Identifying asymptomatic infections of visceral leishmaniasis in non-endemic regions with the associated risk factors in Gedaref state, Sudan

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Objectives
Visceral Leishmaniasis (VL) can be symptomatic and asymptomatic infection. In this study we aimed at investigating the prevalence of asymptomatic infections and to study risk factors of VL in non-endemic regions in Gedaref state, Sudan. A descriptive cross-sectional study conducted during 2014. Blood samples were collected to serological and molecular analysis. Sticky traps, knockdown spray and CDC miniature light traps were used for the collection of sandflies.

Results
Ninety-Five participants were included; 52 from Abukishma, 15 Algadamblia Tirfa, 25 Abualnaja and 3 were from Algadamblia Aljabal. Most of the study participants were belonging to the Belala tribe (74.3%). The most frequent reported age was above 40-years old (9.5%). Females were (61.1%) and males were (38.9%). B. aeygptica was the most planted tree in/around the houses (46.3%). 73 (76.8%) of the participants bred more than two types of animals in the house. DAT test revealed 5 positive participants (5.2%). 4/5 DAT positive were past VL infection. PCR detected 35 (36.8%) positive patients. A total of 31 (32.6%) were considered asymptomatic infections based on PCR detection method. Households planted Balanites/Acacia trees or breed domestic animals were found in high percentages with VL PCR positive participants (60.1%, 91.4%).

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Key words: Visceral Leishmaniasis, Asymptomatic infection, Risk factors, non-endemic areas, Gedaref state, Sudan

Introduction
Visceral leishmaniasis (VL), also called kala-azar, is a disease transmitted through the inoculation of protozoan parasites of the Leishmania donovani complex by phlebotomine sandfly vectors. It is endemic throughout southern Europe, Latin America, Asia, and Africa [1]. For this parasite, the role of animals as reservoirs differs considerably from one area to another [2, 3]. VL infections are clinically defined as symptomatic infection with a characteristic form of illness, consisting of prolonged persistent fever (i.e. longer than 2 weeks) and wasting with progressive spleen enlargement [4, 5], although they can also present as sub-clinical infections with immune-mediated febrile illness or a delayed nonspecific infection that consequently advances to apparent illness [6]. Although VL has long been endemic to Sudan, it continues to be a significant public health problem [7-9]. Sudan is considered an essential endemic country with high incidence, morbidity and mortality rates, Sudanese endemic areas extend from Sudan-Ethiopia borders in the central east to the White Nile state in the west [1]. Many sandfly species have been found in Sudan, including Phlebotomus orientalis, Phlebotomus papatasi, and five other species. Despite this variety, P. orientalis is the only confirmed vector responsible for transmitting VL [10]. In eastern Sudan, there are consistently high rates of infection, with up to 16% of deaths in the state attributed to VL [7]. However, in endemic regions, 20% of the infected population will demonstrate the classical form of the disease [11], while most remain clinically healthy and may progress to clinical VL or may resolve the infection in less than two years without intervention [12]. The vast majority of asymptomatic VL patients are considered to be a potential reservoir for transmission [13]. Investigation and analysis of VL risk
Factors have been conducted in various VL foci, including the Indian subcontinent [11], Ethiopia [14, 15], Iran [13] and Brazil [16, 17]. This study aimed at investigating the prevalence of asymptomatic VL infections in non-endemic regions of Gedaref state, Sudan, and to identify possible risk factors linked with VL.

**Materials And Methods**

**Study design and study area characteristics**

A descriptive cross-sectional hospital-based study conducted in non-endemic regions for VL in Gedaref state Sudan during October 2014. In this study Four regions have been chosen due to reports of the presence of 4 new cases (Unpublished data) in each of the selected districts from the Leishmaniasis Control Program, Ministry of Health, Gedaref state, Sudan. The four villages across Gedaref state which were chosen for the study are Abukishma (14.05328°N, 035.12329°E), Algadamblia Tirfa (14.01715°N, 035.00059°E) Algadamblia Aljabal (14.02141°N, 035.00466°E) and Abualnaja (13.97850°N, 035.30479°E) (Supplementary File 1). These villages are located in the eastern part of Sudan and considered as non-endemic areas of VL in Gedaref state.

**Participants and qualitative data collection**

Based on the reports of Leishmaniasis control program, Gedaref state, on the four reported cases in the areas under study (unpublished reports) during October 2014, a total of 95 participants were recruited; 52 from Abukishma, 15 Algadamblia Tirfa, 25 Abualnaja and 3 from Algadamblia Aljabal. Participant suffering from chronic diseases and those who refused to provide informed consent were excluded from the study. All participants’ demographical data, household information and insect control strategy used were collected using a semi-structured questionnaire.

**Sample collection and processing**

An experienced phlebotomist collected three milliliters of venous blood from each participant using disposable syringes from the antecubital vein, after cleaning the skin with the disinfectant. Specimens were slowly poured into K3 EDTA containers to prevent coagulation. Then, each specimen was gently and adequately mixed by inverting the container to avoid hemolysis, clotting, or platelet aggregation [18]. Blood samples were preserved in 4° C refrigerator for 4-5 hours after collection when they were
centrifuged at 3000 rpm for 5 minutes to separate the serum and buffy coat. Red blood cells were discarded, whereas the buffy coat and serum were collected in separate tubes and stored at -20°C until molecular and serological analysis.

Serological analysis
The serological analysis was done using the direct agglutination test (DAT). DAT was performed in the Biomedical Research Laboratories of Ahfad University for Women, Sudan. The antigen was prepared from the local endemic strain (strain MHOM/68/1-S; an isolate from a VL case in Sudan) following the protocol described by El Harith et al. [19]. The procedure followed for DAT antigen preservation was described by El Harith et al. [20]. The procedures of the serological analysis performed is described in supplementary file 2.

Molecular analysis
Genomic DNA from the buffy coats was extracted using the guanidine hydrochloride extraction method described by Ciulla et al. (1988) [21]. Detection of VL infection was achieved using primers 18S-LEISH: 5´GCTGTGCAGGTTTGTTCCTG´3 and 18S-LEISH: 5´GGACGCCTAAACCCCTCAA´3, which amplifies a band of 357 bp within the 18S rRNA gene of L. donovani. PCR was performed in one step (single tube) with a 25µl reaction volume using iNtRON's Maxime PCR PreMix Kit (iNtRON i-Taq, South Korea) according to the manufacturer's instructions. PCR was performed on a thermocycler (SensoQuest brand, Germany) with thermal conditions described in supplementary file 3.

Entomological survey
Sandfly collection was carried out simultaneously inside and outside the houses of the participants from 18.00 - 06.00 hours on four subsequent nights during October 2014. Sticky oil traps and CDC miniature light traps were situated at 30 cm above ground level inside and outside the houses. Also, the knockdown spray was used for sandfly collection in the period of 06.00 – 09.00 hours indoors (inside the rooms). Collected sandflies were preserved in RNAlater solution (iNtRON biotechnology, South Korea). The identification process was carried out using the identification keys published previously by Lewis [22].

Statistical analysis
Statistical analysis was performed using the statistical package for social sciences (SPSS. Version 16). Demographical data including age, gender, tribe, occupation, residency, forest visits, household information, the use of bed nets, insect bites frequency and the serological and molecular results underwent t-test and frequency distribution analysis. Variables considered as risk factors, including ethnic groups of the participants, vegetation in/around the house, breeding animals at the house, insect bites and insect control methods used, were analyzed using Chi-square test.

Results

Participants' demographic data

The 95 participants recruited during October 2014 from the study areas mostly belonged to the Belala tribe (74.3%). The majority of participants 51 (54.3%) were farmers. Most of the participants had been residents for more than five years (82.1%). The most frequent reported age was above 40-years old (9.5%). 61.1% of participants were female and 38.9% were male. Almost one-third of the participants visited forested areas (32.6%) and more than half had thatched houses with windows (55.8%). B. aeygyptica was the most planted tree in and around the houses (46.3%). Approximately three-quarters of the participants bred animals inside the house (76.8%). Bed nets were the most frequently used insect control method (66.3%). Complaints of nocturnal and crepuscular insect bites were reported by 70.5%.

Serological tests

There were five positive VL cases (out of 95) (5.2%) determined by DAT test using serum samples. Four of these cases had a titer of > 1:6400 and one had a titer of 1:3200. Most of the respondents had not had VL infection previously (95.8%), whereas Four of those with a positive DAT titer had been previously infected with VL.

Molecular analysis

L. donovani DNA was found in 35 out of 95 samples (36.8%) with a male/female ratio of 40%:60%. Five of the samples were found to be positive using the DAT test were also positive by PCR. The distribution of VL infected (positive) and non-infected (negative) participants detected by PCR and DAT techniques according to the location of sample collection is illustrated in Table 1.
A Representative positive samples of PCR amplifications of 18S rRNA gene of *L. donovani* is shown in supplementary file 4.

Sandfly findings

A total of 220 sandflies were collected. All the collected sandflies were identified as *P. papatasi*, and none of the flies were identified as *P. orientalis*.

Risk factors analysis

Out of the four factors included in the household information, two factors were found to be shared for VL PCR-positive participants; 60.1% of VL positive households planted *Balanites/Acacia* trees and 91.4% bred domestic animals. No statistically significant association was observed for VL and the following variables; the ethnic group of the participants, vegetation in/around the house, breeding animals at the house, insect bites, having a bed net and sleeping on bed (not on the ground) and the control methods used (Table 2). Furthermore, t-test was not statistically significant for PCR positive VL participants and the age of participants, participants' family number and residence status of participants in any of the villages (Table 3).

Discussion

In this study, the results of the socioeconomic survey showed that most cases of VL were distributed among people living in thatched houses and having *Acacia/Balanities* trees in/around the house and domesticated animals (mainly bovine), which is in agreement with other studies [23-27]. Previous studies also identified the low financial status and mud/thatched houses or splintered houses' walls as risk factors for VL [28-33]. However, in other studies, no association was found between individual socioeconomic factors and VL risk [17, 34-36]. Some of the factors which are associated with the disease may change over time, resulting in conflicting reports of their effect on *L. donovani* infection, such as the use of bed nets or the role of domesticated animals or implementation of insecticides. In some studies, herding of animals in or around the house was found to be a risk factor, while it was considered to have a protective role in other studies, with the domesticated animals acting as a barrier from sandfly bites as sandflies shifted to feed on the animals [29, 37]. Insecticides may not eradicate sandflies, and they can persist inside the houses.
Additionally, the application of repellents might interrupt the attempts of sandflies to feed on humans and eventually alter their host preference behavior towards an exposed host, such as an animal, in a place which has not been treated with an insecticide. Although there have been no such reports on the behavior of phlebotomine sandflies, the existence of vector in a specified area can be misleading. Their presence alone does not prove VL transmission, which is affected by strain, behavior, seasonal activity, and density of the vector [29, 37].

In this study, the detected PCR-positive individuals for whom clinical VL did not develop are considered to be asymptomatic, and they have no history of previous VL infections or treatment, with no clinical signs or symptoms. However, these asymptomatic cases might act as reservoirs for the *Leishmania* parasite [38] or sustain VL transmission in non-endemic regions, as proposed by mathematical modeling [39]. Although the actual estimate of asymptomatic cases and their prospective role in the transmission of VL in endemic areas is difficult to assess [30], this may be an escalating challenge for disease control [40].

VL is challenging to diagnose despite the accessibility of numerous diagnostic techniques. A single diagnostic method is not satisfactory to detect all positive VL infections and the results obtained through multiple diagnostic methods vary from one region to another. The variable diagnostic performance of these methods in VL endemic regions is reflective of the origin of the test-antigen [40-42].

The proportion of *L. donovani* infected patients who may act as a reservoir for *Leishmania* parasites in Sudan is insufficiently documented as this requires extensive prospective epidemiological studies. However, no evidence exists which demonstrates that individuals with asymptomatic *L. donovani* infection in Gedaref state are not reservoirs of infection. Thus, the assessment of those with asymptomatic infections by screening and up to one-year follow-ups is beneficial in early VL detection.

**Conclusion**

In this study, DAT and PCR diagnostic methods were used as tools for screening of VL in regions neighboring endemic areas, leading to early treatment of VL-infected patients, especially for patients
suffering from VL-like symptoms, which could eventually help to reduce disease morbidity and mortality.

Limitations:

Although a small number of participants have been recruited, it also provided insights on the status of asymptomatic VL infections. Therefore, a more extensive study scale is needed to give a clear situation of VL infections in Sudan.

DAT detected a small number of VL infections since it tends to be negative in asymptomatic cases. Meanwhile, further follow-up for patients who are PCR positive is required.

Declarations

Ethics approval and consent to participate

All participants included in the study received comprehensive information concerning the study. Participants were included after signing an informed consent form, agreeing verbally in the case of illiterate participants or in case of children, their parents or legal guardians did so. The study was approved by the community leaders, Gedaref state Ministry of Health and the Department of Parasitology and Medical Entomology, Faculty of Medical Laboratory Sciences, University of Khartoum, Sudan.

Consent to publish

Not Applicable.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

No competing interests to disclose.

Funding

Not Applicable.

Authors' contributions

NSM and AHE provided conceptual framework for the study, guidance for interpretation of the data,
performed data analysis. NSM, HAO, AOM and AHE performed the field and laboratory work. MSM, EES, AMS and AA performed the statistical analysis. NSM, EES, HAO and AHE participated in the manuscript preparation, revision and coordination. All authors read and approved the final manuscript.

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