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Short Communication

Screening and Co-Morbid Association of Visceral Leishmaniasis and Diabetes in Osun State, Nigeria

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ABSTRACT

Visceral leishmaniasis (VL) is the most severe form of leishmaniasis and causes the second-largest parasite-related mortality in the world after malaria. Its treatment with antimonial drugs has been known to also precipitate diabetes in the patients. However, the occurrence of the disease is hitherto quite alien in Southwestern Nigeria. This sero-epidemiology surveillance was carried out to ascertain VL incidence in Osun State, Nigeria, and to investigate the association of the disease co-morbidity with diabetes viz-a-viz implication on biochemical parameters of kidney and liver functions. A total of 272 volunteers across the State were enrolled on this study. All samples were screened for leishmaniasis using capillary, buffy coat and ELISA methods, concurrently. Liver and kidney function parameters were assayed spectrophotometrically, and comparisons were made between the methods, and outcome of parameters among seronegative and seropositive participants. The capillary technique did not detect (0%), the thin buffy coat detected 2 (2.1%) whereas ELISA detected 44 (16.2%) leishmaniasis cases among the participants. There were statistically significant ($p < 0.05$) decreased urea, increased creatinine, elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in Leishmania-infected participants compared to those uninfected. It is interesting to note that urea, creatinine, ALT and AST levels of participants having a co-morbidity of VL-diabetes and VL-diabetic foot ulcer did not change significantly compared to the Leishmania-infected participants without diabetes or the foot ulcer. This study indicated that Leishmania infection may be a singular factor that perturbs the kidney and liver enzymes. Therefore, VL does not show association with diabetes or diabetic foot ulcer co-morbidity in the population studied. There has not been any reported case of VL in Osun State prior to this study. It, therefore, suffices that the infectious disease has hitherto remained un- or mis-diagnosed.

Keywords: *Visceral leishmaniasis, Surveillance, co-Morbidity, Buffy coat, Kidney function, Liver function*

INTRODUCTION

Visceral leishmaniasis is also called black fever, kala-azar as well as Dumdum fever (James *et al.,* 2006). Leishmaniasis is a disease that is caused by protozoan parasites of the Leishmania genus. The genus Leishmania comprises two sub-genera, Leishmania and Viannia, and each subgenus includes many species Leishmaniasis caused by protozoan parasites which is endemic in tropical regions with an estimation of 1.3 million new cases annually (WHO, 2013).

Leishmaniasis is a vector-borne disease that is caused by several species of obligating intra-macrophage protozoan parasite. This vector-borne parasitic and neglected disease is endemic in large areas of the tropics, subtropics and the Mediterranean basin, and is characterized by diversity and complexity (Oryan, 2015). It is caused by about 20 Leishmania species and is transmitted to humans by more than 30 different species of phlebotomine sand flies (Magill, 1995). There are four distinct clinical forms of leishmaniasis including cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), mucocutaneous leishmaniasis (MCL) and post- kala-azar dermal leishmaniasis (PKDL) caused by different Leishmania species (Murray *et al.,* 2005). Within the classical distinction of Old World and New World leishmaniasis, different etiological agents are associated with visceral forms, with L. donovani only involved in the Old World, while L. infantum circulates worldwide. The means of transmission of the two Leishmania species mainly involved in visceral leishmaniasis differs depending on the parasite's preferred reservoir (del Giudice et al., 1998). Leishmaniasis by L. infantum is typically a zoonosis since the vector becomes infected after biting an animal reservoir. In contrast, L. donovani transmission is anthroponotic, as persistent and abundant parasitemia facilitates sand fly infection from humans (Saporito *et al.,* 2013).

Visceral leishmaniasis is known to be a major disease in tropical areas with approximately 500,000 new cases and 50,000 deaths estimated every year (Alvar et al., 2012). It is the main health problem with 67% of the global disease burden and approximately 150 million people are at risk (Hasker et al., 2013), and it is also one of the world's most neglected diseases, it largely affects the poorest people, mainly in developing countries (Boelaert *et al.,* 2009), while it is endemic in 60 countries (Desjeux, 2001).

Diabetes is a chronic, metabolic disease characterized by elevated blood glucose levels, usually with underlying inflammation. It damages the pancreas, blood vessels, liver and kidneys. Hallmarks of diabetes, liver and kidney function tests are also applicable in the diagnosis of visceral leishmaniasis where there has been observed functional derangement of liver and kidneys, showing as a severe life-threatening hepatitis and nephritis (Tesfanchal *et al.,* 2020; Endale *et al.,* 2021).

The treatment of leishmaniasis with the available harsh pentavalent antimonials (Sbv) drugs – including antimony, pentamidine, amphotericin, paromomycin and now the oral drug miltefosine – has been related to predisposition to insulin-dependent diabetes mellitus diabetes and renal toxicity as consequent side effects, as recorded around the world (Radwan *et al.,* 2007; Gouri *et al.,* 2021). It is also plausible that the impact of hyperglycaemia on the sweat pheromones of diabetics attract sandflies, making them suitable baits and object of attack for infectious bites (Zhang *et al.,* 2020).

Even though, no previous studies have been carried out in Osun State, unpublished report on surveillance of hematophagous flies in Osun State by the Vector Biology and Control Group (Osun State University) have indicated intensive biting of Phlebotomus sandflies in different parts of the state. World Health Organization listed VL as one of the neglected tropical diseases targeted for elimination in tropical regions. To achieve this, there is need to have in-depth knowledge on the distribution and transmission of this disease and its associated complications in different parts of the world. In Osun State, the occurrence of VL is hitherto known to be unreported and there is little or known efforts towards its control. It is against this background that the present study was designed to determine the prevalence of VL, its co-morbidity with Diabetes and the implications on hepatic and renal function.

MATERIALS AND METHODS

Sample size: A total of 272 consented participants were enrolled for the study.

Sample collection and storage

Five millimeters of blood was collected aseptically from the participant by venipuncture. Earlier, ethical approval was obtained from the Osun State University Teaching Hospital (formerly Ladoke Akintola University Teaching Hospital), Osogbo, Osun State, Nigeria. All participants in this study consented by filling and signing the Consent Form.

Capillary, Buffy coat and ELISA assessment of leishmaniasis

Glass micro-hematocrit capillary tubes were filled by capillary action and then sealed tightly. The tubes were centrifuged to separate the blood into plasma, buffy coat, and serum. Thin blood film was made after 5 minutes of centrifugation and was focused under ×10 to check for the presence of Leishmania spp in the supernatant plasma. The measurement was performed within 10 minutes to avoid merging of the layers.

Buffy coat was separated following the principle of concentration gradient separation by using Histopaque solution (Histopaque-1119; Sigma-Aldrich). Three millilitres of collected blood was layered onto 3 ml of the Histopaque-1119 solution in a sterile 15 ml centrifuge tube. The tube was capped and then centrifuged in a table-top centrifuge at 4,000 × g for 10 minutes at ambient temperature (Salam et al., 2012). After the centrifugation, the diffuse gray band of leukocytes (buffy coat) in between the Histopaque solution and plasma above the erythrocyte pellet was aseptically removed with a pipette and transferred to a sterile 1.5 ml microcentrifuge tube to be utilized for smear preparation. A thin blood film was made from the buffy coat and stained with Leishman stain diluted in buffer water for 10 minutes and it was fixed in undiluted Leishman stain for 2 minutes. The film was examined carefully for the presence of Leishmania species with the aid of a ×100 objective lens.

ELISA kit from Diagnostic Automation, Inc was used. The Leishmania ELISA kit was used for the screening of serum antibodies, primarily IgG, for visceral leishmaniasis.

Briefly, Wash Buffer was diluted 1 to 20 with distilled water. A 50 µl each of positive control, negative control and specimen was added into their respective wells. Then, 50 µl HRP-Conjugate antibody was added to each well except the Blank. The plate was incubated for 60 minutes at 37˚C and the wells were washed 5 times with Wash Buffer, allowing the micro wells to soak for 30 - 60 seconds. A volume of 50 µl of TMB solution A and 50 µl TMB solution B was added into each well including the Blank, and the plate was incubated at 37˚C for 10 minutes avoiding light. The enzymatic reaction between the TMB solutions and the HRP-Conjugate antibody produced blue colour in positive control and Leishmania antibody positive sample wells. Using a multichannel pipette, 50 µl stop solution was added into each well. Intensive yellow colour developed in positive control and Leishmania antibody positive sample wells. The absorbance was read at 450 nm, using 630 nm as reference wavelength. The cut-off value for the batch was determined and individual results were interpreted as negative (<0.9), positive (>1.1) and equivocal (0.9 - 1.1). All equivocal samples were repeated for proper classification, as appropriate (Rajasekariah et al., 2016).

Biochemical assessment tests

Liver (alanine aminotransferase and aspartate aminotransferase) and kidney (urea and creatinine) function tests were carried out spectrophotometrically using Randox commercial kit from Randox, Anthrim, UK.

Haematocrit evaluation

Capillary tubes were filled to a ¾ length of the blood specimen and sealed with wax as a sealer at the other end. The tubes were placed in the groove of micro-hematocrit centrifuge exactly opposite each other and centrifuged at 13000 rpm (Qater Al-nada et al., 2015). The hematocrit reader was used to find the value of hematocrit.

Statistical evaluation

The data values were presented as Mean ± SE and analyzed using ANOVA (SPSS version 22.0). A two-tailed p-value less than 0.05 was considered significant and the magnitude of association was expressed as odds ratio with a 95% confidence interval (CI).

RESULTS

Demographic data of the participants

A total of 272 consented participants were enrolled for the study. The demographic data of the study participants revealed that out of the 272 participants, 178 (65.4%) were males while most of the study participants were within the age group of 21-30 years (26.5%), followed by 31-40 years (21.3%) while age group ⩽10yrs (0.7%) constituted the least.

Assessment of the prevalence of visceral leishmaniasis using thin film, buffy coat and ELISA diagnostic methods

The prevalence of visceral leishmaniasis was determined by using capillary, buffy coat, and ELISA diagnostic techniques. Out of 272 participants screened for visceral leishmaniasis, the buffy coat technique detected the infection only in 2 (0.7%) participants, the capillary technique did not detect the infection and ELISA detected Leishmania infection in 44 (16.2%) participants. With buffy coat examination, sensitivity (2.3%), its specificity (99.6%), negative predictive value (84.1%) and positive predictive value (50%) (Table 1).

Assessment of biochemical parameters of the participants infected with leishmania parasite and participants not infected with leishmania parasite

The urea level is slightly lower in the VL patients compared to the non-infected participants while the creatinine level is slightly elevated in VL patients; whereas the urea and creatinine levels were significantly affected by VL ($p<0.05$) (Table 2).

Assessment of biochemical parameters of the Leishmania-infected participants with diabetic wound in comparison with VL participants without diabetic wound

The results of the biochemical analyses carried out on the blood samples of participants with co-morbidity of VL and wound showed lower urea and creatinine levels in the group having wound compared to the infected participants without a wound. However, as this was not statistically significant ($p<0.05$), wound and visceral leishmaniasis appear to be independently associated. The presence of wound also was not related to changes in the renal enzyme markers in the group with visceral leishmaniasis infection. The ALT and AST levels in participants with VL were higher than in those without a wound. This was also not statistically significant (Table 3). Therefore, co-morbidity of VL and wound also has no effect on the renal enzyme markers.

Assessment of biochemical parameters of the Leishmania-infected participants with diabetes

Comparison of the biochemical parameters of participants with co-morbidity of VL and diabetes showed the assessment of biochemical parameters of the participants having co-morbidity of VL and diabetes indicates that the urea, creatinine, ALT, and AST of participants with co-morbidity of VL-diabetes is elevated than that of participants with VL without diabetes although not statistically significant (Table 4). This could indicate that the high blood glucose level of the patients can contribute more to the elevation of the renal and hepatic enzyme markers. Many diabetic patients are prone to renal dysfunction (Zijlstra, 2016) as well as hepatic problems and also visceral leishmaniasis patients. The observations suggest that the co-morbidity of diabetes with Leishmania infection increases the risk of nephropathy faster. This can be due to the fact that the patients are immuno-compromised and this can be life-threatening (Saha et al., 2006).

Table 1 Diagnosis of Visceral leishmaniasis using three clinical methods

Variable	Negative (%)	Positive (%)
Thin film	270 (99.3)	2 (0.7)
Buffy Coat	272 (100.0)	-
ELISA	228 (83.8)	44 (16.2)

Table 2. Levels of biomarkers in apparently healthy individuals and those with Visceral Leishmaniasis infection

ELISA	Urea (mmol/L)	Creatinine (µmol/L)	ALT (U/L)	AST (U/L)
VL Negative	4.56 ± 0.71	80.05 ± 9.72	5.42 ± 2.23	7.74 ± 1.88
VL Positive	4.46 ± 0.322	84.43 ± 8.71	7.86 ± 4.54	9.14 ± 4.47
Total	4.55 ± 0.67	80.67 ± 8.81	5.77 ± 2.78	7.94 ± 2.44
p value	0.583	0.089	0.002*	0.045*

Table 3. Levels of biomarkers in visceral leishmaniasis with the presence or absence of diabetic wound

ELISA	Urea (mmol/L)	Creatinine (µmol/L)	ALT (U/L)	AST (U/L)
VL without wound	4.41 ± 0.97	73.93 ± 13.86	4.71 ± 1.49	8.55 ± 1.99
VL with diabetic wound	3.98 ± 0.89	70.61 ± 9.00	5.17 ± 1.79	8.89 ± 2.22
Total	4.33 ± 0.97	73.32 ± 13.12	4.79 ± 1.55	8.6122
p value	0.085	0.336	0.262	0.524

Table 4. Levels of biomarkers in visceral leishmaniasis with the presence or absence of diabetes

ELISA	Urea (mmol/L)	Creatinine (µmol/L)	ALT (U/L)	AST (U/L)
VL without diabetes	4.85 ± 1.35	77.48 ± 17.13	4.92 ± 1.99	8.25 ± 2.21
VL diabetic	5.30 ± 0.95	82.33 ± 10.33	4.98 ± 1.44	8.55 ± 1.49
Total	4.92 ± 1.29	82.33 ± 10.33	4.97 ± 1.91	8.40 ± 2.11
p value	0.276	0.347	0.912	0.920

DISCUSSION

The results of this study showed that although the buffy coat diagnostic method (least recommended) is specific, but its sensitivity is grossly compromised and many Leishmania-positive patients will be missed. The buffy coat technique (sensitivity: 2.3%, specificity: 99.6%, negative predictive value: 84.1%, positive predictive value: 50%) is therefore considered unfit for the screening purposes. This is in agreement with the research carried out by Zijlstra (2016) revealing that the thin film diagnostic method has low sensitivity but very high specificity and therefore cannot be used as the sole diagnostic technique for detecting leishmaniasis infection. The capillary method did not detect the presence of Leishmania parasite in the sera (sensitivity and specificity = 0%). This is in support of research carried out by Menike et al. (2016) where all patients tested were negative for Leishmania promastigotes in similar method. However, ELISA detected 44 (16.2%) leishmaniasis cases among the participants. It is the gold standard and can be relied upon for best diagnosis.

Studies carried out on renal involvement in visceral leishmaniasis revealed that nephrotoxicity is one of the outcomes of VL infection (Stanley and Engwerda, 2007; Radwan, 2008). Even when creatinine levels appeared to be normal, microalbuminuria has been detected in more than 40% of patients with visceral leishmaniasis (Oryan and Akbari, 2016). Therefore, not finding a direct increase in creatinine in our study may be due to the fact that the participants are asymptomatic or might be at the early stage of infection and as such their immune response was still low, and have not developed a renal impairment (Oliveira et al., 2010). Therefore, the low level of creatinine recorded in the present study could be that most of the participants are asymptomatic or the infection is at pre-patent stage with low immune response and least renal impairment. Even though study had shown that leishmania drugs do induce renal dysfunction in treated individuals (da Silva Junior *et al.,* 2014), the fact that none of the participants sampled in the present study was under treatment eliminated the possibility of renal impairment due to chemotherapy (Elnojomi *et al.,* 2010). The participants who tested positive for the presence of Leishmania parasites have elevated ALT and AST which are statistically significant ($p<0.05$) when compared to participants not infected with Leishmania parasite. Thus, it appeared that VL infection causes high expression of liver enzyme markers, hepatosplenomegaly and cirrhosis. The increase of ALT and AST may also be in response to the hepatomegaly caused by the parasites (Romero *et al.,* 1995; Medhi, 2008; Bhattacharyya and Hati, 2004; Naseralla et al., 2015). Studies have shown that a high prevalence of functional liver disarrangement is common in leishmania

patients (Mathur *et al.,* 2008; Taher *et al.,* 2015) and liver involvement is not unusual in kala-azar as cirrhosis, hepatosplenomegaly, chronic liver disease occurs when not treated, but these can be reversed if treated early with available drugs such as the oral miltefosine (Avasthi *et al.,* 2009).

CONCLUSION

This study indicated that Leishmania infection may be a singular factor that perturbs the kidney and liver enzymes. Therefore, VL may not show association with diabetes or diabetic foot ulcer co-morbidity in the population studied. There has not been any reported case of visceral leishmaniasis in Osun State prior to this study. It, therefore, suffices that the infectious disease has hitherto remained under- or misdiagnosed. There has not been any reported case of visceral leishmaniasis in Osun State prior to this study. It, therefore, suffices that the infectious disease has hitherto remained un- or misdiagnosed.

AUTHORS' CONTRIBUTIONS

Conceptualization and methodology, EIOA, MAA and MAM; validation, EIOA, MAA and MAM; formal analysis, All Authors; investigation, FVO, AJA and MAM; resources, EIOA and MAA; data curation, MAM; writing—original draft preparation, FVO and AJA; writing—review and editing, EIOA, AU, MAA and MAM; supervision, EIOA, MAM and MAM; project administration, EIOA, MAA and MAM; funding acquisition, EIOA. All authors have read and agreed to the published version of the manuscript.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

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