BRIEF REPORT

Genetic Markers for SSG Resistance in *Leishmania donovani* and SSG Treatment Failure in Visceral Leishmaniasis Patients of the Indian Subcontinent

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The current standard to assess pentavalent antimonial (SSG) susceptibility of *Leishmania* is a laborious in vitro assay of which the result has little clinical value because SSG-resistant parasites are also found in SSG-cured patients. Candidate genetic markers for clinically relevant SSG-resistant parasites identified by full genome sequencing were here validated on a larger set of clinical strains. We show that 3 genomic locations suffice to specifically detect the SSG-resistant parasites found only in patients experiencing SSG treatment failure. This finding allows the development of rapid assays to monitor the emergence and spread of clinically relevant SSG-resistant *Leishmania* parasites.

Leishmania is a protozoan parasite that is transmitted by sand flies and can cause cutaneous, mucocutaneous, or visceral disease in Latin America, the Mediterranean Basin, East Africa, and Asia. Visceral leishmaniasis (VL), thought to be lethal if left untreated, is the systemic form of the disease and is caused by *Leishmania infantum* (syn *L. chagasi*) and *Leishmania donovani*—the latter being responsible for VL in the hyperendemic regions of the Indian subcontinent and East-Africa [1]. Antimonials (SSG) have long been the first-line treatment for leishmaniasis throughout the world, but an increasing rate of SSG treatment failure has been reported in the Indian subcontinent [2, 3]. This was shown to be associated among others with SSG resistance of the parasites [4, 5].

So far, the gold standard for determining the SSG susceptibility of *Leishmania* strains is an in vitro test in which the survival of the intracellular form of the parasite in infected macrophages is microscopically evaluated at various concentrations of SSG [6]. However, this test is difficult to standardize, labor-intensive, and therefore not suitable for routine testing of a large amount of samples (eg, for epidemiological purposes). Furthermore, the current in vitro assays do not necessarily identify clinically relevant SSG resistance phenotypes, as shown by a previous clinical study in Nepal in which in vitro SSG-resistant *L. donovani* were not only found in all SSG-nonresponder patients but also in 65.0% of the SSG-cured patients [5]. This poor accuracy might be explained by, among others, the absence of an immunological environment, which is known to be crucial in determining SSG clinical outcome, in these in vitro assays and thus the presence of undetected parasite epi-phenotypes that may influence such host-pathogen interactions.

Laboratory tools that are easier to use and able to identify the clinically relevant SSG-resistant phenotype are thus pivotal to correctly monitor the further dynamics of drug resistance in natural populations. Several candidate markers at gene expression level have been proposed (reviewed elsewhere [7]), but they have yet to be validated in a large sample or were not uniformly present in all natural SSG-resistant strains analysed. Here, 5 single-nucleotide polymorphisms (SNPs) selected from a previous whole genome study [8] were tested on a larger sample of clinical *L. donovani* strains from the Indian subcontinent to evaluate their performance and predictive value for (1) the in vitro SSG-resistant phenotype of the parasite and (2) SSG treatment failure of the infected patient.

METHODS

Written informed consent was obtained from all patients prior to enrolment and in the case of children from their parents or guardians. The LeishNatDrug study in which

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the Nepalese strains (further labeled with the prefix *BPK*) were isolated was approved by the institutional review boards of (1) the Nepal Health Research Council, Kathmandu, Nepal, and (2) the Institute of Tropical Medicine (ITM), Antwerp, Belgium. The Kaladrug-R study in which the Indian strains (further labeled with the prefix *BHU*) were isolated was approved by the institutional review boards of (1) the Faculty of Medicine, Banaras Hindu University, Varanasi, India, and (2) the ITM, Antwerp, Belgium.

Mouse care and experimental procedures were performed under approval of the animal ethics committee of the Institute of Tropical Medicine Antwerp and were in compliance with the national and international laws for the protection and welfare of animals.

A list of all strains, their metadata, and their World Health Organization codes are available as supplementary data. The BPK *L. donovani* strains were isolated from bone marrow aspirates from 36 patients with confirmed VL recruited at the B. P. Koirala Institute of Health Sciences, Dharan, Nepal, during the LeishNatDrug project in 2002–2003. The BHU *L. donovani* strains were isolated from spleen aspirates from 14 patients with confirmed VL recruited at the Kala-Azar Medical Research Centre in Muzaffarpur, India, during the Kaladrug-R study in 2009–2010. All but 4 of the Nepalese BPK patients discussed here received a full supervised treatment course of sodium antimony gluconate of 20 mg/kg/day intramuscularly for 30 days and were followed-up for clinical and parasitological evaluation at the end of treatment and at 3, 6, and 12 months after start of treatment. All sampled Indian patients in this study received a treatment other than sodium antimony gluconate. Initial cure was defined as a VL case with absence of fever, decreased spleen size, and negative parasitology at the end of a full treatment course. Nonresponders were cases with positive parasitology after a full treatment course. Definite cure was defined as a patient with initial cure who showed no signs and symptoms of relapse at the 12-month follow-up visit. Relapse was defined as a patient with initial cure but reappearance of clinical signs and positive parasitology during follow up. Treatment failure was defined as either nonresponse or relapse [5].

The clinical isolates were cryopreserved after isolation and sent (under controlled conditions) to ITM for backup and monitored storage until the onset of the experiments. All isolates were identified as *Leishmania (Leishmania) donovani* by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis of cysteine proteinase b [9]. Clones were derived from isolates using the microdrop method to obtain homogenous working parasite populations [10]. The SSG susceptibility was evaluated in an in vitro amastigote model as described elsewhere [5, 11].

Genome sequences of 17 *L. donovani* strains (10 SSG-susceptible and 7 SSG-resistant) were previously screened for homozygous SNPs occurring solely in SSG-resistant strains. However, SSG-resistant *L. donovani* may have emerged on different genetic backgrounds, and a single SNP shared by all SSG-resistant phenotypes in the sample was not found [8]. From the SNPs specific to the previous SSG-resistant strains, 5 were selected and here used in a multilocus sequence typing (MLST) approach to screen the present collection of clinical isolates (Supplementary Figure 1). SNPs were detected in DNA isolated from promastigotes either by screening whole genome sequencing data as described elsewhere [8] or by PCR amplicon sequencing of the region around the SNP (primers and conditions are available as supplementary data).

RESULTS AND DISCUSSION

Each of the 61 *L. donovani* strains was assigned a haplotype determined by its base composition at the 5 selected SNPs (haplotypes 1–8) (see Table 1). The distribution of the

Table 1. Observed Haplotypes of *Leishmania donovani*

	Location				
Haplotype	chr13 pos442924	chr24 pos26882	chr35 pos1192217	chr35 pos1619958	chr35 pos1656169
1[a]	A	C	C	G	C
2	[b]	T	[b]	A	[b]
3	[b]	C/T	[b]	A/G	[b]
4	[b]	C/T	[b]	[b]	[b]
5	[b]	[b]	T	[b]	[b]
6	[b]	[b]	C/T	[b]	[b]
7	[b]	[b]	[b]	[b]	T
8	T	[b]	[b]	[b]	[b]

Accession codes: chromosome 13, FR799600.1; chromosome 24, FR799611.1; chromosome 35, FR799622.1.

[a] Haplotype 1 is considered to be the reference haplotype (*L. donovani* BPK282/0cl4).

[b] Same base as the reference.

Table 2. Frequency of Each Haplotype for In Vitro and In Vivo Phenotypes to Antimonials (SSG)

Haplotype	In Vitro SSG Sensitivity (n = 20)	In Vitro SSG Resistance (n = 29)	SSG Definite Cure (n = 23)	SSG Nonresponse (n = 5)	SSG Relapse (n = 4)
1	16	11	21	0	2
2	2	10	0	3	1
3	1	2	0	0	0
4	0	1	0	0	0
5	0	1	1	0	0
6	1	2	0	1	1
7	0	1	1	0	0
8	0	1	0	1	0

haplotypes was assessed in the context of (1) the in vitro SSG susceptibility phenotype (as determined by the current in vitro assay) and (2) the treatment outcome of the patient. All clones showed identical haplotypes as the mother isolate from which they were derived (the haplotypes of each individual strain and summarizing tables are available as supplementary data).

Assessing the haplotypes of strains with a known in vitro SSG phenotype (20 SSG-susceptible and 29 SSG-resistant) showed that the majority of in vitro SSG-susceptible strains had haplotype 1 (16 of 20) whereas most SSG-resistant strains had haplotype 1 or 2 (11 and 10 of 29, respectively) (Table 2). The distribution of haplotypes 2–8 (taken together) and haplotype 1 was significantly different between in vitro SSG-resistant and SSG-susceptible strains (Fisher exact test; $P = .0078$). The presence of haplotypes 2–8 in a strain showed a sensitivity of 62.1% (95% confidence interval [CI], 42.4%–78.7%), a specificity of 80.0% (95% CI, 55.7%–93.4%) and a positive and negative predictive value of 81.8% (95% CI, 59.0%–94.0%) and 59.3% (95% CI, 39.0%–77.0%), respectively, for in vitro SSG resistance. This low negative predictive value is due to the high prevalence of haplotype 1 among the SSG-resistant strains. Further screening for additional SNPs might reveal other loci, allowing resolution of the latter SSG-resistant strains from SSG-susceptible ones. This is consistent with the hypothesis that SSG resistance has multiple origins [8, 12, 13]. Alternatively, SSG-resistant strains that carried haplotype 1 could still be phenotypically different from the other SSG-resistant strains at an as yet uncharacterized level. This hypothesis is supported by the fact that all haplotype 1 SSG-resistant strains were isolated from patients that were cured with SSG. These parasites possibly represent a subpopulation of in vitro SSG-resistant parasites that lack the epi-phenotype that allows them to survive SSG treatment in vivo—an epi-phenotype that remains hidden in the current in vitro test, likely due to the lack of host immune components [5]. In this context, the haplotypes of the strains should also be directly correlated with the SSG treatment outcome of the patients from which they were isolated, irrespective of the in vitro SSG phenotype of the infecting strain.

Thirty-two Nepalese patients enrolled in this study received a full course of SSG treatment and completed the 12 month follow-up—only strains isolated from these patients were assessed in this SSG treatment outcome analysis (23 SSG definite cures, 5 SSG nonresponders, and 4 SSG relapses) (Table 2). Haplotype 1 was found in strains from 21 definite cures and 2 relapses, and haplotypes 5 and 7 were found in the 2 remaining strains isolated from definite cures. Haplotypes 2, 6, and 8 occurred only in strains isolated from patients who failed treatment (both relapses and nonresponders). The presence of haplotypes 2, 6, or 8 in a strain showed a sensitivity of 77.8% (95% CI, 40.2%–96.1%), specificity of 100.0% (95% CI, 82.2%–100.0%), and a positive and negative predictive value of 100.0% (95% CI, 56.1%–100.0%) and 92.0% (95% CI, 72.5%–98.6%), respectively, for SSG treatment failure of the patient. The distribution of haplotypes 2, 6, and 8 (taken together) relative to the other haplotypes was significantly different between strains isolated from SSG treatment failures and strains isolated from SSG definite cures (Fisher exact test; $P < .0001$).

The presence of haplotypes 2, 6, or 8 in a given strain, identifiable by sequencing the amplicon of 3 PCR reactions, proved to be a significantly better marker for SSG treatment failure of the patient compared with the previously described in vitro defined SSG-resistant phenotype of the parasite, which showed a sensitivity of 66.7%, a specificity of 35.0%, and a positive and negative predictive value of 31.6% and 30.0%, respectively [5]. However, testing these haplotypes on a larger number of strains isolated from patients with well-documented SSG treatment outcomes can result in a more accurate estimation of the power and performance of these haplotypes to detect and predict SSG treatment failure of the patient.

Several studies aimed to identify markers for in vitro SSG resistance in clinical *L. donovani* isolates by targeting the thiol metabolism of the parasite, which is important for activation of the drug and defense against its action mechanism, and

import/export mechanisms for the drug (reviewed elsewhere [14]). However, these candidate markers have yet to be validated on a larger sample or were not uniformly present in all SSG-resistant strains. This study describes the first attempt (to our knowledge) to validate genetic markers (MLST), earlier identified by full genome sequencing, for clinically relevant SSG resistance of *L. donovani*. It highlights the limits of the current in vitro SSG susceptibility assays and provides a simple and biologically more robust alternative. In this study, just 3 PCR reactions and amplicon sequencing were sufficient to identify clinically relevant SSG resistance markers. Moreover, once these PCR reactions are optimized for use in clinical samples, line-probe assays similar to those used for the detection of drug resistance in *Mycobacterium tuberculosis* [15] could be designed to simplify the application of these markers and allow faster detection of parasites that contribute to treatment failure. Whether in a diagnostic, epidemiological, or experimental research setting, such a simplified format would be easily applicable in laboratories with limited PCR facilities.

One of the limitations of our analysis is that predictive values depend on prevalence: the lower the prevalence, the lower the positive predictive value of a positive result. Therefore, the current markers should be further validated on larger, prospective series from the Indian subcontinent and East Africa. In the future, these markers can also be complemented with new ones stemming from running genome sequencing projects. This MLST approach represents a significant asset for control programs and other epidemiological studies aiming to monitor the emergence and spread of clinically relevant SSG-resistant *Leishmania* parasites. It is also relevant in regions where SSG was abandoned, such as in the Indian subcontinent, to follow the disappearance (if any) of SSG-resistant parasites and determine their impact on future VL control strategies.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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