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1 Review

2 **Meta-analysis and discussion on challenges to translate**

3 ***Leishmania* drug resistance phenotyping into the clinic**

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18

19 **Abstract**

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

36 Leishmaniasis are caused by obligate unicellular eukaryote parasites belonging to the genus
37 *Leishmania* (Trypanosomatida: Trypanosomatidae). There are twenty-one *Leishmania* species with
38 documented pathogenicity in humans (Akhoundi et al., 2016). These digenetic or heteroxenous
39 parasites have a life cycle that involves two hosts, a vertebrate and an invertebrate (the sandfly).
40 Infection is initiated when metacyclic promastigotes are regurgitated by sandflies having a blood meal
41 on a vertebrate host. The clinical manifestations of leishmaniasis range from simple or diffuse
42 cutaneous leishmaniasis (CL), mucocutaneous (MCL), mucosal (ML), post-Kala-azar dermal (PKDL)
43 and visceral (VL) leishmaniasis (Akhoundi et al., 2017). These diseases rank after malaria in term of
44 annual incidence and affect 98 countries and territories worldwide. Visceral leishmaniasis kill between
45 20 000 to 30 000 persons annually, 1 millions cutaneous leishmaniasis cases reported during the past 5
46 years and, over 1 billion peoples living in endemic area at risk of infection (Alvar et al., 2012;
47 <http://www.who.int/leishmaniasis/en/>).

48 Heterogeneity in clinical presentation, disease progression, complications and the response to
49 treatment with various etiological agents of patients with leishmaniasis, makes the choice of therapy
50 **sometimes problematic**. Guidelines for the treatment of the different clinical forms are published and
51 include only a few a drugs: meglumine antimoniate, sodium stibogluconate, amphotericin B,
52 miltefosine, pentamidine and paromomycin (Aronson et al., 2016; Gradoni et al., 2017; Uliana et al.,
53 2017; Alves et al., 2018). The broad antimicrobial activity of Sb-containing compounds was
54 investigated at the same time as the discovery of their antileishmanial and antitrypanosomal activities
55 (Camac 1911); these compounds are currently the first-line drugs in most parts of the world. In 1997,
56 the first indication of therapeutic failure related to leishmania drug-resistant isolates was reported in
57 the northern Bihar province of India are now problematic (Lira et al., 1999). Amphotericin B (AmB),
58 initially described as an alternative to Sb for the treatment of MCL, has also been successfully used to
59 treat severe VL and HIV/*Leishmania* coinfection (Uliana et al., 2017). Therapeutic failure or relapse
60 after treatment is reported but currently rare (Purkait et al., 2012; Pandey et al., 2012). Currently, AmB
61 (especially in its liposomal form) is now recommended by the World Health Organization and by the

62 main scientific societies as the first line drug to treat any form of VL worldwide, not only severe or
63 HIV-associated conditions but also in children and immunocompetent adults. Miltefosine is the sole
64 registered oral drug effective against leishmaniasis. Since its registration in India in 2002, a clinical
65 decrease in its efficacy has been reported (Dorlo et al., 2012). Pentamidine is a second-line drug for the
66 management of Sb-resistant VL cases, but its cure rate is inferior to that of amphotericin B (Mishra et
67 al., 1992). Additionally, the toxicities associated with pentamidine (cardiac toxicity, diabetes mellitus,
68 hypotension, and gastrointestinal side effects) limit its use. Paromomycin, an aminoglycoside
69 antibiotic, was rediscovered as an antileishmanial agent for the treatment of VL in the 1980s (Jha et al.,
70 1998), but its clinical use remains limited.

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

81

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

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

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

89 To carry AST, isolates from needle aspirates, punch biopsies, or blood samples are usually
90 subjected to initial cultivation in a diphasic agar medium, such as NNN medium (reviewed in Evans
91 DA, 1987) (Figure 1). Once established in this medium, parasites must be transferred into a semi-
92 defined culture medium (reviewed in Schuster and Sullivan, 2002). Considering the workflow
93 described in Figure 1, 4 to 30 days after biopsy are required to isolate promastigotes in culture. If the
94 *in vitro* test is performed on the promastigote stage of the parasite, then another 3 days is required. For
95 tests performed on intracellular amastigotes, after the initial proliferation phase in biphasic medium,
96 parasites are transferred into a semi-defined medium, to obtain homogeneous populations of
97 metacyclic promastigotes that are used to infect host macrophages, at a determined host cell/parasite
98 ratio (Inocência da Luz et al., 2009). Infected macrophages are then incubated for 5 days (Maes et al.,
99 2013).

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

103

104 3.1. Molecular marker

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

113 prompted the emergence of resistant leishmania strains (Marquis et al., 2005). The level of AQP1
114 activity as an Sb transporter is abolished in isolates with a homozygous two-base pair insertion in the
115 AQP1 sequence; this insertion is linked to some highly Sb-resistant field isolates (Marquis et al., 2010).
116 Hybrid strains with intermediate Sb susceptibility are heterozygous for the AQP1 insertion (Imamura
117 et al., 2016). Experimental selection for miltefosine resistance identified inactivating mutations or
118 deletions in the MIL translocation machinery (LMT and/or LROs3) (Perez-Victoria et al., 2006).
119 Mutations in LMT (2 sense mutations) are documented in *L. donovani* (Mittal et al., 2007; Cojean et al.,
120 2012) and *L. infantum* (Mondelaers et al., 2016). Amphotericin B-resistant field isolates are scarce,
121 mainly because of the high efficacy of this molecule in treating leishmania infection (Cojean et al.,
122 2012). Experimental selection for amphotericin B resistance highlighted a change in the sterol
123 composition of the membrane of drug-resistant organisms (Mbongo et al., 1998).

124

125 3.2. Antimicrobial susceptibility test (AST)

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

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

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

148 *Leishmania* can be grown in vitro as amastigotes under axenic conditions, i.e., without the
149 requirement for a host cell (Serenio et al., 1997; Monte-Alegre et al., 2001; Guptan et al., 2001). These *in*
150 *vitro*-cultured amastigotes have biochemical, morphological and immunological similarities to
151 intracellularly cultured ones (Saar et al., 1998; Li et al., 2008; Pescher et al., 2011; Brotherton et al.,
152 2012). Nevertheless, not all *Leishmania* species are amenable to culture as amastigotes in axenic
153 condition, therefore no extensive studies have been performed on field isolates.

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

160

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

162 Notably, the methodology used to ascertain the drug susceptibility of isolate must be 1)
163 amenable to standardization, 2) inexpensive, 3) easy to handle, and 4) easy to interpret and normalize.

164 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?

168

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

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

201

202 3.2. How to delineate drug resistant isolates

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

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

217 multiplicity of AST protocols described in the literature reveal that guidance and SOPs previously
218 published are not followed (Table 2). We further investigated if the amastigote ECOFF value can be
219 deduced from AST performed on promastigote. Using the linear equation of the regression line
220 (Figure 2), we calculated the IC₅₀ of amastigote and then ascertained ECOFF (see Figure 2 and Table 3).
221 The computed ECOFF reflects well those obtained on intramacrophagic amastigotes for Miltefosine
222 and to a lesser extent for Antimony (Table 3). In conclusion AST performed on promastigotes reflect
223 well the susceptibility status of intracellular amastigotes, giving the opportunity to compute the basal
224 susceptibility level of the 21 human pathogenic leishmania species, but also to search for geographic
225 variation in leishmania drug susceptibility and finally to be used as a parasite drug resistance
226 diagnostic tool.

227

228 3.3. How to translate drug-resistance diagnosis into clinical setting

229 ECOFFs, pharmacokinetic (PK) and pharmacodynamic (PD) cutoffs and clinical cutoffs defined
230 together clinical breakpoints (Box 1) that defines the clinical phenotypes: S (high likelihood of clinical
231 success), I (uncertain clinical success) or R (high likelihood of therapeutic failure). Leishmania
232 susceptibility is often defined by the IC₅₀ and less frequently by the IC₉₀ (concentrations of drug
233 required to inhibit 50% or 90% of growth). Unfortunately, to compute these values, it is necessary to
234 gather drug response information for at least 5 concentrations. This is time-consuming and do not
235 provide information on the minimal critical concentration that kills or completely inhibits the growth
236 of the parasite population, an important parameter for the clinician. From, the experience gained from
237 medical bacteriology, methods that use agar or liquid dilution for the determination of the MIC
238 (minimal inhibitory concentration) would be a faster and easier alternative to IC₅₀ or IC₉₀ calculation.
239 In addition, the effort undertaken to standardize interpretation of AST results for the disc diffusion
240 test (Kronvall and Smith, 2016) will be beneficial for leishmania drug resistance diagnosis, if amenable
241 with this micro-organism.

242

243 4. Conclusions

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

252

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255 **References**

- 256 Aït-Oudhia, K., Gazanion, E., Sereno, D., Oury, B., Dedet, J.P., Pratlong, F., Lachaud, L. 2012. *In vitro*
257 susceptibility to antimonials and amphotericin B of *Leishmania infantum* strains isolated from dogs in a
258 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).
259
- 260 Akhoundi, M., Downing, T., Votýpka, J., Kuhls, K., Lukeš, J., Cannet, A., Ravel, C., Marty, P.,
261 Delaunay, P., Kasbari, M., Granouillac, B., Gradoni, L., Sereno, D. 2017. Leishmania infections:
262 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).
263
- 264 Akhoundi, M., Kuhls, K., Cannet, A., Votýpka, J., Marty, P., Delaunay, P., Sereno D. 2016. A Historical
265 Overview of the Classification, Evolution, and Dispersion of *Leishmania* Parasites and Sandflies. *PLoS.*
266 *Negl. Trop. Dis* 10, e0004349. doi: [10.1371/journal.pntd.0004349](https://doi.org/10.1371/journal.pntd.0004349).
267
- 268 Alvar, J., Vélez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., den Boer, M., WHO
269 Leishmaniasis Control Team. 2012. Leishmaniasis worldwide and global estimates of its incidence.
270 *PLoS. One.* 7, e35671, doi: [10.1371/journal.pone.0035671](https://doi.org/10.1371/journal.pone.0035671).
271
- 272 Alves, F., Bilbe, G., Blesson, S., Goyal, V., Monnerat, S., Mowbray, C., Muthoni Ouattara, G., Pécoul,
273 B., Rijal, S., Rode, J., Solomos, A., Strub-Wourgaft, N., Wasunna, M., Wells, S., Zijlstra, E.E., Arana, B.,
274 Alvar, J. 2018. Recent development of visceral leishmaniasis treatments: successes, pitfalls, and
275 perspectives. *Clin. Microbiol. Rev.* 31, e00048-18. PMID: [30158301](https://pubmed.ncbi.nlm.nih.gov/30158301/).
276
- 277 Aronson, N., Herwaldt, B.L., Libman, M., Pearson, R., Lopez-Velez, R., Weina, P., Carvalho, E.M.,
278 Ephros, M., Jeronimo, S., Magill, A. 2016. Diagnosis and Treatment of Leishmaniasis: Clinical Practice
279 Guidelines by the Infectious Diseases Society of America (IDSA) and the American Society of Tropical
280 Medicine and Hygiene (ASTMH). *Clin. Infect. Dis.* 63, 1539-1557. PMID: [27941143](https://pubmed.ncbi.nlm.nih.gov/27941143/).

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282
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286
287
288
289
290
291
292
293
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295
296
297
298
299
300
301
302
303
304
305

Berman, J.D. 1984. *Leishmania tropica*: quantitation of *in vitro* activity of antileishmanial agents by Giemsa staining, viability, and 3H-formycin B incorporation. *J. Parasitol.* 70, 561–562, PMID: 6502359.

Bhandari, V., Kulshrestha, A., Deep, D.K., Stark, O., Prajapati, V.K., Ramesh, V., Sundar, S., Schonian, G., Dujardin, J.C., Salotra, P. 2012. Drug susceptibility in *Leishmania* isolates following miltefosine treatment in cases of visceral leishmaniasis and post kala-azar dermal leishmaniasis. *PLoS. Negl. Trop. Dis.* 6, e1657, doi: 10.1371/journal.pntd.0001657.

Bilbao-Ramos, P., Dea-Ayuela, M.A., Cardenas-Alegría, O., Salamanca, E., Santalla-Vargas, J.A. 2017. Leishmaniasis in the major endemic region of Plurinational State of Bolivia: Species identification, phylogeography and drug susceptibility implications. *Acta. Trop.* 176, 150-161. doi: 10.1016/j.actatropica. 2017.07.026.

Brotherton, M.C., Racine, G., Ouameur, A.A., Leprohon, P., Papadopoulou, B., Ouellette, M. 2012. Analysis of membrane-enriched and high molecular weight proteins in *Leishmania infantum* promastigotes and axenic amastigotes. *J. Proteome. Res.* 11, 3974-3985. doi: 10.1021/pr201248h.

Budczies, J., Klauschen, F., Sinn, B.V., Schmitt, W.D., Darb-Esfahani, S. 2012. Cutoff Finder: a comprehensive and straightforward web application enabling rapid biomarker cutoff optimization. *PLoS. One.* 7, 1–7. doi: 10.1371/journal.pone.0051862.

Camac C.N. 1911. Intramuscular and intravenous injections of antimony in trypanosomiasis. *Br. Med. J.* 2, 104-105. PMID: 20765726.

306 Cojean, S., Houzé, S., Haouchine, D., Huteau, F., Lariven, S., Hubert, V., Michard, F., Bories, C.,
307 Pralong, F., Le Bras, J., Loiseau, P.M., Matheron, S. 2012. Leishmania resistance to miltefosine
308 associated with genetic marker. *Emerg. Infect. Dis.* 18, 704-706. doi: [10.3201/eid1804.110841](https://doi.org/10.3201/eid1804.110841).
309

310 De La Llave, E., Lecoeur, H., Besse, A., Milon, G., Prina, E., Lang T. 2011. A combined luciferase
311 imaging and reverse transcription polymerase chain reaction assay for the study of *Leishmania*
312 amastigote burden and correlated mouse tissue transcript fluctuations. *Cell. Microbiol.* 13, 81-91, doi:
313 [10.1111/j.1462-5822.2010.01521.x](https://doi.org/10.1111/j.1462-5822.2010.01521.x).
314

315 Deep, D.K., Singh, R., Bhandari, V., Verma, A., Sharma, V. 2017. Increased miltefosine tolerance in
316 clinical isolates of *Leishmania donovani* is associated with reduced drug accumulation, increased
317 infectivity and resistance to oxidative stress. *PLoS. Negl. Trop. Dis.* 11, e0005641, doi:
318 [10.1371/journal.pntd.0005641](https://doi.org/10.1371/journal.pntd.0005641).
319

320 Dorlo, T.P., Balasegaram, M., Beijnen, J.H., de Vries, P.J. 2012. Miltefosine, a review of its
321 pharmacology and therapeutic efficacy in the treatment of leishmaniasis. *J. Antimicrob. Chemother.*
322 67, 2576-2597, doi: [10.1093/jac/dks275](https://doi.org/10.1093/jac/dks275).
323

324 Dube A, Gupta R, Singh N. Reporter genes facilitating discovery of drugs targeting protozoan
325 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).
326

327 Eddaikra, N., Ait-Oudhia, K., Kherrachi, I., Oury, B., Moulti-Mati, F., Benikhlef, R., Harrat, Z., Sereno,
328 D. 2018a. Antimony susceptibility of *Leishmania* isolates collected over a 30-year period in Algeria.
329 *PLoS. Negl. Trop. Dis.* 1, e0006310, doi: [10.1371/journal.pntd.0006310](https://doi.org/10.1371/journal.pntd.0006310).
330

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 leishmaniases in the WHO European Region. World Health Organization Regional Office for Europe,
348 Copenhagen.
349
350 Guptan, 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 Schönian, 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 Kronvall, G., Smith, P. 2016. Normalized resistance interpretation, the NRI method: Review of NRI
385 disc test applications and guide to calculations. APMIS. 124, 1023-1030. doi: [10.1111/apm.12624](https://doi.org/10.1111/apm.12624).

386

387 Kulshrestha, A., Bhandari, V., Mukhopadhyay, R., Ramesh, V., Sundar, S., Maes, L., Dujardin, J.C.,
388 Roy, S., Salotra, P. 2013. Validation of a simple resazurin-based promastigote assay for the routine
389 monitoring of miltefosine susceptibility in clinical isolates of *Leishmania donovani*. Parasitol. Res. 112,
390 825-828. doi: [10.1007/s00436-012-3212-3](https://doi.org/10.1007/s00436-012-3212-3).

391

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

395

396 Kumar, D., Kulshrestha, A., Singh, R., Salotra, P. 2009. *In vitro* susceptibility of field isolates of
397 *Leishmania donovani* to Miltefosine and amphotericin B: correlation with sodium antimony gluconate
398 susceptibility and implications for treatment in areas of endemicity. Antimicrob. Agents. Chemother.
399 53, 835-838, doi: [10.1128/AAC.01233-08](https://doi.org/10.1128/AAC.01233-08).

400

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

404

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

407

408 Leprohon, P., Légaré, D., Raymond, F., Madore, E., Hardiman, G. 2009. Gene expression modulation is
409 associated with gene amplification, supernumerary chromosomes and chromosome loss in antimony-
410 resistant *Leishmania infantum*. Nucleic. Acid. Res. 37, 1387-1399. doi: [10.1093/nar/gkn1069](https://doi.org/10.1093/nar/gkn1069).
411

412 Li, Q., Zhao, Y., Ni, B., Yao, C., Zhou, Y., Xu, W., Wang, Z., Qiao, Z. 2008. Comparison of the
413 expression profiles of promastigotes and axenic amastigotes in *Leishmania donovani* using serial
414 analysis of gene expression. Parasitol. Res. 103, 821-828. doi: [10.1007/s00436-008-1048-7](https://doi.org/10.1007/s00436-008-1048-7).
415

416 Lira, R., Sundar, S., Makharia, A., Kenney, R., Gam, A. 1999. Evidence that the high incidence of
417 treatment failures in Indian kala-azar is due to the emergence of antimony-resistant strains of
418 *Leishmania donovani*. J. Infect. Dis. 180, 564-567, doi: [10.1086/314896](https://doi.org/10.1086/314896).
419

420 Maes, L., Cos, P., Croft, S.L. The Relevance of Susceptibility Tests, Breakpoints, and Markers. In: Ponte-
421 Sucre A., Diaz E., Padrón-Nieves M. (eds) Drug Resistance in Leishmania Parasites. 2013; Springer,
422 Vienna.
423

424 Maia, C., Nunes, M., Marques, M., Henriques, S., Rolão, N., Campino, L. 2013. *In vitro* drug
425 susceptibility of *Leishmania infantum* isolated from humans and dogs. Exp. Parasitol. 135, 36-41, doi:
426 [10.1016/j.exppara.2013.05.015](https://doi.org/10.1016/j.exppara.2013.05.015).
427

428 Marquis, N., Gourbal, B., Rosen, B.P., Mukhopadhyay, R., Ouellette, M. 2005. Modulation in
429 aquaglyceroporin AQP1 gene transcript levels in drug-resistant *Leishmania*. Mol. Microbiol. 57, 1690-
430 1699. doi: [10.1111/j.1365-2958.2005.04782.x](https://doi.org/10.1111/j.1365-2958.2005.04782.x).
431

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., Papadopoulou, 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](https://doi.org/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](https://doi.org/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](https://doi.org/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](https://pubmed.ncbi.nlm.nih.gov/9763285/).

505

506 Schuster, F.L., Sullivan, J.J. 2002. Cultivation of clinically significant hemoflagellates. *Clin. Microbiol.*

507 *Rev.* 15, 374-389. PMID: [12097246](https://pubmed.ncbi.nlm.nih.gov/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., Adai, 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

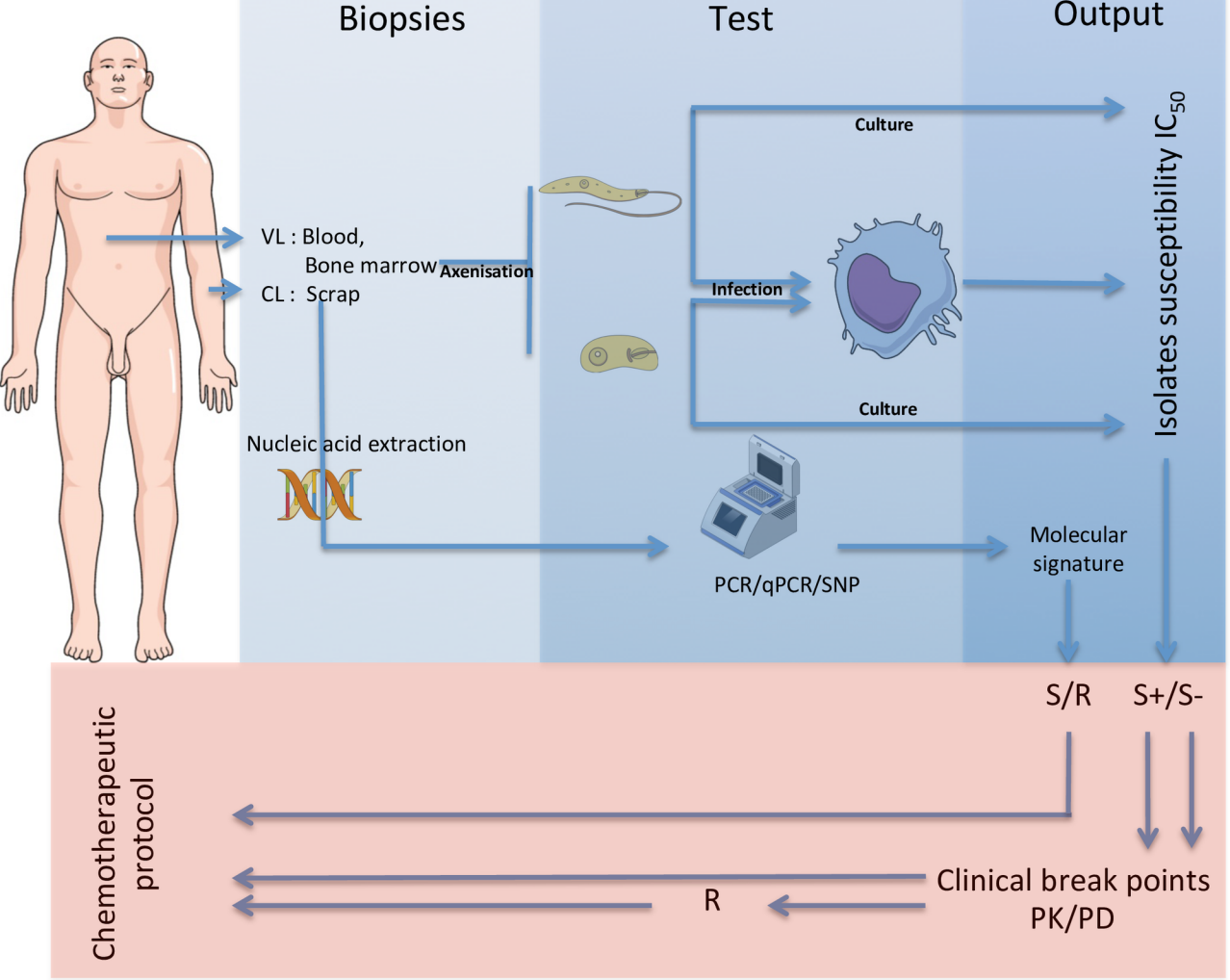
555 **Legends to the figures**

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

566

567 **Figure 2:** Exemple of linear correlation between promastigote and intracellular amastigote drug
568 susceptibility. The identification of isolates included in the analysis of Miltefosine and Amphotericin b
569 is provided in the figure. The regression line equations are given in the figure after being computed by
570 forcing the regression to pass through the origin, dashed lines represent the 90% confidence interval
571 values. Data were extracted from Kumar and collaborators (2009) and Eddaikra and collaborators
572 (2018).

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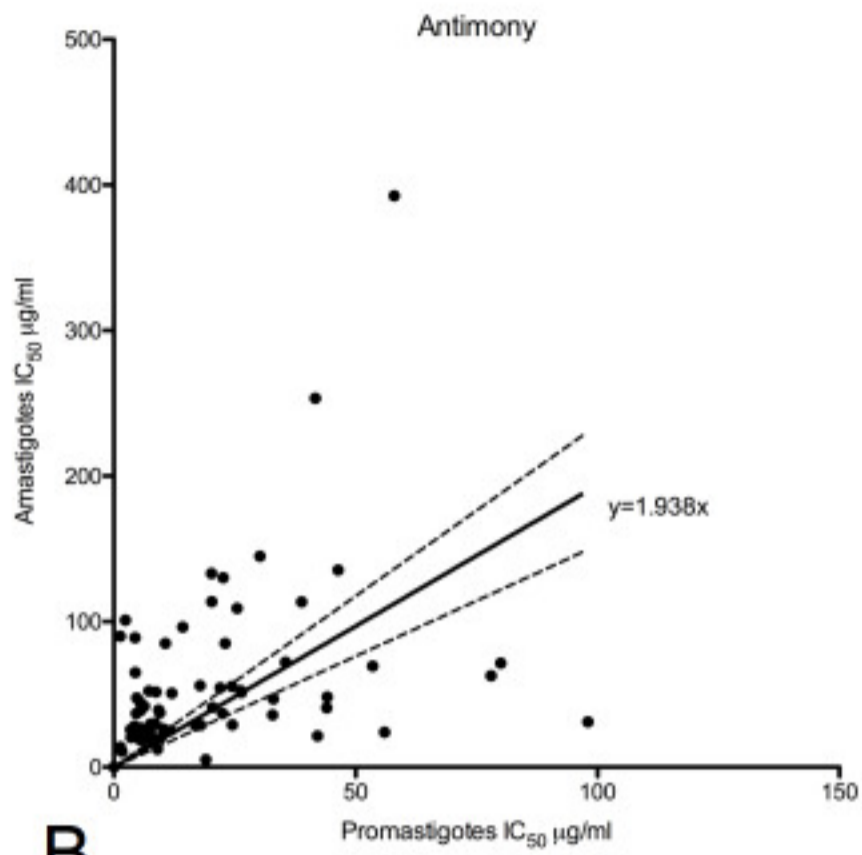
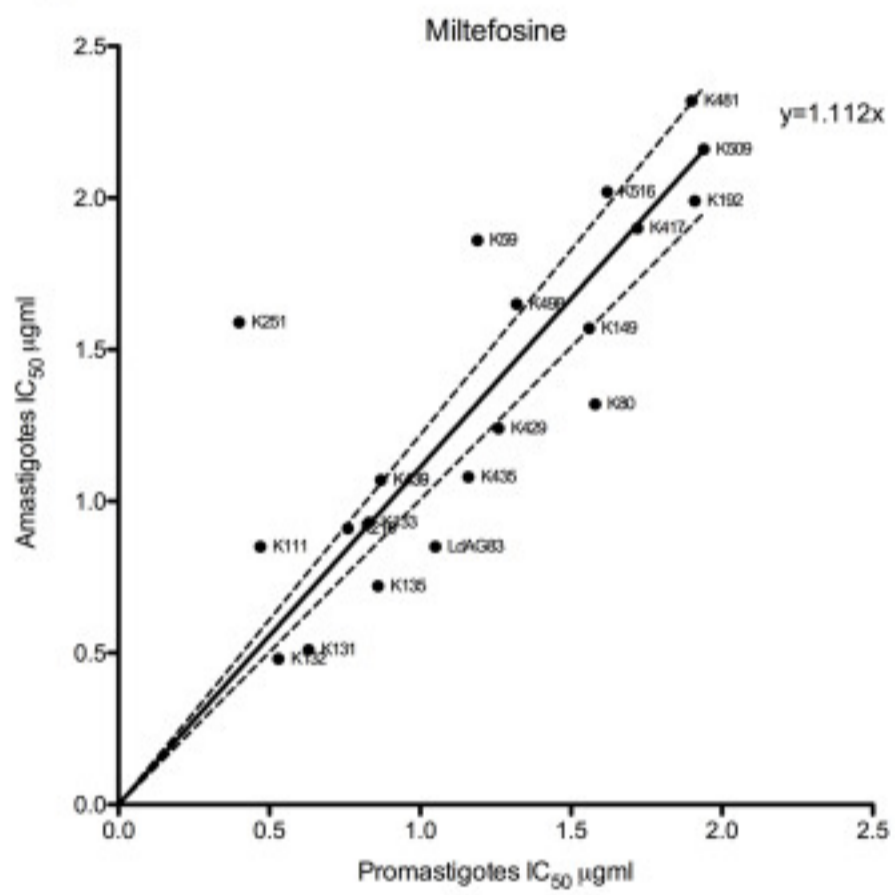
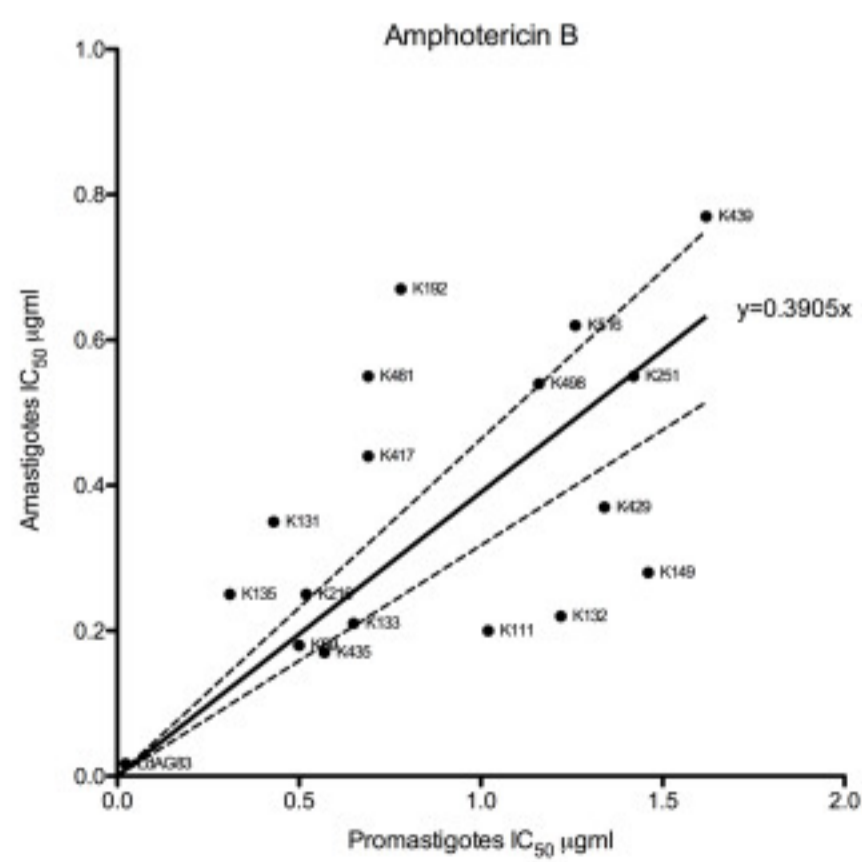
A**B****C**

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	Ait-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 R^2 are given. NS not significant, NA not available, not tested in the study.

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

Drugs	Leishmania 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/Counting	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/Counting	1.36	ND	Kulshrestha et al., 2011
Paromomycin (µM)	<i>L. donovani</i>	20	M199/3d/Counting	J774A1/2d/Counting	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 *ECOFF-(S+/S-) – N	Amphotericin B *ECOFF-(S+/S-) – N	Antimony *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.