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

Thierry Laurent [a], Suman Rijal [b], Vanessa Yardley [c], Simon Croft [c], Simonne De Doncker [a], Saskia Decuypere [a,f], Basudha Khanal [b], Rupa Singh [b], Gabriele Schönian [d], Katrin Kuhls [d], François Chappuis [e], Jean-Claude Dujardin [a,*]

[a] *Department of Parasitology, Unit of Molecular Parasitology, Institute of Tropical Medicine, Antwerp B-2000, Belgium*
[b] *B.P. Koirala Institute of Health Sciences, Dharan, Nepal*
[c] *Department of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London WC1E7HT, UK*
[d] *Institut für Mikrobiologie und Hygiene, Charité Universitätsmedizin Berlin, Dorotheenstr. 96, D-10098 Berlin, Germany*
[e] *Department of Community Medicine, Travel and Migration Medicine Unit, Hopitaux Universitaires de Geneve, Geneva, Switzerland*
[f] *Department of Biomedical Sciences, University of Antwerp, Antwerp B-2080, Belgium*

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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

* Corresponding author. Tel.: +32 3 2476355; fax: +32 3 2476359.
E-mail address: jcdujard@itg.be (J.-C. Dujardin).

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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*

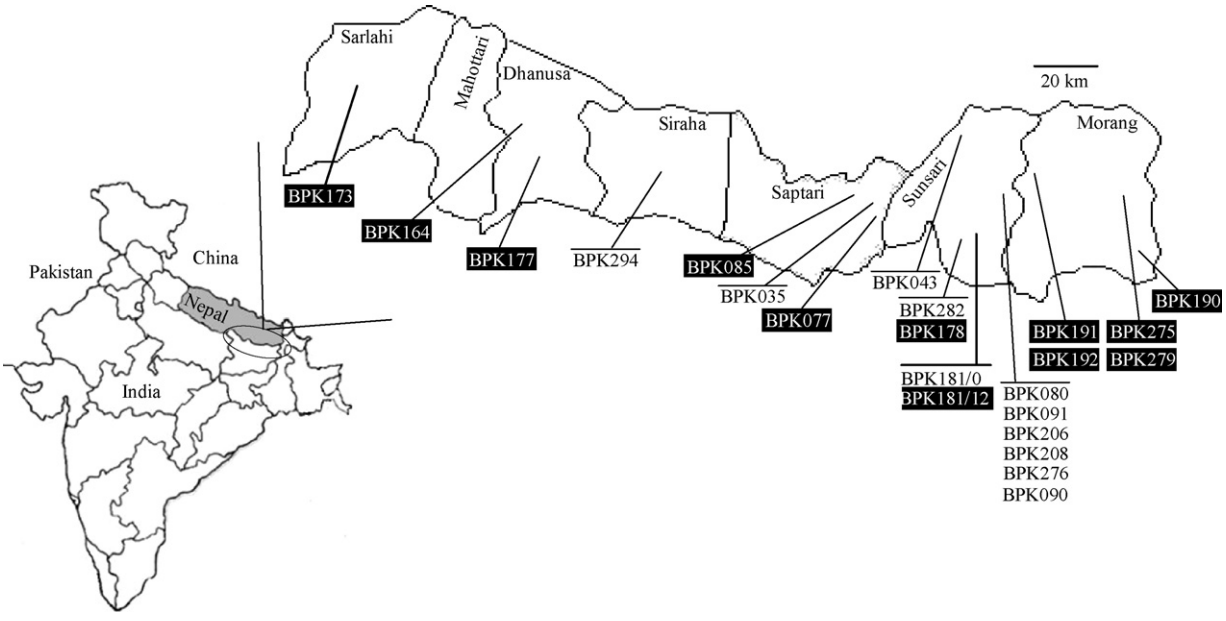

Fig. 1. Geographial distribution (districts of Terai region, Eastern Nepal) of the *L. donovani* isolates studied here: black boxes, SbV-resistant parasites.