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Full Length Research Paper

Detection of *Leishmania* in human blood and tissue exudate ingested by *Tunga penetrans*

Elizabeth Sentongo[1*], Samuel Kalungi[2] and Jacinto Amandua[3]

[1]Department of Microbiology, School of Biomedical Sciences, College of Health Sciences, Makerere University, P. O. Box 7072, Kampala, Uganda.
[2]Department of Pathology, Mulago National Referral Hospital P. O. Box 7051 Kampala, Uganda.
[3]Department of Clinical Services, Ministry of Health P. O. Box 7272, Kampala, Uganda.

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Abstract

Communities in Uganda are experiencing a multiparasitism, blood microscopy often reveals *Leishmania* and microfilaria in patients with anaemia, haemorrhage and splenomegaly. Many are likely asymptomatic and probably exposed to insect vectors. We examined the blood and tissue exudate ingested by *Tunga penetrans*, from relatively healthy eastern Ugandans who had been afflicted by sand fleas in 2010. Comparison was thereafter made with Giemsa-stained blood and bone marrow aspirate smears from patients admitted to Mulago National Referral Hospital. In histology preparations of formalin-preserved paraffin-embedded enucleate stained with haematoxylin and eosin, were cross sections with columnar lining containing a pink substance. Within the substance were numerous lobular and circular organelles, representing nuclei of polymorphonuclear and mononuclear white blood cells. Clustered about the nuclei were translucent spheres containing two spots; one large and dark, the other small and red. No microfilaria were seen. In patients' blood smears, where microfilaria were also demonstrable, the spheres clustered inside monocytes and in bone marrow aspirate smears they were Leishman-Donovan bodies. The findings, besides indicating an inflammatory reaction of the host, implied a cutaneous and/or systemic leishmanial infection. Persons with *Leishmania* in their body fluids can infect female sand flies or develop visceral, cutaneous or mucocutaneous disease.

Key words: Tungiasis, leishmaniasis, haematophagy, asymptomatic carriage.

INTRODUCTION

Communities in Uganda are experiencing a multiparasitism which includes leishmaniasis and filariasis. Since 2015, microscopy of peripheral blood smears from severely ill patients admitted to the medical wards at Mulago National Referral Hospital has frequently revealed *Leishmania* and microfilaria (Sentongo et al., 2020a). Most notable was blood from patients with differential diagnoses of unexplained anaemia, aplastic anaemia, human immunodeficiency virus (HIV)- or azidothymidine (AZT)-induced anaemia if they were HIV co-infected, bleeding diathesis, idiopathic thrombocytopenic purpura (ITP), hyper-reactive malarial splenomegaly (HMS), haematological malignancy and lymphoma, respectively. *Leishmania* were also seen in patients' bone marrow aspirates and biopsies. In areas where bancroftian filariasis, mansonelliasis and loiasis are endemic, some individuals suffer mild or self-limiting disease; many of the infected remain asymptomatic despite being filaraemic or antigenaemic (Kar et al., 2017; Bouyou Akotet et al., 2015; Onapa et al., 2005). Likewise, communities where systemic or visceral leishmaniasis is endemic have asymptomatic individuals who harbour the parasite. In neighbouring countries where visceral leishmaniasis is highly endemic,

Corresponding Author. E-mail: besimensi@gmail.com
Tel: +256 772 697 373

asymptomatic infection has been established using serological recombinant K39 (rK39) antigen-based strip test and direct agglutination test (DAT), the cell mediated immunity evaluating Leishmanin skin test (LST) and molecular polymerase chain reaction test (Mohamed et al., 2019; Custodio et al., 2012; Schaefer et al., 1995; Shiddo et al., 1995). The necessity to demonstrate the parasites often arose in clinical practice when serological test results were indeterminate despite high suspicion of visceral leishmaniasis, where response to antileishmanial treatment was inadequate and as a test of cure (TOC) before patient release (MoH South Sudan, 2012; FMoH Ethiopia, 2013; MoH Kenya, 2017; MoH Uganda, 2019). *Leishmania* amastigotes would then be sought for in spleen, bone marrow or lymph node aspirates but hardly in blood.

Leishmaniae were found in peripheral blood smears of eastern African patients with visceral disease in the then Republic of Sudan (Rohrs, 1964), in Kenya (Chulay et al., 1985), Ethiopia (Diro et al., 2017) and in a Somali patient (Clement and Li, 2017). Similar observations were made on patients in southern Asia's Bangladesh (Chandan et al., 2009; Salam et al, 2012), India (Rahman et al., 2015) and Nepal (Ghimire et al., 2019); southern Europe's France (Moniot et al., 2018) and Spain (Delgado, 1997) and South America's Brazil (Martinez et al., 1993). The asymptomatic microscopic parasitaemia was documented in India (Sharma et al., 2000) and, by use of more elaborate analyses on buffy coat and peripheral blood mononuclear cells, inferred amongst blood donors in southern France (Fichoux et al., 1999), the Balearic Islands of Spain (Riera et al., 2008) and Midwest Brazil (França et al., 2020), as well as in southern Iranian children (Gigloo et al., 2018). Other means of determining infection in human blood have used *Phlebotomus* and *Lutzomyia* vectors in xenodiagnosis (Mondal et al., 2018; Ferreira et al., 2018), sometimes involving rodent models (Sadlova et al., 2015) or animal reservoirs (Magalhães-Junior et al., 2016). In Uganda's north-eastern focus of visceral leishmaniasis, the acknowledged reservoirs are humans infected with *Leishmania donovani* which is transmitted by *Phlebotomus martini* (MoH, 2013). Presently, leishmaniasis is no longer confined to that part of the country, a multiparasitism complicates the clinical picture and, the reservoirs and mode of transmission may be different. For instance, antileishmanial antibodies were detected in domestic dogs from south-western Uganda (Millán et al., 2013).

Communities in eastern Uganda had been severely afflicted by *Tunga penetrans* (MoH Uganda, 2010), a tissue-embedding haematophagous ectoparasite. Normally sand fleas are enucleated from the affected body parts by the infested person or their caretaker, using a thorn or safety pin. In October 2010, female sand fleas were hygienically enucleated from the feet of some residents in Jinja and Kamuli districts to relieve heavy infestation and to provide specimens for a definitive diagnosis (Sentongo et al., 2020b). The donors, eight females and seven males, ten of whom were primary school-age children, had been naturally infested. Save for the localised inflammation of the toes and soles of the feet, there was no record of systemic illness. This article presents the result of scrutinising the luminal content of the arthropod's digestive tract.

METHOD

Histology slides of formalin-preserved paraffin-embedded gravid sand fleas, stained with haematoxylin and eosin, as previously described (Sentongo et al., 2020b), were examined by light microscopy. Cross-sections of the arthropod's exoskeleton and internal organs were displayed. Within the coelom the sections with lumina were identified and based on the morphology of their boundaries characterised as sections with an acellular and those with a cellular wall. Of the latter, the sections with a tortuous innermost layer were earmarked. Using high magnification including the oil-immersed objective, the luminal lining and content were scrutinised. Comparison was thereafter made with air-dried methanol-fixed Giemsa-stained peripheral blood and bone marrow aspirate smears from patients who had been admitted to the National Referral Hospital.

RESULTS

The external acellular and inner cellular layers of the exoskeleton enclosed the coelom of the arthropod, within which were cross-sections of the internal organs (Figure 1). Acellular eosinophilic rings enclosing clear space represented respiratory tubes and tubules that transport air. Of the sections with a cellular boundary, those with an even luminal lining contained eosinophilic anisocytic spheres representing ova within the reproductive tract. The sections with a convoluted innermost layer were of three types. One category had filamentous lining and a generally collapsed lumen containing clear space (Figure 2), likely fluid within the filtration-excretory system. The other two categories had columnar lining, characteristic of digestive epithelia, and lumina containing a pink substance, representing blood and tissue exudate that had been ingested by the sand flea. Where the lining was dense, prolific and deeply invaginated the lumen was narrower and the pink substance was relatively homogeneous (Figure 3A.) However, where the lining had short spread out cylindrical cells the lumen was spacious and the pink substance had many organelles representing nuclei of polymorphonuclear and mononuclear white blood cells (Figure 3B). About the nuclei were clusters of translucent spheres with spots inside them (Figure 4). At higher magnification the sphere was a clearness around two spots, one large and dark, the other small and red. Other spheres clustered on yellow globular particles, some were solitary. No microfilaria-like

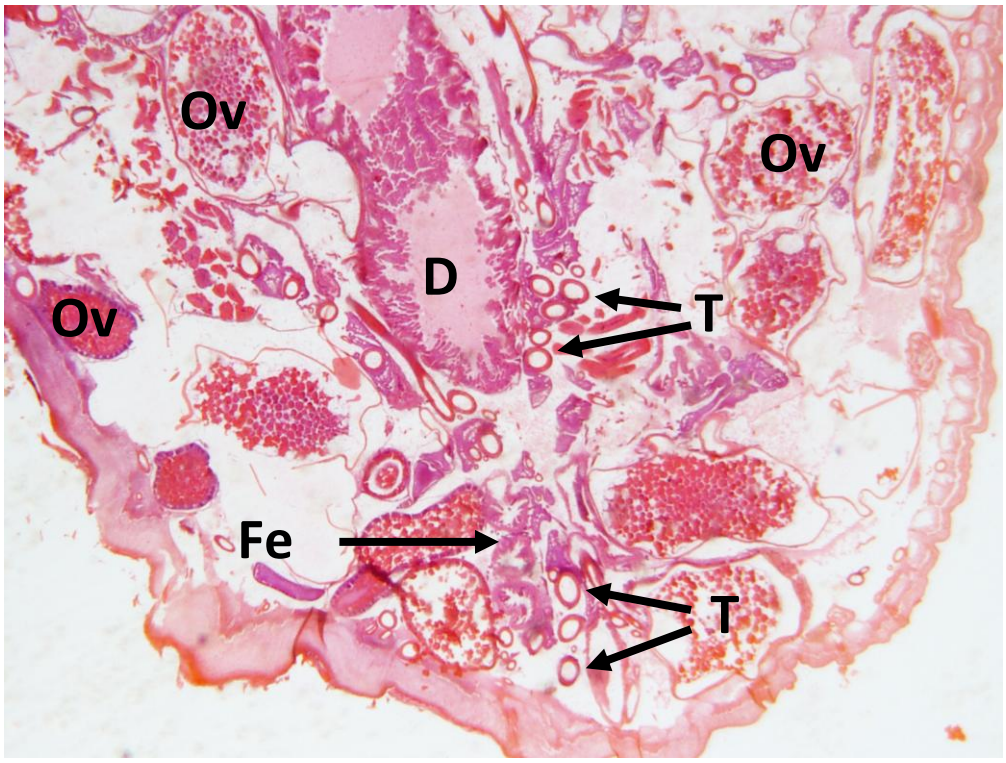

Figure 1. A section through the arthropod's coelom showing cross-sections of respiratory (**T**), reproductive (**Ov**), filtration-excretory (**Fe**) and digestive (**D**) organs (X10).

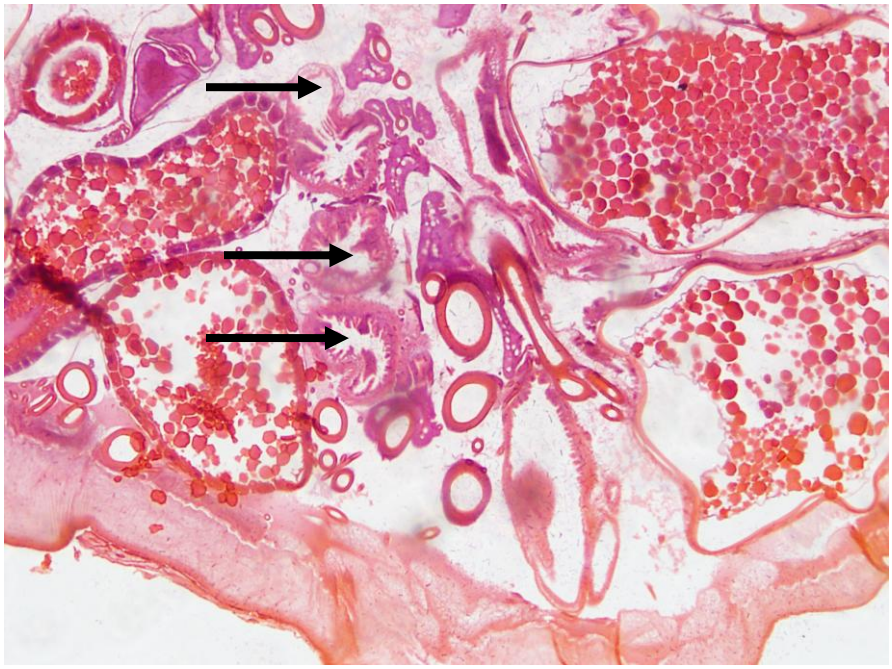

Figure 2. The filtration-excretory sections with a highly convoluted lining, arrows point to the clear content in the lumen (X40).

structures were seen within the pink substance. On cross-reference, blood smears from patients had microfilariae, sometimes found at the periphery (Figures 5A and B), as well as spheres, at times dark blue and red and other times translucent, clustered within monocytic white blood cells (Figures 6A and B). In smears of bone marrow aspirates from patients, the spheres were clearly seen as Leishman-Donovan (LD)

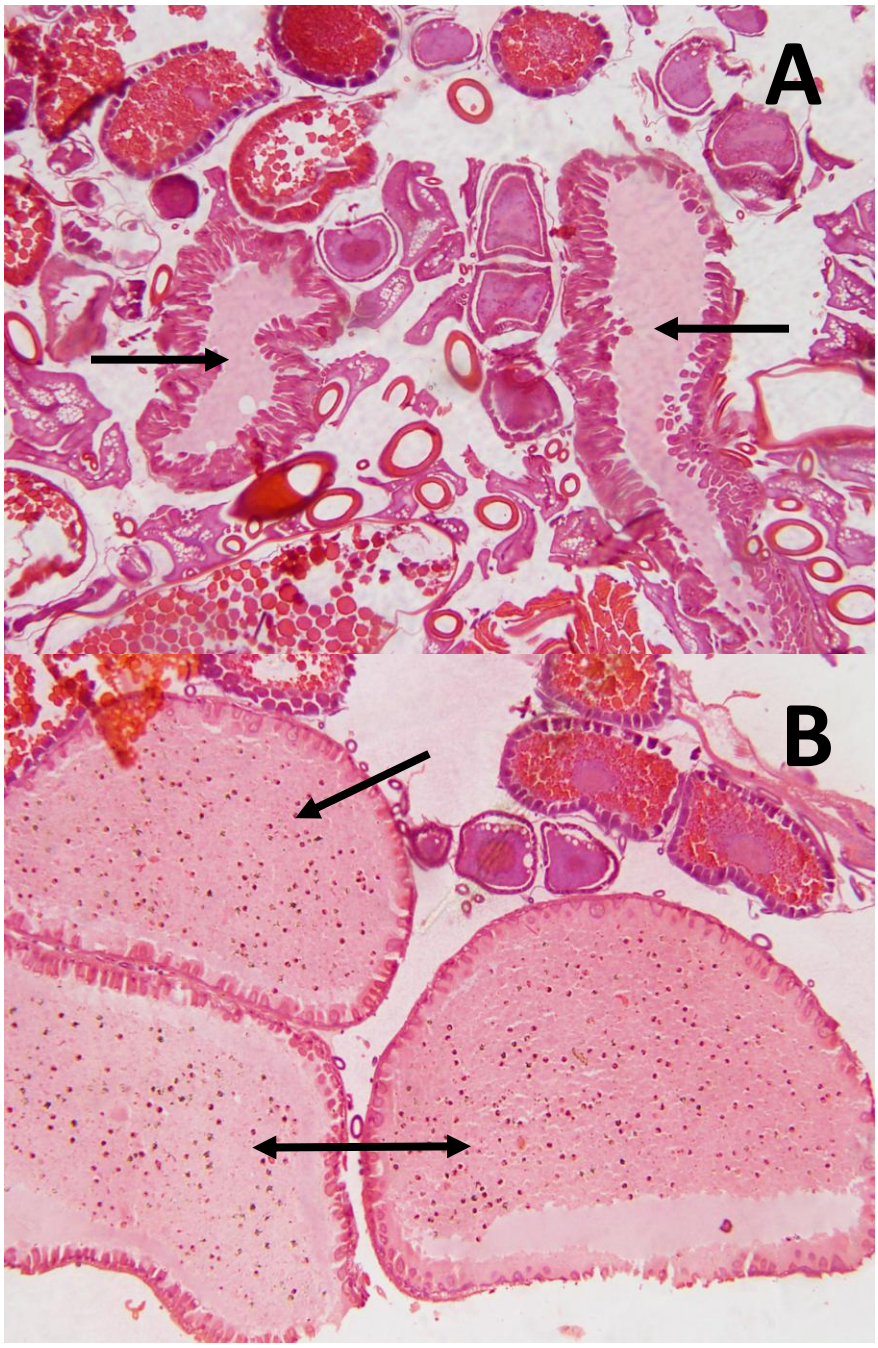

Figure 3. The digestive sections with columnar lining, arrows point to the homogeneous (**A**) and speckled (**B**) pink substance inside the lumina (X20).

bodies with a dark nucleus and red kinetoplast (Figure 7).

DISCUSSION

The various cross-sections surrounded by a multi-layered exoskeleton could identify with the respective internal organs of the arthropod, based on the morphological features of their boundaries and the luminal content. At all levels, abundant respiratory and reproductive cross-sections inferred the bifurcating or winding nature of the organs. The filtration-excretory and digestive cross-sections on the other hand were conspicuous for their tortuous luminal lining, suggestive of an absorptive-excretory function. Inside digestive lumina the pink substance matched the characteristics of the blood and tissue exudate that would have been ingested by the sand flea. Usually in Giemsa-treated blood smears, red blood cells stain pale to rich pink, white blood cell cytoplasm stains pale blue to light purple and the nuclei stain dark blue to deep purple (WHO, 2003). The exudate, which in human tungiasis

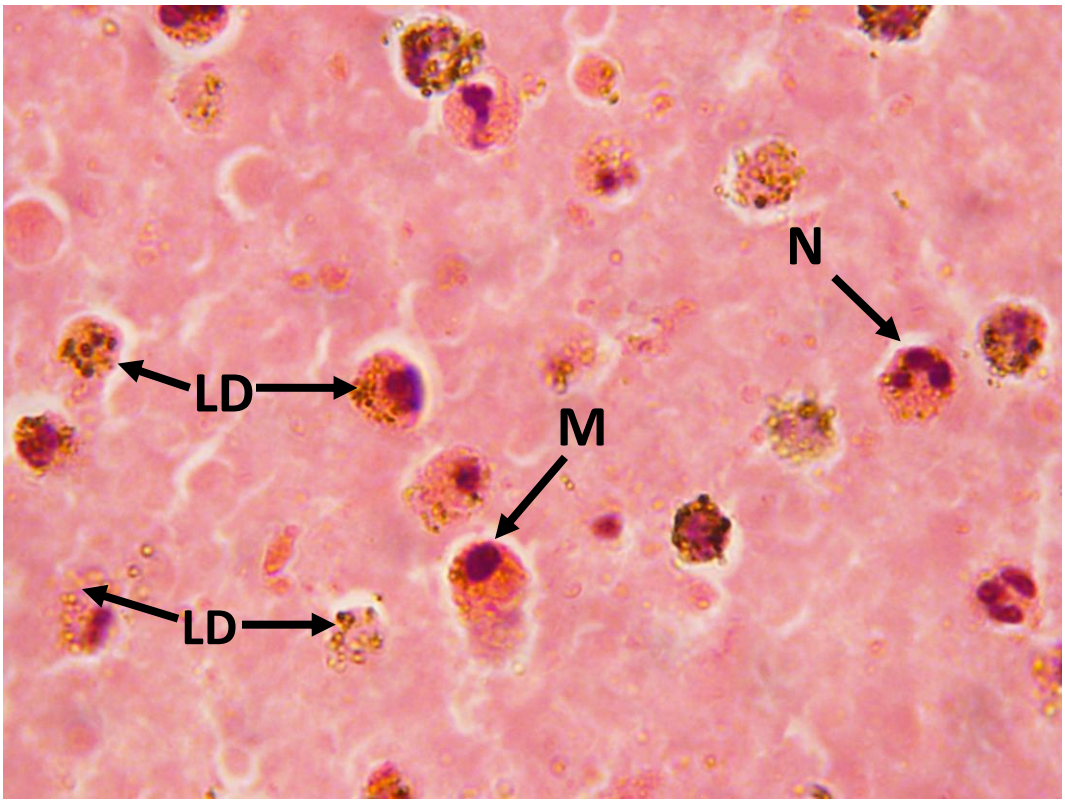

Figure 4. The pink substance containing nuclei of polymorphonuclear (**N**) and monocytic (**M**) leukocytes, about which clustered translucent spheres (**LD**). A nucleus and kinetoplast are seen in some spheres (x100).

ranges from serous to sanguinous, serosanguinous and purulent, consists of plasma, blood proteins and white blood cells. Because female sand fleas burrow under the skin and hypertrophy (Geigy and Herbig, 1949), a foreign body effect is created within tissue. This and the frequent secondary bacterial infections (Zziwa, 2009) provoke an influx of granulocytic neutrophils and agranular monocytes which serve antimicrobial and tissue repair purposes. In the pink substance therefore, an inflammatory reaction of the human host was suggested by the plentiful white blood cell nuclei. Both polymorphonuclear and mononuclear cells showed intracytoplasmic bodies which were suspected to be unicellular microorganisms, since two minute organelles could be identified inside them. The bodies had probably been endocytosed or released, as several others were also seen in space far from the nuclei.

The identification of translucent spheres, now recognised as LD bodies, was retrospective given that the initial intention had been to describe histological features of the sand flea. This followed observation of similar microstructures within monocytic cells first in bone marrow aspirate smears from patients suspected with haematological malignancy, then in tissue biopsies from patients suspected with lymphoma or Kaposi sarcoma and subsequently in peripheral blood smears from patients with anaemia or haemorrhagic tendency. The clustering of the spheres about cell nuclei implied an intracellular confinement that is characteristic of *Leishmania* particularly in tissue macrophages (WHO, 1991). On the other hand, the solitary spheres and others adherent onto adipose-like globules suggested an extracellular existence of the microorganism, possibly following white blood cell degeneration or rupture. That the nuclei and spheres were absent from portions of the flea's digestive tract, may have been consequent to processing in the midgut. However, the total absence of microfilaria which on light microscopy were frequently demonstrable in patients' blood, urine and faeces was more difficult to explain. It was possible that the haematophagy excluded the much larger helminths (Figure 6A) or that they were also degraded by the digestive process or even removed during the numerous staining and washing stages of tissue processing. For instance, the staining and washing of blood smears tended to displace microfilaria to the periphery (Figures 5A and B). Subsequently, they could escape recognition if microscopy focussed only on the body of the smear. Microfilaria were as well hardly seen at the tail-end of a blood smear, while multitudes of solitary LD bodies were often found at tail-ends of bone marrow aspirate smears, particularly in severely ill patients (Figure 7).

The sand flea's carriage of LD bodies had epidemiological significance, since neither leishmaniasis nor *Phlebotomus* were known in eastern

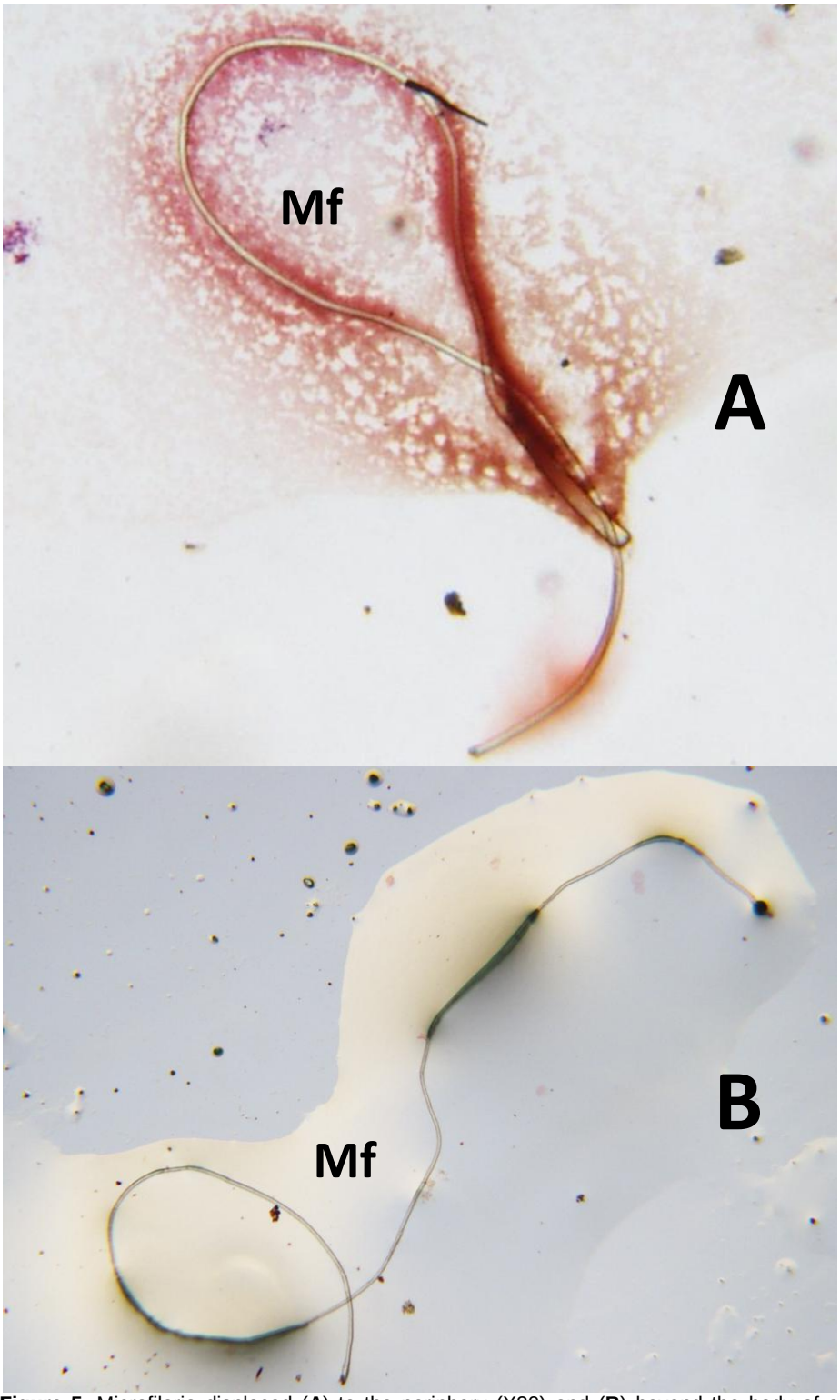

Figure 5. Microfilaria displaced (**A**) to the periphery (X20) and (**B**) beyond the body of a blood smear following the Giemsa-staining process (X10).

Uganda. Questions arose about either species involved, potential reservoirs and possible modes of transmission. The gravid sand fleas were end stages which fed exclusively on humans, hence the presence of LD bodies in their meal was diagnostic of a cutaneous and/or systemic leishmanial infection in the donors. In no way did this indicate an effective parasitisation of the sand fleas or that they and their off-host feeder counterparts could transmit the protozoa. It is within *Phlebotomus* and *Lutzomyia*, in the Old and New Worlds respectively, that *Leishmania* replicate, develop and migrate appropriately to produce a transmi-

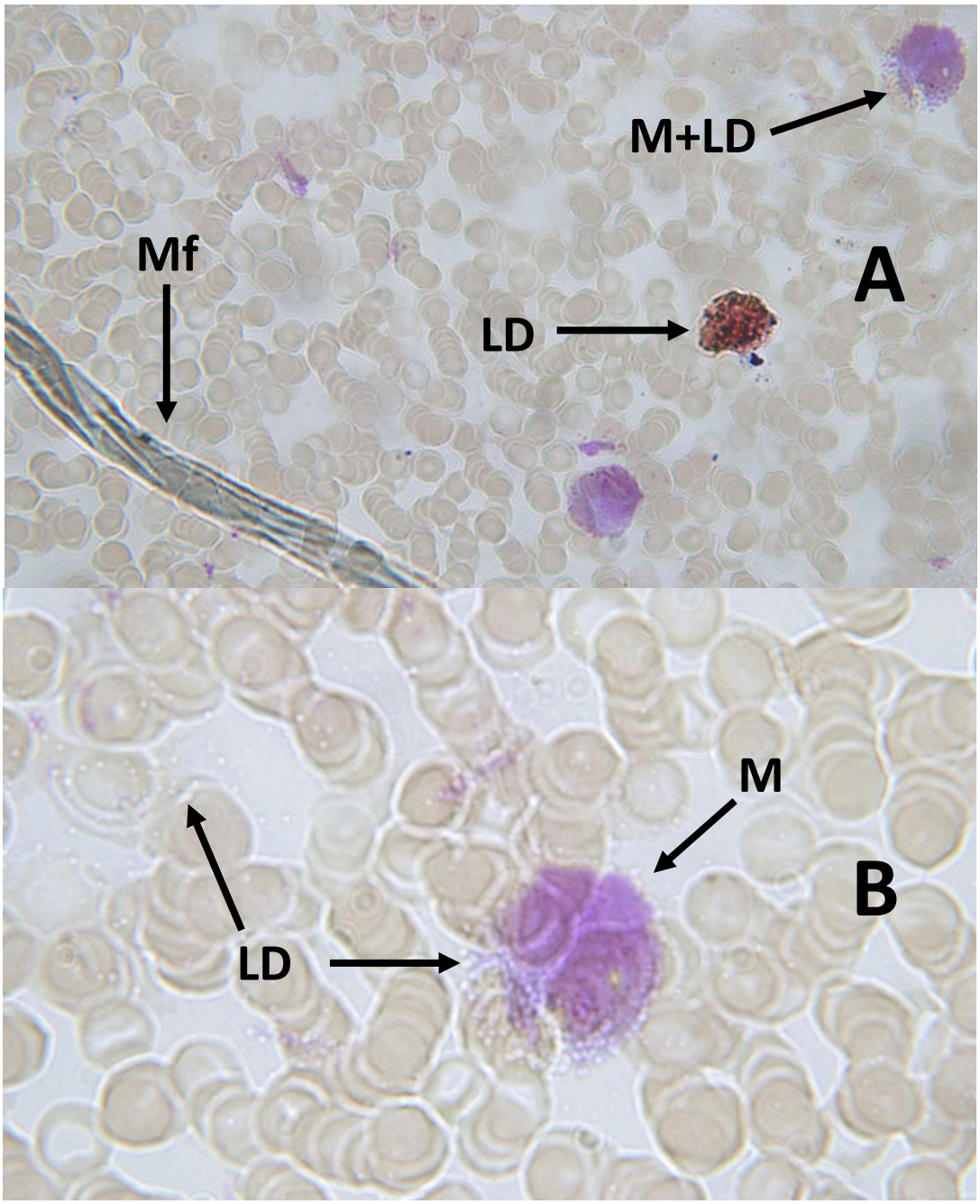

Figure 6. A: A Giemsa-stained blood smear with part of a microfilarium (**Mf**), mononuclear leukocyte with clustered dark blue and red spots (**M+LD**) and the spots (**LD**) clustered onto an adipose-like globule (X40). **B:** Translucent spheres clustered within a monocyte (**M**) and several extracellular others (X100).

issible infection (WHO, 2010). In South America where human and canine leishmaniasis are endemic, ticks, lice and synanthropic fleas were found with leishmanial nucleic acids (Colombo et al., 2011; de Morais et al., 2013), making them suspects as vectors. Like the phlebotomines, the ectoparasites inhabit peridomestic environments, feed alternatively on birds, rodents and animals and can transmit other parasites. Human babesiosis for instance, has been documented (Lee et al., 1997) and *Babesia* was detected in cow and dog ticks in Uganda (Nakayima et al., 2014; Proboste, 2015). The cestodes *Hymenolepis* and *Dipylidium* which cause paediatric disease (Buzigi, 2015; Chappel et al., 1990) can be transmitted by *Ctenocephalides* dog and cat fleas, and the dog louse *Trichodectes* can transmit *Dipylidium* (Chappel et al., 1990). Still, despite a closer proximity than the phlebotomines to hosts which might be infected, the ability of the ectoparasites to transmit leishmaniae requires further evaluation (Otranto and Dantas-Torres, 2010).

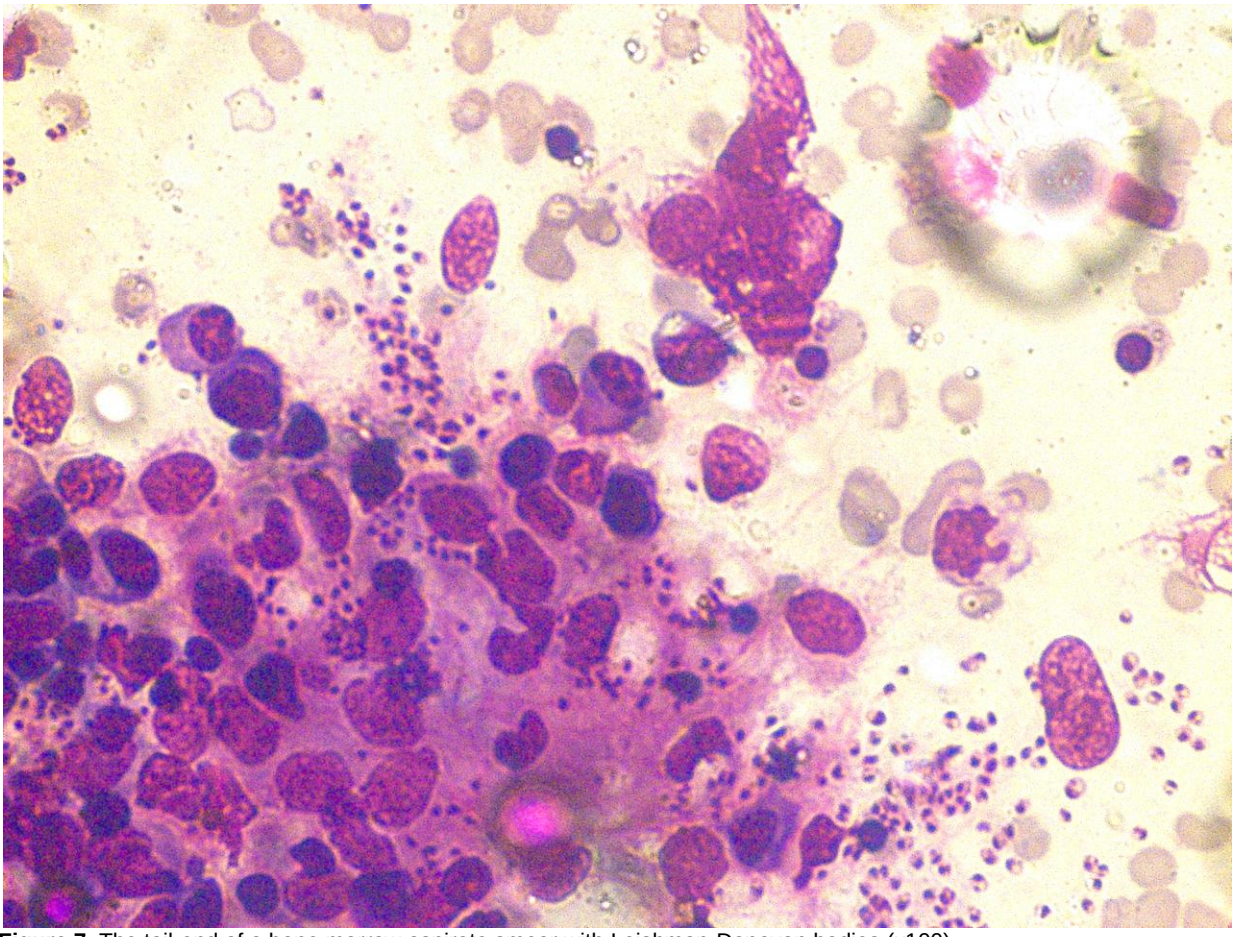

Figure 7. The tail-end of a bone marrow aspirate smear with Leishman-Donovan bodies (x100).

More significantly, the human carriage of LD bodies had clinical and epidemiological implications. The enucleate donors, most of them very young, were reasonably healthy save for the normally self-limiting tungiasis. Nonetheless, they could eventually develop full-blown cutaneous, mucocutaneous or systemic leishmaniasis. In endemic communities, asymptomatic infection has been characterised by parasite-specific immune responses or the presence of *Leishmania* or their nucleic acids in blood (Alvar et al., 2020). Because the donors were not in a known endemic area, they probably would not qualify as asymptomatically infected. Even then, their existence hinted that many others were similarly affected, an occurrence in our setting for which vector-borne transmission might be responsible. Such persons, besides creating foci of disease, can act as reservoirs of infection for the vectors (Sentongo et al., 2018). Because they tend to be more in numbers than those with disease, symptom-free individuals may also contribute considerably to parasite transmission. Namely, they could facilitate parenteral transmission via trans-placental (Eltoum, 1992), intravenous inoculation (Pineda et al., 2001), blood product infusion (Manthur and Samantaray, 2004) and blood transfusion (Dey and Singh, 2006) routes. These alternative modes of parasite transmission would seriously challenge the current control strategy for visceral leishmaniasis in Uganda (MoH Uganda, 2013), which is intensified case management.

CONCLUSION

The blood and tissue exudate which had been ingested by the enucleated gravid sand fleas had numerous leukocytes, indicating an inflammatory reaction of the human host. The phagocytic neutrophils and monocytes therein contained Leishman-Donovan bodies, suggesting a cutaneous and/or systemic infection in the donors. Though the relatively healthy mostly paediatric humans were not in a known endemic area, the findings implied that many individuals were similarly affected. With *Leishmania* in their blood and tissue, such persons can infect female sand flies, develop visceral, cutaneous or mucocutaneous leishmaniasis and facilitate parenteral modes of parasite transmission.

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