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Original paper

Low serum zinc concentrations in Sudanese patients with visceral leishmaniosis does not impair the anti-*Leishmania* antibody response

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ABSTRACT. Visceral leishmaniosis (VL) is a poverty-related disease, affecting poor peoples in the developing countries. For unknown reasons, the rK39 rapid test has poor diagnostic sensitivity in some endemic areas such as East Africa. Here, the hypothesis was tested whether micronutrient deficiency is associated with low *Leishmania*-specific antibody responses and consequently decreased diagnostic sensitivity. Serum zinc concentrations in 107 human sera of VL and controls that were HIV-negative were measured using atomic absorption spectroscopy. Anti-*Leishmania donovani* antibodies were detected quantitatively by rKLO8 ELISA. The influence of low serum zinc concentrations on the amount of anti-*Leishmania* antibody and outcome of the rK39 rapid test was tested. Serum zinc concentrations were significantly ($p < 0.0001$) reduced in VL sera (mean 0.41 ± 0.15) as compared to healthy control groups (mean ≥ 0.75 ± 0.13). Interestingly, the majority (92.2%) of the VL patients had low serum zinc concentrations (< 0.6 mg/l) whereas all healthy controls showed normal levels. Unexpectedly, VL sera with normal (0.94–0.6 mg/l) or low (< 0.6 mg/l) zinc concentrations demonstrated no significant difference in amounts of *Leishmania* antibodies. In addition, VL sera with positive or negative rK39 rapid test results demonstrated similar serum zinc concentrations; mean values of 0.39 ± 0.14 mg/l and 0.38 ± 0.1 mg/l for VL sera of positive or negative rK39 results, respectively. Low serum zinc concentration seems not to play an important role in lowering anti-*L. donovani* antibody titers observed among Sudanese VL patients and doesn't affect rK39 rapid test results.

Keywords: visceral leishmaniosis, zinc deficiency, HIV

Introduction

Visceral leishmaniosis (VL), caused by the protozoan *Leishmania (L.) donovani*, is a life-threatening disease worldwide, with most infections (90%) occurring in remote, unstable and poor communities in East African and South-East Asian regions. East Africa has the second highest global disease burden. The disease is considered one of the most dangerous neglected tropical diseases and one of the deadliest parasitic infections after malaria [1]. *L. donovani* infection ranges from severe to mild and can be asymptomatic. These asymptomatic cases appear to have a role in the transmission and pose a significant challenge in the diagnosis and control of the disease [2–4].

Symptoms of VL are generally non-specific and must be confirmed in the laboratory. According to national guidelines of the Sudanese ministry of health, laboratory confirmation is done by microscopic detection of parasites in organ aspirates or by antibody based sero-diagnostic. The latter includes direct agglutination test (DAT) and rK39 rapid tests, which are useful in rural setting. Both tests are simple blood tests used for the field diagnosis in endemic areas of Sudan and other endemic areas of East Africa [5–7]. Furthermore, the simple format of the rK39 rapid test makes it highly suitable for the field use. However, the test showed un-explained varying sensitivity in different endemic countries, having significantly lower sensitivity in East Africa than in other endemic countries. This phenomenon was explained to be associated with the low amounts of anti-*Leishmania* antibodies in Sudanese VL patients [8–9].

Malnutrition is a common health problem,

affecting rural communities in developing countries. It can cause a state of immunodeficiency, rendering individuals susceptible for many diseases [10–12]. Micronutrients, such as zinc, are essential for normal immune functions and its deficiency can lead to susceptibility to infections and cancers [13–14]. In murine experimental models, the ability of the immune system to generate normal antibody and cellular immune responses is significantly influenced by zinc deficiency [15–16]. Zinc deficiency may lead to severe immune dysfunction characterized by impaired humoral and cell-mediated immunity and individuals with this condition can suffer from opportunistic infections and often show a shorter life span [17]. Zinc has been used as nutritional supplement to enhance immunity in people with several infections including leishmaniosis. Zinc supplementation can effectively improve zinc status and reverse the impairment of the immune system, reducing infectious disease morbidity and mortality [18].

Data on zinc deficiency among VL patients in Sudan are limited and reliable information on nutritional status of individuals at the VL endemic areas of East Africa are scarce [19]. Since nutrient restriction is common in African countries, it was assumed that the varying sensitivity of the rK39 rapid diagnostic test in East Africa is a consequence of zinc deficiency. Lack of these nutrients may interrupt immune responses, causing reduction of anti- *L. donovani* antibody responses and thus may influence rK39 rapid test result. In this study, we determined serum zinc concentration with regard to the amount of specific antibody responses and its consequence for reliable sero-diagnosis of VL.

Materials and Methods

Ethics statement

The study was performed at Laboratory for Biomedical Research of Ahfad University for Women in accordance with the ethical standard for research involving human subjects. The Research Ethics Committee of the Ministry of Health in Sudan approved the study (06-2005). The study involved analysis of collected sera that have been used previously [20,21]. These samples were obtained after obtaining formal informed consent.

Sample collection and study population

In total, 107 human serum samples were obtained from the serum bank at Laboratory for Biomedical Research, Ahfad University (Omdurman-Sudan). At the time of diagnosis, blood samples were collected in vacutainer tubes by venipuncture. Sera were separated and stored at –20°C in containers free of zinc-containing heparin. The samples included 77 sera of confirmed VL from the hyperendemic area in Eastern Sudan, 15 sera of symptomatic VL suspected cases (VLS) with unconfirmed diagnosis and 15 control sera from healthy individuals resident at Doka village – Eastern Sudan (an endemic area for VL, n=5) or Khartoum (a non-endemic area, n=5). The control sera of healthy endemic and non-endemic individuals were collected at the same time as for the VL sera. Control specimens included also sera of healthy German individuals (n=5). All patients' sera and diseased controls were collected before administration of treatment and none of cases were receiving any zinc or other micronutrient supplements before sample collection.

Diagnostic tests and algorithms

Definitive diagnosis of VL was done by microscopic detection of amastigote stages of the parasite in inguinal lymph node aspirates. Patients with positive smear were considered as confirmed VL. Symptomatic patients with negative smears were defined as VL suspects. All sera of confirmed and unconfirmed VL cases were screened for HIV infection according to guidelines of Sudan National AIDS and STI control program of the Federal Ministry of Health. HIV testing was performed with a serial HIV testing algorithm using HIV 1 and 2 simple/rapid diagnostic tests (Biorex Diagnostics, UK) and fourth generation HIV ELISA (1+2) Ag/Ab ELISA kit (Fortress Diagnostic, UK). All test procedure was according to the manufacturer's instructions. A complete and detailed description of the testing algorithm is recently published by us [22]. In this study, we included only VL sera that were HIV negative.

Determination of serum zinc levels

Measurement of zinc in serum specimens was carried out using Perkin Elmer™ 400 atomic absorption spectroscopy. Sera of patients and controls were diluted to a ratio of 1:5 in deionized water. Analysis was performed against standard solution of glycerol using a blank solution consisting of 5% (v/v) glycerol as per instructions of the manufacturer. VL positive and negative sera were blind tested and testing was repeated at least

Table 1. Serum zinc concentration in patient and control sera from endemic and non-endemic areas

Study group	Mean ± SD	Range	Serum zinc < 0.6 mg/l
			Number/total (%)
VL (n=77)	0.41 ± 0.15	0.24–1.12	71/77 (92.2%)
VLS (n=15)	0.44 ± 0.12	0.25–0.72	13/15 (86.7%)
Endemic controls (n=5)	0.76 ± 0.09	0.68–0.87 0/5	(0%)
Non-endemic controls (n=5)	0.75 ± 0.13	0.67–0.97	0/5 (0%)
German controls (n=5)	0.97 ± 0.14	0.75–1.14	0/5 (0%)

Explanations: VL, visceral leishmaniosis; VLS, visceral leishmaniosis suspects; SD, standard deviation; n, number of used sera

twice. Serum samples with any sign of hemolysis were not acceptable for zinc analysis. Normal range of serum zinc was regarded to be 0.60–1.20 mg/l [23,24].

Anti-Leishmania donovani antibody detection by rKLO8 ELISA

Indirect rKLO8 ELISA was used as previously described [25]. rKLO8 is a recombinant protein of 249 amino acid sequence cloned from *L. donovani* and expressed in an *E. coli* system. The protein was expressed as 6 × His-tagged fusion protein using 1 mM IPTG (Roth, Germany). Purification of the recombinant protein was carried out with Ni^{2+}–NTA matrix (Qiagen GmbH, Germany). Protein was stored at constant temperature of –80°C until used. ELISA was carried out in high binding 96-well microtiter plates (Nunc., Roskilde, Denmark). Plates were coated with 5ng/100µl of rKLO8 in 0.1 M $NaHCO_3$ (pH9.6) and incubated overnight at 4°C. The coated plates were blocked with bovine serum albumin (3% BSA in PBS buffer with 0.05% tween 20) for 60 min at R/T. After being washed with washing buffer (PBS containing 0.05% tween 20), sera diluted 1:800 were transferred to each well and incubated for 45 min at R/T. After washing step, plates were incubated with peroxidase-conjugated donkey anti-human IgG (Jackson ImmunoResearch Laboratories, Inc., USA) diluted 10,000 in 3% BSA buffer. After further washing step, the plates were incubated with hydrogen peroxidase and tetamethylbenzidine substrate (R&D System Inc., USA) for 10 min at R/T and the optical density was measured at 450 nm.

rK39 Rapid Diagnostic Test

Commercial available IT LEISH rapid diagnostic tests manufactured by Bio-Rad Laboratories Inc. (France) were performed as recommended by the manufacturer. Ten µl serum specimens were added to the test device with a drop of the buffer. The appearance of red test and control lines indicated a positive test whereas absence of test lines was regarded as negative. Testing was done without prior knowledge of serum zinc results.

Data analysis

Statistical analyses were performed using GraphPad Prism 8 (GraphPad Prism Inc.). Results were expressed as mean serum zinc concentrations ± SD of the patient and control groups. Unpaired Student's t-test was used to compare means of zinc concentrations at 95% confidence intervals. Serum zinc value of 0.6 mg/l was considered a normal lower limit [23,24].

Results

Low serum zinc concentration is common among VL patients in Sudan

A total of 77 sera of HIV-negative VL and 15 sera of clinical VL suspects (VLS) were tested for low serum zinc concentrations (< 0.6 mg/l). Results were compared to control sera from endemic and non-endemic areas. As shown in Table 1, 92.2% VL and 86.7% VLS sera had serum zinc concentrations below the low limit of 0.6 mg/l. The mean zinc concentrations of VL sera was 0.41 ± 0.15 mg/l, whereas controls from the same endemic revealed normal levels of zinc (0.76 ± 0.09 mg/l) demonstrating that VL sera show significantly decreased amounts of serum zinc (Fig. 1). Further, sera of VLS revealed low serum zinc levels with a mean value of 0.44 ± 0.12 mg/l as compared to normal controls. No differences were observed between Sudanese healthy controls from endemic and non-endemic areas (mean values of 0.76 ± 0.09

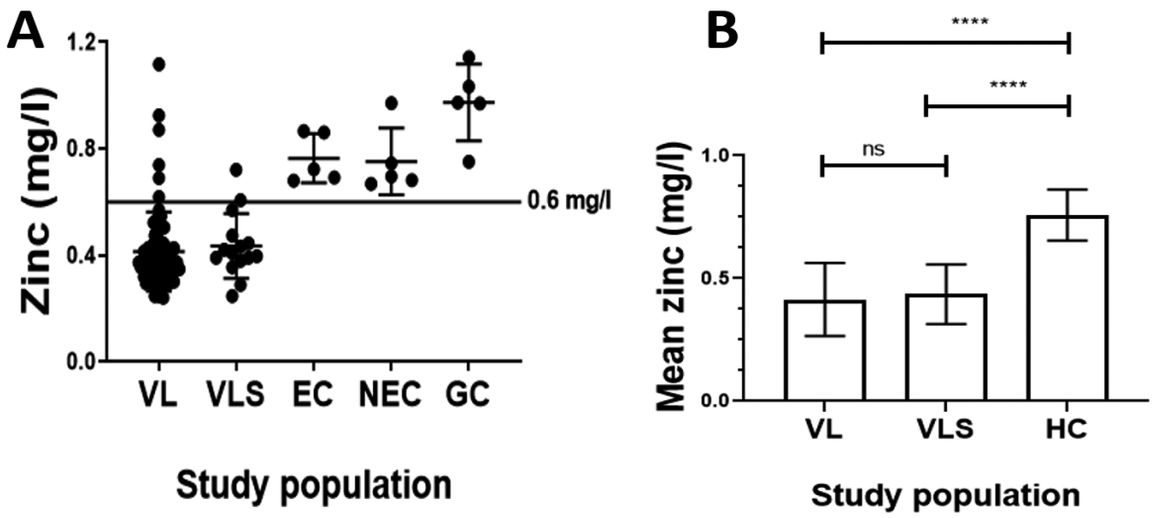

Figure 1. Serum zinc concentration in sera of VL and controls. Serum concentrations of zinc were determined by atomic absorption spectroscopy using a group of patients and controls, including visceral leishmaniosis (VL, n=77), visceral leishmaniosis suspects (VLS, n=15), healthy endemic controls (EC, n=5), healthy non-endemic controls (NEC, n=5) and healthy German controls (GC, n=5). (A) Serum zinc concentrations of individual patients and control sera. Horizontal line indicates a value of 0.6 mg/l zinc, the lower limit of normal. (B) Mean serum zinc concentrations of the patients and controls. Results are expressed as mean ± SD. Unpaired Student's *t* test was used to compare mean of zinc concentration. ****, $P < 0.0001$; *ns*, not significant.

and 0.75 ± 0.13 for endemic and non-endemic controls, respectively) and healthy controls from Germany (mean values of 0.97 ± 0.14). It's important to note that none of the control groups showed serum zinc concentrations lower than the normal (0.6 mg/l).

Low serum zinc does not affect anti-Leishmania donovani antibody responses

Anti-*L. donovani* antibody responses were determined by rKLO8 ELISA using 33 randomly chosen VL sera with varying serum zinc concentrations. A total of 28 VL sera (84.8%) showed presence of anti-*Leishmania* antibodies (at cut off 0.12). To determine whether low serum zinc concentration affect anti-*Leishmania* antibody level, we compared anti-*Leishmania* antibody responses in VL sera of normal or low zinc concentrations. Unexpectedly, low serum zinc concentration did not affect the anti-*L. donovani* antibody response, demonstrating similar humoral responses in sera from patients with normal (0.94–0.6 mg/l) or low (<0.6 mg/l) zinc concentrations ($P > 0.17$, Fig. 2).

Low serum zinc doesn't affect performance of Leishmania serology

Serum zinc concentrations were determined in VL sera with positive (n=28) or negative (n=4) rK39 rapid test results. As shown in Figure 3, serum zinc concentrations were found to be similar in the two patient groups (mean values of 0.39 ± 0.14 mg/l and 0.38 ± 0.1 mg/l, respectively for VL sera of positive or negative rK39 results). However, of the 28 sera of rK39 rapid test positive results, serum zinc concentrations were > 0.6 mg/l in two sera. Such high serum zinc concentrations were not observed with the sera of negative rK39 test.

Discussion

Micronutrient deficiency can compromise immune responses, causing a decline in antibody formation and thus may influence serological test reactivity [8,9]. In this study the role of zinc with respect to antibody formation during infection with VL was studied. In contrast to healthy individuals, low serum zinc was often observed in patients with VL in endemic areas. This finding suggests that low serum zinc is a potential risk for developing VL. Significantly reduced concentrations of zinc were documented in VLS sera compared to healthy controls; 86.7% VLS sera showed serum zinc concentrations below the limit. Thus, low serum zinc concentrations < 0.6 mg/l may be predictable factor for VL in endemic areas.

The prevalence of low serum zinc in our study is similar as in other VL endemic areas. Mishra et al. [26] reported significantly lower zinc concentrations in sera of VL in India, with a prevalence rate of 75%.

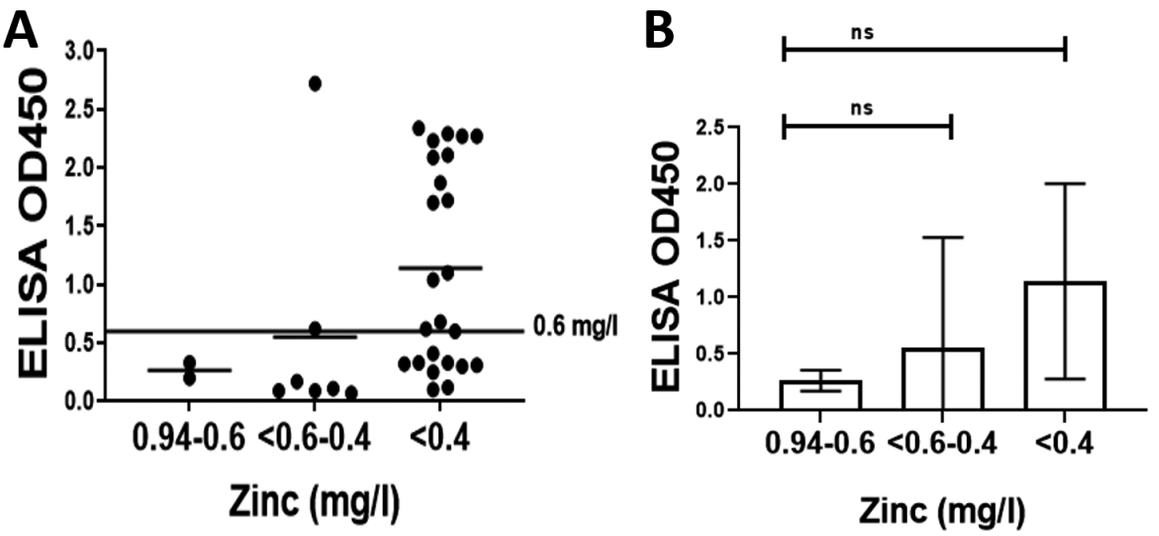

Figure 2. Comparison of anti-*Leishmania* antibody levels in VL patients with varying serum zinc concentrations. The rKLO8 protein of *L. donovani* was used to measure anti-*L. donovani* antibodies in 33 sera of VL by ELISA. Sera were divided into 3 groups based on zinc concentrations; 0.94–0.6 mg/l, normal; < 0.6–0.4 mg/l, low; < 0.4 mg/l, very low. (A) ODs of individual sera with varied serum zinc concentrations. Horizontal line indicates a zinc value of 0.6 mg/l. (B) Mean ODs ± SD of VL sera with varied zinc concentrations. Unpaired Student's *t* test was used to compare mean of anti-*Leishmania* antibodies. *ns*, not significant.

Furthermore, decreased zinc concentrations in plasma have been reported in all forms of leishmaniosis, including VL, and among tuberculosis patients as compared to health controls [27,28].

Notably, in average, serum zinc concentrations among healthy Sudanese populations were only slightly but not significantly reduced when compared to healthy German individuals. It remains unclear whether the slightly lower zinc concentration is of any clinical importance. It has to be mentioned that endemic healthy controls from Bihar (a hyperendemic area in India) also revealed significantly lower serum zinc concentrations, comparable to that of VL patients [26]. Similarly,

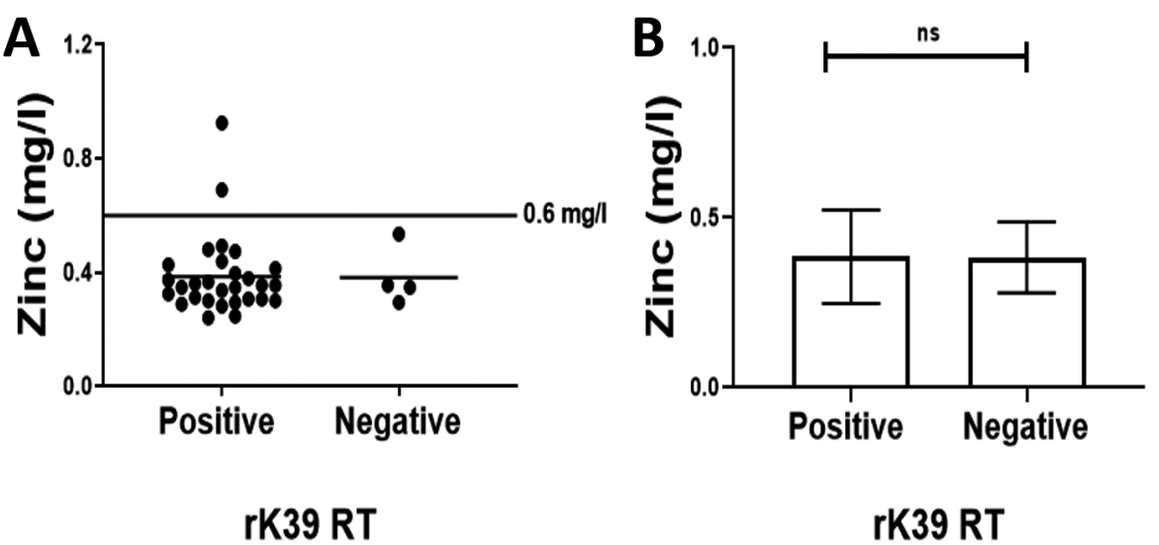

Figure 3. Effect of serum zinc concentration in VL patients on serological test results. Zinc concentrations of 33 VL sera were compared with rK39 rapid test results. Sera were divided into 2 groups based on the rapid test results. (A) Zinc concentrations of individual sera. Horizontal line indicates a value of 0.6 mg/l zinc. (B) Mean zinc concentrations ± SD (unpaired Student's *t* test) of sera with positive or negative rK39 test results.

more than 50% of the healthy population in VL endemic area in Bangladesh had zinc deficiency [29].

Animal models have documented the effect of malnutrition on the immune system. Anstead et at. [30] reported that malnutrition can impair the function of lymph nodes in *Leishmania* infection, characterized by decreased interleukin-10 and nitric oxide. A study from Brazil demonstrated significantly lower IFN-γ production by malnourished BALB/c mice following *L. chagasi* infection [31]. Malnutrition has also been shown to increase the risk of visceralization by *L. donovani* and reduce the number of lymph node phagocytes, demonstrating that the lymph node function as effective barrier for parasite dissemination, was impaired [32].

Unexpectedly, we showed that neither rKLO8 ELISA nor rK39 rapid tests are influenced by the low serum zinc concentrations. There was no difference in the performance of rK39 rapid test for detection of VL. Sera of VL patients with normal or low zinc concentrations exhibited similar amounts of *Leishmania* antibodies as measured by rKLO8 ELISA. The sera of VL with positive or negative rK39 rapid test results demonstrated similar serum zinc concentrations. However, we observed increased (> 0.6 mg/l) serum zinc concentrations in two sera that had positive rK39 rapid test results. Such high zinc concentrations were not observed in sera of negative rK39 test.

Previous studies with TB showed that micronutrient deficiency may influence the laboratory diagnosis. Mukherjee et al. [33] have demonstrated that zinc deficiency reduces the diagnostic sensitivity of QuantiFERON-test for TB diagnosis. This observation is contradictory to our finding and most likely reflects differences of the two test systems. The QuantiFERON-TB is based on detection of IFN-γ while the rK39 is an antibody-based test. The possible effect of low serum zinc concentrations on accuracy of diagnostic tests raise an interesting concern, taking into consideration the high rates of infectious diseases and micronutrient deficiencies in developing countries. Both infection and malnutrition can impair cellular immunity, which is important to control infectious diseases, creating a state of immunodeficiency [12].

The adverse effect of malnutrition and micronutrient deficiency on diagnostic test outcome has been studied in animal models. In Brazil, Cuevas et al. [34] have reported that high serum zinc concentration after zinc supplement was often associated with a positive tuberculin test. Conversely, zinc deficiency can lead to false tuberculin results and zinc cream supplement can boost adequate immune responses to *Mycobacterium,* sensitive enough to enhance TB detection [35]. However, another study showed no association between tuberculin positivity and the zinc status in hemodialysis patients [36].

In many endemic areas, VL mainly affects children and young adolescents [37,38]. While many people are exposed to *Leishmania*, only few are susceptible and develop clinical disease. The factors that increase susceptibility to VL remain unknown. Since nutrient restriction is a frequent problem in rural areas where VL is endemic, we assume that malnutrition and nutrient restriction increases susceptibility of individuals in endemic areas to VL and contributes to the development of the disease in children. It is clear that nutritional status contributes in determining immunity or susceptibility to *Leishmania*. Animal studies have confirmed the high susceptibility for VL in malnourished host. Serafim et al. have demonstrated that malnutrition diminishes immune responses to *L. chagasi* infection and favors susceptibility [31].

In conclusions, the prevalence of zinc deficiency in Sudan is very high among VL patients but not in the endemic, healthy populations. This finding indicates the possible association of VL and zinc deficiency, with low serum zinc being a potential consequence of the disease. Surprisingly, low serum zinc does not seem to affect the amount of anti-*L. donovani* antibodies and rK39 rapid test results. Thus, the cause of the inefficient sero-diagnostic of VL in East Africa has to be further investigated.

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