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Research Article

Rapid Assessment of Patterns of *Leishmania donovani* Infection in Eastern Sudan: Immune Surveillance and Application of Geographic Information System

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Abstract

Visceral Leishmaniasis (VL) is a fatal disease that predominantly affects children in Sudan. Recent epidemics demonstrated that the health system is neither adequate nor prepared. Control of visceral Leishmaniasis is hampered by the elusive nature of the vector and failure to identify a reservoir host. Case detection and treatment with antimonial drugs contribute to disease control.

A rapid epidemiological survey (RES) and spatial analyses were conducted to map VL clinical phenotypes, identify population at risk of VL and molecularly characterize *L. donovani* isolates.

Eight villages were randomly selected for total population census demographic and clinical data collection, simple immunological techniques (leishmanin skin test, DAT) and parasite culture and molecular characterization of the *Leishmania* isolates.

The use of clinical interview combined with simple immunological tests can give valuable information about the pattern of *L. donovani* infection and predict future prevalence in a short time. Leishmanin non-reactive individuals are a useful piece of data to plan for future vaccine efficacy studies. RES can provide useful information in a short time (~10-14 days) about disease phenotypes, population at risk and target population for future vaccination campaigns. The data could be depicted in map formats to give an enhanced visual impact and can act as a nidus for the development of a Pan-Sudan/Pan-Africa VL map.

ABBREVIATIONS

VL: Visceral Leishmaniasis; GIS: Geographic Information System; CL: Cutaneous Leishmaniasis; LRG/Sudan: Leishmania Research Group/Sudan; PKDL Post Kala-Azar Dermal Leishmanisis; DAT: Direct Agglutination Test; LST: Leishmanin Skin Test; SSG: Sodium Stibogluconate; COII: Cytochrome Oxidase; NNN: Novy-Macneal-Nicolle Medium; FCS: Fetal Calf Serum; PCR: Ploymerase Chain Reaction; HAD: Heteroduplex Analysis; DCL: Difuse Cutaneous Leishmaniasis

INTRODUCTION

Visceral leishmaniasis (VL) is endemic in the Sudan and has been reported by Neave [1]. The main endemic area is the eastern part of the country that stretches from the western bank of the White Nile to the Sudanese-Ethiopian borders in the east and in the north from the upper reaches of the Atbara River to the Sudan-South Sudan borders. The disease is associated with *Acacia* forests and deeply cracked black clay soil [2]. Epidemics that claimed hundreds of thousands of lives showed that the health services in Sudan were inadequate in dealing with such disasters that are more likely to occur from time to time [3,4]. A devastating epidemic of VL occurred in Sudan from 1984 to 1994. As this was the first epidemic in the area, the population was highly susceptible. Some studies have estimated that the disease caused 100 000 deaths in a population of around 300,000 in the western upper Nile area of the country during this period. In some villages, more than half of the population died from the disease. The epidemic in 1997 in Sudan caused an increase of cases of VL by 400% over the previous year. Treatment centers were overwhelmed and stocks of first-line drugs were depleted. The migration of seasonal workers and large population movements caused by civil unrest carried the epidemic into Eritrea and Ethiopia (WHO, 2013). The state of war that is raging in eastern and southern Sudan forced immunological naïve individuals to enter VL endemic areas with grave consequences. In VL endemic area in eastern Sudan, regular epidemiological studies were carried out in the human population, as well as in mammals and sand flies. *Leishmania* strains were isolated from patients and mammalian animals. Characterization of isolated strains

(by starch gel electrophoresis and isoelectrofocusing) identified zymodemes of *Leishmania donovani*, *L. infantum* and of *L. archibaldi* complexes from patient samples and zymodemes of *L. donovani*, three of *L. infantum* and two of *L. archibaldi* complexes from dog samples [5,6]. Control of the infection includes active case detection and use of impregnated bed nets [4,7].

The objective of rapid mapping is to ascertain rapid, non-invasive techniques applied to those most at risk, whether VL is present in the community under study and, if so, to obtain a measure of its endemicity level. However, in the absence of a simple, affordable technique for identifying and mapping out high risk communities, governments have been unable to put to best use their scarce resources and the assistance offered by the NGOs for national control programs. The advent of GIS has greatly facilitated the mapping process and, in particular, enables maps to be updated rapidly and presented in different ways. CL and VL could possibly be mapped using GIS and database of the incidence and/or prevalence rates in a selected area using census data and official CL/VL records from the available health centres or peripheral hospitals. Furthermore, rapid epidemiological mapping (REM) has also been applied in some African countries to evaluate the endemicity of onchocerciasis using the same criteria [8,9].

In this study a rapid assessment scheme was introduced using a cross-sectional survey employing clinical interview, clinical examination and simple immunological tests. The clinical interview was introduced to overcome some of the limitations of cross-sectional surveys, by looking into the past medical history of volunteers. We also used more sophisticated moleculobiological and *in vitro* culture techniques to try to identify the *Leishmania* isolates and to look into the more complicated problem of drug resistance.

METHODS

Study area, volunteers and patients

Eight villages (Hillat Hashim, Aradaiba, Rugab, Dalaib, Jammam, Sangir, Ali Babiker and Salala) within the VL endemic region, eastern Sudan were selected for this study. The area located between coordinates 12°55.040 and 12°47.580 north and 035°03.664 and 035°16.671 east. The total population of these villages is about 12700 individuals as was shown by the census carried out by the Leishmaniasis Research Group of Sudan (LRG/Sudan) in 2015. The first leishmaniasis survey in the area was conducted in 1990 by the LRG/ Sudan. Seven hundred and ninety seven individuals were randomly selected using Epi Info 7 version 7.0.9.34 software using Random (Not cluster) sampling for cross-section population surveys. Demographic and clinical data (VL, PKDL, subclinical infection) were collected. 44 parasitologically confirmed VL patients attending the LRG/ Sudan clinic at the study area during the period of the survey were enrolled had their lymph node/bone aspirates cultured for parasitological confirmation. Re-checking was carried out by independent pathologist and patients were followed up to the end of treatment.

Detection of VL infection

Past VL infection was detected by direct agglutination test (DAT) as described by Harith et al. [10]. Leishmanin skin test (LST) was carried out using leishmanin antigen and diluents (Pasteur Institute, Iran, lot No. 111). The antigen was administrated separately at a dose of 0.1 ml intradermally on the volar surface of the forearm. The test was read after 48-72 hours using the ballpoint pen method [11]. The occurrence of induration +/- erythema of any size was considered a positive reaction [12].

Parasite isolation and characterization

NNN medium was used for primary isolation of *Leishmania* parasites. Bone marrow and/or lymph node aspirates from patients who were parasitologically confirmed were inoculated. Isolated were then transferred into 10-15 ml of RPMI-1640 supplemented with 10% fetal calf serum (FCS) and kept at 27°C until the promastigotes growth reached stationary phase.

Parasite DNA was extracted and field isolates were characterized based on the size of their mini-circle DNA as described by Smyth et al. 10 ng of parasite DNA was amplified using two primers (AJS3 and DB8). The primers' position is on the conserved region of the mini circle and encompassing part of the sequence of origin of replication. On amplification these primers yield a whole length minicircle sequence, which differ between the different *Leishmania* species. Another PCR was

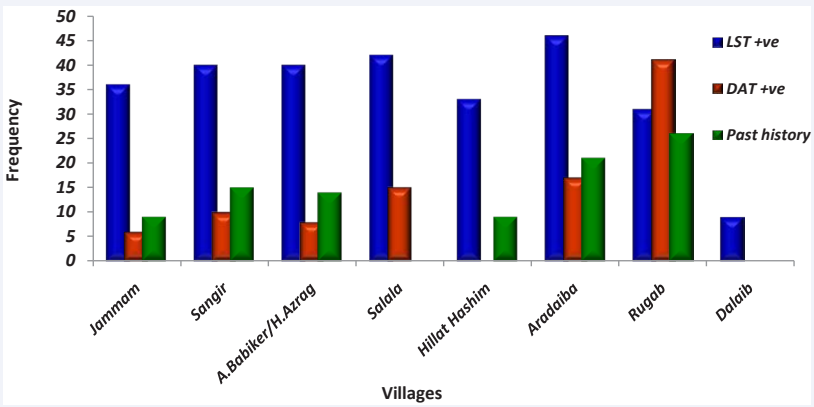

Figure 1 LST and DAT positive results versus past history of VL infection.

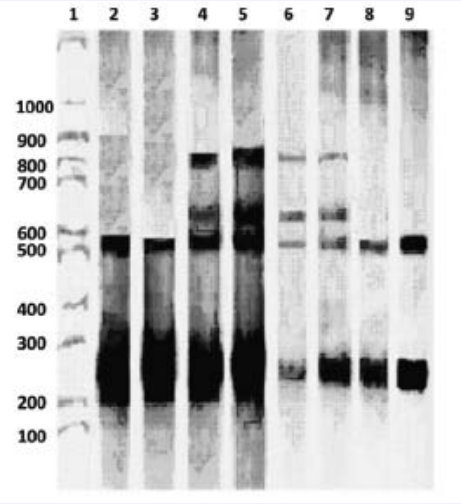

Figure 2 Results of heteroduplex analysis of 2 *L.d..infantum* and 3 *L.dononvani* isolates from Sudan. Lane 1: 1Kb DNA marker; Lane 2: *L.donovani* 2+ *L.donovani* 3; Lane 3: *L.donovani* 1 *L.donovani* 2; Lane 4: LEM307+ *L.donovani* 3; Lane 5: LEM307+ *L.dononvani* 2; Lane6: *L.donovani* 2 + REB2; Lane 7: LEM307+REB2; Lane 8: REB2; Lane 9: LEM307.

performed, in which the COII gene was targeted. The sequences of the primers were designed to target the cytochrome oxidase II (COII) gene are:

F: 5' GGCATAAATCCATGTAAGA 3' and R: 5' TGGCTTTTATAT-TATCATTTT 3'

The PCR products were analyzed in 1.5% agarose gel, stained with ethidium bromide, visualized under UV illumination and photographed using Polaroid film [13].

In vitro sensitivity screening of Leishmania strains

The *in vitro* sensitivity of *L. donovani* was evaluated using J774; a murine macrophage-like cell line by the method described by Gebre-Hiwot et al., with modifications. Briefly, J774 cells were subcultured in RPMI- 1640 medium supplemented with 10% FCS. Cells in the logarithmic phase of growth were differentiated (into non-dividing adherent monolayers) by incubation for 2 days at 37 _C and 5% CO_2 in medium containing 20 ng of phorbol myristate acetate (PMA, Sigma) per ml, which induced differentiation and caused the cells to become adherent. J774 cells treated with PMA were washed and then infected with stationary phase extracellular amastigotes in eight-chamber Lab Teck chamber slides (Nunc) at a host cell/parasite ratio of 1:5 at 37°C with 5% CO_2. After 48 h incubation, non-internalized parasites were removed. Serial dilutions of each drug were freshly made in RPMI-1640 medium supplemented with 10% FCS and were dispensed into the wells. After 3 days of drug exposure, wells containing adherent, differentiated J774 cells were washed. The cells were fixed with methanol and stained with Giemsa stain. The number of infected host cells and the number of intracellular amastigotes/100 infected host cells were measured. Therefore, *in vitro* sensitivity and drug activity were assessed by determining (i) the percentage of infected macrophages and (ii) the parasite survival index (PSI), the mean number of intracellular parasites in an infected macrophage. PSI calculated as 100 -(number of amastigotes/100 infected macrophages in treated wells)/ (number of amastigotes/100 infected macrophages in untreated wells) [14,15].

Mapping of *L. donovani* patterns of infection

ArcView GIS ESRI, USA was used to construct and draw the proposed project map (leishmania.apr). Some relevantly-selected files were downloaded from www.maproom.psu.edu site. These files contained the layer coverages e.g. political boundaries; populated areas; drainage network, drainage points and roads network (Figure 5). Unzipped files were imported to the Arc View GIS software as the following files: Dnpoint.e01, Dnnet.e01, Icpoly.e01, Ponet.e01, Ofline.e01 andRdnet.e01. All coverage layers (converted as themes) were placed in one view. Tables of leishmanin reactivity, DAT titres, and age and sex distribution for the selected villages were added to the table appropriate (Figure 6,7). Charts were also made in the project. Different layouts were constructed to show map themes and study area were constructed. Monthly total rainfall, relative humidity, monthly minimum and maximum temperatures for the years 1996 to May 2001 was collected from the National Metrological Centre, Khartoum, Sudan.

RESULTS

The mean age of the study volunteers was 19.0 ± 13 years, and children below 15 years were 55% of the total population surveyed. Preliminary data from the clinical interview revealed that past history of VL (treated at the Kala-Azar centre or at the local dispensary) was reported in 94/800 (8.5%) and history of skin rash following treatment was report in 70% of patients. Eighty individuals declared their history of parasitologically confirmed VL within the previous twelve months. Some of these patients (mean age 7.7 ± 7.0 years) presented and were diagnosed during the survey period. All were non-reactive in the leishmanin skin test and their DAT titres were above the diagnostic cutoff titer of 6,400. LST screening (Figure 1) showed that the majority of volunteers (65.6%) did not react to the leishmanin antigen and those above 15 years of age were more likely to be leishmanin reactive (induration>=5mm) compared to children. Although males are more likely to be leishmanin reactive compared to females, this was statistically insignificant (P=0.3). DAT detected anti-*L.donovani* antibodies above the diagnostic cut-off titer in sera of only 10% plus 2% of the volunteers who had boarder titers of 3,200.

Thirty-six parasites were isolated from confirmed-positive and who were seen during the study period. Patients were followed up for their response to SSG treatment. Seventy-two patients responded clinically to treatment with sodium stibogluconate, while eight VL patients failed treatment with repeated courses. The parasite isolates were characterized using heteroduplex analysis (HAD) as a direct and easy to perform method for characterizing *Leishmania* strains. The PCR amplification with two maxicircle primers (MAX2N and MAX3), yielded bands of approximately 540 bp as shown in Figure 2, which corresponding to the main portion of the cytochrome oxidase II (COII) gene of *L. donovani*. Following HDA between samples from the field and two *L. d. infantum* strains (LEM307

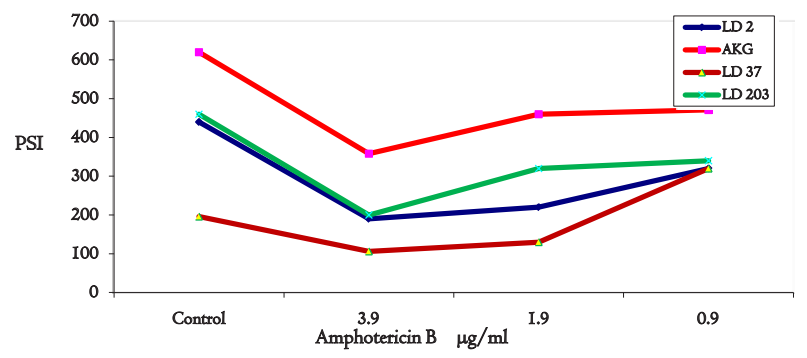

Figure 3 Effect of pentostam on the survival index of four *L. donovani* amastigotes infecting a J774 macrophage-like cell line.

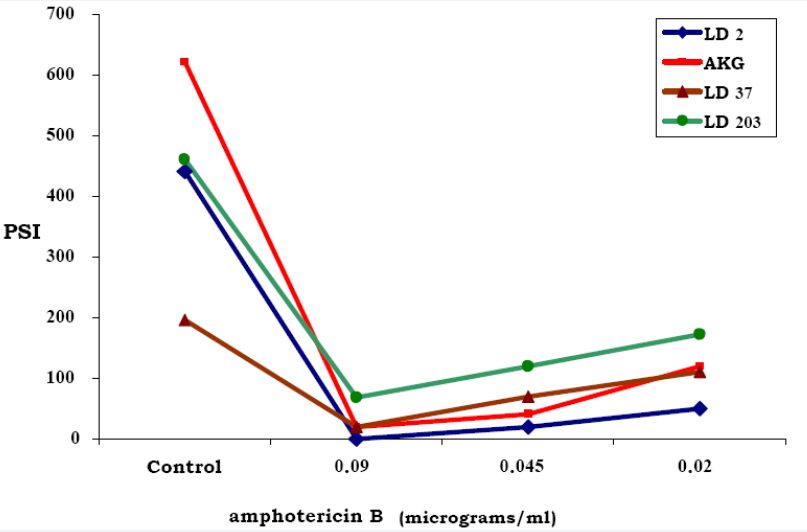

Figure 4 Effect of amphotericin B on the survival index of four *L. donovani* amastigotes infecting a J774 macrophage-like cell line.

Figure 5 Completed Sudan map with political boundaries, drainage network, road network and study area.

and REBEL02) taken as wild types, extra bands were recorded. In contrast, the reference strains produced heteroduplexes as shown in same figure.

Four *L. donovani* isolates were *in vitro* tested for their response to Pentostam and Amphotericin B in their amastigote form using J 774 murine macrophage-like cell line. All the isolates tested, were highly infective to the cells (80%-99%) and exhibited moderate to high intracellular replication potentials. It was noted that the number of parasites in infected macrophages decreased steadily at drug concentrations of 0.9 µg/ml to 3.9 µg/ml (in pentostam) and 0.02 µg/ml to 0.09 µg/ml (in Amphotericin B) and the capacity of parasites to replicate was also affected, Figures 3 and 4. The survival index is less steep in pentostam compared to amphotericin B.

The constructed project (preparation of base maps) was named Leishmania.apr. The project contained views that were formed from political boundaries, drainage network, populated areas and road network themes. Tables of age groups, LST reactivity, DAT titres and VL/PKDL cases were also added to the project. Charts were constructed from the tables and layouts for printing were formed.

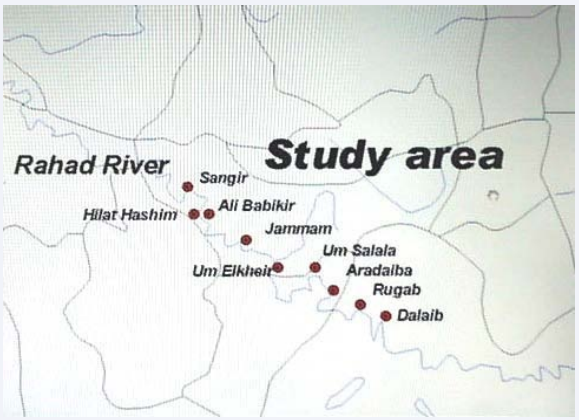

Figure 6 Study villages in Rahad Province, Gedarif State, eastern Sudan.

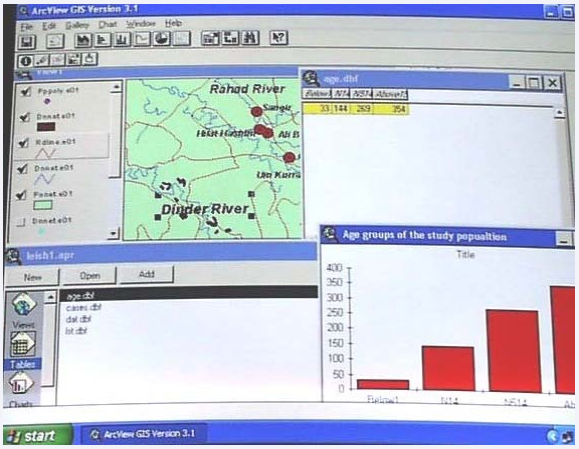

Figure 7 Complete leishmania.apr with views (with themes), tables and charts.

DISCUSSION

In this study the clinical interview was introduced to overcome some of the limitations of cross-sectional surveys, by looking into the past medical history of volunteers. We also used more sophisticated moleculobiological techniques to identify the *Leishmania* isolates and to look into the more complicated problem of drug resistance. The demographic data showed that the communities under study are mainly from young age groups with equal number of male and female VL patients as was previously reported in longitudinal studies carried in the same area [16,17]. The calculated overall incidence rate of VL over the past year was 7.9/1000 persons per year (LRG/Sudan, unpublished data). This incidence is lower than that noted by other investigators working in the same area [16]. This fluctuation does not mirror changes in weather phenomena over the last four seasons. The drop in cases could be due to intervention methods carried out in the area by the Sudan Leishmaniasis Research Group and MSF-Holland. But this fact could not be fully true because the vaccine efficacy was 6% and that the drop of cases was also noted in villages where impregnated bed nets were introduced [4,17].

The number of PKDL cases seen during the survey probably reflects those with persistent lesions rather than the actual incidence of the disease. Many PKDL cases presented with transient rash that heals spontaneously with a small proportion that continues to have chronic lesions .The parameter of past history was introduced based on the very good knowledge about VL in studied areas as well as people's diagnosis and knowledge about the disease which is fairly accurate. Past history was lower than the rate of LST conversion; this may simply point to the possibility of subclinical infection [18]. Moreover, LST positivity increased with age indicating that as individuals get older they will be increasingly exposed; some will develop overt disease while others remain asymptomatic (Subclinical infection). Positive LST was not significantly different in males (18.2%) compared to females (16.3%) (P= 0.24). This finding is in contradistinction to that reported from across the borders in Ethiopia, where males have a higher degree of leishmanin positivity [19,20]. This dissimilarity may simply reflect behavioral disparity rather than divergence in immunological responses. 10% of volunteers had DAT titers above the cutoff of 6,400; this figure closely matches volunteers who gave past history of VL. As seroconversion is lower than LST conversion, all those patients had truly past VL. DAT titre will remain high for many years following successful treatment. Infected individuals with infected sand flies are classified into 4 groups; a; naïve individuals whose immune system go toward Th2 form of response, they fail to eliminate the parasite and also develop liver, spleen, fever and expulsion of B cell stimulation with huge amounts of antileishmanial antibodies that detected by DAT. b; those who eliminate the parasites but at the same time they produce memory cells and T cells. When injected with LST, they produce IL-17 and B cells, T cells, NK cells and neutrophils recruited and this is well reflected in LST induration. c; individuals who are not LST+ve and have no DAT reactivity, but if their cells been stimulated in the whole blood with Leishmania antigen, they will produce interferon "those whose LST +ve do not produce either IL-10 or interferon". d; VL patients who produce IL-10 and never produce interferon [21]. The contribution of confirmed VL cases to the total incidence rate of infection is limited and that the true incidence of infection is much higher when all categories of both clinical and subclinical VL are taken into account. Moreover, infection takes place in presumed immune individuals (LST positive) as reflected by seroconversion in the DAT. Apparently these individuals have transient parasitaemia resulting in a humoral immune response as measured by the DAT. None of these individuals develop clinical VL, which supports the observation that primary VL only occurs in LST negative individuals; few relapse cases of VL in patients with a positive LST have also been seen [22].

We employed HDA for characterizing *Leishmania* isolates to obtain maximum information of the infra-specific variation of *L. donovani* species complex. The method of HDA is sensitive in detecting sequence differences down to a single base substitution [13,23,24]. The presence of extra bands is taken as an evidence for difference in the gene sequence by at least a single base pair. Indeed the amount of mobility shift should be proportional to the number of bases in which they differ.

The present study demonstrated that *in vitro* drug sensitivities did not match clinical unresponsiveness in the strains that were

tested for SSG sensitivity. Such a lack of correlation has previously been reported [14,15]. Four isolates (3 resistant; 1 susceptible) that were tested in a macrophage system were highly infective with satisfactory replication potentials in the non-treated controls. Addition of pentostam or amphotericin B markedly affected the replication potential and the infectivity of the parasites. There was no difference in macrophages' infectivity or replication potential between the resistant and sensitive isolates. It was shown by this, that *in vivo* unresponsiveness doesn't necessarily mean primary parasite resistance. As was reported previous by Khalil et al. [25], that concomitant diseases may render patients unresponsive, this simply means before declaring a patient unresponsive a thorough search should be carried out to exclude concurrent illnesses. We also showed that amphotericin B is a suitable second line drug if patients can tolerate it toxicity. The acquired resistance of *Leishmania* to antimony has generated intense research on the mechanisms involved but the question has not yet been resolved. Drug efflux in *Leishmania*, is largely dependent on the number of efflux pumps an isolate can express. After exposure to three antileishmanial drugs, *in vitro,* all isolates were resistant to Glucantime but susceptible to Miltefosine, whilst Amphotericin B was more effective on the "susceptible" isolates. The MDR gene, expressing the transmembrane efflux pump Pgp 170, appears to play a key role in the phenomenon of drug resistance [26]. The immune status of leishmaniasis patients has long been known to affect drug efficacy. This is of particular importance in relation to pentavalent antimonial treatment of diffuse cutaneous leishmaniasis (DCL) and co infections with HIV in the visceral form where there is an absence of a specific T-cell mediated immune response and mutual exacerbation of infection [27,28]. Significant differences were observed between patients in the elimination rate of antimonials and area under the curve analysis suggested that differences in the length of exposure to antimony could influence clinical response in CL treatment [29].

The GIS constructed interactive map where epidemiological information was linked to locations allows easy access to information and helps in graphical display. The information can easily be updated according to the current situation of the disease. The map can easily be shared through the Internet, which allows faster transfer of the latest assessment of the disease in Sudan and/or other neighboring countries.

In conclusion: the use of clinical interview combined with simple immunological tests could give valuable information about the pattern of *L. donovani* infection and predict future incidences of VL in a short time and with minimum cost in other places. The information obtained was not different from that obtained through longitudinal surveys, and can be used for epidemiological projections in other places and at future times. Leishmanin non-reactive individuals are a useful piece of data to plan for future vaccine efficacy studies. It is also clear that *in vivo* drug unresponsiveness does not correlate well with the *in vitro* sensitivity. The interactive map that was produced in the GIS can be used as a starting point for a more comprehensive map for Sudan and other surrounding countries that are endemic for visceral leishmaniasis.

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