Epidemiology of Leishmaniasis in the Time of Drug Resistance

Jean-Claude Dujardin and Saskia Decuypere

Introduction

Leishmaniasis is a group of communicable diseases widespread around the world, including Southern Europe. The disease is essentially endemic in poverty ridden settings, and clinical cases are underreported due to the absence of systematic surveillance systems (Mosleh et al. 2008; Singh et al. 2010). Hence, good consolidated region-specific epidemiological data are frequently unavailable, and we currently only have estimations of population at risk (350 million people worldwide), prevalence (12 million clinical cases worldwide) and incidence (1.5–2 million new cases occurring annually: 1–1.5 million cases of cutaneous leishmaniasis and 500,000 cases of visceral leishmaniasis) (World Health Organization 2010). Infection rates are also underestimated, particularly in species causing visceral leishmaniasis for which it is guesstimated that 10 (*L. donovani*) to 100 (*L. infantum*) asymptomatic infections may occur for 1 clinical case (Ostyn et al. 2011; Pampiglione et al. 1975). However, particular care should be taken to avoid inflating existing epidemiological data, and this further highlights the need for good (both at qualitative as quantitative levels) and updated data.

The epidemiology of leishmaniasis is dynamic, and the disease is reported to (re-)emerge and spread in many regions (Desjeux 2001; Dujardin 2006). Control is challenged by three major escalating risk factors: human-made and environmental changes, immune status (essentially because of *Leishmania*/HIV co-infection), and

J.-C. Dujardin (✉)
Molecular Parasitology Unit, Institute of Tropical Medicine, Nationalestraat 155, 2000 Antwerp, Belgium

Department of Biomedical Sciences, University of Antwerp, 2080 Antwerp, Belgium
e-mail: jcdujardin@itg.be

S. Decuypere
Molecular Parasitology Unit, Institute of Tropical Medicine, Nationalestraat 155, 2000 Antwerp, Belgium

A. Ponte-Sucre et al. (eds.), *Drug Resistance in Leishmania Parasites*,
DOI 10.1007/978-3-7091-1125-3_4,

treatment failure (TF) and drug resistance (Desjeux 2001; Dujardin 2006; Schonian et al. 2008). Drug resistance is the aim of this book, but its epidemiology cannot be dissociated from other factors that threaten the control of the disease. Indeed, the different risk factors have a reciprocal influence on each other and can vary from region to region (Dujardin 2006). This specific synergy between risk factors defines the character of drug resistance epidemiology and the nature of the challenges they pose for the local control programs.

In the present chapter, an update of various aspects of leishmaniasis epidemiology is presented, with a particular emphasis on their relation with parasite drug resistance. The focus of this chapter is on antimonials since we have most experience in the field and in the lab with this drug. Although antimonials are currently being abandoned as first-line treatment in several countries of the Indian subcontinent, we can still draw many lessons from the experience with this drug with respect to (1) speculation of the future of the few other available drugs and (2) the design and implementation of adequate surveillance strategies to monitor their efficacy. Major gaps and confusing issues currently existing in our epidemiological knowledge of drug resistance will be addressed, with a particular attention for the ambiguous interpretation of the concepts of drug resistance and treatment failure. Existing and needed tools relevant for epidemiological surveillance (at the levels of primary health centers, reference hospitals and laboratories) and the potential impact of this surveillance on local drug policies will be reviewed as a guide to orient further research activities and inspire funding agencies.

Epidemiology of Drug Resistance and Treatment Outcome

Treatment outcome is a complex phenomenon with a potentially multifactorial origin. This clinical phenotype may be determined by (1) host factors, such as genetics, immunological response (Maurer-Cecchini et al. 2009), characteristics, and clinical presentation of the patients (Nacher et al. 2001; Palacios et al. 2001); (2) treatment features, such as drug quality (Franco et al. 1995), duration of therapy, and compliance; and (3) parasite characteristics, such as variable intrinsic susceptibility (species) (Allen and Neal 1989) and drug resistance (Lira et al. 1999). The relative importance of parasite drug resistance is still unclear, but in many scientific communications, the terms "drug resistance" or "resistance" are chronically (ab-) used for both parasite and clinical phenotypes. In this paper, we will use (1) the term "drug resistance" to refer to the parasite phenotype characterized by a decreased susceptibility to a given drug, acquired following successful molecular adaptations under drug pressure and detected – till now – by an in vitro susceptibility test and (2) the term "treatment failure" for the clinical phenotype of a patient not responding to a given treatment or presenting a relapse within a specific time-window following treatment. We strongly encourage paying a particular attention to this issue, which is not only of semantic nature.

Literature survey on drug resistance reveals a severe imbalance between the number of papers providing information on (1) drug resistance of clinical isolates and (2) experimental studies, mostly on laboratory strains artificially induced. Detailed analysis of these reports (Table 1) reveal a lack of standardization in (1) clinical protocols (e.g., durations of follow-up or definitions of treatment outcome), (2) sampling procedures (e.g., parasite isolation before the onset of chemotherapy or at the time of treatment failure), and (3) laboratory procedures for testing in vitro susceptibility (e.g., the type of macrophages used in the model to test drug susceptibility), which makes it very difficult to compare the findings of the respective studies. Conditions are thus suboptimal for a correct assessment of the epidemiology of antimony (Sb^V) resistance worldwide.

Anyway, based on the current available data, we can extract the following information on the status of parasite antimonial resistance in endemic regions. First of all, isolates defined as Sb^V-resistant have been encountered so far in India [Muzzafarpur, Bihar, (Lira et al. 1999; Singh et al. 2006)], Nepal [Eastern Terai, (Rijal et al. 2007)], Iran (Hadighi et al. 2006), Eastern Sudan [Gedaref, (Abdo et al. 2003)], France (Faraut-Gambarelli et al. 1997), Peru [Amazonian jungle, (Yardley et al. 2006)], and Colombia (Rojas et al. 2006). The reported frequency of drug-resistant parasites is biased toward specific regions where treatment failure was observed; hence, their conclusions should not a priori be generalized to wider endemic regions or other regions. Secondly, the Sb^V-resistant phenotype was identified in seven different *Leishmania* species, including a species associated with zoonotic transmission (see below). Thirdly, we found that the frequency of Sb^V-resistant parasites in a particular region can be strikingly high despite testing a similar number of isolates from antimonial cured and nonresponding patients. Fourthly, the relationship between parasite phenotype and clinical phenotype varies between different regions: some studies report a good correlation between the two phenotypes (Lira et al. 1999), but others report that the in vitro drug susceptibility of the parasite has a poor predictive value for clinical treatment outcome (Rijal et al. 2007; Yardley et al. 2006). Altogether, this shows that our knowledge on the epidemiology of drug resistance in leishmaniasis is still very limited, which contrasts with other parasitic diseases, like malaria (Wongsrichanalai et al. 2002). This is possibly due to the neglected character of leishmaniasis and the lack of coordination among the few groups involved in the study of drug resistance in a clinical context but also to the complexity of *Leishmania*'s biology which impedes the development of tools to identify and study drug resistant (DR) parasites.

Literature survey on treatment outcome is more abundant (Amato et al. 2007; Gonzalez et al. 2008; Gonzalez et al. 2009; Olliaro et al. 2005; Tuon et al. 2008); but again, evidence base for comparative evaluation of antimonial treatment outcome and epidemiological mapping has many limitations due to poor design and reporting of many clinical trials (Romero and Boelaert 2010; Gonzalez et al. 2009). However, a series of key aspects emerge from the few comparable reports (see Table 2 for a selection of them). First of all, long-term treatment efficacy data is only available for antimonials in North Bihar (India) and shows how treatment

Table 1 Summary of published reports on antimonial susceptibility of clinical isolates and link with treatment outcome

Report	Species, origin (sampling period)	Number of tested isolates by clinical category [a]	Proportion Sb^V resistant isolates by clinical category [b]	Mean ED_{50} (μg/mL) or activity index (AI) [c]
Rijal et al. 2007	*L. donovani*, Nepal, Eastern Terai (2002–2004)	20 from cure	65%	AI = 3.8 +/− 2.3 [d]
		9 from TF	66.6%	AI = 4.6 +/− 2.3 [4]
Lira et al. 1999	*L. donovani*, India, Bihar, Muzzafarpur (1995–1998)	9 from cure	n.a.	ED_{50} = 2.4 +/− 2.6
		15 from TF	n.a.	ED_{50} = 7.4 +/− 3.7
Singh et al. 2006	*L. donovani*, India	7 from VL cure	14.3%	ED_{50} = 5.1 +/− 2.9
		3 from VL TF	100%	ED_{50} = 15.07 +/− 3.7
		9 from PKLD cure	0%	ED_{50} = 4.6 +/− 1.3
		4 from PKDL TF	100%	ED_{50} = 19.7 +/− 6.8
Abdo et al. 2003	*L. donovani*, Gedaref, eastern Sudan (2001–2003)	22 from TF	27.3%	n.a.
Faraut-Gambarelli et al. 1997	*L. infantum*, France, immunocompetent	6 from cure	n.a.	ED_{50} = 33.7 +/− 18.6
		9 from TF	n.a.	ED_{50} = 76.8 +/− 83.6
Faraut-Gambarelli et al. 1997	*L. infantum*, France, immunocompromised	1 from cure	n.a.	ED_{50} = 19
		20 from TF	n.a.	ED_{50} = 62.1 +/− 39.1
Hadighi et al. 2006	*L. tropica*, Iran	165 from cure	n.a.	ED_{50} = 4.1 +/− 1.6
		16 from TF	n.a.	ED_{50} = 18.9 +/− 3.2
		4 from TF	n.a.	ED_{50} = 48.6 +/− 1.9
Yardley et al. 2006	*L. braziliensis*, Peru	13 from cure	84.6%	AI = 4.3 +/− 1.5 [e]
		10 from TF	80%	AI = 5 +/− 2.1 [5]
Yardley et al. 2006	*L. guyanensis*, Peru	4 from cure	50%	AI = 4 +/− 2.4 [5]
Yardley et al. 2006	*L. lainsoni*, Peru	4 from cure	75%	AI = 4.7 +/− 2.5 [5]
Rojas et al. 2006	*L. panamensis*, Colombia	16 from TF	12.5% before treatment	ED_{50} = 21.8 +/− 41.7
		(Before and after treatment)	36.5% after TF	ED_{50} = 51.8 +/− 61.3

[a] *TF* Treatment failure, *PKDL* post-Kala-Azar dermal leishmaniasis

[b] *n.a.* not available

[c] Activity index is a criterion introduced by Yardley et al. 2006 to normalize ED_{50} results of a clinical isolate by comparison of the ED_{50} of a reference strain introduced in each assay. A value of 1 means that the isolate has an ED_{50} equal to that of the reference strain; 6 means that ED_{50} is 6 times higher than the reference strain. ED50 is defined as described by Croft in chapter "The relevance of susceptibility tests, breakpoints and markers" as the drug concentration resulting in a decrease in replication of 50% compared to untreated controls

[d] Activity index of 1 corresponds an ED_{50} = 7–18 μg/mL, an activity index of 6 corresponds to ED_{50} > 60 μg/mL

[e] Activity index of 1 corresponds an ED_{50} = 4–15 μg/mL, an activity index of 6 corresponds to ED_{50} > 60 μg/mL

Table 2 Selection of reports on the outcome of antimonial treatment in different clinical forms and regions (excluding if possible HIV-positive patients)

Report	Clinical form (species), country	No. of patients	Treatment regimen[a]	Period of recruitment	Treatment failure rate
Sundar et al. 2000	VL, India, Bihar	209	SSG, 20 mg/kg/day	1994–1997	65%
Sundar et al. 2000	VL, India, Uttar Pradesh	111	SSG, 20 mg/kg/day for 30 days	1994–1997	14%
Rijal et al. 2010	VL, Nepal, Eastern Terai	169	SSG, 20 mg/kg/day for 30 days	2001–2003	9.5%
Altaf et al. 2005	VL, Pakistan, Muzaffarabad	61	MA, dosis not specified, 21 days	1999	3.3%
Verma et al. 2007	VL, India, Uttarakhand	9	SSG, 20 mg/kg/day for 4 weeks	2004–2006	0%
Melaku et al. 2007	VL, Southern Sudan	1,178	SSG, 20 mg/kg/day for 30 days	2002–2005	7.6%
Moore et al. 2001	VL, Kenya	51	SSG, 20 mg/kg/day for 30 days	1997–1998	17%
Moore et al. 2001	VL, Kenya	51	MA, 20 mg/kg/day for 30 days	1997–1998	4%
Ritmeijer et al. 2001	VL, Ethiopia	112	SSG, 20 mg/kg/day for 30 days	1998–1999	7.9%
Toumi et al. 2007	VL, Tunisia	16	MA, 20 mg/kg/day for 25 days	1983–2002	0%
Llanos-Cuentas et al. 2008	CL (*L. braziliensis*), Peru	29	SSG, 20 mg/kg/day for 20 days	2001–2004	31%
Llanos-Cuentas et al. 2008	CL (*L. Peruviana*), Peru	63	SSG, 20 mg/kg/day for 20 days	2001–2004	28.6%
Llanos-Cuentas et al. 2008	CL (*L. guyanensis*), Peru	27	SSG, 20 mg/kg/day for 20 days	2001–2004	7.4%
Romero et al. 2001	CL (*L. braziliensis*), Brazil	52	MA, 20 mg/kg/day for 10–20 days	1996	41.2%
Romero et al. 2001	CL, (*L. guyanensis*), Brazil	59	MA, 20 mg/kg/day for 10–20 days	1996	73.7%
Firdous et al. 2009	CL (*L. major*) Pakistan	207	MA, 20 mg/kg/day for 20 days	2005–2007	19%
Padovese et al. 2009	CL and MCL, Northern Ethiopia, Tigray	167[b]	MA, 20 mg/kg/day for 28–30 days	2005–2007	28%
Uzun et al. 2004	CL, Turkey	890	MA, 10–20 mg/kg/day for 15–20 days	1998–2002	3.9%

[a]*SSG* Sodium Stibogluconate (Pentostam), *MA* Meglumine antimoniate (Glucantime)
[b]Including 5.7% HIV positive patients

efficacy can decline in two decades: from 1.5% to 14% of treatment failure in 1981–1982 (Thakur et al. 1984) to 65% in 2000–2001 as is described in chapter "Visceral Leishmaniasis" in the present volume. Secondly, substantial variations in treatment efficacy can be observed at regional level. This is best illustrated by the situation in the Indian subcontinent where treatment failure rates for antimonials range from 65% in Muzzafarpur (North Bihar, India) to 14% in Uttar Pradesh (India), 9.5% in Nepalese Eastern Terai, and 0% in Uttarakhand (India) (Sundar et al. 2000; Rijal et al. 2007; Verma et al. 2007). Hence, it is incorrect to perceive that a high antimonial treatment failure rate is a general feature of the Indian subcontinent or even India (often mentioned in scientific communications).

Generalization of region-specific results is done too often and causes misconceptions about the nature of leishmaniasis treatment failure. Thirdly, high treatment failure rates are encountered not only in endemic regions with anthroponotic leishmaniasis but also in areas with zoonotic leishmaniasis (see below). Fourthly, studies evaluating treatment efficacy should type the species of the infecting parasite as various species can have an intrinsic difference in tolerance to a particular drug (Allen and Neal 1989). This point is of particular relevance for Latin America where several species circulate which were shown to have significant differential tolerance to the treatments used in that region and concomitant variations in treatment outcome were observed. This feature is described in chapter "American Tegumentary Leishmaniasis" in the present volume. Interestingly, regional differences also occur as illustrated by the treatment outcome of infections with *L. braziliensis* and *L. guyanensis*: in Peru, it was the former species which was associated with higher treatment failure (Llanos-Cuentas et al. 2008), while in Brazil, it was the latter one (Romero et al. 2001). Fifthly, treatment failure rates are much lower than the frequency of drug resistant isolates: e.g., 31% TF vs 84.6% DR in Peru (Llanos-Cuentas et al. 2008; Yardley et al. 2006) or 9.5% TF vs 66.7% DR in Nepal (Rijal et al. 2007; Rijal et al. 2010). This raises the question on the significance of the current definition of parasite drug resistance which is based on in vitro susceptibility results (discussed later) and also indicates that treatment outcome data is currently still more relevant to guide control strategies. Since resources are limited for clinical research of neglected diseases, there is a need for giving the priority to properly designed clinical trials. Therefore, it was suggested to create an international strategy to improve the quality and standardization of future trials for a better evidence-based strategic approach in the future (Gonzalez et al. 2009).

Transmission Patterns

Leishmaniasis is characterized by two major transmission patterns, of anthroponotic and zoonotic natures, respectively. In anthroponotic forms, parasites are reported essentially to circulate between humans, without any known animal reservoir. In contrast, in zoonotic leishmaniasis, parasites circulate essentially among animals (wild or domestic), while humans are considered accidental and dead-end hosts. The latter is described in detail in chapter "The Role of Reservoirs: Canine Leishmaniasis" in the present volume. The nature of the reservoir is theoretically very important for the emergence and spreading of drug resistance. In anthroponotic leishmaniasis, the parasite is theoretically submitted to a relatively constant drug pressure (present in each host), and drug resistance may emerge and spread rapidly. In contrast, in case of zoonotic transmission, drug pressure should be absent in the wild animal reservoir; hence, drug-resistant parasites could only emerge in treated humans and be transmitted with difficulties to animals. If drug-resistant parasites do manage to be transmitted to animals, they should not have a selective advantage in animals (no drug pressure present),

except if the mechanism leading to drug resistance has a broad impact on the physiology of the parasites and change their global fitness as is described in chapter "The concept of fitness and drug resistance in *Leishmania*" in the present volume. Thus, theoretically, in zoonotic leishmaniasis, the prevalence of drug-resistant parasites before the onset of treatment (primary resistance) is expected to be very low. However, some studies indicate that in Latin America, this is not necessarily true for antimonial treatment. Some believe this is due to a shift from zoonotic to anthroponotic transmission (Rojas et al. 2006). We believe that this primary Sb^V-resistance phenotype found in zoonotic context is not the result from previous contact with the drug but a secondary effect from the adaptation to host cell stress (Yardley et al. 2006). The demonstration of cross-resistance to antimony and nitric oxide (Holzmuller et al. 2005) supports this possibility and should be further explored. In case of zoonotic visceral leishmaniasis, which involves a domestic animal reservoir (the dog), drug pressure is also present in the reservoir. Given the intense treatment courses needed to treat dogs, they could rapidly represent another epicenter for emergence and spreading of drug resistant strains. This was shown clearly in Italy, where parasites isolated from dogs treated with meglumine antimoniate were 8- to 41-fold less susceptible to the drug after treatment compared to before treatment (Gramiccia et al. 1992). Based on these observations, recommendations have been made to forbid the use of similar treatments in dogs and humans (Dujardin et al. 2008; Gramiccia et al. 1992). This important theme is described in chapter "The Role of Reservoirs: Canine Leishmaniasis" in the present volume.

There are several recent reports highlighting that the true nature of drug pressure in the different transmission modes is far more complicated than the clear-cut theory outlined above. For anthroponotic leishmaniasis, the role of asymptomatic human cases and possibly unknown animal reservoirs in VL epidemiology has been largely disregarded till now but should be examined carefully as they might have an impact on the epidemiological dynamics of drug resistance. Asymptomatic human infections are more frequent than clinical cases [up to 10 times more in a recent study in Nepal in anthroponotic VL foci (Ostyn et al. 2011)]. These people are not treated because of the cost and/or toxicity of existing drugs. Animals were recently recognized as having a possible role in the epidemiology of anthroponotic VL. In a recent emerging focus of VL in Nepal, asymptomatic *Leishmania* infections were found at higher rates among goats (16%) than humans (6.1%) (Bhattarai et al. 2010). The exact role of these infected animals and asymptomatic human carriers as reservoir is still unknown, but these findings highlight the need for further explorations. Similarly, in the context of anthroponotic cutaneous leishmaniasis of the Old World, a recent study in two foci of Northern Israel demonstrated rock hyraxes (*Procavia capensis*) to be reservoir hosts of *L. tropica* (Svobodova et al. 2006).

In zoonotic leishmaniasis, the nature of the reservoir is also questioned. The presence of *Leishmania (Viannia) sp.* parasites in unaffected skin and peripheral-blood monocytes in a high proportion of patients even after treatment and the infectivity of these subjects as shown by the acquisition of infection by sand flies

support the plausibility of anthroponotic transmission of American cutaneous leishmaniasis (Vergel et al. 2006). It is currently not known if these various reports concern exceptional situations or if they highlight serious gaps in our knowledge of the transmission patterns of leishmaniasis. Alternatively, these observations might be the consequences of changes in the epidemiology of leishmaniasis.

Human-Made and Environmental Changes of the Epidemiology

Human-made and environmental changes have a major impact on (1) the appearance of new foci and (2) transmission pattern changes, and this is confirmed by several reports.

First of all, as a consequence of global warming, leishmaniases are likely to spread into currently temperate zones where increased average temperatures may allow extension of sand fly breeding seasons, or into areas where overwintering in larval stage was so far prevented by low temperatures (Schonian et al. 2008). This is well documented in Italy, where comparisons with historical data showed that *P. perniciosus* and *P. neglectus* have increased in density and expanded their geographic range in northern continental Italy (Maroli et al. 2008). More recently, the analysis of a randomized sample of 526 healthy adults from north-western Italy (which is traditionally not considered an area of endemicity) showed a seropositivity of 7.41% and an asymptomatic infection rate (as evidenced by PCR) of 53.8% among seropositives (Biglino et al. 2010).

Secondly, increase in the worldwide mobility is also causing changes in the epidemiological picture. The best example comes from the post-Conquista era during which *L. infantum* from the Mediterranean basin was brought to Latin America (Mauricio et al. 2000), as is described in chapter "Epidemiology of leishmaniasis in the time of drug resistance" in the present volume. *L. infantum* successfully colonized local sandflies and is now causing a serious public health problem [> 3,500 cases of VL per year in Brazil (Miles et al. 1999)]. Trans-Atlantic migration of strains can still occur with potential consequences for spreading of drug resistance. For instance, miltefosine, one of the few available antileishmanial drugs, has been recently launched in the market for canine leishmaniasis treatment in Portugal, Spain, Italy, Greece, and Cyprus. Given the long half-life of the drug and the fact that dogs are never cured parasitologically, rapid emergence of drugs resistance is expected in these dogs. If dogs infected with miltefosine-resistant parasites were to migrate to Latin America, where several countries have registered the drug for human use [currently Colombia, Guatemala, Argentina, Venezuela, Paraguay, Ecuador, and Honduras; (World Health Organization 2007)], there might be epidemiological consequences (Dujardin et al. 2008). Mobility of strains also carries the additional risk of bringing new parasite phenotypes in a given region. Even if *Leishmania* is thought to reproduce essentially clonally, allogamic sexual recombination may not be excluded (Rougeron et al. 2009), and examples of successful hybrids were already shown like *L. braziliensis/L. peruviana* in Peruvian

inter-Andean valleys (Dujardin et al. 1995; Nolder et al. 2007). This might obviously contribute to the horizontal transfer of drug resistance genes, but this was not yet documented.

Thirdly, population movements for economic reasons such as the development of agroindustrial projects or the seeking of safe haven from civil unrest may contribute to increased density of susceptible human hosts with epidemiological consequences. This is illustrated by the last epidemics of VL in Sudan, where an estimated 100,000 people (out of 300,000) died from VL in Western Upper Nile State (Zijlstra and El Hassan 2001).

Fourthly, urbanization and domestication of zoonotic transmission cycles is also increasingly reported. We already reported the case of a suburban emerging focus of anthroponotic VL around the big city of Dharan in Eastern Terai (Nepal) (Bhattarai et al. 2010). However, this phenomenon is best illustrated by the situation in Latin America. In undisturbed Neotropical forests, where (muco) cutaneous leishmaniasis was for long characterized by a zoonotic profile, *Leishmania* are transmitted among sylvatic mammals by the bite of phlebotomine sand flies. The close association between forest, wild mammal reservoirs, and sand flies has previously led to predictions that deforestation would lead to local eradication of some of the most important *Leishmania* species (Esterre et al. 1986). However, as a consequence of anthropogenic environmental changes (deforestation and urbanization), new vectors and reservoir hosts may adapt and interact at the interface with humans (Garcia et al. 2007b), resulting in new pathogenic complexes tending to synanthropic zoonoses, if not anthroponoses (Rotureau 2006).

Epidemiology and Immune Status

Leishmaniasis has been identified as an opportunistic infection in immunosuppressed individuals, especially in those with human immunodeficiency virus (HIV) infection and less frequently in individuals that had organ transplant, chemotherapy for malignancy, or suffer from immune-mediated disorders (Alvar et al. 2008). The theme of HIV co-infection is described in chapter "Co-infection with HIV" in the present volume. Immunosuppression is one of the factors responsible for increased susceptibility to primary *Leishmania* infection or reactivation of a silent infection. Among the various sources of immunosuppression, HIV co-infections and its epidemiology have been best documented. *Leishmania*/HIV co-infection has emerged as a result of the increasing overlap between leishmaniasis (mainly visceral) and AIDS, which is due to the spread of the AIDS pandemic to rural areas and of visceral leishmaniasis to suburban areas. Historically, this was first described in the early 1990s in Southern Europe, when the typical clinical pediatric leishmaniasis profile was shifting due to an increasing number of HIV co-infected adults. A dedicated surveillance network was established in 1994 and revealed, by early 2001, a cumulative number of cases peaking at 1,911 (Desjeux and Alvar 2003). A clear decrease of the incidence of co-infection was observed later on in

Europe, which is likely attributed to the routine use of highly active antiretroviral therapy (HAART) (Alvar et al. 2008). Currently, HIV co-infection also affects the three major foci of visceral leishmaniasis, e.g., the Indian sub-continent, East Africa, and Brazil. In India, HIV infection is concentrated in the south and to a lesser degree in the northeast (including the district Bihar) where overlap with leishmaniasis occurs (Alvar et al. 2008). There, the problem of co-infection seems to be exacerbated by economic migrants who acquire HIV in urban settings and then return to their rural homes in VL endemic areas, where a new *Leishmania* infection can be acquired or an old infection reactivates due to declining immunity (Alvar et al. 2008). In a clinical setting of Bihar, the VL-HIV co-infection rate was shown to increase from 0.88% in 2000 to 2.18% in 2006 (Alvar et al. 2008). In Brazil, where a surveillance network exists, 2% of VL patients were shown to be co-infected compared to 0.1% in patients with cutaneous leishmaniasis (Maia-Elkhoury et al. 2007). In East Africa, the worst situation reported so far was in Humera (northwest Ethiopia), where the proportion of VL patients who were co-infected with HIV increased from 18.5% in 1998–1999 to 40% in 2006 (Alvar et al. 2008).

There are few clinical trials analyzing the efficacy of the different treatments for *Leishmania*/HIV co-infected patients as discussed in chapter "Co-infection with HIV" of this volume. In general, these patients have lower cure rates, higher drug toxicity rates, and higher fatality rates for leishmaniasis compared to immunocompetent patients. Following multiple relapses, the patient often becomes unresponsive to all the previously used drugs (Alvar et al. 2008). Furthermore, co-infected patients, which can have high parasite loads in the bloodstream, were confirmed to be a source of infection for the sand fly or for other humans (through sharing syringes among intravenous drug users) (Alvar and Jimenez 1994). Co-infected patients might thus become a true reservoir for zoonotic VL, especially in an urban setting (Ritmeijer et al. 2001). In this context and considering the very long treatment schemes in case of co-infection, it is feared that HIV co-infected patients might be a source for the emergence of drug resistance. This was well documented in a study of French patients infected with *L. infantum*. Relapses were observed in all HIV infected patients, and the susceptibility of isolated parasites was shown to decrease progressively during successive courses of meglumine antimoniate treatments (Faraut-Gambarelli et al. 1997). In contrast, in a similar follow-up study of HIV co-infected patients treated with amphotericin-B, successive isolates from individual patients did not show decrease in susceptibility, which led to the suggestion that amphotericin-B will remain a useful drug against VL, even when used as a prophylactic or in repetitive treatment courses (Lachaud et al. 2009). In the case of miltefosine, there are no follow-up studies available so far in the context of HIV co-infection. However, given the short half-life of the drug (about 1 week) and the ease of selecting for parasite miltefosine resistance in vitro (Seifert et al. 2003), there is a serious risk of emergence of resistance in relapsing patients (Berman et al. 2006). These observations illustrate the relevance of HIV co-infection surveillance networks but also strengthen the importance of implementing surveillance of drug resistance among the identified patients.

Tools for Epidemiological Surveillance of Drug Resistance and Treatment Outcome

Considering the fact that only few drugs are available, with a low number in the pipeline, it is essential to safeguard the effectiveness of existing drugs. Combination regimens are under clinical development (van Griensven et al. 2010), but the drug policy will take several more years to change. Meanwhile, the uninterrupted supply of quality drugs, the promotion of treatment adherence, and the monitoring of treatment effectiveness and drug resistance will be pivotal. There is currently no systematic surveillance of these critical issues in leishmaniasis, as existing, for instance, for malaria (Guerin et al. 2009), which is – among others – due to the lack of adequate tools.

Monitoring leishmaniasis treatment effectiveness is complicated by the fact that the parasites persist after clinical cure of a primary symptomatic episode (Bogdan 2008), and it is clinically well documented that there is a persisting risk of relapse in the first 6–12 months after treatment. Consequently, clinicians only consider patients definitely cured if this posttreatment period passes uneventful (Murray 2004; Murray et al. 2005). Given the window of time required to assess clinical cure, it has been and still is difficult to standardize the clinical definitions of the major treatment outcomes cure, nonresponse, and relapse for the different forms of leishmaniasis (Modabber et al. 2007). An adequate laboratory test of cure is also not available because it is currently unclear which indicators have the best predictive value of definite clinical cure. Serum antibodies can decrease after successful treatment as shown with the rk39 test (Braz et al. 2002; Kumar et al. 2001), but they remain detectable up to several years after cure (De Almeida et al. 2006); hence, VL relapse cannot be diagnosed by serology (Chappuis et al. 2007). PCR is extremely sensitive to detect current infections but was shown to be a better marker of infection than of disease (Deborggraeve et al. 2008); thus, a positive PCR at the end of treatment would have a low predictive value of treatment outcome. Quantification of the parasite load by QPCR allows determining infection/disease thresholds and was proven to have some potential to address this problem, but further studies are needed to evaluate this tool in various clinical situations (presentations, treatments, and infecting parasite species) (Antinori et al. 2009; Mary et al. 2006). Antigen detection tests like the Katex represent a promising avenue for tests of cure (Sundar et al. 2005), but similarly as with PCR, a too high sensitivity could decrease their performances. In absence of an adequate laboratory test of cure, treatment outcome is currently assessed by microscopic evaluation of parasite load in tissue smears at the end of treatment and subsequent regular patient follow-up for 6 up to 12 months posttreatment. Hence, monitoring treatment effectiveness in routine conditions is difficult, and compliance of patients to the follow-up visits can be poor. Hence, there is a need to develop new approaches to monitor treatment effectiveness at the program level, like the retrospective cohort analysis used in tuberculosis programs (Mukherjee et al. 2004).

Monitoring drug resistance suffers from the same limitations as outlined above for monitoring treatment efficacy, i.e., there is an acute lack of knowledge and tools. The in vitro intracellular amastigote-macrophage model is currently commonly used for testing drug susceptibility of *Leishmania* clinical isolates and is essential for drugs like antimonials, which are only active on the amastigote form of the parasite (Vermeersch et al. 2009). The screening entails a complex, labor-intensive, and time-consuming protocol, which involves in vitro infection of primary macrophages with an infective stage of *Leishmania* parasites (metacyclic promastigotes, axenic amastigotes, or ex vivo amastigotes), 3–7 days Sb^V exposure and a final step of microscopical evaluation of the different infections (Neal and Croft 1984; Vermeersch et al. 2009).

This test should not be considered as a golden standard since it is plagued by many implementation problems. First of all, there are several standardization problems with these assays, making interlaboratory comparisons difficult. Protocols differ at the level of used type of host cells, tested drug concentrations, timing of drugging, duration of drug exposure, and inclusion (or not) of reference strains for interassay comparison. Furthermore, the significance of the results must be interpreted with extreme care. In vivo, the response of the infected cells to antimonials leads to a more substantial involvement of the host immune system to attack the parasites (Mookerjee et al. 2006), resulting in a synergistic activity between antimonials and the specific T-cell response of the host (Murray et al. 2000). In comparison, the in vitro system used for susceptibility assays does not include any immune components and is somehow reductionist. Accordingly, the parasites may develop various epi-phenotypes under in vivo antimonial pressure that remain hidden in the in vitro susceptibility assays. This could concern, for instance, (1) adaptation to the macrophage effectors in the immunological context of the clinical infection (absent in the in vitro susceptibility assays) or (2) resistance to the reduced form of the drug, Sb^{III} (Rijal et al. 2007; Yardley et al. 2006). These epi-phenotypes might explain the incongruence between parasites' in vitro antimonial susceptibility and clinical treatment outcome reported elsewhere (Abdo et al. 2003; Rijal et al. 2007; Yardley et al. 2006). Further efforts are definitively needed to standardize these intracellular amastigote assays and possibly for upgrading them: activation of the macrophages with cytokines could be an option, but the system would still be miles away from the immune involvement in vivo. In parallel, susceptibility assays on extracellular promastigotes, which are relatively easily grown in vitro, should be developed and standardized for new drugs like miltefosine that have similar activity on all life stages of *Leishmania*. This is currently being explored in the frame of the Kaladrug-R project (see www.leishrisk.net/kaladrug), and if successful validation ensues, this would seriously facilitate the epidemiological surveillance of parasite resistance against that specific drug, under conditions of standardization and quality control.

Molecular assays represent a third category of tools relevant for epidemiological surveillance of treatment failure and drug resistance.

First, as highlighted above, since the infecting *Leishmania* species is a risk factor for treatment failure, it is highly recommended to perform species typing, especially in regions where different species are endemic. While multilocus enzyme electrophoresis is still considered as the reference method for species typing, we

highly recommend implementing the new PCR-based assays which are (1) much simpler to use, (2) better standardized, and (3) directly applicable on clinical samples without losing sensitivity. The *hsp70* PCR-RFLP is currently being disseminated for this purpose (da Silva et al. 2010; Garcia et al. 2007a; Montalvo et al. 2010) and might become the future reference method.

Strain fingerprinting assays represent a second class of molecular tools that can also be relevant for monitoring treatment outcome. Theoretically, they could be used to distinguish relapse from reinfection in clinical secondary symptomatic cases, a phenomenon which is poorly studied in leishmaniasis, in contrast to malaria, for instance (Collins et al. 2006). PCR-RFLP analyses of kinetoplast DNA are very useful for this purpose because they generate strain-specific patterns (Laurent et al. 2007). In a recent study on naturally infected dogs treated with antimonials (da Luz et al. 2009), we found different parasite genotypes in each dog, and the genotype of a particular dog did not change significantly after successive treatments. The apparent stability of the genotype strongly contrasted with the decreasing in vitro SSG susceptibility of the corresponding parasite isolates. This study provides convincing evidence that short-term treatment of dogs with antimonial leads to enhanced selection of decreased susceptibility.

Molecular assays are also expected to facilitate the detection of drug-resistant parasites. However, not much is known about the molecular adaptations acquired by drug resistant parasites, and this impedes the design of such tools. Most molecular studies on *Leishmania* drug resistance were done on in vitro-induced resistant parasites. The identified mechanisms and markers in these "artificial" drug-resistant parasites cannot a priori be extrapolated to the "natural" drug-resistant parasites emerging in endemic regions (Maltezou 2010). In natural *Leishmania* populations under treatment pressure, it seems that drug resistance is emerging frequently through independent events (Laurent et al. 2007), and the essential molecular adaptation process may not necessarily be uniform throughout a parasite population. We assessed the molecular heterogeneity of an antimonial-resistant *L. donovani* population in Nepal and found that the SSG-resistant phenotype is marked by a distinct set of molecular features in two genetic subpopulations. The identified molecular features further suggested a possible relation between antimonial tolerance and oxidative stress tolerance, and this was confirmed through a battery of in vitro susceptibility stress tests (Decuypere 2007). In *L. braziliensis* from Peru, we found that the expression of two genes, *ODC* (ornithine decarboxylase) and *TRYR* (trypanothione reductase) was significantly higher in some, but not all, Sb^V-resistant parasites. Interestingly, putative markers correlated better with treatment outcome than with the in vitro susceptibility phenotype (Adaui et al. 2011). We found a similar result in our *L. guyanensis* study in Brazil, where *GSH1* (encoding gamma-glutamylcysteine synthetase) was 3.9-fold overexpressed in isolates from therapeutic failure patients (11) compared to isolates from clinical cure patients (14) (Torres et al. 2008). We hypothesized that genetically distinct parasite populations acquire a different set of molecular adaptations under antimonial treatment pressure, which could complicate the design of widely applicable molecular surveillance tools (Decuypere 2007; Laurent et al. 2007). The success of

the targeted molecular studies done so far largely depend on a good foreknowledge of candidate cellular pathways that may be modified in drug-resistant parasites. However, there are still many hiatuses in our knowledge on the mode of action of antileishmanial compounds, the cellular pathways they affect, and the protective mechanisms the parasite can muster in defense against them. Hence, we believe that untargeted approaches might be more adequate for studying *Leishmania* drug resistance, and recent technological developments have brought some new perspectives in that respect. The new high-throughput sequencing technologies and latest mass-spectrometry techniques offer great potential to screen the whole genome, transcriptome, and metabolome for molecular adaptations that correlate with drug resistant phenotypes (Dujardin 2009; Scheltema et al. 2010; t'Kindt et al. 2010). Furthermore, this molecular exploration should not only focus on the identification of markers of the in vitro drug susceptibility phenotype (with all the possible biases associated with it) but also – and maybe essentially – on the clinical phenotype. At the end of the day, this is the feature to which health professionals are confronted in first line.

Conclusions and General Recommendations

Surveillance of treatment effectiveness and drug resistance is a major contributor to the understanding of the epidemiology of leishmaniasis and is pivotal in the control of this disease. This chapter highlights the importance of integrating its study in a broad context; a mathematical modeling approach is definitively needed to assess the complexity of its dynamics. However, our literature survey also demonstrates how limited our knowledge is on the epidemiology of treatment effectiveness and drug resistance. The lack of standardization of study methods is a major problem and burdens all levels from clinical to experimental research. Research and coordination platforms are therefore needed. The recently launched Kaladrug-R initiative (see www.leishrisk.net/kaladrug) is such a platform and aims to develop, evaluate, and disseminate new tools for the assessment of drug resistance in *L. donovani* and innovative methodologies for monitoring Kala-Azar treatment effectiveness under routine conditions. By providing knowledge and tools relevant for monitoring the effectiveness of the existing few drugs, this type of initiative should contribute to their "protection" and establish the bases for their longer-term and more rational use.

Acknowledgments The work of JCD and SD here mentioned was supported by the EC (projects Leishnatdrug-R, contract ICA4-CT-2001-10076; LeishepinetSA, contract INCO-CT2005-015407; Kaladrug-R, contract 222895), the Directorate General for Development Cooperation of the Belgian Government (framework agreements 02 and 03) and the GeMInI initiative of the Institute of Tropical Medicine.

References

Abdo MG et al (2003) Antimony-resistant *Leishmania donovani* in eastern Sudan: incidence and *in vitro* correlation. East Mediterr Health J 9:837–843

Adaui V et al (2011) Comparison of gene expression patterns among *Leishmania braziliensis* clinical isolates showing a different *in vitro* susceptibility to pentavalent antimony. Parasitology 138:183–193

Allen S, Neal RA (1989) The *in vitro* susceptibility of macrophages infected with amastigotes of *Leishmania* spp. to pentavalent antimonial drugs of other compounds with special relevance to cutaneous isolates. In: Hart D (ed) Leishmaniasis. Plenum Publishing Cooperation, New York, pp 711–720

Altaf et al (2005) Childhood visceral leishmaniasis in Muzaffarabad, Azad Jammu and Kashmir: frequency and response to treatment in 61 cases. J Pak Med Assoc 55:475–478

Alvar J, Jimenez M (1994) Could infected drug-users be potential *Leishmania infantum* reservoirs? AIDS 8:854

Alvar J et al (2008) The relationship between leishmaniasis and AIDS: the second 10 years. Clin Microbiol Rev 21:334–359

Amato VS et al (2007) Treatment of mucosal leishmaniasis in Latin America: systematic review. Am J Trop Med Hyg 77:266–274

Antinori S et al (2009) Is real-time polymerase chain reaction (PCR) more useful than a conventional PCR for the clinical management of leishmaniasis? Am J Trop Med Hyg 81:46–51

Berman J et al (2006) Miltefosine: issues to be addressed in the future. Trans R Soc Trop Med Hyg 100(Suppl 1):S41–S44

Bhattarai NR et al (2010) Domestic animals and epidemiology of visceral leishmaniasis, Nepal. Emerg Infect Dis 16:231–237

Biglino A et al (2010) Asymptomatic *Leishmania infantum* infection in an area of northwestern Italy (Piedmont region) where such infections are traditionally nonendemic. J Clin Microbiol 48:131–136

Bogdan C (2008) Mechanisms and consequences of persistence of intracellular pathogens: leishmaniasis as an example. Cell Microbiol 10:1221–1234

Braz RF et al (2002) The sensitivity and specificity of *Leishmania chagasi* recombinant K39 antigen in the diagnosis of American visceral leishmaniasis and in differentiating active from subclinical infection. Am J Trop Med Hyg 67:344–348

Chappuis F et al (2007) Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nat Rev Microbiol 5:873–882

Collins WJ et al (2006) The use of genotyping in antimalarial clinical trials: a systematic review of published studies from 1995-2005. Malar J 5:122

da Luz RI et al (2009) *In vitro* sensitivity testing of *Leishmania* clinical field isolates: preconditioning of promastigotes enhances infectivity for macrophage host cells. Antimicrob Agents Chemother 53:5197–5203

da Silva LA et al (2010) Sequence analysis and PCR-RFLP profiling of the hsp70 gene as a valuable tool for identifying *Leishmania* species associated with human leishmaniasis in Brazil. Infect Genet Evol 10:77–83

De Almeida SL et al (2006) Immunologic tests in patients after clinical cure of visceral leishmaniasis. Am J Trop Med Hyg 75:739–743

Deborggraeve S et al (2008) Diagnostic accuracy of a new *Leishmania* PCR for clinical visceral leishmaniasis in Nepal and its role in diagnosis of disease. Trop Med Int Health 13:1378–1383

Decuypere, S. (2007) Antimonial treatment failure in anthroponotic visceral leishmaniasis: towards improved tools and strategies for epidemiological surveillance and disease control. Doctoral Thesis. Dissertation, University of Antwerp

Desjeux P (2001) The increase in risk factors for leishmaniasis worldwide. Trans R Soc Trop Med Hyg 95:239–243

Desjeux P, Alvar J (2003) *Leishmania*/HIV co-infections: epidemiology in Europe. Ann Trop Med Parasitol 97(Suppl 1):3–15
Dujardin JC (2006) Risk factors in the spread of leishmaniases: towards integrated monitoring? Trends Parasitol 22:4–6
Dujardin JC (2009) Structure, dynamics and function of *Leishmania* genome: resolving the puzzle of infection, genetics and evolution? Infect Genet Evol 9:290–297
Dujardin JC et al (1995) Putative *Leishmania* hybrids in the Eastern Andean valley of Huanuco, Peru. Acta Trop 59:293–307
Dujardin JC et al (2008) Spread of vector-borne diseases and neglect of Leishmaniasis, Europe. Emerg Infect Dis 14:1013–1018
Esterre P et al (1986) Evaluation of a cutaneous leishmaniasis control program in a forest village of French Guyana. Bull World Health Organ 64:559–565
Faraut-Gambarelli F et al (1997) *In vitro* and *in vivo* resistance of *Leishmania infantum* to meglumine antimoniate: a study of 37 strains collected from patients with visceral leishmaniasis. Antimicrob Agents Chemother 41:827–830
Firdous et al (2009) Efficacy of glucantime in the treatment of Old World cutaneous leishmaniasis. Int J Dermatol 48:758–762
Franco MA et al (1995) Antimony oxidation states in antileishmanial drugs. Am J Trop Med Hyg 52:435–437
Garcia AL et al (2007a) American tegumentary leishmaniasis: direct species identification of *Leishmania* in non-invasive clinical samples. Trans R Soc Trop Med Hyg 101:368–371
Garcia AL et al (2007b) Epidemiological monitoring of American tegumentary leishmaniasis: molecular characterization of a peridomestic transmission cycle in the Amazonian lowlands of Bolivia. Trans R Soc Trop Med Hyg 101:1208–1213
Gonzalez U et al (2008) Interventions for Old World cutaneous leishmaniasis. Cochrane Database Syst Rev (4):CD005067
Gonzalez U et al (2009) Interventions for American cutaneous and mucocutaneous leishmaniasis. Cochrane Database Syst Rev 2009(2):CD004834
Gramiccia M, Gradoni L, Orsini S (1992) Decreased sensitivity to meglumine antimoniate (Glucantime) of *Leishmania infantum* isolated from dogs after several courses of drug treatment. Ann Trop Med Parasitol 86:613–620
Guerin PJ, Bates SJ, Sibley CH (2009) Global resistance surveillance: ensuring antimalarial efficacy in the future. Curr Opin Infect Dis 22:593–600
Hadighi R et al (2006) Unresponsiveness to Glucantime treatment in Iranian cutaneous leishmaniasis due to drug-resistant *Leishmania tropica* parasites. PLoS Med 3:e162
Holzmuller P, Sereno D, Lemesre JL (2005) Lower nitric oxide susceptibility of trivalent antimony-resistant amastigotes of *Leishmania infantum*. Antimicrob Agents Chemother 49:4406–4409
Kumar R et al (2001) Enzyme-linked immunosorbent assay for recombinant K39 antigen in diagnosis and prognosis of Indian visceral leishmaniasis. Clin Diagn Lab Immunol 8:1220–1224
Lachaud L et al (2009) Parasite susceptibility to Amphotericin-B in failures of treatment for visceral leishmaniasis in patients co-infected with HIV type 1 and *Leishmania infantum*. Clin Infect Dis 48:e16–e22
Laurent T et al (2007) Epidemiological dynamics of antimonial resistance in *Leishmania donovani*: Genotyping reveals a polyclonal population structure among naturally-resistant clinical isolates from Nepal. Infect Genet Evol 7:206–212
Lira R et al (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
Llanos-Cuentas A et al (2008) Clinical and parasite species risk factors for pentavalent antimonial treatment failure in cutaneous leishmaniasis in Peru. Clin Infect Dis 46:223–231
Maia-Elkhoury ANS et al (2007) Co-infecção da leishmaniose visceral e AIDS no Brasil. Rev Soc Bras Med Trop 40:124
Maltezou HC (2010) Drug resistance in visceral leishmaniasis. J Biomed Biotechnol 2010:617521

Maroli M et al (2008) The northward spread of leishmaniasis in Italy: evidence from retrospective and ongoing studies on the canine reservoir and phlebotomine vectors. Trop Med Int Health 13:256–264
Mary C et al (2006) Reference values for *Leishmania infantum* parasitemia in different clinical presentations: quantitative polymerase chain reaction for therapeutic monitoring and patient follow-up. Am J Trop Med Hyg 75:858–863
Maurer-Cecchini A et al (2009) Immunological determinants of clinical outcome in Peruvian patients with tegumentary leishmaniasis treated with pentavalent antimonials. Infect Immun 77:2022–2029
Mauricio IL, Stothard JR, Miles MA (2000) The strange case of *Leishmania chagasi*. Parasitol Today 16:188–189
Melaku et al (2007) Treatment of kala-azar in southern Sudan using a 17-day regimen of sodium stibogluconate combined with paromomycin: a retrospective comparison with 30-day sodium stibogluconate monotherapy. Am J Trop Med Hyg 77:89–94
Miles MA et al (1999) Canine leishmaniasis in Latin America: control strategies for visceral leishmaniasis. In: Killick-Kendrick R (ed) Canine leishmaniasis: an update. Hoechst Roussel Vet, Barcelona, pp 46–53
Modabber F et al (2007) Consultative meeting to develop a strategy for treatment of cutaneous leishmaniasis. Institute Pasteur, Paris. 13–15 June, 2006. Kinetoplastid Biol Dis 6:3
Montalvo AM et al (2010) Heat-shock protein 70 PCR-RFLP: a universal simple tool for *Leishmania* species discrimination in the New and Old World. Parasitology 137:1159–1168
Mookerjee BJ et al (2006) Sodium antimony gluconate induces generation of reactive oxygen species and nitric oxide via phosphoinositide 3-kinase and mitogen-activated protein kinase activation in *Leishmania donovani*-infected macrophages. Antimicrob Agents Chemother 50:1788–1797
Moore et al (2001) Comparison of generic and proprietary sodium stibogluconate for the treatment of visceral leishmaniasis in Kenya. Bull World Health Organ 79:388–393
Mosleh IM et al (2008) Cutaneous leishmaniasis in the Jordanian side of the Jordan Valley: severe under-reporting and consequences on public health management. Trop Med Int Health 13:855–860
Mukherjee JS et al (2004) Programmes and principles in treatment of multidrug-resistant tuberculosis. Lancet 363:474–481
Murray HW (2004) Progress in the treatment of a neglected infectious disease: visceral leishmaniasis. Expert Rev Anti Infect Ther 2:279–292
Murray HW et al (2000) Interleukin-12 regulates the response to chemotherapy in experimental visceral Leishmaniasis. J Infect Dis 182:1497–1502
Murray HW et al (2005) Advances in leishmaniasis. Lancet 366:1561–1577
Nacher M et al (2001) Influence of clinical presentation on the efficacy of a short course of pentamidine in the treatment of cutaneous leishmaniasis in French Guiana. Ann Trop Med Parasitol 95:331–336
Neal RA, Croft SL (1984) An *in-vitro* system for determining the activity of compounds against the intracellular amastigote form of *Leishmania donovani*. J Antimicrob Chemother 14:463–475
Nolder D et al (2007) Multiple hybrid genotypes of *Leishmania (viannia)* in a focus of mucocutaneous Leishmaniasis. Am J Trop Med Hyg 76:573–578
Olliaro PL et al (2005) Treatment options for visceral leishmaniasis: a systematic review of clinical studies done in India, 1980–2004. Lancet Infect Dis 5:763–774
Ostyn B et al (2011) Incidence of symptomatic and asymptomatic *Leishmania donovani* infection in highly endemic foci in India and Nepal: a prospective study. PLoS Negl Trop Dis 10:e1284
Padovese et al (2009) Cutaneous and mucocutaneous leishmaniasis in Tigray, northern Ethiopia: clinical aspects and therapeutic concerns. Trans R Soc Trop Med Hyg 103:707–711
Palacios R et al (2001) Treatment failure in children in a randomized clinical trial with 10 and 20 days of meglumine antimonate for cutaneous leishmaniasis due to *Leishmania viannia* species. Am J Trop Med Hyg 64:187–193
Pampiglione S et al (1975) Studies in Mediterranean leishmaniasis. 3. The leishmanin skin test in kala-azar. Trans R Soc Trop Med Hyg 69:60–68

Rijal S et al (2007) Antimonial treatment of visceral leishmaniasis: are current *in vitro* susceptibility assays adequate for prognosis of in vivo therapy outcome? Microbes Infect 9:529–535
Rijal S et al (2010) Clinical risk factors for therapeutic failure in kala-azar patients treated with pentavalent antimonials in Nepal. Trans R Soc Trop Med Hyg 104:225–229
Ritmeijer K et al (2001) Ethiopian visceral leishmaniasis: generic and proprietary sodium stibogluconate are equivalent; HIV co-infected patients have a poor outcome. Trans R Soc Trop Med Hyg 95:668–672
Rojas R et al (2006) Resistance to antimony and treatment failure in human *Leishmania (Viannia)* infection. J Infect Dis 193:1375–1383
Romero GA, Boelaert M (2010) Control of visceral leishmaniasis in latin america-a systematic review. PLoS Negl Trop Dis 4:e584
Romero GA et al (2001) Comparison of cutaneous leishmaniasis due to *Leishmania (Viannia) braziliensis* and *L. (V.) guyanensis* in Brazil: therapeutic response to meglumine antimoniate. Am J Trop Med Hyg 65:456–465
Rotureau B (2006) Are New World leishmaniases becoming anthroponoses? Med Hypotheses 67:1235–1241
Rougeron V et al (2009) Extreme inbreeding in *Leishmania braziliensis*. Proc Natl Acad Sci USA 106:10224–10229
Scheltema RA et al (2010) The potential of metabolomics for *Leishmania* research in the post-genomics era. Parasitology 29:1–12
Schonian G et al (2008) Leishmaniases in the Mediterranean in the era of molecular epidemiology. Trends Parasitol 24:135–142
Seifert K et al (2003) Characterisation of *Leishmania donovani* promastigotes resistant to hexadecylphosphocholine (Miltefosine). Int J Antimicrob Agents 22:380–387
Singh R et al (2006) Visceral leishmaniasis, or kala azar (KA): high incidence of refractoriness to antimony is contributed by anthroponotic transmission via post-KA dermal leishmaniasis. J Infect Dis 194:302–306
Singh VP et al (2010) Estimation of under-reporting of visceral leishmaniasis cases in Bihar, India. Am J Trop Med Hyg 82:9–11
Sundar S et al (2000) Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. Clin Infect Dis 31:1104–1107
Sundar S et al (2005) Detection of leishmanial antigen in the urine of patients with visceral leishmaniasis by a latex agglutination test. Am J Trop Med Hyg 73:269–271
Svobodova M et al (2006) Distinct transmission cycles of *Leishmania tropica* in 2 adjacent foci, Northern Israel. Emerg Infect Dis 12:1860–1868
t'Kindt R et al (2010) Metabolomics to unveil and understand phenotypic diversity between pathogen populations. PLoS Negl Trop Dis 4:e904
Thakur CP et al (1984) Comparison of regimens of treatment with sodium stibogluconate in kala-azar. Br Med J (Clin Res Ed) 288:895–897
Torres TT et al (2008) Gene expression profiling by massively parallel sequencing. Genome Res 18:172–177
Toumi et al (2007) Demographic, clinical and therapeutic features of adult visceral leishmaniasis at the Rabta hospital in Tunis (tunisia) from 1983 to 2002. Bull Soc Pathol Exot 100:282–286
Tuon FF et al (2008) Treatment of New World cutaneous leishmaniasis–a systematic review with a meta-analysis. Int J Dermatol 47:109–124
Uzun et al (2004) Clinical Features, Epidemiology, and Efficacy and Safety of Intralesional Antimony Treatment of Cutaneous Leishmaniasis: Recent Experience in Turkey. J Parasitol 90:853–859
van Griensven J et al (2010) Combination therapy for visceral leishmaniasis. Lancet Infect Dis 10:184–194
Vergel C et al (2006) Evidence for *Leishmania (viannia)* parasites in the skin and blood of patients before and after treatment. J Infect Dis 194:503–511
Verma SK et al (2007) Sodium stibogluconate-sensitive visceral leishmaniasis in the non-endemic hilly region of Uttarakhand, India. Trans R Soc Trop Med Hyg 101:730–732

Vermeersch M et al (2009) *In vitro* susceptibilities of *Leishmania donovani* promastigote and amastigote stages to antileishmanial reference drugs: practical relevance of stage-specific differences. Antimicrob Agents Chemother 53:3855–3859

Wongsrichanalai C et al (2002) Epidemiology of drug-resistant malaria. Lancet Infect Dis 2:209–218

World Health Organization (2010) Control of the leishmaniases. World Health Organ Tech Rep Ser xii-186, back

World Health Organization (2007) Progress 2005-2006:eighteenth programme report. World Health Organization, Geneva

Yardley V et al (2006) American tegumentary leishmaniasis: is antimonial treatment outcome related to parasite drug susceptibility? J Infect Dis 194:1168–1175

Zijlstra EE, El Hassan AM (2001) Leishmaniasis in Sudan. Visceral leishmaniasis. Trans R Soc Trop Med Hyg 95(Suppl 1):S27–S58