

Antimony-resistant *Leishmania donovani* in eastern Sudan: incidence and in vitro correlation

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الليشمانية الدونوفانية المقاومة للأنتيمون في شرق السودان. معدل الوقوع وارتباطه بالنتائج المخبرية منال عبده، وليد الأمين، الطاهر خليل، ماوية مختار

الخلاصة: أجريت دراسة طولانية في منطقة موطونة ببدء الليشمانيات في شرق السودان خلال الفترة بين تشرين الثاني/نوفمبر 2001 وشباط/فبراير 2003 لمعرفة معدل وقوع فشل المعالجة بستيبوغلوكونات الصوديوم. وقد شملت الدراسة 820 مريضاً تأكدت إصابتهم ببدء الليشمانيات الحشوي، وعولجوا جميعاً بمركب ستيبوغلوكونات الصوديوم بمعدل 20 ميلي غرام لكل كيلو غرام من وزنهم لمدة لا تقل عن 28 يوماً. وقد استُفردت الطفيليات من رُشافات العقد اللمفية من 22 ممن عرفوا بأنهم مرضى ناكسون. وقد حددت جميع الأنماط لدى المرضى على أنها من الليشمانية الدونوفانية، باستخدام التفاعل السلسلي للبوليميراز الذي يتم فيه تضخيم الدنا الخاص بمنشأ حركة طفيلي الليشمانيا. وفي المختبر، أظهرت ستة طفيليات مقاومة تجاه ستيبوغلوكونات الصوديوم، وذلك باستخدام طريقة اختبار البلاعم المأخوذة من الجرذ J774 والأطوار اللاسوطية، فقد أظهرت المستفردات المقاومة مرتسمات مختلفة الاقتران عندما تم هضم نواتج تضخيم دنا منشأ حركة الطفيلي نتيجة للتفاعل السلسلي للبوليميراز بإنزيم الاقتران ALU1، مما يشير إلى أن المقاومة تُعزى إلى نساقل مختلفة من الطفيليات.

ABSTRACT A longitudinal study was done in a leishmaniasis-endemic region in eastern Sudan during the period November 2001–February 2003 to determine the incidence of failure of sodium stibogluconate treatment. We studied 820 confirmed visceral leishmaniasis patients. All were treated with sodium stibogluconate, 20 mg/kg body weight for at least 28 days. Parasites were isolated from lymph node aspirates from 22 participants identified as relapsed patients. All isolates were typed as *Leishmania donovani* based on polymerase chain reaction (PCR) amplification of parasite kDNA. Six parasites showed in vitro resistance to sodium stibogluconate using murine J774 macrophage amastigote testing method. The resistant isolates showed different restriction profiles when the amplified kDNA PCR products were digested with ALU1 restriction enzyme, indicating that resistance was mediated by different parasite clones.

Résistance à l'antimoine de *Leishmania donovani* au Soudan oriental : incidence et corrélation *in vitro*

RESUME Une étude longitudinale a été réalisée dans une région d'endémie de la leishmaniose au Soudan oriental entre novembre 2001 et février 2003 afin de déterminer l'incidence de l'échec du traitement au stibogluconate de sodium. Nous avons étudié 820 patients atteints de leishmaniose viscérale confirmée. Tous les patients ont été traités par le stibogluconate de sodium à raison de 20 mg/kg pendant 28 jours au minimum. Les parasites ont été isolés dans les échantillons prélevés par aspiration dans les ganglions lymphatiques de 22 participants identifiés comme patients ayant rechuté. Tous les isolats ont été typés comme *Leishmania donovani* sur la base de l'amplification en chaîne par polymérase (PCR) de l'ADNk du parasite. Six parasites ont montré une résistance *in vitro* au stibogluconate de sodium en utilisant le test de détection des amastigotes dans les macrophages J774 murins. Les isolats résistants ont présenté des profils de restriction différents lorsque les produits de l'ADNk amplifiés par PCR ont été digérés par l'enzyme de restriction ALU1, ce qui indique que la résistance était médiée par différents clones des parasites.

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Introduction

Visceral leishmaniasis (kala-azar) is a chronic protozoan infection in humans that is associated with high morbidity and mortality of untreated patients. The causative organism is *Leishmania donovani*. At present, first line treatment is with pentavalent antimony compounds, sodium stibogluconate and meglumine antimonite, for all forms of the infection [1,2]. Emergence of sodium stibogluconate-resistant strains of *L. donovani* in leishmaniasis-endemic areas in Asia and Africa is becoming a serious health problem. Lack of response to the initial course of pentavalent antimony drug treatment is generally around 10% in most areas, but this seems to be increasing [3]. Second-line agents such as pentamidine and amphotericin B are used when sodium stibogluconate or meglumine antimonite fail [2,4] but these drugs are highly toxic and too expensive for widespread use in developing countries. In some cases, clinical resistance to amphotericin B and pentamidine has been observed [5].

The mechanism of resistance to pentavalent antimony compounds has not always been clear, and factors such as changes in the pharmacokinetics of the drug, drug purity, suboptimal concentrations and host immune status have been cited as causes of apparent resistance or failure to respond to treatment [6]. There is evidence, however, that an inherent lack of susceptibility and/or the development of resistance can also contribute to parasite unresponsiveness to drugs [7].

Furthermore, studies on *L. donovani* in mice demonstrated the need for a Th1 response for parasite clearance with the pentavalent antimony treatment, suggesting that the in vivo efficacy of such treatment is at least partially immune based. Approximately 25% of leishmaniasis patients co-infected with HIV relapse following treat-

ment with antimonials, and 50% of such patients do not respond to antimonial drugs [8–10].

Current control of visceral leishmaniasis is based on case detection and treatment. The failure rate of sodium stibogluconate treatment in eastern Sudan is increasing and is alarming (Médecins Sans Frontières, unpublished report, 2002). No detailed study has been done on resistance of *L. donovani* to this drug in the country. Our study aimed to determine the incidence of resistance of *L. donovani* to sodium stibogluconate in visceral leishmaniasis patients in eastern Sudan.

Methods

A longitudinal study was carried out in a leishmaniasis-endemic area in Gedaref state, eastern Sudan, during the period November 2001–February 2003.

We randomly recruited 820 visceral leishmaniasis patients to the study from three VL treatment centres (Tabarak Allah, Kassab, and Doka) in Gadaref state, eastern Sudan. All patients gave informed consent to take part. The patients were diagnosed on the basis of direct agglutination tests, confirmed by detection of parasites in lymph node aspirates. When parasites were not detected, patients were not treated and were re-examined 4 weeks later. Patients in whom the parasites were detected were treated with sodium stibogluconate injection BP (Albert David Ltd., Calcutta, India), concentration of pentavalent antimony 100 mg/mL at a dose of 20 mg/kg for at least 28 days. Patients were followed up for case detection every 2 months by visits to villages to determine the incidence of relapse. Patients in whom parasites were still evident after the complete course of treatment (test of cure was done by parasite detection in lymph node

aspirates) and patients who developed symptoms of visceral leishmaniasis (e.g. fever, weight loss, epistaxis, splenomegaly) during the active follow-up period of 16 months were defined as relapsed patients, while patients who still had detectable parasites at the test of cure were defined as uncured.

Uncured and relapsed patients were re-treated until no parasite was detectable in the lymph node aspirates (minimum duration of treatment 28 days).

Parasites were isolated by inoculating lymph-node aspirate from the relapsed patients into biphasic Novy–MacNeal–Nicolle (NNN) medium consisting of a solid phase agar mixed with defibrinated rabbit blood (10%) and a liquid phase of RPMI 1640 medium supplemented with 10% fetal calf serum (Sigma-Aldrich, USA). Primary cultures were incubated at 26 °C for 4 days and then grown in RPMI 1640 medium containing 25 mM Hepes, pH 7.4, 10% heat-inactivated fetal calf serum, streptomycin and penicillin at 5 U/mL in 25 mL culture flasks.

Isolates were characterized by polymerase chain reaction (PCR) technique. We used the Chelex® extraction method to extract DNA from cultured parasites and PCR was carried out using genus-specific primers for mini-circle kinetoplast DNA (kDNA) (AJS3, 5' GGGGTTGGTGTA-AATAGGCG-3' and DBY 5' CCAGTTTC-CCGGCCCCGAg-3') as described by Smyth et al. [11]. The amplification was run for 35 cycles on a PCR machine (Perkin-Elmer Thermal Cycler Model 480, Torrance, California, USA) at an annealing temperature of 64 °C for 1 minute.

For determination of restriction fragment length polymorphism (RFLP), 13 µL of amplified PCR products were digested with 1 U ALU1 restriction enzyme (Biolone, London) in a total volume of 20 µL. The

digestion was done at 37 °C for 4 hours. The restriction profile was determined by electrophoretic separation of 10 µL of the digested product on 2% agarose at 80 V, 100 mA.

For in vitro sensitivity testing of the isolates to sodium stibogluconate, we prepared monolayers of cultured murine macrophage cell line J774. Growing cells were added to 24-well tissue culture plates (1 mL/well) containing glass coverslips 13 mm diameter. The plates were incubated overnight at 37 °C in a 5% CO₂ atmosphere. The cells were infected with stationary-phase promastigotes at a ratio of 10–15 promastigotes/cell. Infected cultures were incubated overnight at 37 °C in a 5% CO₂ atmosphere. Excess promastigotes that did not infect the macrophages were washed with RPMI 1640. We added 1 mL sodium stibogluconate injection BP to 2 wells to give a final concentration of 5mg/mL pentavalent antimony; 1 mL RPMI 1640 medium was added to 2 wells as controls. Each parasite isolate was tested in 4 separate wells. The plates were re-incubated for 3 days at 37 °C in a 5% CO₂ atmosphere. The coverslips were then removed, air-dried, fixed in 70% methanol and stained with 10% Giemsa stain. To calculate the percentage of infected cells and the number of parasites/cell, 100 macrophages were examined under oil immersion light microscopy at 100 × magnification.

The chi-squared test was used to compare the proportion of surviving parasites in the presence and absence of sodium stibogluconate.

Results

The age of the participants ranged between 4 and 60 years. Adults (age ≥ 16 years) comprised about 40% of all treated pa-

tients. All patients were negative for HIV antibodies (using an enzyme-linked immunosorbent assay test) and none had pulmonary tuberculosis.

Of 820 patients who were treated with a complete course of sodium stibogluconate,

22 (2.7%) were identified as relapsed patients on the basis of detection of parasites in lymph node aspirates. Recurrence (confirmed by detection of parasites in lymph node aspirate of patients) occurred 2–6 months after treatment with sodium stibogluconate (Table1). The age range of these patients was 5–32 years.

All isolates were identified by PCR as *L. donovani* on the basis of the amplified minicircle band 800 bp (Figure 1).

The restriction fragment length polymorphism analysis of the PCR product showed the presence of common profile patterns in all isolates with band sizes of 290 bp and 580 bp (Figure 2). Comparison with the sensitive isolates showed a band size of 316 bp in the resistant isolates. A band size of 750 bp was detected in both resistant and sensitive isolates.

No differences were found in the electrophoresis patterns of the pairs of resistant isolates before and after treatment (VLR4B-VLR4A, VLR5B-VLR5B and VLR6B-VLR6A) (Figure 2).

Of the isolates from the 22 patients identified as relapsed, 6 (27.3%) showed a significant increase in the number of infected macrophages and the number of parasite/macrophage ($P < 0.05$) compared with sensitive field isolates and *L. donovani* reference strain (LD 1S) (Figures 3 and 4) in the presence of 10 mg/mL sodium stibogluconate.

A significant difference was detected between resistant and sensitive reference isolates when comparing parasite survival in the presence of sodium stibogluconate ($P < 0.005$).

Discussion

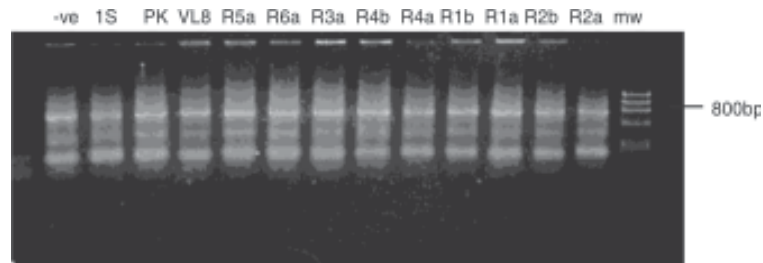
The observed increase in the clinical resistance of visceral leishmaniasis to sodium stibogluconate treatment in eastern Sudan

Table1 Characteristics of 22 leishmaniasis patients classed as relapsed after first treatment with sodium stibogluconate

Patient ID	Age (years)	Sex	Relapse (days ^a)	Duration of second treatment ^b (days)
12	7	Male	60	80
28	12	Female	60	79
44	13	Female	160	28
113	8	Male	70	118
176	5	Female	90	28
190	9	Female	60	144
272	8	Female	90	81
308	8	Male	75	121
415	16	Male	150	28
551	32	Male	140	28
776	8	Male	180	28
3	9	Male	150	28
121	16	Male	160	60
214	12	Female	65	80
283	22	Male	100	28
460	8	Female	120	28
621	7	Male	160	56
634	6	Male	180	28
668	13	Male	150	28
711	20	Male	60	66
742	11	Female	80	54
812	18	Female	160	28

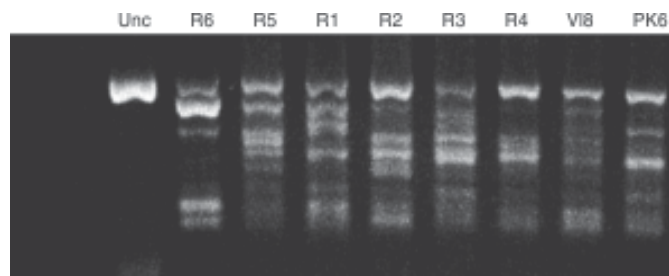
^aReport of relapse in days after the first sodium stibogluconate injection.

^bDose 20 mg/kg body weight of sodium stibogluconate.



1S = reference strains.
 R*b = resistant isolate before second treatment.
 R*a = resistant isolate after second treatment.
 Mwt = molecular weight marker.

Figure 1 Characterization of Leishmania isolates by amplification of parasite kDNA using AJS3/DBY primers



Unc = undigested.
 R* = restriction fragment length polymorphism if *in vitro* resistant isolate.
 V1 = restriction fragment length polymorphism if *in vitro* sensitive visceral isolate.
 PK = restriction fragment length polymorphism if *in vitro* post-Kala azar dermal leishmaniasis isolate.

Figure 2 Diversity of *in vitro* sodium stibogluconate resistant Leishmania isolates following Alu1 restriction of PCR amplified kDNA fragment

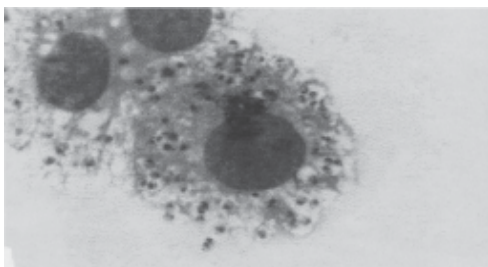


Figure 3 J744 murine cell line infected with resistant leishmania isolate in the absence of sodium stibogluconate

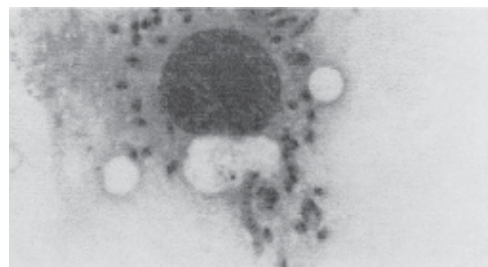


Figure 4 J744 murine cell line infected with resistant leishmania isolate in the presence of 100 mg/kg sodium stibogluconate

is alarming, but no accurate records of the incidence of resistance are available. Especially alarming was the increase in the number of adults diagnosed with visceral leishmaniasis in this area. The disease has been known as disease of children, and adults were relatively immune [12]. In our study, 820 visceral leishmaniasis patients were recruited and followed up for 16 months after completion of their treatment courses.

The incidence of clinical resistance during the period of the study was calculated as 2.7%. All relapsed patients were tested for HIV infection and pulmonary tuberculosis and were negative, therefore, neither HIV nor tuberculosis contributed to the resistance. Parasites were successfully isolated from lymph node aspirates of relapsed patients and all were typed as *L. donovani*. Other reports of typing of isolates from visceral leishmaniasis patients from the same area have identified *L. donovani* and *L. infantum* as the main causes of visceral leishmaniasis [13]. More than one RFLP profile was detected, indicating that resistance was mediated by more than 1 parasite clone [14].

To determine genuine parasite resistance to sodium stibogluconate, all isolates were tested in vitro using J774 macrophage cell line infection assay. This assay allowed

testing of sensitivity of parasite amastigotes stages to sodium stibogluconate. Amastigotes are known to provide a more reliable indication of resistance compared to promastigotes [15]. Isolates showed variable relative resistance to sodium stibogluconate: 6 showed relative resistance in vitro to sodium stibogluconate compared with sensitive strains. These isolates were considered genuine resistant parasites.

In conclusion, sodium stibogluconate-resistant isolates from an endemic area in Gedaref state, Eastern Sudan, were classified as belonging to the *L. donovani* complex on the basis of minicircle DNA amplification using AJS3/DBY primer. According to our findings, different *L. donovani* clones might be responsible for the current drug failure in this endemic region.

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