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ScienceDirect
Infection, Genetics and Evolution 7 (2007) 206–212
Infection, Genetics and Evolution
www.elsevier.com/locate/meegid

# Epidemiological dynamics of antimonial resistance in *Leishmania donovani*: Genotyping reveals a polyclonal population structure among naturally-resistant clinical isolates from Nepal

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Received 17 May 2006; received in revised form 30 August 2006; accepted 31 August 2006
Available online 27 September 2006

**Abstract**

Pentavalent antimonials (SbV) are the first line drug against leishmaniasis worldwide, but drug resistance is increasingly reported, particularly in the Indian sub-continent, where it represents a major threat for the control of anthroponotic visceral leishmaniasis (VL). In order to understand the epidemiological dynamics of antimonial resistance in anthroponotic VL, we analysed here the population structure of 24 *Leishmania donovani* stocks isolated from anthroponotic VL-patients from Eastern Nepal: 13 SbV-naturally resistant and 11 SbV-sensitive, as demonstrated by *in vitro* drug susceptibility assays. The parasites were genotyped by PCR-RFLP analysis of kDNA minicircles and by microsatellite analysis and the encountered polymorphism revealed a polyclonal structure among resistant isolates. Furthermore, analysis of paired samples obtained from the same patients before treatment and after failure revealed primary as well as acquired resistance. The hypothesis of independent events of drug resistance emergence is proposed and confronted to alternative explanations. Our results show the dynamics of drug resistance epidemiology and highlight the importance of surveillance networks.


*Keywords:* *Leishmania donovani*; Kala-Azar; Antimony resistance; kDNA; Microsatellite; Population structure

## 1. Introduction

Visceral leishmaniasis (VL) or Kala-Azar (KA) has been reported from 51 countries around the world with an annual incidence of 500,000 cases and up to 59,000 deaths each year, occurring mainly in Sudan, Bangladesh, India, Nepal and Brazil (Desjeux, 1996). In the Indian sub-continent, an anthroponotic form of the disease (anthroponotic VL) is encountered; it is caused by *Leishmania donovani*, which is transmitted by the sandfly *Phlebotomus argentipes*, without a known animal reservoir (Bora, 1999). Because of the anthroponotic character of the disease in that region, control is essentially based on (i) early diagnosis followed by adequate treatment and (ii) by vector control when it is possible (Ghosh et al., 1999; Boelaert et al., 2000). Pentavalent antimonials (SbV) have been the first line drugs for VL in most endemic countries for over 60 years (Croft et al., 2006). Only a few alternative drugs exist: paromomycin, amphotericin B and its lipid formulation (Ambisome®), and a very promising oral drug, miltefosine (Impavido®) (Guerin et al., 2002).

In this context, treatment failure and drug resistance represent a major threat for the control of anthroponotic VL, and both are well documented for SbV. The most alarming reports came from the province of Bihar (India), where over

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doi:10.1016/j.meegid.2006.08.005

60% of anthroponotic VL-patients are unresponsive to SbV treatment (Sundar, 2001). In other provinces of India or in neighbouring countries like Nepal, the documented situation is currently less dramatic: in the South East of Nepal (Terai), a recent study showed a general SbV-unresponsiveness rate of 11.4% during the period 2001–2004 (Rijal et al., 2003), but more therapeutic failures (24%) were reported in the districts bordering Bihar (Rijal, unpublished data). The geographical and temporal grouping of SbV treatment failures suggests the existence of SbV-resistant isolates. Indeed in Muzaffarpur (Bihar) SbV-resistant *Leishmania* isolates were identified and a correlation was found between clinical outcome of SbV treatment and *in vitro* SbV susceptibility of corresponding *L. donovani* isolates (Lira et al., 1999). However, the way by which drug resistance is emerging and spreading is not at all understood. Under conditions of anthroponotic transmission (in which the parasite is likely to be under a stronger drug pressure than in zoonotic leishmaniasis), we might hypothesise that once a resistant parasite has emerged (f.i. by mutation), clonal spreading would play a major role. Accordingly, in anthroponotic VL, the population structure of resistant parasites would be expected to be monoclonal, or at least all resistant parasites should cluster together in a group of genetically similar isolates. A population genetics approach using highly discriminatory DNA fingerprinting methods is required to address this issue.

We aimed here to identify the population structure of SbV-sensitive and SbV-resistant *L. donovani* isolates for understanding the epidemiological dynamics of drug resistance in anthroponotic VL. Twenty-four isolates (13 SbV-resistant and 11 SbV-sensitive, as demonstrated by *in vitro* drug susceptibility assays) isolated from Nepalese patients with Kala-Azar were genotyped by PCR-RFLP analysis of kDNA minicircles and by microsatellite analysis. Polymorphism was mostly encountered with the former method, and phenetic analysis revealed a polyclonal structure among resistant isolates. The hypothesis of independent events of drug resistance emergence is proposed and is confronted to alternative explanations.

## 2. Materials and methods

### 2.1. Patients

Ethical clearance was obtained from the ethical committees of the Health Research Council, Kathmandu, Nepal and the Institute of Tropical Medicine, Antwerpen, Belgium. Informed consent was obtained from patients or their parents or guardians. Clinical cases with VL in Nepal were recruited from November 2002 to September 2003 at the B.P. Koirala Institute of Health Sciences (Dharan, Nepal), a 650-bed referral hospital for eastern Terai (Fig. 1). Individuals less than 2 years old were excluded from the study. VL cases were suspected on the base of fever for 14 days or longer with splenomegaly, and were confirmed by bone marrow aspiration and visual identification of parasites. All patients received 20 mg $Sb^V$/kg/day i.m. × 30 days (sodium antimony gluconate, Albert David Ltd., Calcutta, India). Unresponsive patients subsequently received amphotericin B (amphotericin B deoxycholate) treatment.

### 2.2. Parasites

The 24 isolates here used (Table 1, Fig. 1) were isolated from bone marrow aspirates of confirmed VL cases. Most of isolates were obtained before treatment (marked/0 in Table 1), but three isolates were obtained 1 or 12 months after treatment (marked/1 or/12, respectively): for two of them, the pre-treatment paired isolate was available (BPK173/0 and/1, and BPK181/0 and/12). Patient material was isolated, grown directly on Tobie's blood agar medium (Tobie et al., 1950) at 26 °C and typed by *cpb*

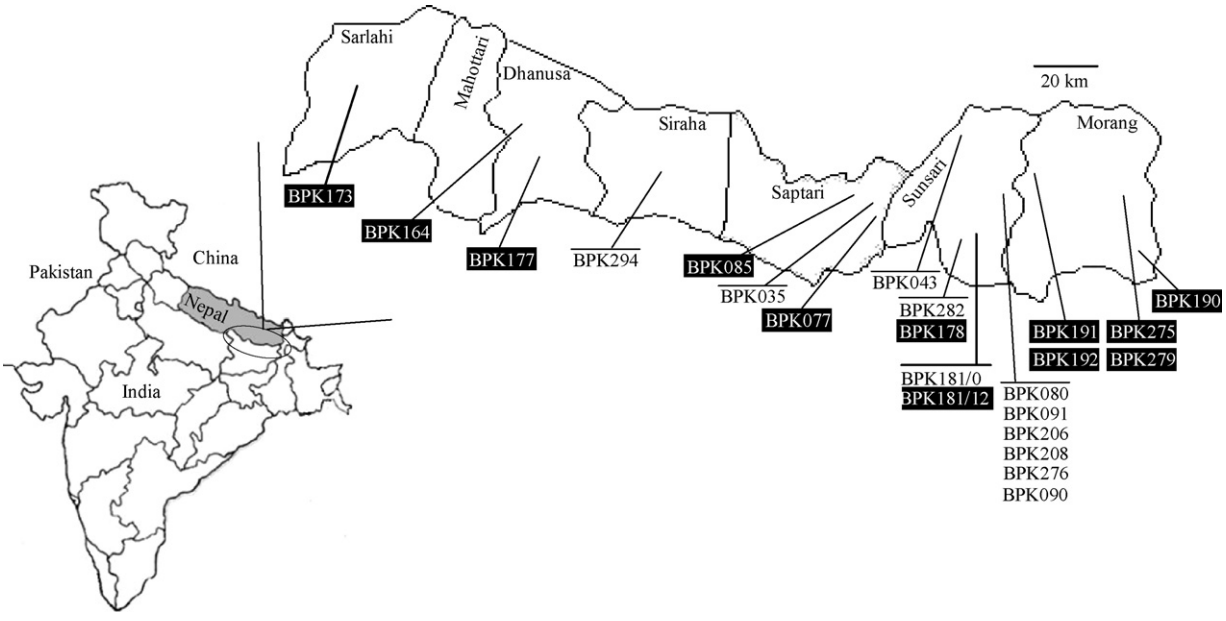


Fig. 1. Geographial distribution (districts of Terai region, Eastern Nepal) of the *L. donovani* isolates studied here: black boxes, SbV-resistant parasites.

Table 1
List of Nepalese *L. donovani* isolates here analysed; /0, /1 and /12: isolated before treatment, 1 or 12 months after, respectively; bold, paired isolates obtained from a same patient before treatment and at the time of treatment failure; SbV, pentavalent antimonials; SbIII, trivalent antimonials

| Study code | SbV susceptibility | SbIII susceptibility | District, village | Sampling date |
|---|---|---|---|---|
| BPK 035/0 | Sensitive | Sensitive | Saptari, Bhagani | March 2002 |
| BPK 043/0 | Sensitive | n.d. | Sunsari, Chatra | April 2002 |
| BPK 077/0 | Resistant | n.d. | Saptari, Phattepur | June 2002 |
| BPK 080/0 | Sensitive | n.d. | Sunsari, Ithari | June 2002 |
| BPK 085/0 | Resistant | n.d. | Saptari, Kamalpur | June 2002 |
| BPK 090/0 | Sensitive | n.d. | Sunsari, Ithari | June 2002 |
| BPK 091/0 | Sensitive | Sensitive | Sunsari, Ithari | June 2002 |
| BPK 164/1 | Resistant | n.d. | Dhanusa, Dharapani | November 2002 |
| **BPK 173/0** | Resistant | n.d. | Sarlahi, Pirari | December 2002 |
| **BPK 173/1** | Resistant | n.d. | Sarlahi, Pirari | January 2003 |
| BPK 177/0 | Resistant | n.d. | Dhanusa, Sarsa | December 2002 |
| BPK 178/0 | Resistant | n.d. | Sunsari, Inurwa | December 2002 |
| **BPK 181/0** | Sensitive | n.d. | Sunsari, Dumraha | January 2003 |
| **BPK 181/12** | Resistant | n.d. | Sunsari, Dumraha | January 2004 |
| BPK 190/0 | Resistant | Resistant | Morang, Govindpur | March 2003 |
| BPK 191/0 | Resistant | n.d. | Morang, Sundarpur | March 2003 |
| BPK 192/0 | Resistant | n.d. | Morang, Dulari | March 2003 |
| BPK 206/0 | Sensitive | n.d. | Sunsari, Ithari | April 2003 |
| BPK 208/0 | Sensitive | n.d. | Sunsari, Ithari | April 2003 |
| BPK 275/0 | Resistant | n.d. | Morang, Sanischare | August 2003 |
| BPK 276/0 | Sensitive | n.d. | Sunsari, Ithari | August 2003 |
| BPK 279/0 | Resistant | n.d. | Morang, Sanischare | August 2003 |
| BPK 282/0 | Sensitive | Sensitive | Sunsari, Inurwa | August 2003 |
| BPK 294/0 | Sensitive | Sensitive | Siraha, Bishnupur | September 2003 |

PCR-RFLP as described elsewhere (Quispe Tintaya et al., 2004). Frozen stocks were sent to the London School of Hygiene and Tropical Medicine, London (LSHTM), where the intracellular amastigote susceptibility to sodium stibogluconate (Sb(V), GSK, UK) was tested *in vitro* within eight passages from isolation, in order to decrease the potential risk of losing infectivity (Santos-Gomes and Abranches, 1996) or resistance phenotype (Gazola et al., 2001) during long-term *in vitro* maintenance. Late stage promastigotes were used to infect starch-induced murine peritoneal macrophages at a ratio of 7 promastigotes to 1 macrophage in Labtek™ 16-well tissue culture well slides (VWR Ltd., UK), in quadruplicate, and kept at 37 °C, 5% $CO_2$/air mix. Twenty-four hours after infection, one slide was methanol fixed and Giemsa stained, to determine the initial level of infection. If infection level was higher than 80%, the infected cultures were exposed to sodium stibogluconate (Sb(V), GSK, UK) over a dose range of 60, 20, 6.6, and 2.2 μg/ml. After five days, the percentage of infected macrophages in each well was determined by microscopy. From a comparison of counts from treated with untreated cultures, the percentage inhibition was calculated by sigmoidal regression analysis (MS *xl*fit™) and $ED_{50}$ ($ED_{90}$) values were determined. The strain *L. donovani* MHOM/ET/67/HU3, a WHO reference strain sensitive to sodium stibogluconate and meglumine antimoniate, was included in each assay as a reference. The ratio of the $ED_{50}$ of a tested strain versus the $ED_{50}$ of the reference strain, here defined as the activity index (A.I.), was used to express the *in vitro* susceptibility of that tested strain and to compare easily the results obtained from different series of experiments. An A.I. of 1 meant that the tested isolates showed a similar $ED_{50}$ as that of the reference sensitive strain of that experiment (ranging from 7 to 18 μg SbV/ml according to the experimental series), while an A.I. of 6 meant that $ED_{50}$ of the tested isolate was six times higher than that of the reference and corresponded to $ED_{50}$ higher than 60 μg SbV/ml (maximal measurable value because of toxicity to the macrophage). Eleven isolates with an A.I. of 1–2 were considered as SbV-sensitive (SbV-S); 13 isolates with an A.I. of 6 or higher were considered as SbV-resistant (SbV-R). For some of the resistant isolates, data of *in vitro* susceptibility to Triostam (SbIII, Burroughs Wellcome) were also available (Table 1). SbIII was used over a dose range of 30–1.1 μg SbIII/ml, and as for SbV, an A.I. was defined by comparison with HU3. Comparable concentrations to SbV could not be used due to host cell toxicity at higher concentrations. Animal experimentation guidelines of London School of Hygiene and Tropical Medicine were followed. Finally, a SbV-R clone was derived from the SbV-R isolate BPK190/0 by the microdrop method (Van Meirvenne et al., 1975). This cell line was maintained for more than 1 year *in vitro*, in order to test the stability of the resistance phenotype and the fingerprinting pattern.

### 2.3. kDNA/PCR-RFLP and microsatellite analysis

DNA was extracted from cultivated promastigotes with the QIAmp DNA mini Kit (Qiagen, Hilden, Germany); for all strains, harvesting was done at a similar and low subinoculation number (about 20) since isolation, in order to avoid the risk of kDNA drift during long-term *in vitro* maintenance. The PCR reactions targeting the kDNA were realized as described elsewhere (Morales et al., 2002), except

that *Taq* silverstar polymerase and buffer (Eurogentec, Belgium) were used for the amplification. Before the digestion, the PCR products (800 bp) were ethanol-precipitated and re-suspended in 20 μl of water. A single pool of PCR products, coming from two PCR amplifications made in the same run in the same machine was used for all digestions. After quantification, PCR products (300–400 ng) were digested overnight in a total volume of 20 μl, with an excess of restriction enzyme, as recommended by manufacturer (*Rsa*I, *Hae*III and *Hpa*II from MBI-Fermentas, St. Leon-Rot, Germany). The digestion was stopped by the addition of 1 μl of EDTA at 0.5 M. The analysis of the restriction fragments was realized by capillary electrophoresis with the Bioanalyser 2100 (Agilent technology) using the DNA 1000 labChip Kit and the manufacturer's guidelines (1 μl of restriction product). As bands below 100 bp can be confused with primer dimers and bands above 700 bp can be undigested amplicons, only the bands ranging from 100 to 700 bp were considered as belonging to the restriction pattern. Multilocus microsatellite typing (MLMT) has been performed using a standard set of 13 markers: (i) 11 used for discrimination of *L. infantum* MON-1 strains as described elsewhere (Ochsenreither et al., 2006): Li41-56, Li46-67, Li22-35, Li23-41, Li45-24, Li71-33, Li71-5/2, Li71-7, Lm2TG, Lm4TA, TubCA and (ii) 2 developed later from a genomic library based on an Indian strain of *L. donovani* (MHOM/IN/01/BHU20140, unpublished data): CS19 and CS20.

### 2.4. Tree building

Phenetic trees were built with the PHYLIP software package (http://evolution.gs.washington.edu/phylip/software.html), using the following programmes: RESTDIST (restriction fragments distance, modification of Nei and Li restriction fragments distance method, Nei and Li, 1979), UPGMA (unweighted pair group method with arithmetic averages) and DRAWGRAM (interactive plotting of phenograms).

## 3. Results

### 3.1. Genotyping

MLMT was applied on most isolates, but very few sequence size polymorphism was observed. Microsatellite variation was only found with one marker, Li 23-41, for five isolates. These were (i) the isolates BPK035/0, BPK043/0, BPK276/0 and BPK294/0 (all SbV-S), with 1 or 3 more repeats than all other isolates and (ii) BPK191/0 (SbV-R) which had 19 repeats less than the others (data not shown). The discriminatory power of kDNA PCR-RFLP was higher: with the 3 restriction enzymes, a total of 41 fragments were scored and the pattern of these characters allowed a fine discrimination among the analysed isolates (see an example in Fig. 2). The stability of the kDNA PCR-RFLP patterns over time was tested with the cloned strain BPK190/0cl3 maintained *in vitro*: no difference was observed after 1 month of maintenance, while 4/41 characters (for RFLP with three restrictions enzymes) changed after 1 year of continuous sub-inoculations (twice a week). This variation concerned fragments of weak intensity (data not shown) and was probably due to drift among the kDNA minicircle populations (see Section 4). During this whole period of time, this cloned strain remained SbV-R.

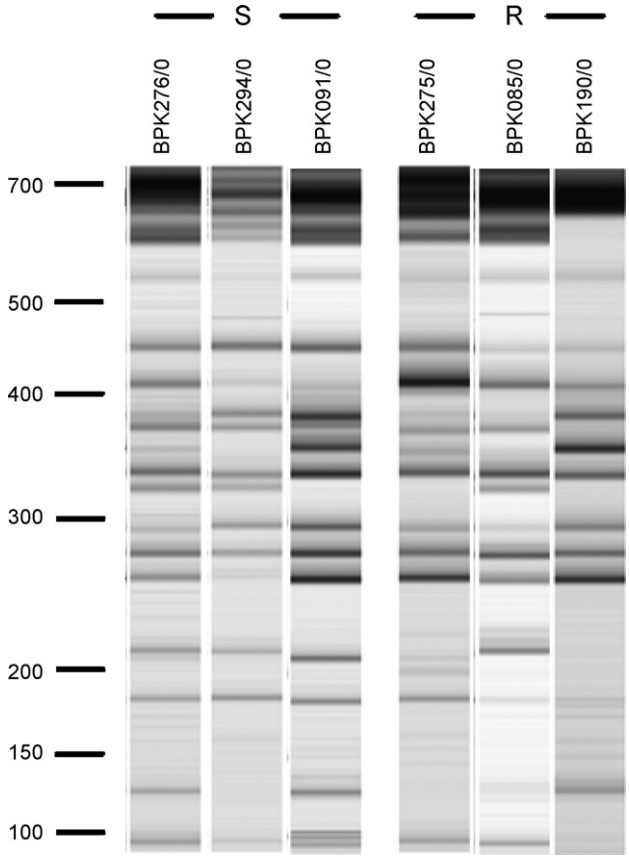


Fig. 2. kDNA PCR-RFLP with *Rsa*I (gel-like image after capillary electrophoresis): polymorphic patterns of SbV-sensitive (S) and SbV-resistant (R) *L. donovani* isolates from each of the three clusters observed after phenetic analysis (see Fig. 3); size is expressed in base pairs.

### 3.2. Phenetic analysis

Data generated by kDNA PCR-RFLP analysis of all isolates were gathered and processed by phenetic analysis. We also introduced as reproducibility control, the results of duplicate analysis of three isolates (BPK275/0, BPK091/0 and BPK085/0, labelled with a dot on Fig. 3). On the total of the patterns obtained with the three restriction enzymes, very few differences were observed in these controls: 0/41 for the BPK091/0 duplicate, 1/41 and 2/41 for BPK085/0 and BPK275/0 duplicates, respectively, always concerning fragments of weak intensity (data not shown). Hence, in further analyses, only the isolates differentiated by 3/41 characters or more were thus considered as different. The reproducibility threshold is indicated on the dendrogram by a dotted line (Fig. 3, labelled Repro). A second threshold was introduced for phenetic analyses, the stability threshold, corresponding to the level of genetic variation observed in a cloned strain maintained 12 month *in vitro* (Fig. 3, labelled 12-m S).

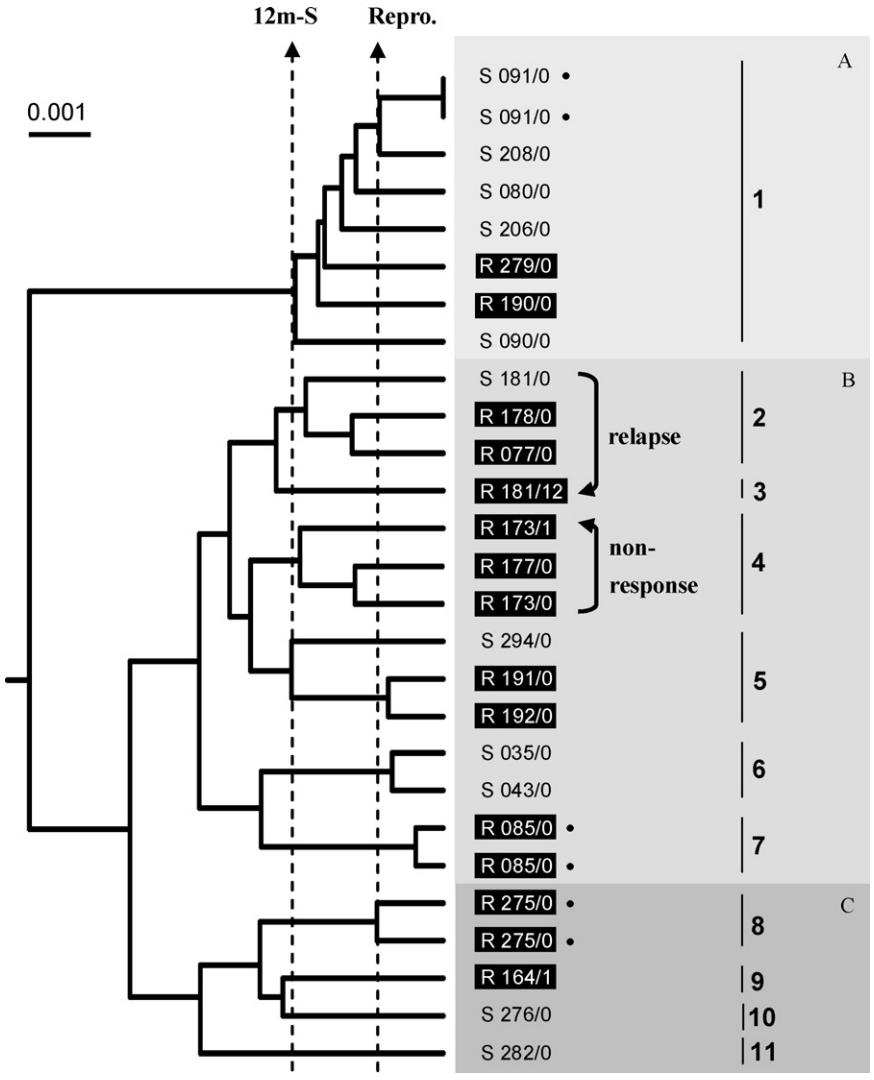


Fig. 3. Phenetic tree (UPGMA) built-up from data of kDNA PCR-RFLP (3 restriction enzymes), and showing the dispersion of SbV-sensitive (S) and SbV-resistant (R, black boxes) *L. donovani* isolates among 3 clusters (A–C); dots, duplicates of 3 isolates, introduced in the analysis to define the reproducibility threshold (Repro); 12-m S, stability threshold corresponding to the level of genetic variation observed in a cloned strain maintained 12 month *in vitro* (use to define 11 kDNA-types, i.e. groups of parasites branching up to this threshold); arrows, paired isolates obtained from a same patient before treatment and at the time of treatment failure.

Using the reproducibility threshold, we defined 21 different genotypes among the 24 isolates; this corresponded to 11 groups of isolates or single isolates branching up to the stability threshold. We considered only these 11 'kDNA-types' as relevant for further interpretation: 3 of them were constituted only by SbV-S parasites (6, 10 and 11 in Fig. 3), 5 by SbV-R ones (3, 4 and 7–9 in Fig. 3) and 3 had a mixed composition (1, 2 and 5 in Fig. 3). The 11 kDNA-types were distributed in 3 major clusters, in which SbV-resistant and SbV-sensitive isolates were interspersed. The dendrogram was also analysed in a geographical context: cluster A contained only isolates from the two Eastern districts of Sunsari and Morang, but the other two contained isolates from all the districts, suggesting circulation of *L. donovani* isolates along west-east axis of the country. There was no association between the sampling date and the clusters (see Table 1).

The phenetic tree also allowed visualizing the genetic similarity among the paired isolates obtained before and after treatment from the same patients. The first case corresponded to a non response at the end of 1-month treatment (couple BPK173/0-BPK173/1). Both isolates were SbV-R and they were considered the same kDNA-type (4) as defined above. The second pair was a relapse, 12 months after the initial treatment (couple BPK181/0-BPK181/12). The pre-treatment isolate was SbV-S, while the post-treatment one was SbV-R; the two isolates were considered as different kDNA-types (2 and 3, respectively), and clustered just above the 12-months stability threshold.

## 4. Discussion

Besides environmental changes and HIV/co-infection, drug resistance constitutes one of the major risk factors for the (re-)emergence of leishmaniasis worldwide. This phenomenon is well documented with respect to antimonial treatment (Sundar, 2001; Croft et al., 2006), but so far poorly understood. On the

one hand, the mode of action of antimonials is unknown and the molecular mechanisms of resistance recently begin to be addressed in naturally resistant isolates from patients (Croft et al., 2006). On the other hand, the epidemiological dynamics of SbV resistance remains a question mark: in particular, what is the relative importance of emergence and spreading factors in the current resistance landscape? We addressed the latter question in an anthroponotic context, by analysing the population structure in a sample of SbV-R and SbV-S *L. donovani* isolates from Nepal.

Out of the two genetic markers here used – kDNA minicircles and microsatellites – the former ones were the most informative. Our results indicated the presence of three major clusters in our sample, but each of them contained resistant and sensitive isolates. Despite a general low resolution of MLMT, genetic heterogeneity among the resistant population was supported by one microsatellite marker which distinguished one R isolate (BPK191/0) from the others. This polyclonal structure of the resistant parasites might theoretically have four explanations.

(i) As the segregation of the kDNA minicircles is not fully conservative during the cell division, this marker is more subjected to a genetic drift at short term (Gao et al., 2001), with ensuing fluctuations of the different populations of minicircles. This is illustrated in present study by the small variations observed during *in vitro* maintenance of a cloned strain over more than 1 year (4 changes in a total of 41 characters, here used as 12-months stability threshold). This might produce short branches as the one observed within each of the three kDNA clusters, and we cannot exclude that the dispersion of resistant isolates within a cluster (f.i. BPK190/0 and BPK279/0 in cluster A) is not influenced by this drift. However, this dispersion was also observed between the three clusters, where the lengths of the branches correspond to more pronounced variations (f.i. 14/41 changes between BPK164/1 and BPK190/0, albeit isolated at 4 months of interval). This observation does not support the hypothesis of rapid kDNA drift as responsible for the overall polyclonal structure of resistant isolates here observed. Further research with other discriminatory markers should be undertaken to complete our observations.

(ii) The absence of correlation between the kDNA-based population structure and the drug resistance of the parasites might also be explained by sexual recombination. This is generally considered to be exceptional in *Leishmania* (Tibayrenc, 1996), but could have a high epidemiological impact, when involving one resistant strain in the anthroponotic context of anthroponotic VL and under drug pressure. Testing this hypothesis would require a battery of discriminatory co-dominant markers, like microsatellites or single nucleotide polymorphism (SNP).

(iii) Instability of drug resistance during *in vitro* maintenance of the parasites after isolation could also explain the lack of clustering of resistant isolates. However, this was under strict control in current study, as the *in vitro* SbV susceptibility of all the isolates was systematically tested within a same time range of 8 weeks post-isolation. In addition, we observed that the *in vitro* susceptibility of a resistant cloned strain maintained for 1 year in a drug-free medium was not altered. The same observation was reported (Sereno and Lemesre, 1997) but stability was shown to depend on the level of parasite drug resistance. All the strains here analysed were characterized by a similar and high level of SbV resistance ($ED_{50}$ at least six times higher than the one of the reference sensitive strain) and we may assume that they all show a stable resistance phenotype, at least within the limits of our experimental conditions.

(iv) Finally, our results might also reveal independent events of drug resistance emergence among the natural populations of *L. donovani*. On the one hand, this could concern the repetition of a same (epi-)genetic event, like gene amplification which is often reported in experimentally-induced resistant isolates (Haimeur and Ouellette, 1998). On the other hand, this might suggest a pleiotropic answer of *Leishmania* to drug pressure, which is supported by the description of different mechanisms leading to antimony-resistance (reviewed by Croft et al., 2006). The pleiotropism hypothesis is also supported by the observation of two resistance phenotypes among Nepalese strains: (i) resistance to the prodrug SbV and to its reduced and active form, SbIII and (ii) resistance to SbV, but sensitivity to SbIII (Rijal, unpublished data).

The analysis of paired stocks isolated from the same patient before treatment and after failure was also very informative for the dynamics of drug resistance. Indeed, two situations were encountered here. In the first case (non response after 1-month treatment), the patient likely got infected with parasites which were already resistant (primary resistance), and parasites encountered after treatment failure were genetically similar and still resistant. The second case (new VL episode 12 months after the end of the treatment) was totally different; the population of parasites present at the onset of treatment was sensitive, while the one isolated at relapsing time was resistant, showing a kDNA-type very similar to the previous one. Even if re-infection cannot be excluded, this result could be indicative of an acquired resistance. Knowing that parasite population isolated from a same patient can be a mixture of resistant and sensitive cells (Bhattacharyya et al., 2002), selection of initially minor resistant parasites and shift of their frequency might lead to the acquisition of resistance. Induction of the resistance by antimony itself appears unlikely, the drug not being known to be mutagenic, but without the complete elucidation of the genetic background of SbV resistance, this possibility should not be excluded yet.

In conclusion, our results demonstrate a polyclonal structure among SbV-resistant *L. donovani* parasites from Nepal; this could have several explanations, but we favour the hypothesis that drug resistance might emerge several times through independent events. It is unknown if this is also the case in other regions of the sub-continent. However, our findings

demonstrate that the epidemiology of drug resistance is quite dynamic and they highlight the need for implementing drug resistance monitoring networks, like the ones developed for malaria. A particular attention should also be paid to the elucidation of the genetic background of antimonial resistance. Our results suggest that such studies should take into account a possible diversity of mechanisms in natural conditions.

## Acknowledgements

The authors are members of the Leishnatdrug-R consortium (www.leishnatdrug.org) and dedicate this paper to the memory of Anne Maurer, a colleague of the consortium who accidentally died in March 2006. We thank local staff in Nepal and patients for their collaboration. This study received the financial support of the European Commission (contracts ICA4-CT-2001-10076 and INCO-CT-2005-015374).

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