Trop. Med. Hyg. 7, 681-688. PMID:
 444 17426170.

445

446 Moher, D., Liberati, A., Tetzlaff, J., Altman D.G; **PRISMA** Group. 2009. Preferred reporting items for
 447 systematic reviews and meta-analyses: the PRISMA statement. PLoS. Med. 6, e1000097. doi:
 448 10.1371/journal.pmed.1000097.

449

450 Mondelaers, A., Sanchez-Cañete, M.P., Hendrickx, S., Eberhardt, E., Garcia-Hernandez, R., Lachaud,
 451 L., Cotton, J., Sanders, M., Cuypers, B., Imamura, H., Dujardin, J.C., Delputte, P., Cos, P., Caljon, G.,
 452 Gamarro, F., Castanys, S., Maes, L. 2016. Genomic and Molecular Characterization of Miltefosine
 453 Resistance in *Leishmania infantum* Strains with Either Natural or Acquired Resistance through
 454 Experimental Selection of Intracellular Amastigotes. PLoS. One. 11, e0154101. doi:
 455 10.1371/journal.pone.0154101.
 456

457 Monte-Alegre, A., Ouaiissi, A., Sereno D. 2006. *Leishmania* amastigotes as targets for drug screening.
 458 Kinetoplastid. Biol. Dis. 5, 6. doi: [10.1186/1475-9292-5-6](https://doi.org/10.1186/1475-9292-5-6).
 459
 460 Pandey, K., Pun, S.B., Pandey, B.D. 2012. Relapse of kala-azar after use of multiple drugs: a case report
 461 and brief review of literature. Indian. J. Med. Microbiol. 30, 227-229, doi: [10.4103/0255-0857.96703](https://doi.org/10.4103/0255-0857.96703).
 462
 463 Pérez-Victoria, F.J., Sánchez-Cañete, M.P., Seifert, K., Croft, S.L., Sundar, S., Castanys, S., Gamarro, F.
 464 2006. Mechanisms of experimental resistance of *Leishmania* to miltefosine: Implications for clinical use.
 465 Drug. Resist. Updat. 9, 26-39. PMID: [16814199](https://pubmed.ncbi.nlm.nih.gov/16814199/).
 466
 467 Pescher, P., Blisnick, T., Bastin, P., Späth, G.F. 2011. Quantitative proteome profiling informs on
 468 phenotypic traits that adapt *Leishmania donovani* for axenic and intracellular proliferation. Cell.
 469 Microbiol. 13, 978-991. doi: [10.1111/j.1462-5822.2011.01593.x](https://doi.org/10.1111/j.1462-5822.2011.01593.x).
 470
 471 Piñero, J.E., Martínez, S., del Castillo, A., Portús, M., Valladares, B. 2002. *In vitro* susceptibility of
 472 *Leishmania infantum* strains isolated from Spanish HIV-positive patients to Abelcet and Fungizone. J.
 473 Antimicrob. Chemother. 50, 304-306, PMID: [12161420](https://pubmed.ncbi.nlm.nih.gov/12161420/).
 474
 475 Ponte-Sucre, A., Gamarro, F., Dujardin, J.C., Barrett, M.P., López-Vélez, R., García-Hernández, R.,
 476 Pountain, A.W., Mwenechanya, R., Papadopolou, B. 2017. Drug resistance and treatment failure in
 477 leishmaniasis: A 21st century challenge. PLoS. Negl. Trop. Dis. 11, e0006052. doi:
 478 [10.1371/journal.pntd.0006052](https://doi.org/10.1371/journal.pntd.0006052).
 479
 480 Prajapati, V.K., Sharma, S., Rai, M., Ostyn, B., Salotra, P., Vanaerschot, M., Dujardin, J.C., Sundar, S.
 481 2013. *In vitro* susceptibility of *Leishmania donovani* to miltefosine in Indian visceral leishmaniasis. Am.
 482 J. Trop. Med. Hyg. 89, 750-754, doi: [10.4269/ajtmh.13-0096](https://doi.org/10.4269/ajtmh.13-0096).

483

484 Pulido, S.A., Muñoz, D.L., Restrepo, A.M., Mesa, C.V., Alzate, J.F., Vélez, I.D., Robledo, S.M. 2012.
485 Improvement of the green fluorescent protein reporter system in *Leishmania spp.* for the *in vitro* and *in*
486 *vivo* screening of antileishmanial drugs. Acta. Trop. 122: 36-45. doi :1016/j.actatropica.2011.11.015.

487

488 Purkait, B., Kumar, A., Nandi, N., Sardar, A.H., Das, S. 2012. Mechanism of amphotericin B resistance
489 in clinical isolates of *Leishmania donovani*. Antimicrob. Agents. Chemother. 56, 1031-41. doi:
490 10.1128/AAC.00030-11.

491

492 Rai, K., Cuypers, B., Bhattarai, N.R., Uranw, S., Berg, M., Ostyn, B., Dujardin, J.C., Rijal, S.,
493 Vanaerschot, M. 2013. Relapse after treatment with miltefosine for visceral leishmaniasis is associated
494 with increased infectivity of the infecting *Leishmania donovani* strain. MBio. 4, e00611-13. doi:
495 10.1128/mBio.00611-13.

496

497 Rijal, S., Ostyn, B., Uranw, S., Rai, K., Bhattarai, N.R., Dorlo, T.P., Beijnen, J.H., Vanaerschot, M.,
498 Decuypere, S., Dhakal, S.S., Das, M.L., Karki, P., Singh, R., Boelaert, M., Dujardin, J.C. 2013. Increasing
499 failure of miltefosine in the treatment of Kala-azar in Nepal and the potential role of parasite drug
500 resistance, reinfection, or noncompliance. Clin. Infect. Dis. 56, 1530-1538, doi: 10.1093/cid/cit102.

501

502 Saar, Y., Ransford, A., Waldman, E., Mazareb, S., Amin-Spector, S., Plumblee, J., Turco, S.J.,
503 Zilberstein, D. 1998. Characterization of developmentally-regulated activities in axenic amastigotes of
504 *Leishmania donovani*. Mol. Biochem. Parasitol. 95, 9-20. PMID: 9763285.

505

506 Schuster, F.L., Sullivan, J.J. 2002. Cultivation of clinically significant hemoflagellates. Clin. Microbiol.
507 Rev. 15, 374-389. PMID: 12097246.

508

509 Seifert, K., Escobar, P., Croft, S.L. 2010. *In vitro* activity of anti-leishmanial drugs against *Leishmania*
510 *donovani* is host cell dependent. J. Antimicrob. Chemother. 65, 508-511, PMID: 20089542.
511

512 Sereno, D., Cordeiro da Silva, A., Mathieu-Daude, F., Ouaisi, A. 2007. Advances and perspectives in
513 *Leishmania* cell based drug-screening procedures. Parasitol. Int. 56, 3-7, doi:
514 10.1016/j.parint.2006.09.001.
515

516 Sereno, D., Lemesre, J.L. 1997. Axenically cultured amastigote forms as an *in vitro* model for
517 investigation of antileishmanial agents. Antimicrob. Agents. Chemother. 41, 972-976. PMID: 9145854.
518 Sereno, D., Maia, C., Ait-Oudhia, K. 2012. Antimony resistance and environment: Elusive links to
519 explore during *Leishmania* life cycle. Int. J. Parasitol: Drugs. Drug. Resist. 2, 200-203, doi:
520 10.1016/j.ijpddr.2012.07.003.
521

522 Singh, N., Gupta, R., Jaiswal, A.K., Sundar, S., Dube, A. 2009. Transgenic *Leishmania donovani* clinical
523 isolates expressing green fluorescent protein constitutively for rapid and reliable *ex vivo* drug
524 screening. J. Antimicrob. Chemother. 64, 370-374, doi: 10.1093/jac/dkp206.
525

526 Singh, R., Kumar, D., Ramesh, V., Negi, N.S., Singh, S., Salotra, P. 2006. Visceral leishmaniasis, or kala
527 azar (KA): high incidence of refractoriness to antimony is contributed by anthroponotic transmission
528 via post-KA dermal leishmaniasis. J. Infect. Dis. 194, 302-306, PMID: 16826477.
529

530 Srivastava, S., Mishra, J., Gupta, A.K., Singh, A., Shankar, P., Singh, S. 2017. Laboratory confirmed
531 miltefosine resistant cases of visceral leishmaniasis from India. Parasit. Vectors. 10, 49. doi:
532 10.1186/s13071-017-1969-z.
533

534 Sundar, S. 2001. Drug resistance in Indian visceral leishmaniasis. Trop. Med. Int. Health. 6: 849-854.
535 [PMID: 11703838](#).
536
537 Uliana, S.R., Trinconi, C.T., Coelho, A.C. 2017. Chemotherapy of leishmaniasis: present challenges.
538 Parasitology. 20, 1-17, [doi: 10.1017/S0031182016002523](#).
539
540 Utaile, M., Kassahun, A., Abebe, T., Hailu, A. 2013. Susceptibility of clinical isolates of *Leishmania*
541 *aethiopica* to miltefosine, paromomycin, amphotericin B and sodium stibogluconate using amastigote-
542 macrophage *in vitro* model. Exp. Parasitol. 134, 68-75. [doi: 10.1016/j.exppara.2013.01.022](#).
543
544 Vermeersch, M., Inocência da Luz, R., Toté, K., Timmermans, J.P., Cos, P., Maes, L. 2009. *In vitro*
545 susceptibilities of *Leishmania donovani* promastigote and amastigote stages to antileishmanial reference
546 drugs: practical relevance of stage-specific differences. Antimicrob. Agents. Chemother. 53, 3855-3859.
547 [doi: 10.1128/AAC.00548-09](#).
548
549 Yardley, V., Ortuno, N., Llanos-Cuentas, A., Chappuis, F., Doncker, S.D., Ramirez, L., Croft, S.,
550 Arevalo, J., Aducci, V., Bermudez, H., Decuypere, S., Dujardin, J.C. 2006. American tegumentary
551 leishmaniasis: Is antimonial treatment outcome related to parasite drug susceptibility? J. Infect. Dis.
552 194: 1168-1175. [doi: 10.1086/507710](#).
553
554

Legends to the figures

Figure 1: The identification of drug-resistant *Leishmania* isolates. Two experimental protocols can be used to achieve this aim. The first method is based on *in vitro* isolate phenotyping. Living parasites must be collected from biopsies in order to measure their susceptibility to the molecules. For this purpose, various tests are available; easier ones rely on promastigote culture, and more complex ones require intracellular amastigote culture. In addition, other protocols, such as the axenic amastigote test protocol, can be considered. Once isolate susceptibility is measured, the main conceptual limitation is classifying the isolates as susceptible (S+) or non-susceptible (S-) and in conjunction with other clinical breakpoints to infer a success of therapy (S) or (R). An alternative is to characterize a molecular marker of drug-resistant parasites, these marker gives information on the presence of a known marker of resistance.

Figure 2: Exemple of linear correlation between promastigote and intracellular amastigote drug susceptibility. The identification of isolates included in the analysis of Miltefosine and Amphotericin b is provided in the figure. The regression line equations are given in the figure after being computed by forcing the regression to pass through the origin, dashed lines represent the 90% confidence interval values. Data were extracted from Kumar and collaborators (2009) and Eddaikra and collaborators (2018).

A

B

C

Table 1: Linear correlation between promastigotes and intracellular amastigotes susceptibility, as computed from the data extracted from the litterature.

<i>Leishmania</i> sp.	N	AmphoB/Fungizone	Miltefosine	Pentamidine	Paromomycin	Antimony SbIII/SbV	References
<i>L. braziliensis</i> , <i>L. lainsoni</i> , <i>L. mexicana</i> , <i>L. infantum</i> , <i>L. guyanensis</i>	9	NS-0.0549	NS-0.111	NA	NA	NA	Bilbao-Ramos et al., 2017
<i>L. donovani</i>	20	P<0.0018 -0.4249	P<0.0001 -0.615	NA	NA	NA	Kumar et al., 2009
<i>L. donovani</i>	18	NA	P<0.0001 -0.9598	NA	NA	NA	Deep et al., 2017
<i>L. donovani</i>	20	NA	NA	NA	NS -0.01782	NA	Kulshrestha et al., 2011
<i>L. donovani</i>	17	NA	P<0.0001 -0.81	NA	NA	NA	Kulshrestha et al., 2013
<i>L. donovani</i>	21	NA	P<0.005 -0.72	NA	NA	NA	Kulshrestha et al., 2013
<i>L. infantum</i>	5	P<0.0008 -0.9845	NA	NA	NA	NA	Piñero et al., 2002
<i>L. infantum</i>	4	NS -0.4608	NA	NA	NA	NA	Lachaud et al., 2009
<i>L. infantum</i>	6	NS -0.1076	NS -0.4828	NA	NA	NA	Maia et al., 2013
<i>L. infantum</i>	28	P<0.0001 -0.9766	NA	NA	NA	P<0.0001 -0.9032	Aït-Oudhia et al., 2012
<i>L. infantum</i>	50	NA	NA	NA	NA	P=0.0071 -0.1390	Eddaikra et al. 2018a
<i>L. major</i>	12	NA	NA	NA	NA	NS -0.2085	Eddaikra et al. 2018b
<i>L. major</i>	7	NA	NA	NA	NA	P=0.0747 -0.5020	[Eddaikra et al. 2018a
<i>L. tropica</i> (<i>L. killicki</i>)	8	NA	NA	NA	NA	NS -0.00935	Eddaikra et al. 2018a
<i>L. infantum</i> , <i>L. major</i> , <i>L. tropica</i> (<i>L. killicki</i>)	73	NA	NA	NA	NA	P<0.0001 -0.1353	Eddaikra et al. 2018a

N: number of isolates/strains analyzed. **P** (Probability, computed using the Pearson correlation coefficient) and R² are given. NS not significant, NA not available, not tested in the study.

Table 2 : Computed ECOFF values of various leishmania species for Miltefosine, Amphotericine B, Antimony, Stimaquine and Paromomycin.

Drugs	<i>Leishmania</i> sp	N	Promastigote	Amastigote	Promastigote	Amastigote	References
			Medium/Time(d)/Meth	Host-cell/Time(d)/Meth			
Miltefosine (µM)	<i>L. donovani</i>	22	M199/3d/Reazurin	NA	5.55	NA	Rai et al., 2013
	<i>L. donovani</i>	30	NA	J774/2d/Counting	NA	3.93	Bhandari et al., 2012
	<i>L. donovani</i>	54	M199/3d/Reazurin	NA	4.32	NA	Prajapati et al., 2013
	<i>L. donovani</i>	45	M199/3d/Reazurin	J774/2d/Counting	8.71	NA	Rijal et al., 2013
	<i>L. donovani</i>	19	M199/NA/Rh123Cytometry	J774/2d/Counting	1.442	1.513	Kumar et al. 2009
	<i>L. aethiopica</i>	23	NA	CD1M□/5d/Counting	NA	3.285	Utaile et al., 2013
Amphotericin B (µg/ml)	<i>L. infantum</i>	25	SDM79/3d/OD	U937/3d/LUC	0.408	ND	Lachaud et al., 2009
	<i>L. infantum</i>	25	SDM79/3d/ PropidiumCytometry	THP-1/5d/Counting	0.40	0.24	Ait-Oudhia et al., 2012
	<i>L. donovani</i>	19	M199/NA/Rh123Cytometry	J774/2d/Counting	0.97	0.42	Kumar et al. 2009
	<i>L. aethiopica</i>	23		CD1M□/5d/Counting	NA	0.23	Utaile et al., 2013
Antimony (µg/ml) (SbIII/SbV)	<i>L. donovani</i>	37	NA	J774/2d/Counting	NA	6.62(SbV)	Singh et al., 2006
	<i>L. donovani</i>	20	NA	J774A1/2d/Couning	NA	7.13(SbV)	Kulshrestha et al., 2011
	<i>L. donovani</i>	19	NA	J774/2d/Counting	NA	7.08(SbV)	Kumar et al. 2009
	<i>L. donovani</i>	24	M199-C/1d/H ³ Thymidin	BALBcM□/3d/Counting	87.97 (SbV)	3.71(SbV)	Lira et al., 1999
	<i>L. donovani</i>	30	NA	J774/2d/Counting	NA	7.42(SbV)	Bhandari et al., 2012
	<i>L. aethiopica</i>	23	NA	CD1M□/5d/Counting	NA	18.45(SbV)	Utaile et al., 2013
	<i>L. . tropica</i>	25	NA	THP-1/5d/Counting	NA	42.09(SbV)	Hadighi et al., 2006
	<i>L. infantum</i>	26	SDM79/3d/PropidiumCytometry	THP-1/5d/Counting	10.48 (SbIII)	56.65(SbV)	Ait-Oudhia et al., 2012
	<i>L. infantum</i>	50	SDM79/3d/PropidiumCytometry	THP-1/5d/Counting	23.83 (SbIII)	59.09(SbV)	Eddaikra et al., 2018b
	<i>L. infantum</i>	37	NA	BALBcM□/7d/Counting	NA	84.04(SbV)	Singh et al., 2006
	<i>L. major</i>	26	SDM79/3d /PropidiumCytometry	THP-1/5d/Counting	5.86(SbIII)	ND	Eddaikra et al., 2018a
	VL*	24	SDM79/3d /PropidiumCytometry	NA	23.83 (SbIII)	NA	Eddaikra et al., 2018a
	CL*	43	SDM79/3d /PropidiumCytometry	NA	15.91 (SbIII)	NA	Eddaikra et al., 2018a
Stimaquine (µM)	<i>L. donovani</i>	20	M199/3d/Counting	J774A1/2d/Couning	1.36	ND	Kulshrestha et al., 2011
Paromomycin (µM)	<i>L. donovani</i>	20	M199/3d/Counting	J774A1/2d/Couning	3.88	47.72	Kulshrestha et al., 2011
	<i>L. aethiopica</i>	23	NA	CD1M□/5d/Counting	NA	12.43	Utaile et al., 2013

* Leishmania isolates collected from patients suffering of visceral (VL) or cutaneous (CL) leishmaniasis. NA: Not available, not tested in the study. (d) days of incubation

Table 3. Computed ECOFF value from promastigotes, amastigotes and from susceptibility of amastigotes deduced from promastigotes using the linear equation.

	Miltefosine	Amphotericin B	Antimony
	*ECOFF-(S+/S-) – N	*ECOFF-(S+/S-) – N	*ECOFF-(S+/S-) – N
Promastigote	1.442 µM-(70/30)-20	NA	11.48 µg/ml-(54.4/42.6)-68
Amastigote	1.513 µM-(60/40)-20	0.427 µg/ml-(60/40)-20	59.09 µg/ml-(58.8/41.2)-50
Amastigote-cPro**	1.591 µM-(70/30)-20	NA	17.03 µg/ml-(57.4/42.6)-68

* ECOFF and percentage of susceptible (S+) and unsusceptible (S-) isolates. (N) number of sample. NA not available not tested in the study.

** Values were deduced from promastigote AST using the equation of the regression analysis.