Retrospective and ongoing researches on Leishmania antimony resistance in Algeria

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Leishmania are the causative protozoal agents of leishmaniasis, that is a significant cause of morbidity and mortality in more than 88 countries. In the absence of any effective vaccines, the only feasible way to treat leishmaniasis is through the use of medications. The first line drugs is composed of molecules developed in the 1950s, like pentavalent antimony (i.e., Pentostam®), Glucantime®. Currently Leishmania antimony resistance still continues to emerge in various part of the world. In Algeria, as early as 1986, a high rate of treatment failure (48.5%) was recorded during the treatment of cutaneous leishmaniasis caused by Leishmania major. In addition, lower sensitivity to meglumine antimoniate was observed in L. major isolated from Psammomys obesus, a reservoir host. More recently decrease antimony susceptibility was reported in L. infantum strains. The current studies engaged in Algeria on the susceptibility of Leishmania parasites will shed light not only on the occurrence of antimony resistance in this area but also on factors that are involved in the selection of antimony resistance in natural Leishmania populations.

Keywords Leishmania, drug resistance, antimony, life cycle.

1. Introduction

Leishmania are the causative protozoal agents of various forms of leishmaniasis, which is a significant cause of morbidity and mortality in more than 88 countries. The clinical manifestations of leishmaniasis range from various cutaneous forms to a fatal visceral form. In the Mediterranean Basin, anthropogenic cutaneous leishmaniasis (ACL) caused by Leishmania tropica, zoonotic cutaneous leishmaniasis (ZCL) caused by Leishmania major or, less frequently Leishmania infantum and zoonotic visceral leishmaniasis (ZVL) caused by L. infantum are all endemic. Visceral leishmaniasis is widely distributed around the Mediterranean Basin, unlike ZCL and ACL, which are more restricted to meridional and oriental regions. In South America, ZCL caused by various Leishmania species (e.g., Leishmania mexicana, Leishmania (Vianna) peruviana, Leishmania (Vianna) guyanensis, Leishmania (Vianna) panamensis and others), mucocutaneous leishmaniasis caused by Leishmania (Vianna) braziliensis, diffuse cutaneous leishmaniasis caused by Leishmania amazonensis and ZVL caused by Leishmania chagasi are all highly endemic. In the Indian subcontinent, anthropogenic visceral leishmaniasis (AVL) caused by Leishmania donovani poses a major health problem. In several regions of the world, the incidence of leishmaniasis outbreaks has been associated with urbanization, travel, climatic change and social conflict [1, 2, 3].

In the absence of effective vaccines, the only feasible way to treat and control leishmaniasis is through the use of affordable medications. The current chemotherapeutic arsenal consists of molecules that were developed in the 1950s, including pentavalent antimony (SbV) compounds (e.g., Pentostam®, Glucantime®), pentamidine and various formulations of the antifungal Amphotericin B and, more recently, miltefosine [4, 5, 6]. Instead of determining therapeutic protocols based on clinical and biological indications, treatment choice is frequently dictated by economic considerations, pentavalent antimonial compounds being one of the most affordable [7]. Therapeutic failure during antimony treatment remains a well know problem, but antimony resistance is not the only factor responsible to therapeutic failure. Indeed, factors that are linked to the host (e.g., immunosuppression or malnutrition), the drug itself (e.g., drug batch or counterfeit drugs), the Leishmania species, or the practitioner (e.g., incomplete treatment follow-ups) will also play a role in parasite drug resistance and treatment failure [1, 8, 9]. In most parts of the world, the frequency of parasite antimony resistance linked to treatment failure is unknown. This information is crucial for addressing the risk of selection and transmission of drug-resistant parasites, particularly in areas where antimony is the only chemotherapeutic alternative. Here we review current tools available to diagnose Leishmania antimony resistance and after reviewing the current epidemiological situation of leishmaniases, both human and canine, we focused on ongoing researches on antimony resistance in this area.
2. Tools to monitor Leishmania antimony resistance

Various hosts and drug-derived molecules play a role in the antileishmanial activity of SbV in vivo. Therefore, to reflect the in vivo activity of antimonials, the in vitro tests must monitor the antileishmanial activity of the microbicidal compounds involved in the killing pathway of SbV. In addition, the origin of the antimonial batch has been taken into account because commercially available SbV solutions are not pure [4, 10]. In fine in all cases, the results given by the in vitro tests must always be compared with clinical observations.

2.1. Cellular models for in vitro antimonial susceptibility testing

Obviously, the intracellular forms of the parasites (amastigotes) represent the ideal form, because both indirect activity through the host cell and direct activity on the parasite can be assessed. Unfortunately, methods that involve intracellular amastigotes are labor intensive, difficult to standardize, time-consuming and dependent upon the nature of the host cell [11, 12, 13]. The development of the reporter gene technologies has enabled the quantification of Leishmania parasites in host cells and whole mammalian hosts [14, 15, 16, 17]. These technologies have been tested to determine the drug susceptibility of field isolates [18, 19]. However, the time required for transfection and the selection of recombinant parasites certainly affect the composition of the isolates in mixed Leishmania infections [20]. As shown in the table 1, the nature of the host influences the outcome of the test [12]. In fine, the lack of consensus in the standardization of this methodology makes interpretations of potential intra and interspecific variations in antimony susceptibility difficult. In fact, even if this system takes into account all the factors that are involved in intracellular SbV antileishmanial activity, its versatility and cost make its application very impractical for large-scale analysis of parasite antimony susceptibility and resistance surveys.

Table 1 Variability in the susceptibility of various Leishmania species towards SbV antimonial according to the host cell nature and the protocol of determination.

<table>
<thead>
<tr>
<th>Host cell</th>
<th>Leishmania</th>
<th>Days</th>
<th>IC₅₀ (μg/ml)</th>
<th>References</th>
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<tbody>
<tr>
<td>THP-1</td>
<td>L. infantum</td>
<td>NA</td>
<td>272.0</td>
<td>20</td>
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<tr>
<td></td>
<td>L. infantum</td>
<td>NA</td>
<td>13.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L. infantum</td>
<td>5</td>
<td>35.0</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>L. tropica</td>
<td>5</td>
<td>2-40</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>L. donovani</td>
<td>5</td>
<td>85.0</td>
<td>21</td>
</tr>
<tr>
<td>Mouse peritoneal</td>
<td>L. tropica</td>
<td>5</td>
<td>2-50</td>
<td></td>
</tr>
<tr>
<td>macrophages</td>
<td>L. major</td>
<td>5</td>
<td>6-17</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>L. amazonensis</td>
<td>2</td>
<td>30.4</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>L. amazonensis</td>
<td>2</td>
<td>49-99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>L. braziliensis</td>
<td>2</td>
<td>35-77</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>L. infantum</td>
<td>7</td>
<td>40-70</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>L. infantum</td>
<td>7</td>
<td>7-5-45.0</td>
<td>-</td>
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<td>25</td>
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<td></td>
<td>L. donovani</td>
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<td>1-8-28.0</td>
<td>26</td>
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<tr>
<td></td>
<td>L. donovani</td>
<td>3</td>
<td>14.0-18.0</td>
<td>27</td>
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<td>28</td>
</tr>
<tr>
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<td>L. infantum</td>
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<td>10-50</td>
<td>-</td>
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<tr>
<td></td>
<td>L. infantum</td>
<td>2</td>
<td>7.4-7.9</td>
<td>-</td>
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<tr>
<td>J774</td>
<td>L. mexicana</td>
<td>3</td>
<td>29.0</td>
<td>30</td>
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<td>U937</td>
<td>L. panamensis</td>
<td>3</td>
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<td>L. panamensis</td>
<td>3</td>
<td>7.0-14</td>
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</tr>
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</table>

Various Leishmania species can be grown in vitro as amastigotes under axenic conditions [33, 15]. The determination of drug activity is simple, typically inexpensive and does not require host cells, which makes standardization easier [34]. Various studies showed that protein expression in axenic amastigotes is not different from the intracellular ones. In addition axenic amastigotes seems much more infective and grow easily that those isolated from macrophages derived cells. Finally, not all Leishmania species, however, are amenable to being cultured in axenic conditions. Currently, no extensive studies using axenic amastigote systems have been performed to evaluate the antimony susceptibility of field isolates.

The usefulness of promastigotes to ascertain the antimony susceptibility status of Leishmania field isolates has been debated. Because promastigote forms are not very susceptible to pentavalent antimony, trivalent antimonial forms are used. Unfortunately, the susceptibility of promastigotes to trivalent forms of antimony does not always reflect the
sensitivity of intramacrophagic amastigotes to pentavalent antimonial formulations [25, 35, 36, 37]. Nevertheless, various experimental reports that used *L. tropica* [18], *L. infantum* [38] or *L. donovani* [21] support the notion that promastigote trivalent antimony susceptibility partially reflects the intrinsic amastigotes SbV susceptibility in macrophages. Because the host cell is omitted in tests that use promastigote forms, we hypothesized that the discrepancies observed between experiments might reflect the differential role of the host-derived microbicidal factors involved in the SbV-induced intracellular killing. Most of these factors (either drug or host-derived) are currently identified, but tests aimed at simultaneously ascertaining the intrinsic susceptibility of Leishmania promastigotes to the molecules known to play a role in the antileishmanial action of antimonials might be more representative.

2.2. Biomarkers for antimony resistance

The search for resistance biomarker is based on the in-depth understanding of the antileishmanial mode of action of antimony (see figure 1) and resistance associated with *in vitro* selection [39]. Studies on the intracellular mode of action of SbIII demonstrates that thiol buffer capacity of Leishmania is deeply affected by SbIII, through the inhibition of the trypanothione reductase [40] and via a diminution in the intracellular thiol content through an efflux mechanism yet unidentified [41]. These two mechanisms combine to profoundly compromise the thiol reduct potential in drug-sensitive parasites and lead ultimately to the accumulation of reactive oxygen species ROS [41, 42]. Many of the specific genes associated with resistance to antimony have been discovered. These mechanisms generally involved:

- limiting antimony entry to the cell. The entry of Sb(III) occurs through an AQP1 transporter [43], but the route of entry of Sb(V) is currently not identified.
- increasing the expression of the target. Sb(III) inhibits the Trypanothione reductase activity leading to an accumulation of reduced form of trypanothione. Overexpression of the Trypanothione reductase has been characterized in antimony resistant field isolates [44] with some exception [45].
- decreasing the activation of the prodrug form of Sb (i.e., Sb(V)) to the active form Sb(III). This function is assumed by two reductases, TDR1, a thiol dependent reductase, belonging to the Glutathione S-transferase family that shares homology with the *T. cruzi* Tc52 protein [46]. The second reductase characterized, LmACR2, share homology with arsenate reductases [47]. Although, currently, variations in reductase capacity of field isolates of Leishmania resistant to antimony has not been investigated.
- conjugation of drug with thiol containing molecules and sequestration or efflux of the thiol conjugated drug. In Leishmania, the major low molecular mass thiol is trypanothione in contrast with to most other eukaryote organisms, which utilize glutathione. Trypanothione is a glutathione–spermidine conjugate which is formed following several enzymatic steps, via the synthesis of glutathione (GSH) and its subsequent conjugation to spermidine. Steps in the synthesis of spermidine involved ornithine decarboxylase (ODC) and spermidine synthase. The first step in the biosynthesis of GSH is catalyzed by γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthase. The conjugation of GSH to spermidine in the following step is then catalyzed by trypanosome specific enzymes, the glutathionyl-spermidine synthetase and the trypanothione synthase. Therefore the overexpression of genes that leads to an increase in the intracellular level of reduced trypanothione (T(SH)₂), induces resistance towards SbIII [48]. The Sb–trypanothione conjugate that is formed inside the parasite cell is then sequestered within a vacule by the intracellular ABC transporter MRPA [49].

Mutations associated with resistance represent molecular tools largely applied for drug resistance surveillance. As an example, the prevalence of some single nucleotide polymorphisms (SNPs) associated with *Plasmodium* susceptibility is often a good indicator of the level of clinical resistance in a population [50]. Unfortunately, currently no SNPs for genes known to be involved in antimony resistance have been proved to be indicative of antimony resistance in field isolates. In fact, in Leishmania, upregulation of resistance genes is frequently associated with genomic rearrangements, which lead to gene amplification through homologous recombination between repeated sequences [49].
Even if trivalent form of antimony is considered as the active form of commercially available pentavalent antimony compounds, host-derived molecules also play a role in the antileishmanial activity of Sb(V) \textit{in vivo}. Both species of antimony are able to induce the generation of NO and H$_2$O$_2$ that are harmful for intracellular parasites. The entry of Sb(V) into the parasite occurs by an unknown transporter, and entry of Sb(III) through AQP1. Once inside the Leishmania, Sb(V) is reduced into Sb(III) by the action of the reductases, ACR2 (an homolog of the yeast arsenate reductase) and TDR1 (Thiol Dependent Reductase). Such reduction can also take place in the host cell (Glutathione can play the role of reducer). Sb(III) can inhibit various enzymes: the topoisomerase (Topo), the fructose-6 phosphatase (F6P) and the trypanothione reductase (TR). SbIII interferes with the antioxidat demand of the parasite through inhibition of the trypanothione and the formation of Sb(III)-TSH complex that are actively exported. These actions led to a drastic reduction in TSH and concomitantly to an accumulation of reactive oxidant species (ROS) toxic for Leishmania. Other abbreviations: TPx, Tryparedoxin, TSH, reduced trypanothione; TS2, oxidized trypanothione; Sb(TS)2, conjugate of Sb(III) with trypanothione. * Trace of SbIII can be found in pentavalent antimony formulations [4,10].

Therefore, either quantification of copy number or expression of genes known to be involved in antimony susceptibility should represent good biomarkers for addressing antimony resistance [51]. If some of the above-mentioned mechanisms are effectively encountered in resistant lines from the field any rule could be applied [42, 52, 53], because of the presence of additional unrelated and less specific mechanisms [21, 26], or because of other yet unidentified mechanisms of resistance [31].

Recent metabolomic approaches have been used to investigate parasites displaying different susceptibilities to antimony. Interestingly, a hierarchical clustering approach (antimony resistant and susceptible) revealed that differences in the metabolite abundance profiles of the drug-resistant and -sensitive clones could clearly be distinguished. The sample size of this experiment, however, was not large enough to conclusively detect drug-resistant parasites [54]. The extensive procedures involved in culturing the parasites, the high cost, the large amount of time required and the need for highly skilled individuals to perform the metabolomic analysis limit its usefulness for antimony susceptibility surveys, but metabolomic studies might provide information on new metabolic markers for monitoring Leishmania resistance.

3. Human leishmaniasis in Algeria: antimony therapy and resistance

In Algeria, leishmaniasis remains a major public health problem. The population at risk is estimated to be more than 7 millions of persons. Two clinical forms are mainly prevalent.
3.1. Cutaneous leishmaniasis

Three distinct clinical forms are encountered according to bioclimatic stages of Algeria. As shown in the Fig.2, in the Sahara and the highland regions, the zoonotic forms, caused by *L. major* (ZCL), affect more than 30,000 peoples and have an incidence of 93.6 per 100,000 inhabitants. The region of Biskra, Batna and M'sila accounted for more than 88% of the total cases recorded in the country [55]. Overall, Algeria is one of the most affected country of the Old World, Afghanistan being the country where the disease is the more prevalent [56]. Only two variants of *L. major*, zymodeme MON-25 and zymodeme MON-269 are reported in Algeria [57, 58]. The vector for *L. major* is *Phlebotomus papatasi* [59] and the wild rodents, *Psamommys obesus* and *Meriones shawi* are the main reservoir hosts [60, 61]. Recently, a spread of *L. major* from the arid zones in the south towards the semi-arid zones of the northern part of the country was reported, such that new ZCL foci were currently found in the country [62].

In the southern part of the country and particularly in the oasis of Ghardaia, the chronic form of cutaneous leishmaniasis due to *L. killicki* is present. *L. killicki* belongs to the *L tropica* complex and generally occurs in sympatry with *L. major* [63]. The annual incidence of this form of cutaneous leishmaniasis is currently unknown but is estimated to be less than 100 cases per year. The proven vector of *L. killicki* is *Phlebotomus sergenti* [64] and the suspected reservoir host is *Masouretiera mzabi*, a rodent close to *Ctenodactylus gundii* that has been found naturally infected with *L. killicki* in Tunisia [65]. In the northern part of the country a zoonotic cutaneous form of leishmaniasis, caused by *L. infantum*, is sporadic. The average incidence is of 200 new cases each year. Three different zymodemes of *L. infantum* have been shown to cause cutaneous leishmaniasis [57]. The proven vector is *Phlebotomus perfiliewi* [66] and dogs are the main reservoir of *L. infantum* MON-24, the latter is the most frequently isolated in patients or canids [67].

3.2. Visceral leishmaniasis

Visceral form of leishmaniasis occurs mainly in the northern part of the country. The disease is caused by *L. infantum* and an average of 150 new cases are recorded. The active VL foci are located in the region of Kabylie in the north of the country and in the east, the department of Jijel and Constantine. Cases are also reported in the south, in Tassili N'ajjer and Hoggar mountains in Illizi and Tamanrasset respectively. The vector is *Phlebotomus perniciosus* and canids, dogs and jackals are the main reservoir [57, 68]. The mortality rate caused by the disease is still high with 6% of total cases that affect mainly children and immunocompromised adults [69].

3.3. Treatment and drug-resistance

Pentavalent antimonial compounds remain the first line treatment against both cutaneous and visceral forms of leishmaniasis, in North Africa. However, its efficiency is currently threatened by the emergence of antimony resistance. In Algeria, the protocol for the treatment of CL recommended by the Ministry of Health is 20 mg/kg/day for 15 days by intramuscular injection in case of multiple lesions or if the lesion is located on the face. For single lesion the Ministry of Health recommends the intradermic (intraleisional) administration 1.5 to 2 ml of Glucantime twice per week for 4 weeks. Alternative therapy like the use of H2O2 10 vol or the use of cryotherapy is also recommended. For VL, the
treatment regimen applied is the one recommended by the World Health Organization. It consists in the intramuscular injection of 20 mg Sb(V)/kg/day, for 28 days. In case of unresponsiveness, intravenous perfusion of amphotericine B (Fungizone®) in a dose of 1 mg/kg/day for 15 days is used.

Patients with ZCL lesions unresponsive to Sb(V) treatment, have been previously reported in Algeria, in the focus of M’sila in 1986. The ninety-seven children, who received 60 mg/kg/day of meglumine antimoniate for 15 days, did not show any significant response to treatment compared to those receiving placebo. The use of in vitro tests using mouse peritoneal macrophages have confirmed that strains of *L. major* isolated from these children displayed a low susceptibility. In fact, isolates from unresponsive patients were less susceptible to Sb(V) antimony as compared to control parasites. The IC₅₀ values range from 18µg/ml to 52µg/ml and were significantly higher than the IC₅₀ of the reference *L. donovani* strain (6µg/ml) [70]. Several cases of VL or CL patients unresponsive to antimony treatment are currently registered in various areas of Algeria. These observations prompted to initiate studies aimed to assess the in vitro antimony susceptibility of amastigote forms for *L. major, L. tropica and L. infantum* isolated from different patients. We observed that all strains isolated before treatment were resistant towards antimony, and that a course treatment did not reduce the parasite load of the patients [Harrat et al., manuscript in preparation]. Furthermore, parasites isolated from wild rodents (*Psammomys obesus and Meriones shawi*) were found resistant in in vitro test [Harrat et al., personal communication]. All these observations support the occurrence of a primary resistance in strains endemic in Algeria. Primary resistance is distinct from the secondary ones that is induced by the treatment.

Overall, little is known about the incidence of antimony resistance in endemic foci of Algeria or of other countries from Northern Africa. This information will be crucial for addressing the risk of selection and transmission of drug-resistant parasites, particularly in areas where antimony is the main chemotherapeutic alternative like in Algeria.

4. Canine leishmaniasis in Algeria: epidemiology, antimony therapy and resistance

4.1. Epidemiology

Since 1908, it has been recognised that the dog plays a major role in the life-cycle parasite (*Leishmania infantum*) [71]. Various sandfly species of the genus *Phlebotomus* act as vectors of the parasite [72]. Canine leishmaniasis is a cosmopolitan disease and has a worldwide distribution. It is present in majority in tropical regions, but also in temperate ones of North Africa, Europe and Asia [73], especially in the moist semi-humid areas. The Mediterranean basin is particularly affected by this disease, which has been reported in almost all the countries bordering the Mediterranean, with relatively variable prevalence. The first leishmaniasis case in Algeria was described by the Sergent brothers almost a century ago [74]. Since then, several epidemiological surveys have been carried out in Algeria, with the aim of evaluating the prevalence of the human disease or the associated canine leishmaniasis (CanL). Although the general prevalence of CanL recorded in the country fluctuated below 11% for many years, it has recently increased, reaching 25.1% in 2006 [75], and this apparent increase in the prevalence of canine infection has been accompanied by an increase in the incidence of human leishmaniasis. In the Algiers area, for example, the annual number of cases of human visceral and cutaneous leishmaniasis increased by 12.2% between 1990 and 1997 [76]. As shown in the table 2, until the 1980s, the prevalence of the leishmanial infection among dogs of Algiers has been always found below 10%. It is only in the last decade that the prevalence has markedly increased, with a value superior to 20%. This change may mainly be explained by the concurrent and dramatic environmental changes that have occurred in Algeria, particularly the intense urbanization that took place in recent years. This new urbanization has led to a proliferation of the number of dogs around the houses and the removal of much natural vegetation, possibly driving vectors into peridomestic and intradomiciliary habitats.

Six zymodemes were identified in Algeria: MON-1, MON-24 in Algiers [67, 75], MON-34 and MON-77 in Grande Kabylie [83] and MON-281 [75]. In some regions in the world, several other types of canids have been found to be parasitized by *L. infantum* and have been incriminated as wild reservoirs. Infection was described in the jackal *Canis aureus* [86] the fox *Vulpes vulpes* [87], the fennec *Fennecus zerda* [88], the wolf *Canis lupus* [88], the Egyptian mongoose, *Herpestes ichneumon* [89], the European genet *Genetta genetta* and the Iberian Lynx, *Lynx pardinus* [90]. But very few isolates obtained from these wild carnivores were identified by isoenzymes. Other carnivores have been infected in highly endemic areas such as cats *Felis felis* [91].
### Table 2 Prevalence of canine leishmaniasis in Algeria in different surveys from 1910 to 2009

<table>
<thead>
<tr>
<th>Period of the study</th>
<th>Mean prevalence</th>
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<tbody>
<tr>
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<td>7.2 %</td>
<td>74</td>
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<tr>
<td>1912</td>
<td>8.8 %</td>
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<tr>
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</tr>
<tr>
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<td>2002-2005</td>
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</tr>
<tr>
<td>2006-2009</td>
<td>25.1%</td>
<td>75</td>
</tr>
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</table>

#### 4.2. Chemotherapy and drug-resistance

For the veterinarian practitioners, the treatment of canine leishmaniasis remains a problem, given its complex pathogenesis, associated with highly variable clinical signs [92]. All antileishmanial compounds in use to treat in dogs can lead to temporary or permanent remission of clinical signs but no established protocols is effective to eliminate the infection. While therapeutic protocols have evolved the drugs used for treatment haven’t changed. In case of a confirmed diagnosis, the practitioner has firstly to worry about the risks of transmission to man and then about chances of curing of the animal [93]. For this reason in Algeria but also in various parts of the world, veterinarian practitioners euthanize dogs diagnosed as positive in order to prevent the spread of the disease to humans. Infected dogs are often treated with the same drug in use to treat human, meglumine antimonate that still remains the main antileishmanial agent. Treatment with antimony is often long and costly, causing toxicity and serious side effects [94, 95]. In dogs, the treatment objectives are: (i) to induce a general reduction of the parasite load, as far as the treated dogs remain infected and potentially infectious to vectors, (ii) to restore an effective immune response, (iii) to stabilize the clinical improvement induced by drugs and delay possible relapses and (iv) to treat clinical relapses. Before considering the long, heavy and costly canine leishmaniasis treatment, two essential characteristics must be taken into account by the veterinary practitioner [95]: (i) the zoonotic nature of the disease: the canine species is in Algeria the reservoir of the parasite. The attitude of clinicians in the field is potentially difficult and has serious consequences; (ii) the persistence of the parasite in the body: Leishmania by complex mechanisms is able not only to resist to diverse processes of destruction developed by macrophages, but also to multiply within these cells. The current treatment of canine leishmaniasis is the association of meglumine antimonate-allopurinol [95, 96].

**Fig. 3** Enzymatic polymorphism of *L. infantum* canine leishmaniasis according to the countries. The highest polymorphism was found in Algeria and Spain. Nimber correspond to zymodemes MON.
Leishmania antimony susceptibility. This information is crucial for addressing the risk of selection and transmission of drug-resistant parasites, particularly in areas where antimony is the only chemotherapeutic alternative. The zoonotic potential of Canine leishmaniasis, lack of a parasitological cure, and the reported occurrence of parasitic resistance to pentavalent antimonials [98] indicates that it would be best to avoid or minimize the use of the same drugs for therapy of canine and human leishmaniasis. The recent finding that the selection for antimony resistance confers fitness advantages will likely result in the prioritization of studies aimed to understand better the antimony susceptibility of Leishmania parasites [99, 100].

5. Ongoing researches on Leishmania antimony resistance in Algeria

5.1. Transmission associated risks of antimony resistant Leishmania parasites

In Algeria, Sb(V) resistant L. major and L. infantum have been isolated from unresponsive patients and reservoirs (dogs) [38, 70]. Despite reliance on antimonials, high treatment failure rates, up to 66% is suspected in Algeria [Harrat, unpublished data]. The emergence of antimonial therapy failure linked to proven parasite resistance has stressed questions about selective factors as well as transmission risk of drug resistance. The poor knowledge about factors that favor selection of resistant parasites and the multiplicity of the agents that can play a role in the in vivo antileishmanial activity of antimony, contribute to insufficient monitoring of antimony resistance. To assess the relative fitness of an antimony-resistant Leishmania in relation to its drug-sensitive counterpart, we choose a field-based approach that will compare the prevalence of both phenotypes between three decades, over which fitness differences can become evident. We think that it will be the most adequate manner to assess the relevant natural fitness of both phenotypes, the former is essential to study each of the fitness components in more detail and to understand why the relative fitness between phenotypes differs, e.g. due to differences in survival, reproduction or transmission [99, 100].

5.2. Insights into the mechanisms of drug-resistance and identification of resistance markers

Upregulation of genes involved in drug resistance is frequently associated with genomic rearrangement, that lead to gene amplification [49]. Therefore, either quantification of copy number or expression of genes known to be involved in antimony susceptibility should represent good biomarkers for addressing antimony resistance [101]. However, the multifactorial origin of antimony resistance makes the analysis at a single gene level only partially indicative of the antimony status of the parasite. The search for biomarker should therefore include both the analysis of potential genetic mutations and of changes in transcript expression and/or protein expression level. The analysis of genetic mutations is now more easily amenable due to the development of new sequencing technologies. As an example, the whole genome sequencing of 17 L. donovani strains from Nepal (10 susceptible and 7 resistsants), was recently performed [102]. This study identified 4 different groups of resistant parasites that carried specific SNPs. This observation implies that resistance might have multiple origins and highlights the need of a multilocus approach to identify candidate genetic markers of antimony resistance. In Leishmania, both gene expression level and mutations associated with antimony resistance, can be analyzed through global RNA sequencing analysis. RNA seq also called ‘Whole Transcriptome Shotgun Sequencing” refers to the use of high throughput sequencing technologies to sequence cDNA in order to get information about a sample’s RNA content and polymorphism, a technique that is quickly becoming invaluable in the study of various diseases. Thanks to the deep coverage and base level resolution provided by next generation sequencing instruments, RNA-seq provides researchers with efficient ways to measure transcriptome data experimentally, allowing them to get information such as how different alleles of a gene are expressed, detect post-transcriptional mutations or identify gene fusions. This might be of some help to identify potential molecular marker indicative of the Leishmania susceptibility.

Proteomics has yield significant insight into mechanisms associated with: the stage differentiation process of Leishmania, the species differences, virulence determinants or drug resistance pathways [103]. A major focus has been the capability to conduct comparative analysis for the identification of new and specific biomarkers of drug resistance. The production of large-scale proteomic data sets has been made possible by technological advances in mass spectrometry, combined with the availability of the complete genome sequences of various Leishmania species, including L. infantum. Comparative studies of isolates harboring distinct drug resistance phenotypes will help to dissect the complexity of drug resistance pathways [21, 104, 107]. As an exemple, the comparative analysis of genetically pairs of antimony sensitive and resistant L. donovani strains isolated from VL patients have highlighted a number of differentially regulated proteins of them. Two proteins, HSP83 and the small kinetoplastid calpain related proteins, were implicated in the drug resistant cell death phenotype [21]. The comparative proteomic analysis of a genetically related pair of L. infantum selected in vitro for their resistance towards Sb(III) has highlighted a number of proteins differentially expressed in resistant parasites. A higher level of the enzyme argininosuccinate synthetase (ARGG) was observed in drug resistant mutants while a decrease in the expression of the kinetoplastid membrane protein (KMP-11) correlated with the drug resistance phenotype [105]. Currently all these approaches are applied to antimony resistant strains isolated in Algeria with the aim to develop molecular and/or biological marker of antimony resistance.
6. Conclusions

Leishmaniasis is not the priority in the agenda of pharmaceutical companies: because the prevalent one, ie cutaneous leishmaniasis is not fatal and it is endemic mostly in developing countries that do not represent a profitable market. It is thus becoming very difficult today to convince decision makers to fund antileishmanial drug innovation. So, one would have to focus on research and development of new tools aimed to diagnose drug resistance earlier. In Algeria, the antimonials therapy failure linked to proven parasite resistance stresses questions about selective factors and of transmission risk associated with drug resistance. However, research on Leishmania drug resistance remains dependent on: the availability of information of patients refractory to antimonials therapy, the availability of isolates before and after treatment particularly for patients with VL, the use of a standardized technique for the screening of the susceptibility phenotype and finally in the training of personnel on tests for the detection and the analysis of Leishmania drug resistance. For all that the development of resistance biomarkers is urgently needed.

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