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Epidemiology of Visceral Leishmaniasis and Entomological Investigations of Sand fly Vectors in Selected Refugee Camps of Gambella and Benishangul-Gumuz Regional States, Ethiopia

Ph.D. Dissertation

By

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Epidemiology of Visceral Leishmaniasis and Entomological Investigations of Sand fly Vectors in Selected Refugee Camps of Gambella and Benishangul-Gumuz Regional States, Ethiopia

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DECLARATION

I, Habtamu Belay Kassa, hereby declare that I conducted the work described in this dissertation, entitled "Epidemiology of Visceral Leishmaniasis and Entomological Investigations of Sand Fly Vectors in Selected Refugee Camps of Gambella and Benishangul-Gumuz Regions, Ethiopia," and that no portion of the dissertation has been submitted for the award of any academic degree or diploma. This dissertation has properly acknowledged all sources and materials utilized. Intellectual property rights and participants' rights to confidentiality and unrestricted withdrawal from the study were all maintained throughout the research procedure.

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DEDICATION

To my beloved late father, **Belay Kassa Yimer**, even though I was a child, your memory continues to inspire and guide me throughout my life.

TABLE OF CONTENTS

TABLE OF CONTENTS I
ABSTRACT IV
ACKNOWLEDGEMENTS VI
LIST OF FIGURES VIII
LIST OF TABLES IX
LIST OF ANNEXES X
ACRONYMS AND ABBREVIATIONS XI
LIST OF ORIGINAL PUBLISHED PAPERS XIII
CHAPTER 1: INTRODUCTION 1
1.1. Background 1
1.2. Statement of the problem 4
1.3. Significance of the study 5
1.4. Research questions 6
1.5. Objective of the study 7
1.5.1. General objective 7
1.5.2. Specific objectives 7
CHAPTER 2: LITERATURE REVIEW 8
2.1. Overview of leishmaniasis 8
2.2. Epidemiology of Visceral Leishmaniasis 10
2.2.1. Global Epidemiology 10
2.2.2. Distribution of Visceral Leishmaniasis in East Africa 11
2.2.3. Current epidemiology of VL in Ethiopia 14
2.2.4. Epidemiology of VL in refugee camps 18
2.3. Risk factors of visceral leishmaniasis transmission 20
2.4. *Leishmania* parasite 23
2.5. Transmission and Life cycle of *Leishmania* Parasites 24
2.5.1. Transmission of *Leishmania* Parasites 24

2.5.2. Life cycle of *Leishmania* parasites 24
2.6. Pathogenesis of leishmaniasis 26
2.7. Clinical manifestations of VL 27
2.8. Current laboratory diagnosis of leishmaniasis 29
2.8.1 Types of recommended specimens 31
2.8.2. Parasitological investigation 32
2.8.3. Serological testing methods 34
2.8.4. Molecular testing methods 37
2.9. Visceral Leishmaniasis Treatment 40
2.10. Sand fly Vectors of Visceral Leishmaniasis 41
2.10.1. Distribution of sand fly vectors of VL 41
2.10.2. Infection rate in naturally infected sand flies 43
2.11. Prevention and Control of Visceral Leishmaniasis 45
2.11.1. Visceral leishmaniasis prevention and control strategies in global perspective 45
2.11.2. Leishmaniasis control and prevention efforts in Ethiopia 46
CHAPTER 3: MATERIALS AND METHODS 48
3.1. Study sites 48
3.2. Study design and period 50
3.3. Source and study population 51
3.4. Study variables 51
3.5. Operational definitions 52
3.6. Sample size determination 53
3.7. Sampling methods 54
3.8. Survey instrument and data collection tools 56
3.9. Laboratory based data collection 58
3.9.1. Clinical sample collection and processing 58
3.9.2. Direct agglutination test (DAT) 59
3.9.3. Mounting and identification of sand flies 60
3.9.4. DNA extraction from buffy coat of centrifuged human blood 61

3.9.5. Leishmania species DNA extraction from sand flies....62
3.9.6. *Leishmania* kDNA detection by real-time polymerase chain reaction....62
3.10. Data quality assurance....63
3.11. Ethical Statement....64
3.12. Statistical analysis....64
CHAPTER 4: RESULTS....66
4.1. Prevalence of *Leishmania* Infection in Refugee Camps....66
4.1.1. Demographic characteristics of study participants....66
4.1.2. Clinical assessment of study participants....68
4.1.3. Prevalence of *Leishmania* infection....70
4.2. Determinants of *Leishmania* Infection in Refugee Camps....76
4.2.1. Socio-demographic characteristics....76
4.2.2. Behavioral and environmental characteristics....78
4.2.3. Determinants of *Leishmania* infection....80
4.3. Sand fly survey in refugee camps....83
4.3.1. Preliminary assessments prior to actual sand fly collection....83
4.3.2. Sand fly species composition and relative abundance across sites....83
4.3.3. Species composition relative to collection methods....84
4.3.4. Indoor and outdoor collections of sand flies....85
4.3.5. Sex ratio and abdominal status of sand flies....85
4.3.6. Habitat preferences of the sand flies....87
4.3.7. Diversity of sand flies among refugee camps....88
4.4. PCR screening of sand flies for the detection of *Leishmania* DNA....88
CHAPTER 5: DISCUSSION....90
CHAPTER 6: CONCLUSION AND ECOMMENDATIONs....103
6.1. Conclusions....103
6.2. Recommendations....103
References....105
Annexes....129

ABSTRACT

Background: Visceral leishmaniasis (VL) is a neglected tropical disease, transmitted by the phlebotomine sand flies, that causes a severe health threat in East Africa. Ethiopia remains one of the VL-affected countries in East Africa, with Gambella and Benishangul-Gumuz Regional States ecologically recognized as high-risk areas. Refugee camps in these regions harbor vulnerable populations living under conditions conducive to sand fly and VL transmission. There are also ongoing VL case reports to MoH every year. Though data on the burden of infection, exposure factors, and sand fly fauna remain limited in these settings, further epidemiological studies are needed to address these knowledge gaps. So, this study aims to determine the epidemiology of VL and sand fly vectors in refugee camps in Gambella and Benishangul-Gumuz regions to improve VL detection and guide effective control strategies in these settings.

Methods: A community based cross-sectional study was conducted in Kule and Terkidi refugee camps located in Gambella, and Tsore and Sherkole refugee camps located in Benishangul Gumuz regional states from May to August 2023 after ethical approval was granted to assess the distribution of VL and from April to May 2025 for a sand fly vector survey. Sociodemographic, clinical, and *Leishmania donovani (L. donovani)* infection-associated factors were collected using structured questionnaires. Whole blood was collected from each study participant and tested using both the direct agglutination test (DAT) and the *Leishmania* kinetoplast minicircle based rtPCR test. A matched case-control study employed to identify significant explanatory factors associated with the infection. Sand flies were collected using CDC light trap (LT) and sticky trap (ST) from both indoor and outdoor environments. Following collection, sand flies were dissected for species identification using taxonomic keys. Female sand flies were preserved for *Leishmania* parasite rtPCR analysis and to determine infection status. *Leishmania* parasite DNA was extracted individually from *Phlebotomus rodhaini* and in pooled sets of *Sergentomyia* species, followed by real-time PCR targeting *Leishmania* kinetoplast minicircle DNA. Data were analyzed using SPSS v24, with proportions compared using Chi-square tests at a 0.05 significance level. Continuous variables were summarized by median and interquartile range (IQR). Conditional logistic regression was applied to assess associations between independent variables and *Leishmania* infection. Sand fly species diversity was analyzed using the Shannon-Wiener index, and habitat associations were assessed descriptively.

Result: The study included 1,223 participants, most of whom were from South Sudan (66.7%), and the majority were females (56.5%). About 17.8% of the participants had reported fever, with no spleen or liver enlargement and 0.2% lymph node swelling. Participants significantly higher DAT positivity rate observed among participants with no formal education (p=0.024). Among those who reported a rash, rash presence on the face was more associated with VL infection using DAT (p=0.001). Real-time PCR positivity was significantly higher in Tsore camp (14.6%, χ^2 = 21.4, $p < 0.001$). Despite the very small number of cases, the DAT positivity was higher

compared to those who did not report the presence of a rash (p=0.002). A total of 336 participants, 252 controls and 84 cases, were enrolled from all selected refugee camps. The distribution of matching variables among cases and controls by age group, sex, and region of camp location showed that they were similarly distributed (p > 0.05). Significant risk factors included residing in the camp for less than five years (AOR=1.76, 95% CI: 1.06-2.93, p=0.028), sleeping outside during the daytime (AOR=2.38, 95% CI: 1.46-3.89, p<0.001) or nighttime (AOR=1.68, 95% CI: 1.07-2.64, p=0.025), and the presence of bushes around the house (AOR=1.76, 95% CI: 1.12-2.77, p=0.014). A total of 2,196 sand flies representing eight species (one *Phlebotomus* and seven *Sergentomyia*) were collected. *Phlebotomus rodhaini*, the only *Phlebotomus* species detected, accounted for 1.7% of captures. *Sergentomyia antennatus* was the most abundant (50.8%), followed by *S. africanus* (15.8%), *S. schwetzi* (14.3%), *S. bedfordi* (10.9%), *S. clydei* (5.2%), *S. squamipleuris* (1.2%), and *S. adleri* (0.1%). Species richness was highest in Terkidi camp, whereas overall diversity was greatest in Sherkole. Indoor captures were limited (<2.3% of specimens), confirming predominantly exophilic resting behavior. Termite mounds and peridomestic habitats harbored the highest sand fly densities. PCR screening of both pooled *Sergentomyia* (n=92) and individual *Ph. rodhaini* (n=11) specimens showed no evidence of *Leishmania* species infection.

Conclusion: *Leishmania* infection was prevalent in refugee camps in Gambella and Benishangul-Gumuz regions. Asymptomatic cases and low parasite loads were common, highlighting the need for active case detection. Duration in the camp for less than five years, sleeping outside during the daytime and nighttime, and the presence of bushes around the house were reported as determinant factors. This first entomological survey in refugee camps in Ethiopia documents a sand fly fauna dominated by outdoor collection *Sergentomyia* species, with very low abundance of *Phlebotomus* species with no detectable *Leishmania* infection.

Key words: Visceral leishmaniasis, Benishangul-Gumuz, Epidemiology, Direct Agglutination Test, Gambella, Polymerase Chain Reaction, risk factors, *Phlebotomus rodhaini, Sergentomyia* species

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LIST OF FIGURES

Figure 1: Status of endemicity of visceral leishmaniasis (VL) worldwide, 2023 11
Figure 2: Distribution of visceral leishmaniasis in East Africa 13
Figure 3: Visceral leishmaniasis endemic regions in Ethiopia (FMoH, 2025) 16
Figure 4: Distribution of asymptomatic Leishmania infection in Ethiopia 18
Figure 5: Representative scheme of the genus Leishmania classification illustrating 24
Figure 6: Life cycle of Leishmania 25
Figure 7: Pictures A and B showing VL patients with splenomegaly and hepatomegaly 28
Figure 8: Clinical presentation of PKDL 29
Figure 9: Overview of visceral leishmaniasis laboratory diagnosis 31
Figure 10: Map showing sand fly collection sites in refugee camps 50
Figure 11: Schematic presentation of sampling procedure 55
Figure 12: House-to-house data collection by trained local health officers 56
Figure 13: Field sampling methods and habitat characterization for sand fly collection 58
Figure 14: Blood sample collection and processing 59
Figure 15: Result of a direct agglutination test (DAT) 60
Figure 16: Laboratory activities related to sand fly processing 61
Figure 17: A workflow diagram depicting the process from field collection of sand flies 63
Figure 18: Distribution of anti-Leishmania antibody agglutination. 71
Figure 19: Venn diagram of DAT and rtPCR positivity 76
Figure 20: Real-time PCR amplification curve for kinetoplast DNA (kDNA) 89

LIST OF TABLES

Table 1: Summary of commonly used parasitological techniques 33
Table 2: Description of infection rates of phlebotomine sand flies 44
Table 3: Description of independent variables 52
Table 4: Socio-demographic characteristics of study participants 67
Table 5:Baseline characteristics of study participants 68
Table 6: Distribution of clinical signs and symptoms 69
Table 7: Distribution of participants by state and origin of country 70
Table 8: Distribution of Leishmania infection by sociodemographic 73
Table 9: Distribution of Leishmania infection among sociodemographic 75
Table 10: Socio demographic characteristics of participants in matched case-control study 77
Table 11: Distribution of participants by country of origin and state 78
Table 12: Distribution of behavioral and environmental characteristics of participants 79
Table 13: The bivariate association 81
Table 14: Results from multivariable conditional logistic regression model o 82
Table 15: Distribution of sand fly species by refugee camp 84
Table 16: Phlebotomine Sand Flies Collected using LT and ST by sex 85
Table 17: Indoor and outdoor abundance of sand fly species 85
Table 18: Male-to-female ratio of sand flies among refugee camps by collection methods 86
Table 19: Distribution of Blood-Fed (BF) and Gravid Sand Fly Species 87
Table 20: Mean density with Standard Error (±SE) of sand fly species 87
Table 21: The Shannon-Wiener diversity index 88

LIST OF ANNEXES

Annex 1: Diagnosis algorithm for VL in Ethiopia 129
Annex 2: Questionnaire for data collection for a study on epidemiology (English Version) .. 130
Annex 3: Questionnaire for a study on epidemiology (Arabic Version) 133
Annex 4: Research Participant Information sheet (English version) 135
Annex 5: Research Participant Information sheet (Arabic version) 140
Annex 6: Consent form for adults (> 18 years old) (English version) 143
Annex 7: Consent form for adults (> 18 years old) (Arabic version) 144
Annex 8: Assent form for children (8-18 years old) (English version) 145
Annex 9: Assent form for children (8-18 years old) (Arabic version) 146
Annex 10: Consent for parents/guardians (English version) 147
Annex 11: Consent for parents/guardians (Arabic version) 148
Annex 12: Direct Agglutination Test Standard Operational Procedure 149
Annex 13: Additional pictures 154
Annex 14: Potential global distribution of human visceral leishmaniasis (Wu et al., 2024) .. 156
Annex 15: Published Articles 157

ACRONYMS AND ABBREVIATIONS

AAU	Addis Ababa University
BC	Buffy coat
BF	Blood-Fed
CDC	Center for Disease Control
CL	Cutaneous Leishmaniasis
DAT	Direct Agglutination Tests
DCL	Diffused Cutaneous Leishmaniasis
DNA	Deoxyribonucleic Acid
DTH	delayed-type hypersensitivity
DNDi	Drugs for Neglected Diseases initiative
ELISA	enzyme-linked immunosorbent assay
EPHI	Ethiopian Public Health Institute
FAST	Fast Agglutination Screening Test
FA	Fluorescent antibody test
HH	Household
HIV	Human Immunodeficiency Virus
ICT	Immunochromatographic test
IDP	Internally Displaced Persons
IFAT	Indirect Fluorescent Antibody Test
IFNγ,	Interferon Gamma
IL	Interleukin
IRS	indoor residual spraying
ITNs	insecticide-treated bed nets
IVM	Integrated Vector Management
kDNA	kinetoplast DNA
LCL	Localized cutaneous leishmaniasis
LST	Leishmanin Skin Test
LT	Light trap
MCL	Mucocutaneous Leishmaniasis
MCM	Micro-culture method

MOH	Ministry of Health
NNN	Novy-MacNeal-Nicolle
NTD	Neglected Tropical Diseases
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PKDL	Post-Kala-Azar Dermal Leishmaniasis
qPCR	quantitative PCR
RBC	Red Blood Cells
rpm	Revolution per minute
RRS	Refugee Returnee Service
rtPCR	Real time PCR
SSG	sodium stibogluconate
ST	Sticky trap
TGFβ	Transforming growth factor beta
Th1	T-helper 1
TH2	T-helper 2
UNHCR	United Nations High Commissioner for Refugees
VIF	variance inflation factor
VL	Visceral leishmaniasis
WBC	White blood cells
WHO	World Health Organization

LIST OF ORIGINAL PUBLISHED PAPERS

The following publications were generated from the dissertation and published in the reputable international journals:

I. **Belay H,** Abera A, Aklilu E, Abdisa B, Belachew M, Sime H, Pareyn M, Bishaw T, van Henten S, van Griensven J, Tasew G, Erko B. (2025) Prevalence of *Leishmania* infection in refugee camps: A serological and molecular study in Gambella and Benishangul-Gumuz, Ethiopia. **PLoS Negl Trop Dis** 19(7): e0013280. https://doi.org/10.1371/journal.pntd.0013280

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Other related publications:

- **Belay, H.,** Eyelachew, E., Abose, E., Aklilu, E., Gebrewold, G., Tadesse, H., Tadese, A., Belay, R., Belachew, M., van Henten, S., and Bishaw, T., 2025. Serological and molecular analysis of *Leishmania* infection in a recent outbreak of visceral leishmaniasis in South Omo Zone, Ethiopia. ***Transactions of the Royal Society of Tropical Medicine and Hygiene***, *119*(1), pp. 65-76.
- **Belay, H.,** Abera, A., Aklilu, E., Bishaw, T., Alemu, A., Tasew, G., and Erko, B., 2025. Prevalence of asymptomatic *Leishmania* donovani infection and associated factors in Ethiopia: a systematic review and meta-analysis. ***BMC infectious diseases*****,** *25*(1):78.

CHAPTER 1: INTRODUCTION

1.1. Background

Visceral leishmaniasis (VL), also referred to as kala-azar, is a systemic parasitic disease caused by the protozoan parasites of the *Leishmania donovani* complex and transmitted by *Phlebotomus* sand flies (Ready, 2014). Globally, VL remains one of the most severe neglected tropical diseases, producing high morbidity and, if untreated, substantial mortality; it is also closely linked with poverty, malnutrition, and immunosuppression, which increases the risk of clinical disease and relapse (WHO, 2010).

VL is predominantly concentrated in Brazil, East Africa, and India. Annually, an estimated 50,000 to 90,000 new VL cases occur worldwide. However, only 25-45% of these cases are reported to the World Health Organization (WHO) each year (WHO, 2023). Between 2002 and 2024, eighty-four countries reported VL cases at least once to WHO. A 2024 report highlighted that out of 11,793 VL cases reported from 49 countries, the number of cases per country ranged from zero to 3,571. About 90% of VL cases in 2023 were reported from Sudan, Ethiopia, Brazil, Kenya, South Sudan, Somalia, India, Eritrea, Yemen, and Uganda. Among these, the majority of the cases were from East Africa, which represented about 79% of the total reported cases from these countries. Remarkably, Sudan, Ethiopia, and Kenya reported the highest number of cases, each exceeding 1,200 in the latest WHO report (WHO, 2024a).

In East Africa, particularly in Sudan, South Sudan, Ethiopia, Kenya, and Somalia, *L. donovani* transmission is largely anthroponotic and sustained by local sand fly vectors in distinct foci (WHO, 2024a). In this region*, Phlebotomus orientalis, Ph. martini,* and *Ph. celiae* are well-established vectors for the transmission of VL, depending on their ecological preferences (Hailu et al., 2009; Sunyoto et al., 2017; Hammam & Hicks, 2021). Similarly, these vector species have been implicated in VL transmission in Ethiopia; their relative importance varies with ecology and geography. Vector ecology (breeding/resting sites, seasonal abundance) and human environment interactions, therefore, determine local

transmission intensity and the spatial footprint of VL (Gebre-Michael et al., 1993; Pareyn et al., 2020; Aklilu et al., 2023).

Historically, VL in Ethiopia concentrated in specific lowland belts (Humera-Metema in the northwest and some lowland areas of the south) (Fuller et al., 1976, 1979; Ali & Ashford, 1993). Over the past decades, epidemiological investigations have revealed that VL has spread to six recognized endemic regions. This could be partly due to climate change, limited awareness of the transmission of VL, particularly among the migrant daily laborers to the endemic regions, and greater ecological plasticity of key vector species. The vector *Ph. orientalis* in particular has been recorded in a wider range of habitats and at higher elevations than previously thought, which may facilitate the north-south and low- and highland spread of infection risk (Ali et al., 2005; Dawit et al., 2013; Gebresilassie et al., 2015). These shifts have important implications for control and surveillance because previously non-endemic or low-risk areas may harbor undetected transmission.

Seroprevalence studies reported in Benishangul-Gumuz Regional State also revealed the expansion of VL in this area. This region is undergoing significant social and ecological changes due to large-scale development projects attracting many temporary laborers and settlers. Therefore, careful attention is required regarding the potential introduction or emergence of VL transmission in the area (Abera et al., 2016a). Following this case report, the authors extended their VL assessment in the region to further investigate the transmission dynamics and disease burden. The VL in the region is considered an emerging disease, with a reported community-based prevalence of 7.3% (Abera et al., 2016b).

Visceral leishmaniasis is influenced by a range of biological, environmental, and socioeconomic risk factors. Host-related factors such as malnutrition, immunosuppression (particularly HIV co-infection), young age, and male sex increase susceptibility and disease severity (Lemma et al., 2015; Welay et al., 2016). Environmental determinants, including poor housing conditions, close proximity to vector habitats, deforestation, and climate variability, enhance exposure to infected sand flies (Alemayehu & Alemayehu, 2017; Yared et al., 2017). Poverty, limited access to healthcare, and population movements (migration, displacement, or conflict) further exacerbate vulnerability by increasing transmission and

hindering timely diagnosis and treatment (WHO, 2024). Additionally, zoonotic reservoirs, particularly infected dogs in some endemic regions, play a key role in sustaining transmission (Lemma, 2018; Najafi et al., 2021). Together, these complex risk factors shape the epidemiology of VL and highlight the need for integrated control strategies addressing both biological vulnerability and social determinants of health.

Refugee and humanitarian settings faced particular challenges for VL control. Displaced populations frequently live in overcrowded, poorly sheltered camps with limited access to diagnosis and treatment, and they often occupy marginal lands with environmental features favorable to sand flies. Previous studies (Hailu et al., 1996) conducted in Gambella refugee camps have documented measurable *Leishmania* infection, including a substantial proportion of asymptomatic infections detected by serological and leishmanin skin test (LST) methods. In this survey, seroprevalence was 18.1% and 34.4% using LST among refugee populations and a high proportion of asymptomatic infections. These observations underscore the need for active case detection, targeted vector surveillance, and context appropriate interventions in refugee camps.

In addition to sand flies being linked to various ecotypes for resting and breeding (Hoogstraal & Dietlein, 1964; Hailu et al., 1995; Hassan et al., 2007; Hammam & Hicks, 2021;), factors such as seasonal population dynamics, microhabitat heterogeneity within and around human settlements, and human behaviors could influence human and vector contact, thereby increasing the risk of infection. In refugee camps, rapid environmental alteration, settlement layout, and shelter construction can create novel microhabitats favorable to sand flies, but systematic entomological data from many camps remain sparse (Hassan et al., 2007).

Therefore, there is a clear need for comprehensive studies that combine active human case detection (including using molecular diagnostics), systematic entomological surveillance, species identification, abundance and infection rates, and associated factors assessment in refugee camps in Gambella and Benishangul-Gumuz Regional States. Such evidence is essential to refine risk maps, inform targeted vector control and surveillance strategies in humanitarian settings, and support national and regional efforts to reduce the burden of VL

and move towards elimination targets. The present study addresses these gaps by investigating the epidemiology of VL and the sand fly vector in selected refugee camps of Gambella and Benishangul-Gumuz Regional States of Ethiopia, thereby providing data directly relevant to local control planning and regional elimination frameworks.

1.2. Statement of the problem

A strategy for the elimination of VL as a public health problem has now been developed by WHO for East Africa (WHO, 2024b). Ethiopia is committed to eliminating VL in the country. However, the burden of VL is not well known. Efforts for management of VL in Ethiopia have been limited to diagnosis and treatment, partly due to significant knowledge gaps on transmission hotspots and dynamics. Hence, there is a need to understand the epidemiology of VL in every corner of the country, including refugee camps. Moreover, studies revealed that there is a strong relationship between civil unrest and VL transmission (Al-Salem et al., 2016). The VL infection often arises when immunologically naïve migrants are displaced into VL endemic areas or when *Leishmania*-infected individuals move into new areas and establish additional foci of transmission. In East Africa, the situation is further affected by limited access to timely diagnosis and effective treatment, HIV co-infection, food insecurity, malnutrition, and suitable transmission ecotypes (UNHCR, 1997; Al-Salem et al., 2016; Alvar et al., 2012).

The United Nations High Commissioner for Refugees (UNHCR) reported that there are more than 122 million displaced populations; of these, 37.4 million people are refugees across the globe in 2023. The government of Ethiopia opens its borders for refugees, being the third-largest refugee-hosting country in Africa, accommodating more than one million refugees and asylum seekers, mainly from South Sudan, Sudan, Somalia, and Eritrea. These refugees, particularly those from Sudan and South Sudan, have come from VL endemic foci (Al-Salem et al., 2016). Gambella and Benishangul-Gumuz Regional States have hosted nearly half of these refugees (48%) (UNHCR, 2025). Environmental risk assessments of VL transmission defined these two regions as high-risk areas (Tsegaw et al., 2013). Moreover, previous studies in these areas have detected a range from 3.2% to 34.4% prevalence of asymptomatic *Leishmania* infection using serological and LST

methods (Hailu et al., 1996; Abera et al., 2016; Bejano et al., 2021). Furthermore, primary and relapse VL confirmed cases continue to be reported annually to the Ethiopian Ministry of Health (MoH unpublished data). However, the distribution of *Leishmania* infection, factors associated with the VL infection, and systematic entomological surveillance are still limited in refugee settings within the Gambella and Benishangul-Gumuz Regional States. This scarcity of data hinders the development and implementation of evidence-based public health interventions, which are a key component of the WHO's strategy for the elimination of VL as a public health problem in East Africa (Alvar et al., 2021). Therefore, we investigated the epidemiology of VL and the species composition, relative abundance, and habitat preference of sand flies as well as screened natural infections of *Leishmania* DNA in sand flies from refugee camps in Gambella and Benishangul-Gumuz Regional States, Ethiopia.

1.3. Significance of the study

This study emphasizes the involvement of multiple pillars of collaboration among epidemiologists, public health responders, entomologists, researchers, and humanitarian workers to address the complex challenges caused by VL in these refugee camps. These stakeholders will be able to gain a more comprehensive understanding of the disease transmission dynamics and distribution, leading to more effective interventions based on the current study.

Moreover, the finding could serve as baseline information for future research, motivating additional investigations into the socioeconomic and environmental determinants of VL in other similar settings. This may include exploring how climate change, population displacement, and living conditions contribute to the spread of VL in the local area. This type of evidence would not only benefit situations in the refugee camp but also inform the global strategies for managing VL in other high-risk areas. The current report provides the critical need for sustained funding and resource allocation to maintain and expand control measures, particularly in this VL elimination era in the East African region. Advocacy efforts driven by this evidence could help secure the necessary support from donors and organizations in any corner, ensuring that the fight against VL elimination remains a

priority in humanitarian health issues. Additionally, in the long term the lesson learned from this finding could contribute to the development of more resilient health and care systems in the refugee camps that could be capable of addressing not only VL but also other emerging health threats. The current study showed its potential by providing a proactive and evidence-based approach to create a lasting impact on the health of vulnerable populations globally, particularly refugee camps in the study areas.

1.4. Research questions

This study was guided by the following research questions:

- What is the prevalence of *Leishmania* infection among refugee populations in selected camps of the Gambella and Benishangul-Gumuz regions?
- Which socio-demographic (e.g., age, gender, occupation) and environmental (e.g., proximity to vegetation, housing structure) factors are significantly associated with the risk of VL in these settings?
- What are the species composition, density, and distribution of sand flies within the domestic, peridomestic, and sylvatic environments of these refugee camps?
- What are the *Leishmania* infection rates of sand flies in the refugee camps during the study periods?

1.5. Objective of the study

1.5.1. General objective

- The general objective of this study was to assess the epidemiology of visceral leishmaniasis and sand fly vectors in selected refugee camps of the Gambella and Benishangul-Gumuz Regional States, Ethiopia, to provide robust evidence-based support for the development of effective disease control and prevention interventions.

1.5.2. Specific objectives

- To determine the prevalence of *Leishmania* infection in the selected refugee camps in Gambella and Benishangul-Gumuz Regional States of Ethiopia
- To identify the significant explanatory for VL among cases in selected refugee camps in Gambella and Benishangul-Gumuz Regional States of Ethiopia
- To determine the sand fly fauna in the selected refugee camps in Gambella and Benishangul-Gumuz Regional States
- To determine the prevalence of *Leishmania* infection in sand flies using real time polymerase chain reaction (rtPCR) techniques in the selected refugee camps in Gambella and Benishangul-Gumuz Regional States

CHAPTER 2: LITERATURE REVIEW

2.1. Overview of leishmaniasis

Leishmaniasis significantly affects health in poor, remote regions with limited access to healthcare (WHO, 2023). The disease exhibits a broad clinical spectrum, determined by the parasite species, tissue tropism, host immune status, and geographic region. Clinically, leishmaniasis is classified into cutaneous (CL), mucocutaneous (MCL), visceral (VL), post-kala-azar dermal leishmaniasis (PKDL), diffuse cutaneous leishmaniasis (DCL), and viscerotropic or atypical forms. Each form presents distinct clinical manifestations and epidemiological characteristics (Desjeux, 2004; Alvar et al., 2012).

In the clinical term the disease is also known as Kala-azar or black fever or Dumdum fever. It is the most severe form of leishmaniasis, where the visceral organs like bone marrow, spleen, and liver are affected and can be fatal if left untreated (Steverding, 2017). In the old world, *L. donovani* and *L. infantum* are well-documented causative agents for the infection. In the new world, *L. chagasi* is the major causative agent of the disease (Ngure et al., 2009). VL patients may develop a complicated form of the disease called post-dermal leishmaniasis (PKDL). These patients have a public health importance for the transmission of the disease and are known as a risk factor for the community. It is common in Sudan (50%) and India (5-10%), where *L. donovani* is a major causative agent for VL (Kordofani et al., 2001; Pandey et al., 2012). Although this event has been reported in immunocompromised patients, it appears to be rare in immunocompetent individuals. The occurrence time varies from region to region; in Sudan PKDL is manifested within six months after treatment, whereas in India it may take up to three years (Ngure et al., 2009). On the other hand, the occurrence of PKDL in Ethiopia is also common in endemic regions. According to the WHO report, between 1998 and 1999 in northern Ethiopia, PKDL occur in an average of 13% of patients with VL and in 27% of patients coinfected with HIV/VL; however, these proportions do not represent the true number of cases since mild PKDL is often not treated and consequently not reported (WHO, 2012).

The second clinical form of leishmaniasis is cutaneous leishmaniasis (CL). It is widely distributed across the globe. According to WHO, over 95% of new cases of CL were

reported from Brazil, Iran, Colombia, Afghanistan, Syria, Iraq, and Algeria in 2017. Cutaneous leishmaniasis is a relatively less severe and often self-healing form of leishmaniasis compared to VL. Clinically it is characterized as localized (LCL), muco-cutaneous (MCL), and diffused (DCL) type of presentations. The clinical manifestation started from where the sand fly bites and includes chronic ulcers, which may create lifelong scars that may cause social stigmas, cosmetic morbidities, and psychological effects to the patient (van Henten et al., 2018; WHO, 2023). The disease may not be fatal, but there are rare records of mortality due to secondary infection (Karunaweera and Ferreira, 2018). There is a variety of parasite species responsible for the transmission and cause of the disease, including *L. aethiopica, L. major,* or *L. tropica* in the Old World (Karunaweera and Ferreira, 2018; Steverding, 2017). Whereas *L. braziliensis, L. guyanensis, L. panamensis, L. mexicana,* and *L. amazonensis* cause CL in South America. Cutaneous leishmaniasis affecting dogs, also referred to as canine CL, in South America is caused by *L. braziliensis* and *L. chagasi* (Gibson, 1983; Steverding, 2017).

Mucocutaneous leishmaniasis (MCL) is a rarely reported clinical manifestation of leishmaniasis. The disease caused by *L. braziliensis, L. amazonensis*, and *L. mexicana* is known to be a severe form of MCL (Steverding, 2017). It is characterized by its impact on the mucosal areas of the body, leading to either partial or complete destruction of mucosal linings in the nose, throat, and mouth. MCL could be life-threatening with massive lesions that could lead to permanent disfigurement (WHO, 2023; CDC, 2024). This clinical manifestation needs to be tested early and treated appropriately to minimize its impact on the public health. Cases of MCL are endemic in regions of Latin America, especially Bolivia, Brazil, Ethiopia, and Peru. Mucocutaneous leishmaniasis, also known as espundia or mucosal leishmaniasis, is caused mainly by the *Viannia* subgenus but occasionally by other *Leishmania* species. It is most common in the south and west of the Amazon basin, precisely in parts of Bolivia, Peru, and Brazil. The parasites are following the lymphatics and blood to affect the nasopharyngeal tissues from the skin lesion. It may take from months to years after the occurrence of skin lesions to invade the mucosal areas of the body (Ngure et al., 2009; Beldi et al., 2017; Carstens-Kass et al., 2021; Castelli et al., 2021; Gupta et al., 2022).

2.2. Epidemiology of Visceral Leishmaniasis

2.2.1. Global Epidemiology

Visceral leishmaniasis primarily affects impoverished populations in Africa, Asia, and Latin America (Fig. 1). According to the 2023 WHO update, 80 countries are globally endemic for VL. An estimated 50,000 to 90,000 new cases of VL occur each year. The disease puts over 600 million people at risk globally (WHO, 2023). The primary eco-epidemiological hotspots for VL in 2023 are Eastern Africa, including Djibouti, Chad, Eritrea, Ethiopia, Kenya, Somalia, South Sudan, Sudan, and Uganda, accounting for 73% of global cases; Brazil, contributing 12%; and the Indian subcontinent, encompassing Bangladesh, India, and Nepal, which reported 6% of the cases (WHO, 2024a).

According to Wu et al. (2024), the global risk prediction model indicated that the risk distribution map for VL highlighted several high-risk areas. The majority of the population at risk were found in the Mediterranean region, the Arabian Peninsula, Asia, and South America. It was estimated that 5.3 billion people were at risk of developing VL. Among them, 3 billion reside in Asia, mainly in China and India. In Africa, around 594 million individuals are at risk of VL, with a significant portion living in Ethiopia. South America has over 271 million people in areas susceptible to VL, with about 95% of this population residing in Brazil (Wu et al., 2024).

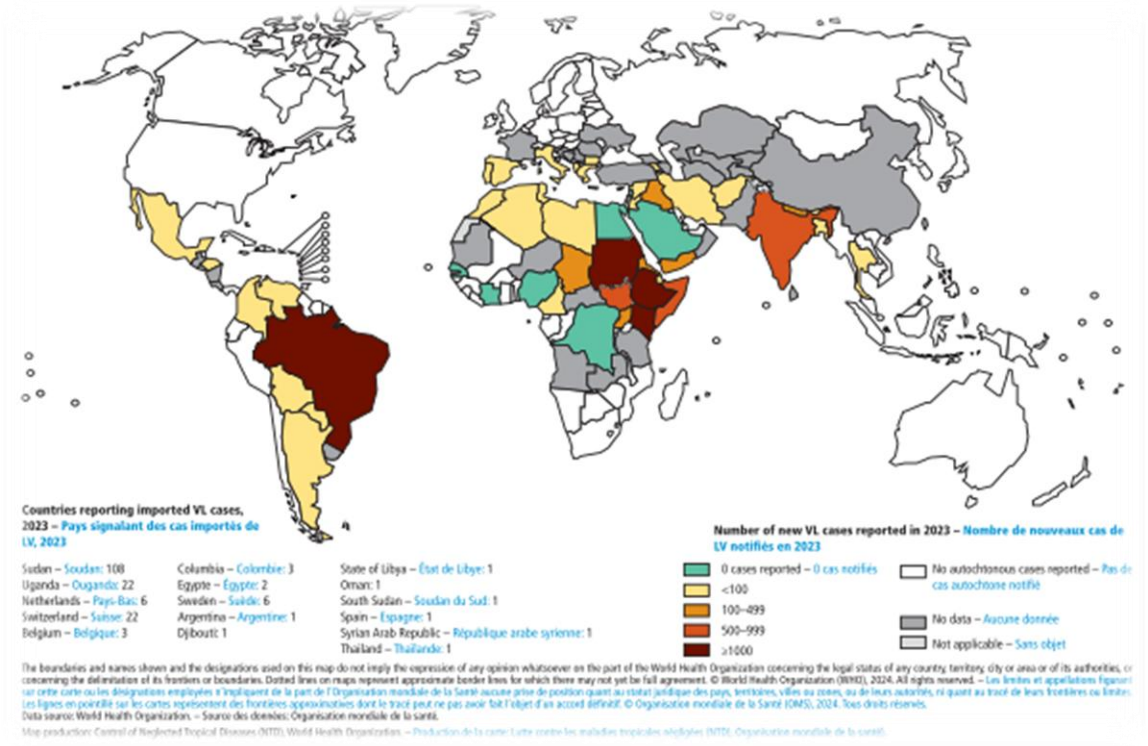


Figure 1: Global distribution and endemicity status of visceral leishmaniasis (VL), 2023 (reported as of November 2024) (Jain et al., 2024)

2.2.2. Distribution of Visceral Leishmaniasis in East Africa

After the successful elimination of VL in the Indian Subcontinent, the global epidemiology of VL shifted to the East African region, which bears the highest global disease burden. The primary causative agent in this region is the *L. donovani* species, with anthroponotic transmission being the most common mode of spread (Alvar et al., 2012; Burza et al., 2018). The epidemiological pattern and potential vectors of VL vary across countries. In Uganda and Somalia, transmission has been reported across multiple regions, although data on VL is limited due to insecurity and insufficient health surveillance systems (Marlet et al., 2003; Kolaczinski et al., 2008; Al-Salem et al., 2016; Alvar et al., 2021). South Sudan has experienced frequent VL outbreaks with the highest VL mortality, the disease being reported particularly in Upper Nile, Jonglei, Unity, and Eastern Equatoria. These outbreaks have largely been driven by refugee movement and civil unrest, with significant occurrence

during the 1980s and 1990s that resulted in tens of thousands of deaths (Ahmed et al., 2016, 2022a, b; Alvar et al., 2021).

Visceral leishmaniasis remains a significant public health challenge in Sudan, with a significant number of cases reported annually to the WHO. In 2023, among the reported cases of VL in East Africa, Sudan accounted for nearly 44% (WHO ,2024a). The country is highly endemic, particularly in regions west of its neighboring country, Ethiopia. In addition, the states of Gedaref, Kassala, and Sennar in Sudan have long been recognized as endemic regions for VL, with significant outbreaks documented since the 20th century. The disease continues to cause a serious public health challenge, contributing to considerable mortality during epidemics. The estimated annual incidence for VL is 15,700-30,300 (Alvar et al., 2012). In 2015, the incidence rate was approximately 33 cases per 100,000 population, with one-fourth of the population at risk of country (Jones & Welburn, 2021). On the other hand, the national VL pooled prevalence was reported as 37.3% (95% CI: 14.6-60.0) (Geto et al., 2024).

Kenya has been significantly impacted by VL compared to other East African countries, reporting approximately 15.3% of cases to the WHO by 2023, ranking after Sudan and Ethiopia (WHO, 2024a). A recent systematic review showed a pooled VL prevalence of 30.1% (95% CI: 19.2-41.1) (Geto et al., 2024). Whereas, the population at risk is more than 5 million people in Kenya, with nearly 30 people per 100,000 population prevalence rates (Jones & Welburn, 2021). In Kenya, outbreaks are often linked to nomadic pastoralist communities and limited access to healthcare services (Marlet et al., 2003; Kolaczinski et al., 2008; Al-Salem et al., 2016; Alvar et al., 2021).

In Ethiopia, VL remains endemic across most of its lowland regions. Its distribution ranges from sporadic occurrences to consistent cases in northwestern Ethiopia, particularly in areas such as Humera, Metema, and Gondar along the Sudanese border, as well as in southwestern Ethiopia (Ali, 1997; Ali & Ashford, 1993, 1994; Ali et al., 2005; Abbasi et al., 2013; Alebie et al., 2019). Ethiopia ranked second among East African countries in reporting VL cases to WHO, with a total number of 1,482 (18.1%), next to Sudan (WHO, 2024a). This figure is considerably lower than previous reports, which ranged between

3,700 and 7,400 cases (Belay et al., 2025c). This decline may be attributed to challenges in case reporting and/or limited access to services in the northern region of the country, an area responsible for over 60% of the reported cases (Abbasi et al., 2013). Previous studies estimated the likelihood of VL cases in Ethiopia at approximately 63 per 100,000 individuals in endemic regions (Jones & Welburn, 2021). Currently, there is no consolidated national prevalence data for VL in Ethiopia. However, a recent pooled analysis revealed a prevalence of 20.6% (95% CI: 13.3-27.8), which is notably lower than the East African pooled estimate (Geto et al., 2024). Before a decade, serological evidence of the disease has been reported in Benishangul-Gumuz region followed a case report (Abera et al., 2016; Bejano et al., 2021) and was previously reported in the Gambella region (Hailu et al., 1996). In addition to the developmental corridor, these areas host a significant number of refugees from various East African countries, majority from South Sudan and Sudan (Fig. 2).

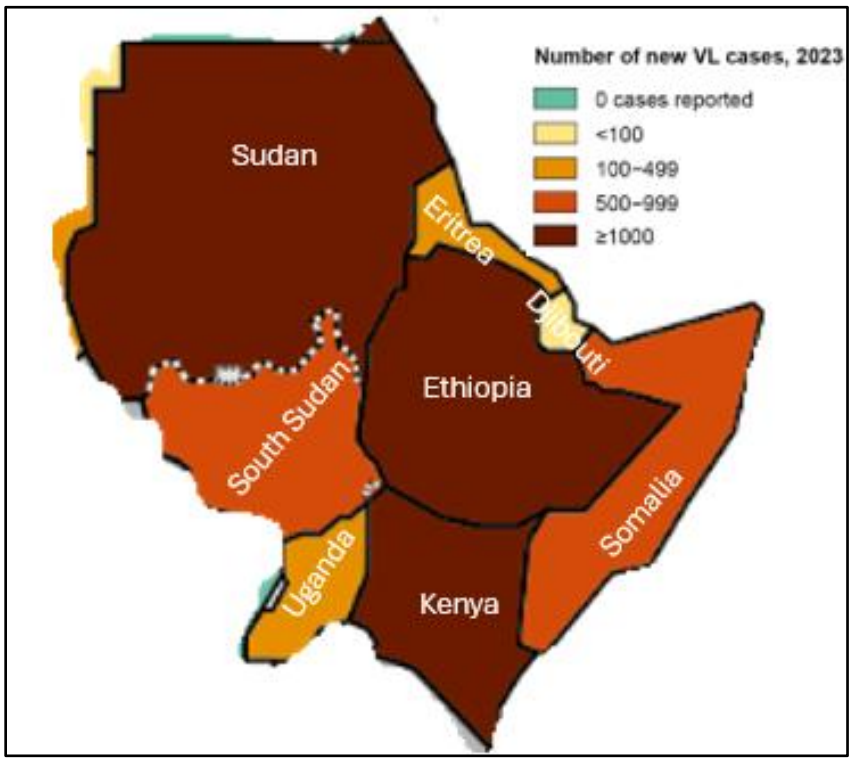


Figure 2: Distribution of visceral leishmaniasis in East Africa (Wu et al., 2024)

2.2.3. Current epidemiology of VL in Ethiopia

Visceral leishmaniasis is an ongoing public health problem in Ethiopia. The burden of the disease, its distribution, the risk of expansion into new areas, and predictions were studied in various regions of Ethiopia, including the known endemic and non-endemic foci (Fig. 3). The annual incidence of VL in Ethiopia is 3700-7400 cases (Alvar et al., 2012). According to the risk map by (Wu et al., 2024), Ethiopia is one of the countries affected by VL, similar to Brazil, Iran, and India. The burden of VL is aggravated by various factors, including comorbidities with HIV (Mengistu & Ayele, 2007; Van Griensven et al., 2019), malaria (Aschale et al., 2019), malnutrition (Ali, 1997; Feleke, 2019), and migration (Yimer et al., 2024).

A study conducted in the Amhara region showed that VL is distributed in low- and highland areas. Facility-based retrospective data from January 2010 to August 2015 in the West Armachiho district showed that 21% were positive for the rK39 antibody test among suspected VL cases and 1.2% for PKDL (Gize et al., 2020). Another retrospective study conducted in Metema and Addis Zemen from 2017 to 2021 showed that 32.4% are serologically (rK39) positive cases (Yimer et al., 2022). Four-year retrospective data from the Waghemra zone of the Amhara region showed that about 23.4% of screened VL-suspected cases are positive for VL (Debash et al., 2023).

Although VL cases have been reported to the MoH, only a few studies conducted in selected sites of the lower Awash Valley area have explored exposure to the *Leishmania* parasite, revealing an overall prevalence of approximately 40%. Among the study sites, the distribution using the leishmanin skin test reaction showed 50.8% in Mile, 37.8% in Dubti, and the lowest, 24.7%, in Asayta. In the region, the ascending positive leishmanin reaction rate with age and higher prevalence in indigenous Afar with a relative majority in males parallels findings in other endemic areas and reflects the relevance of outdoor exposure to VL infection (Ali et al., 2005).

In the Oromia region, a retrospective cohort study was conducted to address the number of VL cases registered over time (2013-2018) and the clinical profile, type of treatment used, and response to treatment in the region from three treatment hospitals, namely, Negelle,

Yabello, and Ginnir hospitals. Out of the 188 VL case charts reviewed, the majority (51.6%) were children, with only three cases identified as VL relapses. Comorbidities were noted among these cases, including severe acute malnutrition (27.1%), tuberculosis (6.4%), and malaria (6.4%). The report indicated that patients originated from 24 surrounding districts. However, the names of the districts were not stated in the report, which limited understanding of the true burden and distribution of the disease in the region (Tekalign et al., 2020).

Southern Ethiopia (formerly known as the Southern Nations, Nationalities, and Peoples' Region (SNNPR)) has been known to be the most affected region in the country. The disease is distributed in many areas of southern Ethiopia: Konso district (Aba Roba) (Hailu et al., 2009), Hamar and Banna-Tsamai districts (Bekele et al., 2018), and other areas including Lake Abaya, Dawa Valley, Galena Valley, and Weyto Valley (Leta et al., 2014). In addition, in the eastern part of Ethiopia, in the Somali region, a VL outbreak was reported in 2001 in the Afder, Liben, Denan, and Hagele areas, bordering Kenya and Somalia (Boussery et al, 2000; Boussery et al., 2001; Alebie et al., 2019). According to the finding, the Shebele zone in the Somali region became a potential new VL-transmission focus in Ethiopia (Alebie et al., 2019).

In the Tigray region, VL cases were found in many areas, including the Humera plains (Kafta Humera and Tsegede, Tahtay Adiabo districts), the Shiraro district, and Raya-Azebo (Fuller et al., 1976; Leta et al., 2014; Berhe et al., 2018). In the lowland areas of Northwestern Ethiopia, where there is intensive migration of the labor force, the incidence of the disease is very high, and the endemic nature of the disease has been reported in many areas of this region.

In addition to the major VL endemic regions, there are also reports indicating the expansion of transmission to other areas in Ethiopia. A study conducted by (Abera et al., 2016a), following a confirmed VL case report from the Benishangul-Gumuz region, revealed a prevalence of 7.3% among 275 participants from the Dangur and Guba districts, based on LST and serological tests. Among the 20 individuals infected with VL, nine reported no history of travel (Abera et al., 2016b). Similarly, another epidemiological assessment

conducted in this region showed that, among 253 individuals tested across six districts, 15 (5.9%) and eight (3.2%) were positive for VL using the DAT and rK39 testing methods, respectively. In addition, 6.0% (72/1,197) of individuals were exposed to *Leishmania* infection as determined by the LST. Notably, among households with LST-positive individuals, five dogs were also seropositive for *Leishmania* infection (Bejano et al., 2021).

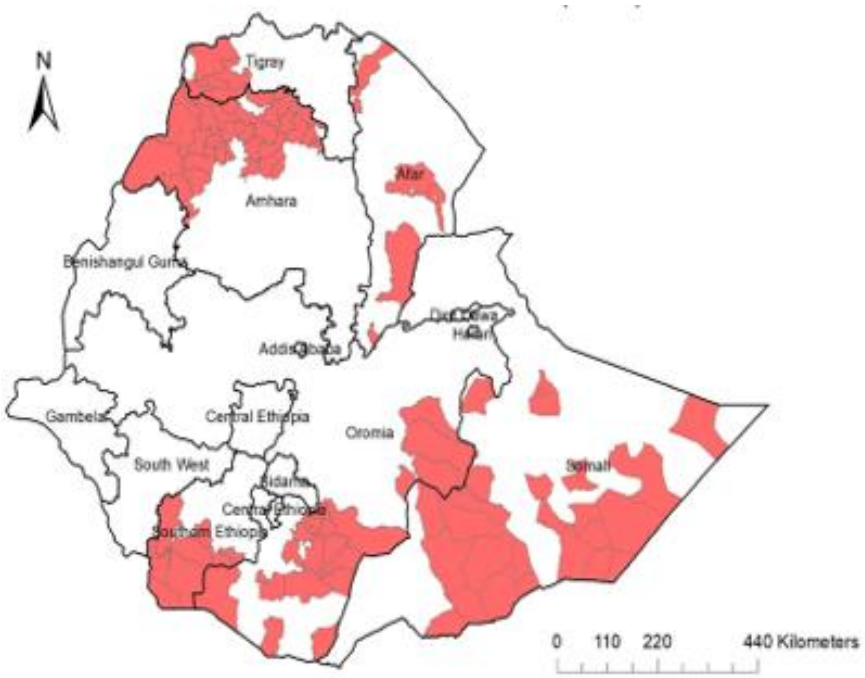


Figure 3: Visceral leishmaniasis endemic regions in Ethiopia (FMoH, 2025)

The clinical features of VL vary significantly across Ethiopia, with distinct clinical presentations and treatment responses in the northern and southern regions. For example, a report from three treatment centers in the Southern Oromia region showed that the most reported symptoms are fever (62.0%), splenomegaly (79.0%), and hepatomegaly (46.0%) (Tekalign et al., 2020). Whereas, reports from northern Ethiopia showed that fever, weight loss, jaundice, bleeding episodes, splenomegaly, and lymphadenopathy (Hurissa et al., 2010; Tarekegn & Tamene, 2021) were the most reported symptoms. But hepatomegaly was not reported from the Amhara and Tigray regions; on the other hand, lymphadenopathy was not reported in the Oromia region (Hurissa et al., 2010; Tekalign et al., 2020; Tarekegn & Tamene, 2021). This could relate to differences in circulating parasite strains, human

genetic background, or prevalence of concurrent conditions such as malnutrition (Tekalign et al., 2020; Tarekegn & Tamene, 2021). A retrospective study conducted in three treatment centers in Oromia region reported that fever (self-reported), splenomegaly, hepatomegaly, and jaundice manifested in many of the VL patients (Tekalign et al., 2020).

Regarding the distribution of asymptomatic *Leishmania* infection in Ethiopia, we generate information to be considered as a challenge for the elimination of the VL initiative as occult transmission in Ethiopia. The overall pooled prevalence of asymptomatic *Leishmania donovani* infection in Ethiopia was estimated to be 9.0% (Belay et al., 2025b). The prevalence varied by region and study population: the pooled estimate was 9.0% in the Amhara region, slightly higher than the 7.0% found in other regions (Benishangul-Gumuz, Oromia, Somali, SNNP, and Tigray), and the highest prevalence was observed among blood donors (12.0%). The study identified two factors significantly associated with a higher likelihood of asymptomatic *L. donovani* infection: living in a household with domestic animals (Odds Ratio [OR]: 2.54; 95% CI: 1.43–3.64) and being male (OR: 2.22; 95% CI: 1.21–3.23). These findings underscore the substantial presence of asymptomatic carriers who can serve as hidden reservoirs for the parasite, potentially complicating VL control and elimination in Ethiopia, leading to the conclusion that regular screening of people living in close contact with animals is essential for disease control and management (Belay et al., 2025b) (Fig. 4).

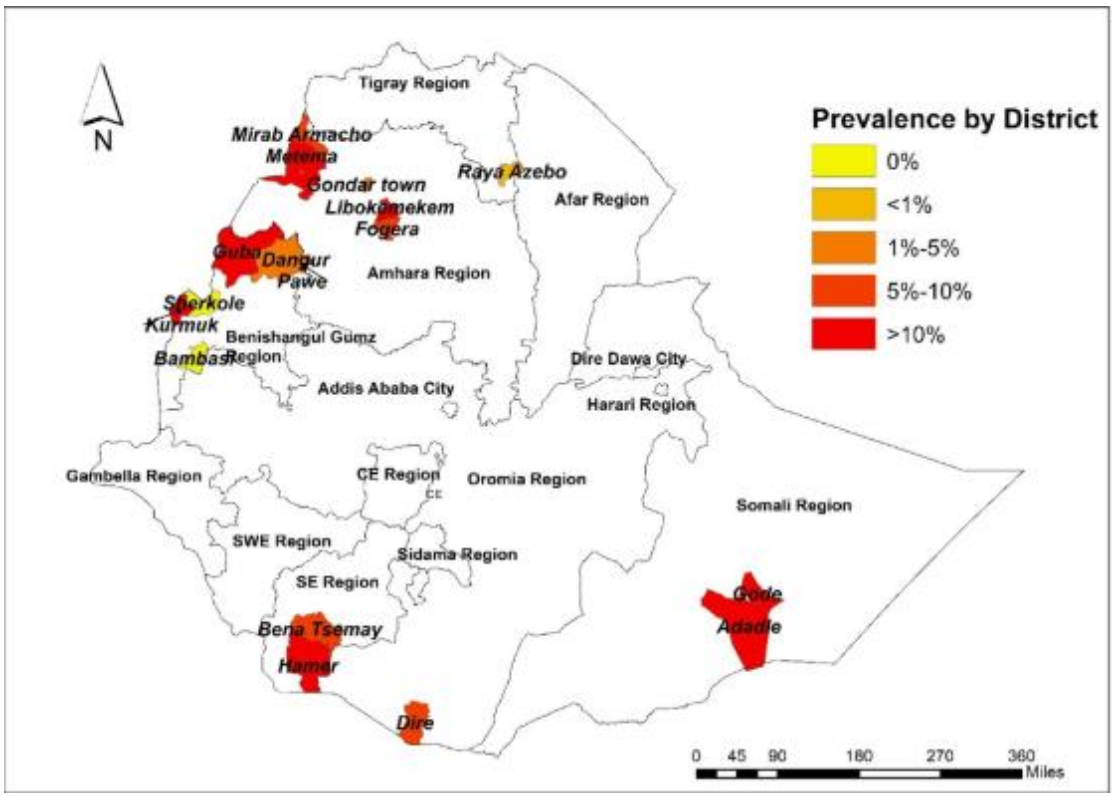


Figure 4: Distribution of asymptomatic *Leishmania* infection in Ethiopia from 2012 to 2024 (Belay et al., 2025b)

2.2.4. Epidemiology of VL in refugee camps

Visceral leishmaniasis disproportionately affects vulnerable populations, including those living in conflict-affected and displaced settings (WHO, 2023). Refugee camps provide unique ecological and social conditions that favor the transmission and persistence of VL. The convergence of susceptible host populations, poor living conditions, and high exposure to sand fly vectors creates an environment where outbreaks are frequent and often severe (UNHCR, 1997; WHO, 2023). The complex epidemiology of VL, involving human hosts, animal reservoirs, and sand fly vectors, is profoundly disrupted by humanitarian crises. Refugee and Internally Displaced Persons (IDP) camps, characterized by overcrowding, malnutrition, and environmental degradation, create a crisis for VL epidemics (UNHCR, 1997).

Limited studies document major VL epidemics in refugee settings. For instance, in South Sudan during the 1980s and 1990s, VL outbreaks in displaced populations caused tens of thousands of deaths, with case fatality rates exceeding 90% in untreated individuals (Seaman et al., 1996; Kolaczinski et al., 2008). In recent years, population mobility has occurred worldwide, including refugee crises affecting especially East Africa. Consequently, VL is spreading globally like other infectious diseases that have significance for public health (Al-Salem et al., 2016; Altare et al., 2019; Shetty, 2019).

A study was conducted to assess the distribution of VL using leishmanin skin tests (LST) and serological surveys for VL in selected locations in refugee camps, resettlements, and host communities in Gambella, Western Ethiopia, between 1989 and 1993. The findings showed that a total of seven cases of VL were detected among refugees. Refugees in this report showed the highest proportion in assessing the previous exposure to VL using LST, about 34.4% positive compared to 7.6% and 1.7% LST positivity among local populations and resettlers from other Ethiopian regions, respectively. Whereas the seroprevalence was also higher among refugees, 18.1% using ELISA/IFAT (Hailu et al., 1996). A study conducted in Turkey showed a 10.6% VL positivity rate among the Syrian refugees settled in the country. The study revealed that *L. donovani* is a causative agent of VL, and this species was not previously reported in Turkey. It was assumed that the emergence of *L. donovani* was partly due to the influx of Syrian refugees (Koltas et al., 2014).

Leishmania was detected from naturally infected sand flies in refugee camps located in Greece. A total of 10 sand fly pools were found positive for *Leishmania* DNA, all originating from the Thessaloniki refugee camps (43% Lagadikia and 52% Diavata). No parasite detection occurred in the 240 pools analyzed from the broader Thessaloniki metropolitan region. Moreover, *Leishmania* parasites were also detected in the dissected sand flies of the species *Ph. perfiliewi, Ph. tobbi,* and *Ph. simici* in these camps, indicating a late-stage transmissible infection (Avgerinou et al., 2019).

Reports showed that there is a documented outbreak of suspected VL that began in April through May 2000 in three refugee camps (Ifo, Dagahaley, and Hagadera) located near Dadaab town in Kenya, which house an estimated 125,000 Somali refugees. Between April

1 and August 31, 2000, twenty-six probable (DAT-positive) VL cases were observed, and 8 additional cases were parasitologically confirmed. The report showed that the overall case-fatality rate was 10 (29.4%) of 34 probable and confirmed VL patients. The median age of the patients interviewed was 15 years. The outbreak involved both Somali refugees and six Kenyan citizens, five of whom were shepherds grazing cattle around Dadaab. Many patients (16 of 26, or 61.5%) were refugees who arrived in the camps after their symptoms began, suggesting they were infected in Somalia, particularly the Lower Juba region (Boussery et al., 2001). A systematic literature review focused on VL cases among refugees and immigrants in Türkiye, the world's largest hosting country for migrants. The analysis systematically reviewed research published between January 1, 2010, and December 31, 2022. Ninety-four positive VL cases were identified, with 19 cases among Syrian refugees. The entry of infected patients into a country where suitable vector species exist increases the risk of transmission from a public health perspective (Şakru et al., 2025).

2.3. Risk factors of visceral leishmaniasis transmission

The transmission of VL is driven by the complex interaction of biological, environmental, socioeconomic, and political factors. Several factors, including environmental changes and population migration between affected and unaffected regions, are thought to influence the spread of *Leishmania* infection. Additionally, the presence of drug-resistant parasite strains and weakened immunity, often due to malnutrition or HIV co-infection, play a significant role (Alvar et al., 2012, 2021). Population migration stands out as a particularly significant factor, especially considering the global scale of displacement reported by the United Nations High Commissioner for Refugees (UNHCR). In 2023, more than 122 million individuals were displaced worldwide, including 37.4 million refugees (UNHCR, 2025). Refugees, due to their frequent movement, inadequate living conditions, and limited access to healthcare, are highly vulnerable to infectious diseases. Visceral leishmaniasis has occasionally been reported in refugee camps (Boussery et al., 2001; Hailu et al., 1996). There is also a report that showed a strong relationship between civil unrest and VL (Al-Salem et al., 2016).

Across East Africa, VL transmission is closely associated with environmental factors, particularly the presence of *Acacia balanitis* forests and black cotton clay soils that provide breeding sites for *Ph. orientalis*. Seasonal agricultural migration, deforestation, and displacement due to conflict have also been implicated in expanding the disease's geographical range (Desjeux, 2004; Aversi-Ferreira et al., 2014; Wamai et al., 2020). The predisposing factors for transmission of VL in Ethiopia are reported in various findings. In areas where VL is endemic, more confirmed cases were distributed among younger age groups compared to the other age categories. This could potentially be due to late development of acquired immunity in this transmission setting. On the other hand, in areas where there are outbreaks or new foci, all age groups are susceptible, with most cases occurring in age groups that have regular contact with the sand fly locations (Ali & Ashford, 1994; Leta et al., 2014). Visceral leishmaniasis case distribution depends on the sex of the individuals. Males are more susceptible to developing VL. This could be due to the cultural and individual responsibility in that males are more engaged in outdoor activities, exposing them to bites of sand flies (Ali & Ashford, 1994; Terefe et al., 2015; Haftom et al., 2021).

Studies conducted in East Africa have identified various exposure factors associated with VL across different study locations and population groups. For instance, sleeping under an acacia tree at night (Argaw et al., 2013; Nackers et al., 2015), the presence of cracked walls, and living in large households have been highlighted as risk factors (Argaw et al., 2013; Leta et al., 2014; Yared et al., 2014). Similarly, findings from Kafta-Humera, a region in northern Ethiopia, indicate that among migrant agricultural workers, a lower level of education significantly increases the risk of infection (Argaw et al., 2013). On the other hand, the use of bed nets has been observed to have a protective effect for VL infection (Argaw et al., 2013). Broadly speaking, social and political influences, such as military activities and agricultural development projects, are major contributors to VL endemicity and outbreaks in both Sudan and Ethiopia (Al-Salem et al., 2016). Despite few reports showing the exposure factors of *Leishmania* infections in these areas, localized and current evidence on specific risk factors such as environmental exposures, behavioral practices, and demographic factors remains scarce in refugee populations. Without a clear understanding of these factors, interventions remain generalized and may fail to reduce the

infection rates effectively. Generating evidence to address this knowledge gap is important for designing effective *Leishmania* infection prevention strategies in refugee camps.

Diets deficient in protein-energy, iron, vitamin A, and zinc heighten the likelihood of infection, which in turn raises the risk of VL manifestation (Dawit et al., 2013; WHO, 2023). Moreover, another study depicted the strong association between severe malnutrition and intestinal parasitic infection and VL. Malnutrition and intestinal helminth infection down-regulate the Th1 cellular immune response. Down-regulation of the Th1 cellular immune response confers susceptibility to VL (Oghumu et al., 2016; Gupta et al., 2022). According to the report from northwest Ethiopia, the prevalence of malnutrition among VL patients was found to be high. The prevalence of severe malnutrition and intestinal parasitic infection among adult VL patients is significantly associated (Mengesha et al., 2014). Other similar findings showed that the prevalence of underweight among VL patients was 82%, and the prevalence of underweight among VL family members was 48.3%. The nutritional status of kala-azar patients was affected by intestinal parasites. The VL treatment guideline should consider supplying nutrients, including iron, as part of the curative intervention (Feleke, 2019).

There are also additional risk factors identified, such as the presence of sand flies and living in houses with cracked walls and waste disposal (Yared et al., 2014; Terefe et al., 2015). Poor housing and poor sanitary conditions may increase sand fly breeding and resting sites, as well as their access to humans. Other studies conclude that owning goats and having a large family size and number of days spent on the farm are associated with the occurrence of VL (Yared et al., 2014). Crowded housing serves as an excellent source of blood meals (WHO, 2023). Sleeping outside or on the ground may increase the risk of infection. This is especially more evident in nomadic populations and in men who work in agricultural or pastoral settings due to increased time spent outdoors and thus higher exposure to the sand fly. Part of a community that lives and/or has frequent contact with acacia trees and termite hills are at increased risk, since acacia trees and termite hills are common breeding and resting sites for certain species of sand flies (Leta et al., 2014).

Overall, VL transmission is not the result of a single determinant but rather the additive effect of parasite-host-vector dynamics, environmental suitability, and socioeconomic vulnerability. Understanding these interlinked determinants is essential for designing integrated interventions, including vector control, improved housing, early case detection, nutritional support, and strengthened health systems in endemic and refugee settings.

2.4. *Leishmania* parasite

Leishmania are protozoan parasites (Class Kinetoplastae, Order Trypanosomatida) with a digenetic life cycle involving an insect vector and a mammalian host. They exist in two principal forms: the motile, elongated promastigote (10-20 μm) and the non-motile, oval-shaped amastigote (3-7 μm in diameter). Promastigotes reside in the sand fly vector, differentiating into the infective metacyclic promastigote form (Bonfim et al., 2022; Thakur et al., 2020; Wamai et al., 2020). The species of the *Leishmania* parasite vary depending on the geographical location. The severity of the disease is influenced by the particular species of *Leishmania* involved. Globally, there are more than 20 species of the parasite that are able to cause a disease (WHO, 2023).

In 1903, Ronald Ross named the new parasite related to kala azar that was found in spleen pulp as *Leishmania donovani* (Gibson, 1983). The genus *Leishmania* has further been classified into two subgenera (Fig. 5). The distinction between the species is based on molecular techniques including zymodemes (iso-enzymes), schizodemes (kDNA analysis with restriction enzymes), serodemes (reactions with monoclonal antibodies), and rapdemes (using PCR with random primers) (Gibson, 1983). Earlier on, the details of parasite development in the sand fly gut were used for the classification. The species causing VL in the India and East Africa regions is *L. donovani*, while in the Mediterranean basin and Latin America it is *L. infantum* (Steverding, 2017).

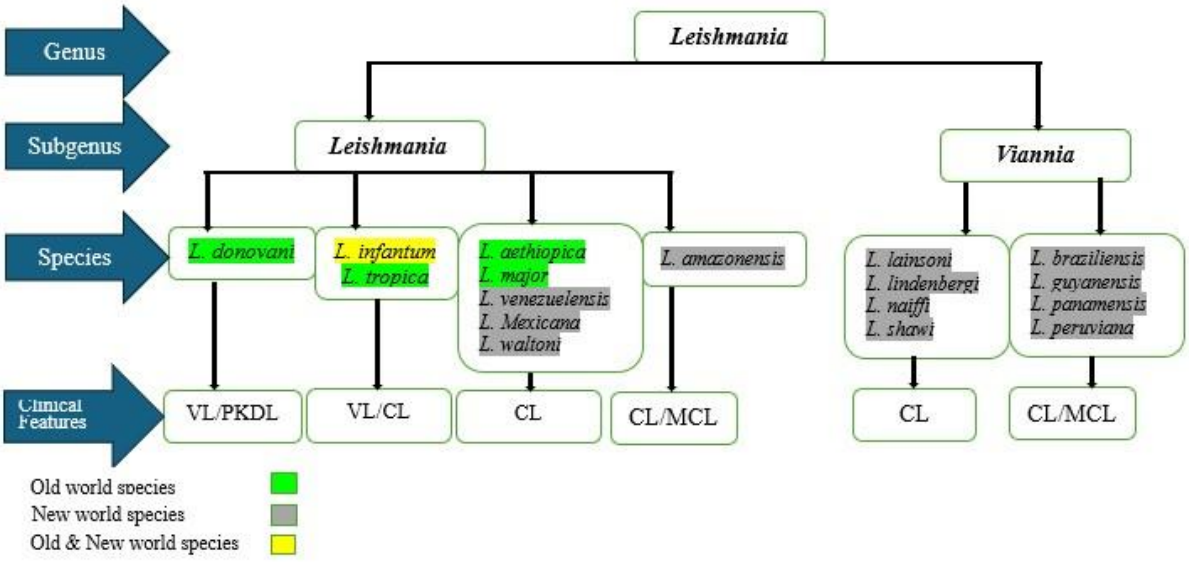


Figure 5: Schematic overview of *Leishmania* genus classification showing two subgenera including selected species commonly studied in biomedical research. (adopted from Steverding,2017, with some modification)

2.5. Transmission and Life cycle of *Leishmania* Parasites

2.5.1. Transmission of *Leishmania* Parasites

Visceral leishmaniasis is zoonotic, involving animal reservoirs like dogs and rodents, primarily in the Mediterranean basin and Latin America, where *L. infantum* is the causative agent. It is also anthroponotic, transmitted from human-to-human by vectors, mainly in India and East Africa, where *L. donovani* is the causative agent (Bern et al., 2008). The disease is mainly transmitted by female *Phlebotomus* spp. and *Lutzomyia* spp. sand flies. However, transmission of the disease is rarely by syringe sharing, blood transfusions, or from mother to fetus (Gupta et al., 2022).

2.5.2. Life cycle of *Leishmania* parasites

Leishmaniasis is transmitted by the bite of infected female phlebotomine sand flies. Infected sand flies transmit promastigotes (flagellated form) into the skin through their proboscis during blood feeding. Promastigotes that reach the puncture wound are phagocytized by macrophages and other types of mononuclear phagocytic cells.

Promastigotes transform within these cells into the intracellular stage of the parasite known as amastigotes. These amastigotes multiply through simple division and subsequently infect additional mononuclear phagocytic cells (monocytes, macrophages and dendritic cells). As the amastigotes continue to multiply, they cause the host cell to burst, facilitating the spread of the infection to neighboring cells. When another sand fly bites the infected person, the infected cells will be ingested. The amastigotes transform into promastigotes and attach to the microvilli of the insect gut. They produce chitinase that degrades the chitin coating between the esophagus and the stomach and weakens the valve. Parasites will backflow into the insect's mouthparts and accumulate in its proboscis within 7-10 days. The insect becomes infectious after consuming infected blood meals for a period of 7 to 10 days and must survive this duration to transmit the infection to another person (Oghumu et al., 2016; CDC, 2024) (Fig. 6).

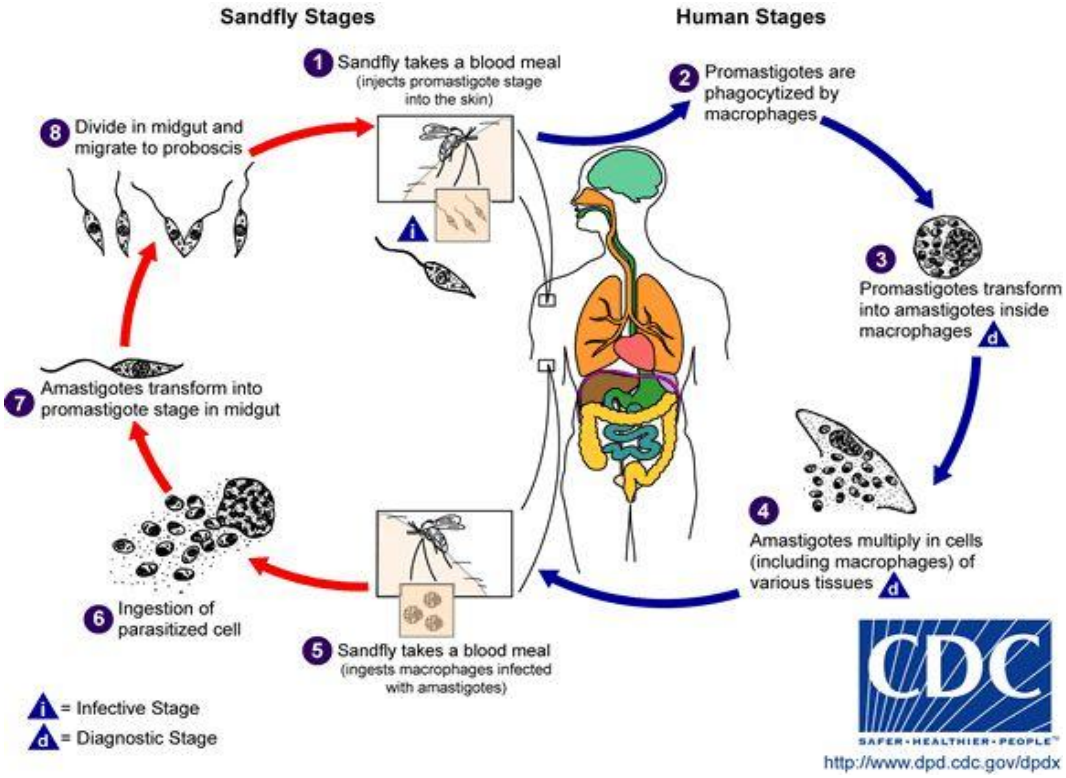


Figure 6: Life cycle of Leishmania (Source: https://www.cdc.gov/dpdx/leishmaniasis/index.html)

2.6. Pathogenesis of leishmaniasis

The pathogenesis of leishmaniasis is a complex process determined by the dynamic interplay between the virulence factors of the infecting *Leishmania* species and the host's immune response (Ejazi et al., 2025). The infective stage of the parasite, flagellated metacyclic promastigotes, is inoculated when an infected female sand fly takes the blood meal. These promastigotes are rapidly phagocytized by resident macrophages and other phagocytic cells, a process facilitated by various parasite surface molecules, such as lipophosphoglycan (LPG) and gp63, which help them evade complement-mediated lysis. Inside the phagolysosome of the macrophage, the parasites transform into the non-flagellated, replicative amastigote form (Bates, 2007; Silva-Almeida et al., 2012). The critical determinant of disease outcome lies in the host's subsequent cell-mediated immune response (Singh et al., 2014).

Most of the *Leishmania* infections remain asymptomatic, detectable early through serological or molecular methods (Fakhar et al., 2008). The factors driving progression to disease are not fully understood, but TH1 and TH2 immune responses are believed to be crucial. A robust T-helper 1 (Th1) response, characterized by the production of interferon-gamma (IFN-γ) and interleukin-12 (IL-12), activates macrophages to produce nitric oxide and other reactive oxygen species, enabling them to kill the intracellular amastigotes and resolve the infection, often resulting in subclinical or localized cutaneous disease. Conversely, a predominant T-helper 2 (Th2) response, with cytokines like IL-4 and IL-10, deactivates macrophage killing mechanisms and suppresses the Th1 response, allowing for uncontrolled parasite replication. This failure to control the infection leads to clinical disease: the amastigotes disseminate via the lymphatic and mononuclear phagocyte systems to the spleen, liver, and bone marrow, causing massive parasitization of macrophages, organomegaly, pancytopenia, and the severe systemic illness characteristic of kala-azar (Costa et al., 2023; Ejazi et al., 2025; Gupta et al., 2022; Oghumu et al., 2016). The complexity of the pathogenesis and immune response is also true in the reports' findings from laboratory animal experimentation (Loeuillet et al., 2016). The chronic immunosuppression seen in VL further predisposes patients to secondary infections (Alvar et al., 1997).

The incubation period may take years to manifest, but it typically ranges from 2 weeks to 18 months, depending on factors such as immune status, age, geographical location, comorbidities, and the parasite species. Severe inflammatory reactions within the viscera usually develop between 2 and 8 months following infection and any initial skin lesions. If left untreated, the disease can lead to death within 2 years, with a fatality rate of 75%-95%. However, spontaneous recovery is also possible in some cases. The parasites primarily proliferate in the spleen and liver cells, causing noticeable enlargement of both organs during the progression of the disease (Afrin et al., 2019; Costa et al., 2023; Martínez-López et al., 2018). Additionally, the invasion of bone marrow cells by the parasite results in reduced production of blood cells, including RBCs, WBCs, and platelets, leading to pancytopenia and subsequent immunosuppression in the patient (Costa et al., 2023). Disease progression can also be influenced by immunosuppressive conditions such as HIV, malnutrition, intestinal helminth infestations, aging, and host genetic factors. Visceral leishmaniasis patients co-infected with HIV exhibit decreased production of macrophage-activating (pro-inflammatory) cytokines (IFNγ, IL12, IL15, IL18) and increased immunosuppressive (anti-inflammatory) cytokines (IL4, IL10, TGFβ) (Ali, 1997; Mengistu & Ayele, 2007; Oghumu et al., 2016).

In the context of refugee populations, factors such as malnutrition, co-infections (e.g., HIV), and psychological stress can skew the immune response towards a non-protective Th2 phenotype, significantly increasing the risk of progression from asymptomatic infection to fulminant, life-threatening disease (Morales et al., 2024). Furthermore, the pathogenesis of the chronic sequelae, Post-Kala-Azar Dermal Leishmaniasis (PKDL), is thought to involve an aberrant immune reconstitution and a dermal inflammatory response to persistent parasites, which subsequently act as a reservoir for continued transmission within crowded camp settings (Bern et al., 2008; Costa et al., 2023).

2.7. Clinical manifestations of VL

Visceral leishmaniasis is the most severe form of leishmaniasis, where the visceral organs like bone marrow, spleen, and liver are affected and can be fatal if not treated (Fig. 7). The name “Kala-azar” is so called in India due to the hypersecretion of the hormone

adrenocorticotropic in VL patients that could lead to skin blackening of the patients (Steverding, 2017). In the old world, *L. donovani* and *L. infantum* are well-documented causative agents for the infection. In the new world, *L. chagasi* is the major causative agent of the disease (Ngure et al., 2009). The clinical manifestation may be explained by asymptomatic to symptomatic diseases that include fever, weight loss, anemia, hypergammaglobulinemia, and hepatosplenomegaly, mainly due to increased parasite burden in these visceral organs (Fakhar et al., 2008). Usually, the symptoms occur a couple of years after infection, when the patients become immunocompromised, which leaves most of the infected persons asymptomatic for VL (Singh et al., 2014) (Fig. 7).



Figure 7: Pictures A and B showing VL patients with splenomegaly and hepatomegaly (Source: https://www.who.int/leishmaniasis)

Post-Kala-azar Dermal Leishmaniasis (PKDL)

Post-kala-azar dermal leishmaniasis is a development of a complication in the VL patients' skin even after treatment. These patients have a public health importance for the transmission of the disease and are known as a risk factor for the community. This clinical manifestation may come up with macules, macupapules, and nodular rashes (Fig. 8). The rashes usually start around the mouth area and disseminate gradually to other parts of the body. This is common in Sudan (50%) and India (5-10%), where *L. donovani* is a major

causative agent for VL (Kordofani et al., 2001; Pandey et al., 2012). In addition, this event is usually reported in immunocompromised patients. The occurrence time varies from region to region; in Sudan PKDL is manifested within six months after treatment, whereas in India it may take up to three years (Ngure et al., 2009). On the other hand, the occurrence of PKDL in Ethiopia is also common in endemic regions. The WHO report indicates that between 1998 and 1999 in northern Ethiopia, PKDL was observed in an average of 13% of VL patients and 27% of those coinfected with HIV/VL. These figures likely underestimate the true number of cases, as mild PKDL frequently goes untreated and unreported (WHO, 2012).



Figure 8: Clinical presentation of PKDL (A): papular rash and (B): nodular type of lesion (WHO, 2012: https://iris.who.int/server/api/core/bitstreams/a5a8b4dd-784d-4d2e-beb2-655f075247e5/content)

2.8. Current laboratory diagnosis of leishmaniasis

The clinical diagnosis of leishmaniasis is challenging to differentiate from other similar symptom-manifested diseases. So, confirmation using the laboratory techniques is

mandatory to avoid mis- and overdiagnosis of leishmaniasis. On the other hand, due to resource limitations and technical issues, diagnosis using laboratory techniques is also challenging, particularly in developing countries like those in East Africa. It can be improved by using a combination of both clinical and laboratory diagnosis approaches with direct parasitology and/or indirect serology confirmations (Fig. 9). As direct evidence is preferred, microscopy, culture, and PCR through correct specimen collection are critical (Boelaert et al., 2008; Vallur et al., 2016; Tabbabi, 2019; Avendaño & Patarroyo, 2020). Selection of laboratory techniques partly depends on the test performance, partly on the availability, and partly on the technical issues.

Using PCR-based methods is more sensitive than culture or microscopy; these are often not standardized, and their availability is limited to health facilities to utilize as point of care (Martín-Sánchez et al., 2004; Abbasi et al., 2013; Sudarshan et al., 2014). For the laboratory diagnosis of VL, test performance depending on the sample collection site with splenic biopsy yields the highest rate of positive specimens (> 90% sensitivity). However, this procedure usually is not recommended for routine use due to a high risk of life-threatening bleeding. Alternatively, bone marrow is the preferred first source despite lower sensitivity (50-80%), whereas other potential sources of tissue specimens include the liver, enlarged lymph nodes, and whole blood, especially in immunocompromised hosts (MoH, 2013). Moreover, indirect detection of the parasites using serological testing methods also plays an important role (Georgiadou et al., 2015). To complement its limitation, a combination of testing methods was applied using microscopy, culture, and PCR. In addition, specimens like whole blood or buffy coat are not recommended for the diagnosis of VL. But it has a good yield for patients coinfected with HIV who may have high parasitemia levels (Fig. 9).

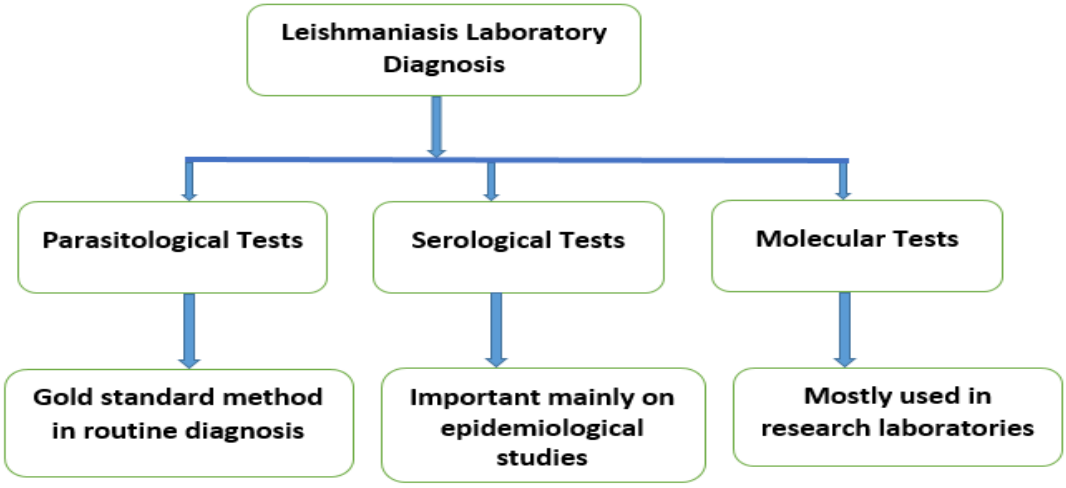


Figure 9: Overview of visceral leishmaniasis laboratory diagnosis

2.8.1 Types of recommended specimens

Aspirations from the spleen, bone marrow, liver, and lymph node are commonly used clinical samples for the diagnosis of VL. The test performance of the specimen depends on the laboratory methods used. The splenic aspirate demonstrates high sensitivity in detecting the parasite. However, the procedure carries a risk of fatal hemorrhage if performed by inexperienced practitioners. While splenic aspiration requires no special equipment and is generally less painful and invasive for the patient compared to a bone marrow aspirate, it has been proven safe and relatively straightforward when conducted by experienced professionals (Reimão et al., 2020; Sakkas et al., 2016; Sundar & Rai, 2002). To mitigate this bottleneck, the specimens should be collected following the hematological profile assessment of the patient that includes a platelet count greater than 40,000 platelets/µl and a prothrombin time not more than 5 seconds over the control (Sundar & Rai, 2002). In addition, rarely can the amastigotes be demonstrated in the buffy coat of peripheral blood. That could be an alternative and non-invasive specimen type for the diagnosis of VL. Such a parasitemia is common in severely immunocompromised patients, such as AIDS patients and patients on immunosuppressive therapy (Singh & Sundar, 2015).

2.8.2. Parasitological investigation

Microscopic

Demonstration of the *Leishmania* parasite with microscopic techniques remains the gold standard and is very crucial in diagnostic and epidemiological studies (Singh, 2006). The technique is advantageous for the following reasons: it is directly able to detect parasite load, it needs no additional laboratory structure or sophisticated setup, and it is inexpensive to perform. In this method the laboratory can be achieved by direct examination of amastigotes in Giemsa-stained specimens (Serafim et al., 2021; Singh, 2006; Thakur et al., 2020). The specificity of this technique is high, although the sensitivity varies depending on the clinical sample used, being higher for spleen (93-99%) than for bone marrow (53-86%), lymph node aspirates (53-65%) or buffy coat (19.5%) and PBMC (33.7%) (Table 1). Results are dependent partly on technical expertise, quality of prepared slides/reagents, and the parasite load in the patient (Srividya et al., 2012). The considerable variation in sensitivity among different clinical specimens indicates that the choice of sample type and testing methods are critical for accurate diagnosis of VL. In settings where highly sensitive specimens such as splenic aspirates cannot be obtained because of safety concerns or limited expertise, combining microscopy with more sensitive serological or molecular diagnostic methods may improve case detection. Therefore, strengthening laboratory capacity and improving technical expertise are essential to enhance the diagnostic performance of microscopy-based methods.

Culture techniques

Cultivation of the parasite is important for the diagnosis of VL and for sequencing the parasite for species identification. This method is rarely used in diagnostic facilities. Parasite culturing balances the detection sensitivity of the microscopic examination (Serafim et al., 2021). There are various types of culture media used for culturing: solid, liquid, and semi-solid media. Solid NNN (Novy-MacNeal-Nicolle) medium containing 20-30% rabbit blood or liquid Schneider's insect culture medium can be used for isolating and culturing the promastigote forms (Singh & Sundar, 2015). To improve diagnostic performance, several new culture methods have been introduced to enhance sensitivity,

including the micro-culture method (MCM). Recent modifications of MCM involve the use of peripheral blood mononuclear cells (PBMCs) and the buffy coat. The specimens from bone marrow and spleen aspiration are good enough to improve the sensitivities of the detection. However, the technique is tedious, time-consuming, and expensive and therefore not easily available at lower health facilities (Table 1) (Elmahallawy et al., 2014; Georgiadou et al., 2015).

Animal inoculation

The parasite can also be demonstrated by inoculating laboratory animals such as hamsters, mice, or guinea pigs with infected specimens. This type of technique is known as xenodiagnosis or animal inoculation (Reimão et al., 2020). However, animal inoculation is not commonly used as a diagnostic test due to the lengthy time required usually several months to obtain a positive result (Singh et al., 2020). Among laboratory animals, the golden hamster is preferred for maintaining the *L. donovani* complex. It can be infected through various routes, including mucous membranes, although intraperitoneal and intrasplenic routes are favored. Following inoculation, the animal is monitored weekly for signs of infection, such as cutaneous lesions, hepatosplenomegaly, or metastatic lesions. Amastigotes can be collected by performing biopsies on the spleen and liver of an anesthetized animal, which is allowed to survive after the procedure to serve as a source of infective parasites (Hirve et al., 2016; Kirstein et al., 2017; Singh et al., 2020).

Table 1: Summary of commonly used parasitological techniques, test performance characteristics, and advantages and disadvantages

Method	Sample type	TAT	Sensitivity	Advantage	Disadvantage	Reference
Microscopy	Spleen aspiration (SA)	<60 minutes	93-99%	• High sensitivity • Short TAT	Sampling is associated with a risk of fatal hemorrhage.	(Elmahallawy et al., 2014)
Microscopy	Bone marrow aspiration	<60 minutes	53-86%	• Lower risk of complications compared to SA • Short TAT	Relatively poor sensitivity, invasive sample collection	(Srividya et al., 2012; Sundar & Rai, 2002; Sundar et al., 2002)
Microscopy	Lymph node aspiration	<60 minutes	53-65%	Short TAT	Poor sensitivity	(Singh, 2006; Sundar & Rai, 2002)
Microscopy	Buffy coat	<60 minutes	19.5%	Shorter TAT	Poor sensitivity	(Diro et al., 2017)
Microscopy	PBMC	<60 minutes	33.7%	Shorter TAT	Poor sensitivity	(Diro et al., 2017)
Culture	Spleen/bone marrow	1-3 weeks	97-100%	• Improve the Sen. • Important for mass production of the parasite	Tedious, time-consuming, expensive, and rarely used for clinical diagnosis	(Elmahallawy et al., 2014; Maurya et al., 2010)

TAT: turnaround time

2.8.3. Serological testing methods

Immunological methods were developed to address the limitations of parasitological diagnostic techniques. These methods rely on detecting specific humoral immune responses. Common diagnostic techniques include enzyme-linked immunosorbent assay (ELISA), western blot, indirect fluorescent antibody tests, and direct agglutination. The sensitivity of these immunological tests primarily depends on the assay and its methodology, while their specificity is determined by the antigen rather than the serological format used (Kobets et al., 2012; Thakur et al., 2020).

Direct agglutination test (DAT)

Direct agglutination test (DAT) is a simple, reliable, cost-effective, and semi-quantitative diagnostic tool. It has been approved for the diagnosis of VL in several VL-endemic countries. The test relies on the interaction between the antigens derived from the promastigote stage of the parasite and anti-leishmanial antibodies, resulting in promastigote agglutination (Srividya et al., 2012; Elmahallawy et al., 2014). Promastigotes and amastigotes have different stage-specific antigenic profiles, but they share several conserved immunodominant antigens, such as gp63, heat shock proteins, and kinesin-related epitopes like rK39. This allows antibodies generated during amastigote infection to recognize antigens derived from promastigotes. DAT is economical and easy to perform both in laboratory settings and in the field. Its sensitivity ranges from 70.5 to 100%, while specificity varies between 53 and 100% (Elmahallawy et al., 2014; Sakkas et al., 2016; Castelli et al., 2021). Furthermore, DAT can be conducted using serum, plasma, and whole blood (Elmahallawy et al., 2014; Singh & Sundar, 2015).

The circulating antibodies persist for several months to years even after treatment; as a result, the test doesn't have prognostic value for the tests of cure of the patient. In addition to this, the long incubation period of the test (18 hours) and requirement of serial dilution of serum or blood make it disadvantageous (Singh et al., 2006). In order to overcome these problems, for quick identification (less than 3 h) of antibodies against *Leishmania* in blood collected on filter paper and in the serum samples, FAST (Fast Agglutination Screening Test) has been developed. The sensitivity and specificity with FAST were reported to be 91.1-95.4% and 70.5%-88.5%, respectively (Hailu et al., 2006).

Fluorescent antibody test (FA)

The principle of the fluorescent marker test involves attaching a fluorescent dye to an antibody, creating a reporter molecule that is fast, easy to detect, and binds to the target molecule with high specificity. This method can be applied through either direct or indirect approaches. In the direct method, the labeled antibody binds directly to the antigen, whereas in the indirect method, a secondary polyclonal antibody binds to the patient antibody that has already attached to the prepared antigen. This technique is commonly

employed to detect anti-*Leishmania* antibodies using promastigotes as the antigen source. Utilizing promastigote antigens helps reduce cross-reactivity with trypanosomal sera (Elmahallawy et al., 2014).

Enzyme-linked immunosorbent assay (ELISA)

This test is considered one of the most sensitive serodiagnostic methods for VL. However, its sensitivity largely depends on the type and source of antigen used. For example, a crude soluble antigen derived from promastigotes through repeated freeze-thaw cycles of live promastigotes demonstrated a sensitivity of 95.1%. Promastigotes of *L. donovani* release excretory, secretory, and metabolic antigens into a protein-free medium, showing a sensitivity range of 67-100%, depending on the parasite strain and the clinical manifestation of the disease. Similarly, the 200 kDa amastigote antigen of *L. donovani* and *Leishmania* amastigote soluble antigen (LASA) exhibited 96.6% sensitivity and 100% specificity. In addition, several recombinant antigens such as rK39, rGBP, Ld-rGBP, and rORFF have been developed to further enhance diagnostic accuracy (Sundar & Rai, 2002; Singh, 2006; Singh & Sundar, 2015; Reimão et al., 2020).

Leishmanin skin test (LST)

The Leishmanin Skin Test (LST), also known as the Montenegro test, is a delayed-type hypersensitivity (DTH) test used to assess cell-mediated immune response to *Leishmania* infection. It involves the intradermal injection of a standardized *Leishmania* antigen (leishmanin) into the forearm skin. After 48-72 hours, the injection site is examined for induration (swelling). The LST is primarily used for epidemiological surveys, post-treatment evaluation, and immunity studies, rather than for diagnosing active VL. Delayed-type hypersensitivity skin reactions are considered positive when the leishmanin skin test (LST) measures >5 mm and negative when the LST measures <5 mm. Following asymptomatic infection or within weeks to months after treatment for visceral leishmaniasis (VL), the test results often turn positive, indicating a healing or protective immune response. The LST demonstrates high sensitivity, ranging from 86.4% to 100%, and is straightforward to administer (Antinori et al., 2012).

Immunochromatographic test (ICT) or immunochromatographic assay (IC)

IC is an accurate, rapid, and straightforward immunochromatographic test based on the rK39 antigen. The sensitivity and specificity of rK39 in VL cases were found to be different among varying populations. Sensitivity and specificity were 90% and 100% in Brazil and 100% and 93-98% in India, respectively (Sundar & Singh, 2018). A study conducted in the Mediterranean region on large sets of patients from rK39 showed the sensitivity of 78% in all VL patients. However, the sensitivity dropped to 67.3% in patients with HIV co-infection (Jervis et al., 2017). These variations in sensitivity were due to the presence of different antibody responses in different groups. This test can be used in fields as well because it is quick, economical, and gives positive results (Elmahallawy et al., 2014).

2.8.4. Molecular testing methods

The traditional parasitological and serological techniques exhibit certain constraints to the diagnosis of leishmaniasis, and these limitations have led to the discovery of molecular methods (de Paiva-Cavalcanti et al., 2015; Tlamcani, 2016). The molecular technique is approved in routine laboratories worldwide due to its feasibility, safety, and reliable molecular tools. Information and knowledge of DNA sequencing have been much used for establishing the PCR based assay for different utilizations in interpreting the parasite and disease (Singh, 2006). Mitochondrial DNA, particularly kinetoplast DNA (kDNA), is preferentially targeted for the diagnosis of *Leishmania* because it exists in thousands of copies per parasite, providing much higher analytical sensitivity than single- or low-copy nuclear DNA targets.

In a study conducted to differentiate *Leishmania* species based on internal transcribed spacers (ITS)-PCR, in contrast to parasitological methods of diagnosing CL, it was observed that ITS-PCR is not only valid for identification of species but also showed a high sensitivity and specificity (98.8% and 100%) as compared to microscopy and culture methods (79.6% and 86.2% sensitivity, respectively) (de Paiva-Cavalcanti et al., 2015).

Conventional PCR

It facilitates the amplification of RNA or DNA through continually repeated cycles. The clarification of some DNA fragments that are species-specific made this procedure feasible for diagnosing leishmaniasis. Also, PCR techniques are more appropriate than parasitological methods of diagnosis (culturing and microscopy), especially for samples that have lower parasite loads, thus being beneficial in observing the disease progression, estimating the result of anti-leishmanial therapy, and describing the species discrimination and drug resistance (Antinori et al., 2012).

A comparative study conducted on different diagnostic methods showed sensitivities of 100%, 96%, and 95.7% for symptomatic, oligo-symptomatic, and asymptomatic groups, respectively, and a specificity of 100% (Moreira et al., 2007). Likewise, in another study, PCR was targeted against kinetoplastid DNA (kDNA) using Uni21/Lmj4 primers, and the results were compared with parasitological methods, cultures, and smears. The results showed that kDNA PCR was most sensitive, i.e., 100%; however, microscopy and cultures were only 71.4% and 54.3% sensitive, respectively (Sundar & Singh, 2018). Another comparative study revealed that PCR, microscopy, and culture showed 87.61%, 53.98%, and 46.90% sensitivity (Al-Salem et al., 2014). Although conventional PCR shows enhanced sensitivity when compared to the parasitological method of diagnosis, an essential drawback of this method is having a high risk of contamination, which takes a longer time to give the results.

Multiplex PCR

In this method, different DNA targets can be amplified simultaneously, and different PCR reactions are performed in a single experiment. Amplicons of specific DNA targets having varying sizes can be produced by using numerous primers set in one PCR mixture only. The annealing temperature of all sets of primers can be developed to perform accurately in one reaction tube, and the size of amplicons should be distinct enough to be anticipated by gel electrophoresis. Different types of markers, like kDNA mini-circles and multicopy SL RNAs (spliced leader RNAs), have been utilized in this method for diagnosis of leishmaniasis (de Paiva-Cavalcanti et al., 2015). This method can be used for clinical

laboratories, but a relative decline in sensitivity is one of the drawbacks of this method as compared to the single PCR method. Utilizing this technique for the detection of infectious agents requires accurate primer design and extensive optimization of the test.

Real-time PCR (quantitative PCR)

This method depends upon analysis by fluorescent signals that are generated during amplification. Fluorescent dye and fluorescent tubes are used to create the fluorescence. RT-PCR is fast and has an extensive dynamic range, as there is no requirement of opening the reaction tube; also, cross-contamination is decreased. RT-PCR is convenient for the diagnosis of PKDL, where it helps in monitoring parasitic load in different tissues throughout and after the treatment (Ghosh et al., 2018). In an experiment, 91 PKDL patients from Bangladesh went for skin biopsies, and both real-time PCR and microscopy were performed. RT-PCR showed the sensitivity of 91.2%, and microscopy showed 50.6% (Ghosh et al., 2018). Quantitative estimation of products, reduced contamination, and high speed are among the significant advantages of this method, but high cost and the need for an expert skilled person to deduce the results are its current significant drawbacks.

2.9. Visceral Leishmaniasis Treatment

According to the Ethiopian National Guideline for the Diagnosis and Treatment of VL, patients are first considered as clinical VL suspects if they present with fever lasting more than two weeks accompanied by splenomegaly or lymphadenopathy, after excluding malaria and previous VL. These patients should undergo an RDT. Individuals with a positive RDT result are diagnosed with VL and are eligible for treatment. If the RDT result is negative, DAT is performed. A positive DAT (titer ≥1:3200) confirms the diagnosis and the patient should receive VL treatment. Borderline DAT results (1:800 or 1:1600) require repeat testing after four to seven days or confirmation by parasitological examination using aspiration from spleen, bone marrow, or lymph node. Whereas, DAT with ≤1:400 is considered as negative for *L. donovani* infection, and alternative diagnoses should be investigated. However, if clinical suspicion remains high, parasitological confirmation or repeat DAT testing may be considered. Patients with parasitologically confirmed infection should receive VL treatment, whereas those with negative parasitological results should be evaluated for other causes of illness or retested if clinically indicated (Annex 1) (MoH, 2013)."

The standard first-line therapy for VL involves a 17-day combination regimen of antimonials (sodium stibogluconate, SSG) and paromomycin. However, due to the potential toxicity of SSG, this regimen is unsuitable for certain groups, including pregnant women, severely ill patients, and individuals over 45 years of age. These patients are instead managed with multiple doses of liposomal amphotericin B. Although cure rates are generally high, patients co-infected with HIV or tuberculosis experience higher rates of relapse and mortality. For those co-infected with HIV, treatment typically consists of a combination of liposomal amphotericin B and miltefosine (MoH, 2013; WHO, 2024b). Post-kala-azar dermal leishmaniasis (PKDL) is often mild and self-limiting and therefore may not require treatment (Gedda et al., 2020); however, therapy is indicated in chronic (lasting more than six months) or severe cases. The diagnosis and treatment of VL should always adhere to current national guidelines and WHO recommendations (MoH, 2013).

Several new therapeutic options are under investigation. A clinical trial evaluating a 14-day regimen combining paromomycin and miltefosine demonstrated good efficacy and safety, with a lower risk of PKDL development, suggesting its potential inclusion in future treatment protocols. Nevertheless, this regimen is contraindicated for women of childbearing age unless effective contraception is used during and after therapy. Additionally, promising new compounds are being developed by the Drugs for Neglected Diseases initiative (DNDi), along with an oral formulation of liposomal amphotericin B from the University of British Columbia (Vancouver, Canada), which could offer shorter, fully oral treatment options. For PKDL, a phase II study assessing combinations of paromomycin with miltefosine and liposomal amphotericin B with miltefosine also reported good efficacy and safety profiles (WHO, 2024b).

2.10. Sand fly Vectors of Visceral Leishmaniasis

2.10.1. Distribution of sand fly vectors of VL

The transmission of *Leishmania* parasites is exclusively through the bite of infected female phlebotomine sand flies. Understanding the taxonomy, ecology, and behavior of these vectors is, therefore, fundamental to controlling the disease. The phlebotomine sand flies belong to the family Psychodidae, subfamily Phlebotominae, of the order Diptera. The subfamily comprises two medically important genera: Lutzomyia in the New World (South and Central America) and *Phlebotomus* in the Old World (Southern Europe, the Mediterranean, Asia, and Africa), which include several species serving as vectors of the different forms of leishmaniasis throughout the world (Cecílio et al., 2022). According to a recent WHO review, about 31 species of *Phlebotomus* and 47 species of Lutzomyia are proven vectors of human leishmaniases. Sand flies normally feed on sugar sources. The female sand fly needs to feed on blood to develop her eggs. They suck blood from both animals and humans. Most species bite at night (from dusk to dawn) (WHO, 2022). Phlebotomine sand flies are characterized by their wing venation (the presence of numerous parallel veins running to the wing margin) and the presence of dense hairs on the wings and thorax. They are also categorized by the presence of short biting mouthparts,

five-segmented palps, 15-segmented cylindrical hairy antennae, hairy bodies, and a characteristic V-shaped wing posture at rest (Killick-Kendrick, 1999).

Global distribution of phlebotomine sand flies spans tropical, subtropical, and some temperate zones, with species composition and vectorial importance varying by biogeographic region. Recent global systematic reviews and species inventories emphasize both the wide geographical coverage and local endemism of individual vector species, highlighting the need for local entomological evidence to guide control (Galati et al. 2025). The sole proven vector in the Indian subcontinent countries, where it is highly anthropophilic and endophilic, is *Ph. argentipes*. Its resting and breeding sites are closely associated with human dwellings and cattle sheds, making indoor residual spraying (IRS) a highly effective control measure (Killick-Kendrick, 1990). The principal vectors in the Mediterranean basin, where VL is primarily a zoonotic disease involving dogs as the main reservoir host, are *Ph. perniciosus* and *Ph. ariasi* (Ready, 2014). In addition, the distribution of the *Lutzomyia longipalpis* complex is mainly mapped into Latin America, where VL is a zoonosis, and *Lu. longipalpis* is the primary vector. This species is highly adaptable to peridomestic environments, and its distribution is strongly linked to the presence of infected dogs (Lainson & Rangel, 2005). The global distribution of VL is intrinsically linked to the ecological niches of these specific sand fly vectors, which are sensitive to climate (temperature, humidity) and environmental changes (Ready, 2014).

In East Africa, the principal vectors of VL are *Ph. orientalis, P. martini,* and, to a lesser extent in some foci, *Ph. celiae*; other *Phlebotomus* species and several Sergentomyia species occur but are generally not implicated as VL vectors (Alvar et al., 2021; Elnaiem, 2011). *Phlebotomus orientalis* is strongly associated with black cotton (vertisol) soils and *Acacia-balanites* woodland and is common in lowland plains of Sudan, South Sudan, and parts of Ethiopia; *Ph. martini* and *Ph. celiae* are often linked to termite mounds and peridomestic habitats in other eastern African foci. Species abundance and seasonal dynamics are highly focal, varying by microhabitat, rainfall pattern, and human activity, which explains the patchy distribution of VL across the region (Elnaiem, 2011).

Phlebotomine sand flies have been studied for more than seven decades in Ethiopia. Until 2023, a total of 65 species from both genera of *Phlebotomus* (20 species) and *Sergentomyia* (45 species) are reported in different leishmaniasis-endemic regions of Ethiopia. Among these, *Ph. orientalis, Ph. martini,* and *Ph. celiae* are the implicated vectors of VL caused by *the L. donovani* complex in different VL foci of the country (Aklilu et al., 2023). Distribution within Ethiopia is highly focal and correlates with lowland ecological zones (vertisol plains, *Acacia-balanites* woodlands) where *L. donovani* transmission occurs (Aklilu et al. 2017a). While several other *Phlebotomus* and *Sergentomyia* species are present but of uncertain epidemiological importance (Pareyn et al., 2020). Recent country-level entomological surveys and species inventories provide updated records and details of species presence and altitudinal ranges (Aklilu et al., 2023), that clearly show gaps remain in many districts, notably within regions where refugee camps are located, underscoring the need for targeted local surveillance to identify the dominant vector(s) and breeding/resting sites relevant for control.

2.10.2.Infection rate in naturally infected sand flies

Several entomological studies have examined *Leishmania* infection in different sand fly species using microscopy, culture, and PCR, revealing generally low infection rates across surveyed populations. *Phlebotomus martini* showed positivity rates of 1.0% (9/863) and 0.7% (16/2326), while *Ph. celiae* exhibited 0.9% (3/355) and 0.3% (3/1044) in studies conducted in southern Ethiopia (Gebre-Michael & Lane, 1996; Gebre-Michael et al., 1993). Infections were also detected at low levels in *Ph. orientalis*, 1.4% (1/70), and *Sergentomyia* species, including *S. clydei*, 1.7% (1/59), and *S. schwetzi* 3.3% (2/60) (Hailu et al., 1995). In contrast, other investigations reported no positive findings in *Ph. orientalis*, *Ph. papatasi*, *Ph. bergeroti, Ph. dubosqi,* and *Ph. rodhaini* using similar diagnostic methods (Aklilu et al., 2018; Gebre-Michael et al., 2007, 2010; Yared et al., 2019). Overall, these data suggest that *Leishmania* infection among sand flies is rare in the known confirmed vectors in endemic regions of Ethiopia, while occasional infections in *Sergentomyia* species may warrant further investigation to clarify their possible epidemiological significance (Table 2).

On the other hand, species of sand flies within the genus *Sergentomyia* are generally considered non-vectors of VL, as many of them do not support the development of *Leishmania* parasites in their midgut (Addo et al., 2023). However, reports in Kenya (Owino et al., 2021), West Africa (Addo et al., 2023; Nzelu et al., 2014), and other regions (Maia et al., 2015; Nikookar et al., 2024) have detected *Leishmania* DNA in *Sergentomyia* spp., whose populations often outnumber those of *Phlebotomus* species, and in the VL endemic areas where *Phlebotomus* species are absent (Mahamoud et al., 2018; Nikookar et al., 2024; Owino et al., 2021). These reports indicated that some *Sergentomyia* species may play a significant role in the transmission of the diseases, although further in-depth studies are required. Similarly, reports from Ethiopia indicated that *Sergentomyia* is the predominant genus in VL endemic areas (Hailu et al., 1995; Lemma et al., 2014; Yared et al., 2017).

Table 2: Description of infection rates of phlebotomine sand flies in various regions of Ethiopia.

Species	No. tested	No, positive.	% positive	Methods used	Reference
Ph. martini	863	9	1.0	Microscopy, culture and PCR	(Gebre-Michael et al. 1993)
Ph. celiae	355	3	0.9		
S. schwetzi	324	0	0.0		
S. multidens	10	1	10.0		
S. clydei	2	0	0.0		
S. bedfordi	3	0	0.0		
Ph. orientalis	70	1	1.4	Microscopy and culture	(Hailu et al. 1995)
S. clydei	59	1	1.7		
S. schwetzi	60	2	3.3		
Ph. martini	2326	16	0.7	Microscopy and culture	(Gebre-Michael & Lane 1996)
Ph. celiae	1044	3	0.3		
Ph. orientalis	607	0	0.0	Microscopy	(Gebre-Michael et al. 2007)
Ph. orientalis	618	0	0.0	Microscopy	(Gebre-Michael et al. 2010)
Ph. papatasi	75	0	0.0		
Ph. bergoroti	25	0	0.0		
Ph. dubosqi	7	0	0.0		
Ph. rodhaini	7	0	0.0		
Ph. orientalis	798	0	0.0	Microscopy and PCR	(Aklilu et al. 2018)
Ph. rodhaini	26	0	0.0		

2.11. Prevention and Control of Visceral Leishmaniasis

2.11.1. Visceral leishmaniasis prevention and control strategies in global perspective

Visceral leishmaniasis control and elimination strategies have been established using various approaches. According to WHO, early diagnosis and complete case management, integrated vector management and vector surveillance, effective disease surveillance through case detection, advocacy, social mobilization and partnership-building, implementation and operational research, and capacity building are the core components that have to be considered to control and eliminate VL (WHO, 2024b). To enhance access to early diagnosis and timely treatment, VL diagnostic and treatment services should be expanded to primary health care facilities, refugee camps, conflict-affected areas, and sentinel sites within VL-endemic districts.

Vector-borne diseases usually coexist, as indicated in a sub-Saharan map of the global distribution of risk for major vector-borne diseases of malaria, lymphatic filariasis, dengue, onchocerciasis, leishmaniasis, human African trypanosomiasis, and yellow fever (Kalitsilo et al., 2025). As defined by WHO, IVM is the control of one or more vector-borne diseases using multiple interventions, either chemical or non-chemical, or both, selected based on good evidence in collaboration with other sectors, including communities. Conversely, effective vector control requires consideration of the behavior, species composition, and abundance of sand flies, as these factors influence the success of insecticide-based interventions and may limit the effectiveness of indoor residual spraying (IRS). In eastern Africa, most sand fly species exhibit exophilic and exophagic behavior, with marked seasonal variations in population density. For example, *Ph. orientalis,* the principal vector in Ethiopia, Kenya, South Sudan, and Sudan, is most abundant during the latter half of the dry season and the onset of the rainy season (March–June) (Elnaiem et al., 2003; Kolaczinski et al., 2008; Jones & Welburn, 2021). In contrast, *Ph. martini* reaches its highest abundance during the rainy season in Ethiopia and Kenya (Gebre-Michael & Lane, 1996; Kolaczinski et al., 2008).

As indoor residual spraying is not an appropriate intervention for exophilic species, the use of targeted outdoor residual spraying of houses and village boundary fencing has been explored in a pilot study in Sudan against *Ph. orientalis*, with highly promising success (Elnaiem et al., 2020). Previous work suggested that transmission can be reduced through health education and behavioral change so that people keep sufficient distance from the vicinities of termite mounds, where exposure to the bites of *Ph. martini* and *Ph. celiae* takes place. Risk communication and community engagement will play a profound role in reducing exposure to VL infection (Leta et al., 2014; Lemma, 2018). The distribution and proper use of insecticide-treated bed nets (ITNs) have proven effective in reducing exposure to sand fly bites and consequently lowering the incidence of VL. For instance, a previous mass distribution campaign of insecticide-treated, sand fly-proof bed nets in Gedaref State, eastern Sudan, led to a significant decline in VL incidence. In addition to ITNs, personal protection measures such as the use of repellents and other protective interventions can further minimize contact with sand fly vectors (Lambert et al., 2002; Nackers et al., 2015).

2.11.2.Leishmaniasis control and prevention efforts in Ethiopia

The National Leishmaniasis Control Program in Ethiopia was established in 2005. The FMOH prepared the first national guideline on the diagnosis and treatment of VL in 2006, which was revised in 2013 and is currently under a second revision. Leishmaniasis control is included in the Ethiopian Ministry of Health's (MoH) Third National Neglected Tropical Disease Strategic Plan for 2021-2025, which stipulates reducing the case fatality rate due to primary VL from 2.7 in 2020 to <1.5. Ethiopia is a signatory to the Nairobi Declaration, where ministries of health from East African countries committed to eliminating VL as a public health problem by 2030. Ethiopia has also signed a comprehensive strategic objective MOU in 2024 that guides the ministry and partners towards VL elimination.

Early diagnosis and prompt treatment of VL remain the cornerstone to control the disease. In Ethiopia leishmaniasis services, particularly for VL, have been decentralized to regional and sub-regional levels. This has led to the establishment of 31 VL treatment centers and 33 diagnostic centers to date. A standardized and accredited training manual for public

health officers, clinicians, and laboratory professionals has been prepared, aiming to improve early diagnosis and treatment; the national control program has actively pursued capacity building and awareness raising since its establishment. The visceral leishmaniasis active case search integrated with other public health priority diseases and regions has started reporting to MoH to support the VL response and control strategies in the country.

CHAPTER 3: MATERIALS AND METHODS

3.1. Study sites

The study was conducted in four refugee camps located in Gambella and Benishangul-Gumuz regions of Ethiopia. There was a total of seven refugee camps in Gambella and five in Benishangul-Gumuz Regional State (UNHCR, 2025). As of 31 May 2025, Gambella hosted 395,203 refugees, and Benishangul-Gumuz hosted 110,606 refugees (UNHCR, 2025). Terkidi and Kule refugee camps are located in Itang district, Gambella Regional State, in southwestern Ethiopia. Whereas, Tsore and Sherkole were located in Homosha district of Benishangul-Gumuz regional state. These camps were randomly selected (Fig. 10).

Itang is a special district that lies in the low-lying floodplain area of Gambella, characterized by a flat to gently undulating landscape. The district is bordered to the south and southeast by the Anuak Zone, to the west by the Nuer Zone, to the north by the Oromia region, and to the northwest by South Sudan, with the Alwero River demarcating part of its southern boundary. The altitude of this district ranges from 350 to 480 meters above sea level (Chelbi et al., 2022). The district's average annual temperature and total rainfall were 29°C and 1000 mm, respectively and June to September is known to be the main rain reason. Temperatures remain high year-round, with peaks in March-May. Vegetation is dominated by savanna woodland, *Acacia seyal*, and extensive grasslands (Rolkier & Yeshitela, 2020). Moreover, there are scattered distributions of small-sized termite hills in the village and natural habitats of the refugee camps.

The Sherkole and Tsore refugee camps are located in Homosha district, Benishangul-Gumuz Regional State, in northwestern Ethiopia. Tsore camp is 28 km from Asosa, the regional and zonal capital, and 10 km from Homosha town, while Sherkole camp is 45 km from Asosa and 8.8 km from Homosha town (Sahldengil & Adugna, 2022). The region borders South Sudan and Sudan. Homosha district is found in eastern parts of the Benishangul-Gumuz Regional State, which is bordered by Kurmuk in the northwest, Menge in the northeast, and Assosa in the south. The study area receives 588-1,549 mm of

rainfall annually, with maximum temperatures of 21.4-31.5°C during the dry season and minimum temperatures of 7.4-17.6°C. In the region June to September is known to be the main rain reason. The soil is deep reddish-brown sandy clay, and the landscape is largely covered by natural vegetation, including shrub grassland, dense woodland, bamboo thickets, and open grassland. Termite mounds are common in the district, particularly in woodland and grassland habitats, but rarely distributed in the refugee camp. These camps have ecological conditions conducive to sand fly survival, increasing the risk of VL transmission (Gadisa et al., 2015), with prior cases reported to the Ethiopian Ministry of Health (unpublished data).

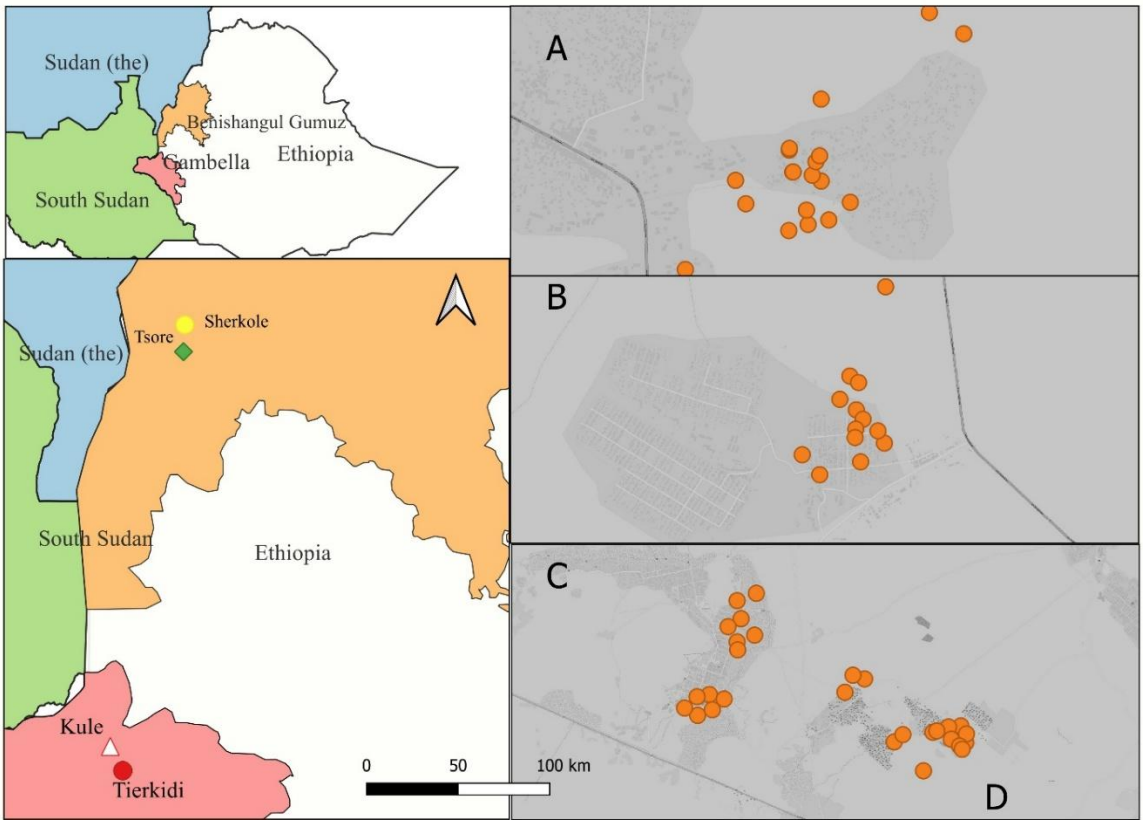


Figure 10: Map showing sand fly collection sites in refugee camps in Gambella and Benishangul-Gumuz regions, Ethiopia [Kule Camp: represented by a white triangle; Terkidi Camp: represented by a red circle; Sherkole Camp: represented by a yellow circle; Tsore Camp: represented by a green diamond]. The sand fly collection sites are represented as orange dots where; A: Sherkole, B: Tsore, C: Kule, and D: Terkidi refugee camp are sand fly collection sites. The maps were built using the free and open source QGIS software version 3.36.3 (QGIS Development Team (2024). QGIS Geographic Information System, version 3.36.3. Open-Source Geospatial Foundation Project. https://qgis.org) and shapefiles were obtained from the free and open-source site https://data.humdata.org.

3.2. Study design and period

A cross-sectional study was conducted to determine the prevalence of *Leishmania* infection in the four refugee camps in the Gambella and Benishangul-Gumuz regional states of Ethiopia. The prevalence and matched case-control studies were conducted from May to August 2023. A matched case-control study design derived from cross-sectional data that was collected to determine determinant factors of *Leishmania* infections in selected refugee camps in Gambella and Benishangul-Gumuz regional states of Ethiopia. The cross-sectional study provided a snapshot of exposures and outcomes during data collection.

Cases were matched to controls based on predefined criteria (age, sex, and region of camp location) for confounding variables. The data collection occurred within a defined timeframe, ensuring consistency in exposure assessment and minimizing recall bias. The study captured exposure and outcome information simultaneously, allowing for an efficient analysis of associations while maintaining the advantages of a case-control approach. Moreover, the entomological survey was conducted cross-sectionally from April to May 2025 in four refugee camps.

3.3. Source and study population

The source population included all individuals living in the refugee camps in Gambella and Benishangul-Gumuz regional states. The study participants were refugees five years of age and older and living in the selected camps. Individuals living in the camp for at least six months and willing to provide assent and/or written informed consent were included in the study. Moreover, the study population consisted of cases and matched controls selected from the study population of the prevalence study participants based on matched criteria. This matching process helped to reduce confounding and enhanced the validity of comparisons. Individuals who were unable to speak, listen, or assent and/or provide written informed consent and who declined to participate or provide blood samples were excluded from the study.

3.4. Study variables

The outcome variable for the objective assessing the prevalence of *Leishmania* infection in refugee camps was *Leishmania* infection using either DAT and/or rtPCR. Independent variables include sex, age group in years, camp name, country of origin, level of education, marital status, family size, and clinical symptoms, including fever, chills, headache, weakness, presence of splenomegaly, hepatomegaly, skin rash, and lymph nodes.

In the case-control study investigation, the factor influencing *Leishmania* infection, the dependent variable, was whether the study participant had been infected with *Leishmania* or not based on the laboratory result using DAT and/or PCR. Independent variables:

stratified as socio-demographic, behavioral, environmental, and housing condition factors as depicted in Table 3.

Table 3: Description of independent variables: stratified as socio-demographic, behavioral, environmental and housing condition factors in a matched case-control study in refugee camps in Gambella and Benishangul-Gumuz regional states, Ethiopia, 2023

Sociodemographic Factors	Behavioral factors	Environmental and housing conditions
• Region • Gender • Age • Country of Origin • Family size • Marital Status • Educational level • Camp duration	• Sleep outside during day time • Sleep outside during night time • Always use bed net while sleeping • Frequency of sleeping outside during dry season	• Bushes available around the house • Termite hills present near house • Presence of cracked wall • Presence of cracked floor near to sleeping place • Presence of termite hills near sleeping place (within 5m)

3.5. Operational definitions

Cases: Cases were operationalized as *individuals infected with Leishmania* as determined by either DAT and/or rtPCR testing methods.

Controls: defined as individuals for whom *Leishmania* was not detected by both DAT and rtPCR testing methods.

Matching: *Leishmania*-infected participants in the refugee camps were matched 1 case:3 controls by age, sex, and refugee camp location with participants who had negative test results using both DAT and PCR testing methods.

Refugee: an individual or group of people who temporarily live in the refugee camp for protection

Refugee camp: A temporary settlement established to accommodate populations displaced by conflict and/or natural disasters

***Leishmania* infection**: Positive laboratory test results for *Leishmania* infection, regardless of whether VL symptoms developed or not

Sleep outside during the daytime: Sleeping behavior at any time during the day, regardless of the duration of sleep.

Sleep outside during nighttime: Sleeping behavior at any time during the night, regardless of the duration of sleep.

Frequency of sleeping outside during the dry season: Participants' experiences/responses are categorized as never (0 nights), sometimes (1-3 nights/week), or always (≥4 nights/week).

Bushes available around the house: The presence/absence (Yes/No) of bushes, shrubs, or dense vegetation in a <50 meter radius of the household compound

Termite hills present near house: Presence of visible termite mounds within a 50-meter radius of the household compound

Presence of cracked wall: the presence or absence of cracked walls of any size

Presence of cracked floor near to sleeping place: the presence or absence of cracked walls of any size near to the sleeping place

Presence of termite hills near sleeping place (within 5 m): presence of termite hills within five meters of the sleeping place

3.6. Sample size determination

Sample size determination for the prevalence study was estimated using a standard formula for household based cross-sectional studies, Nh=((Z^2)2(p)(1-p)(DE)(k))/((P)(n)(e^2)), where Z = 1.96 (95% confidence), p=0.35 (proportion with VL) (Alvar et al., 2007), DE=1.5 (design effect), k=1.1 (non-response multiplier), P=0.95 (target population

proportion), n=4.0 (household size), and e=0.025 (margin of error). This yielded the minimum sample size of 607 households from selected refugee camps in each regional state. Data were collected from 563 households in Gambella and 660 in Benishangul-Gumuz, with a total of 1223, selecting one participant per household.

Sample size determination for the matched case-control study was calculated using the freely available statistical tool "(https://www.openepi.com/SampleSize/SSCC.htm)" based on the proportion of exposure and the odds of availability of bushes around the house. In the previous study conducted in Gedaref State, Sudan (Nackers et al., 2015), it was revealed that the presence of bushes, including some acacia trees, was found to be a risk factor for VL. So that we used the following parameters to estimate the minimum sample size for the current study: 43.5% of the controls reported bushes around the house, with an odds ratio of 2.2, with 80% power and 95% CI and a 1:3 case-to-control ratio; then the sample size was estimated to be 76 cases and 226 controls. Adding a 10% non-response rate, the final sample size was 84 cases and 252 controls, with a minimum total sample size of 336.

3.7. Sampling methods

The sampling procedure for the prevalence study in the refugee camps in Gambella and Benishangul-Gumuz regions was purposely selected because of the high concentration of refugee camps. In Gambella, two refugee camps (Kule and Terkidi) were selected out of a total of seven camps. In Benishangul-Gumuz regional state, similarly, two refugee camps (Tsore and Sherkole) were randomly selected from a total of five camps. This sampling strategy resulted in a total of four refugee camps being included in a study. A simple random sampling technique was applied at the camp level to minimize an unbiased selection process for identifying camps that represent the refugee population adequately. The sampling interval (k=24) was calculated by dividing the total number of households by the desired sample size. The random starting point between 1 and 24 was determined by a lottery method, and the number nine was chosen as the starting point. From this point, every 24^{th} household was systematically selected to ensure a representative and unbiased sample across all camps. This ensures that each camp contributes a sample size

proportionate to its household population. The number of households allocated to each camp was based on its population size (Fig. 11).

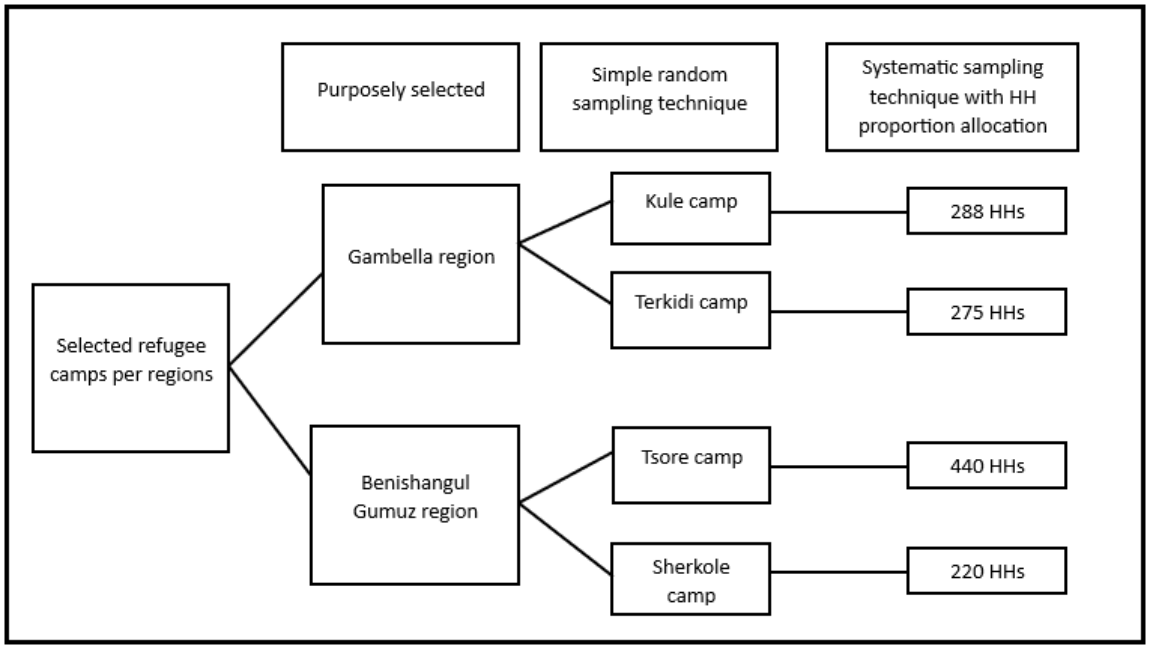


Figure 11: Schematic presentation of sampling procedure (n=1223) in Gambella and Benishangul Gumuz, using different sampling techniques

Whereas, the sampling method for the matched case-control study was based on the selection of cases and controls from the study participants. Cases were recruited from the previously conducted cross-sectional prevalence study. All participants who tested positive using the direct agglutination test (DAT) with a titer 1:1600 concentration (Bekele et al., 2018; Belay et al., 2025c) and/or rtPCR were eligible for recruitment for the study. The cases were distributed in the four refugee camps: Kule, Terkidi, Tsore, and Sherkole. Similarly, controls were selected from the same line list, considering a distribution of age groups, sex, and region of camp location with match tolerance factors of 2, 1, and 1, respectively. This means that cases and controls could differ by up to two years in age but had to be identical in sex and camp location. Controls were also selected based on the negative test to DAT (agglutination <1:1600) and rtPCR. Case-control matching was performed using SPSS, with three controls selected per case according to predefined matching criteria.

3.8. Survey instrument and data collection tools

A structured questionnaire was prepared to assess the determinants of its infection among the refugee populations based on the existing forms (Ayehu et al., 2018; Bekele et al., 2018; Alebie et al., 2019; Tadese et al., 2019). Characteristics of each participant's sociodemographic status (i.e., age, sex, place of residence, and number of family members) and clinical information (e.g., the presence of fever, headache, hepatomegaly, or splenomegaly) were collected (Fig. 12).



Figure 12: House-to-house data collection by trained local health officers using a standardized questionnaire

Preliminary field assessments were conducted before initiating the main sand fly collection in order to evaluate the abundance, distribution, and diversity of sand flies in potential collection sites. The first assessment took place during the fourth week of May 2024 in refugee camps in Gambella Regional State. CDC LT and paper based sticky trapping techniques were employed, and a total of 474 sand flies were collected. Morphological examination at the genus level indicated that all collected specimens belonged to the genus Sergentomyia. Species-level identification was not performed from these collected sand

flies. A second pre-assessment was carried out in Benishangul-Gumuz Regional State during August 2024, using the same trapping procedures. However, no sand flies were captured during this attempt. This absence of sand flies was likely influenced by ecological and seasonal variations such as temperature, rainfall, humidity, and differences in habitat suitability. These preliminary assessments provided baseline information on sand fly presence and distribution in the surveyed areas and guided the selection of sites and timing for the subsequent main collection.

The collection of adult sand flies was conducted by employing two standard techniques: CDC light traps and sticky traps, as detailed elsewhere (Gebre-Michael & Lane, 1996; Aklilu et al., 2017a; Gebresilassie et al., 2015).

CDC light trap (LT): Two fixed sampling points (indoor and household compounds) were established per refugee camp. A single LT was deployed at each point per night, positioned 30-50 cm above ground level and operating from dusk to dawn. A total of 4 LTs were deployed over an eight-night period, amounting to a cumulative total of 32 trap-nights. This LT sampling was performed over two consecutive nights across all collection sites in the refugee camps of the Gambella (April 6-10, 2025) and Benishangul-Gumuz (May 5-9, 2025) Regional States. Specimens were traced and aspirated each morning from the collection LT, inactivated using a combination of cold and mechanical vibration, and subsequently preserved in 95% ethyl alcohol for future morphological and molecular analysis.

Sticky traps (STs): White transparent polypropylene sheets (A4 size) coated with a thin layer of castor oil were used as sticky traps for sand fly collection. In each survey site, four traps were deployed in five habitat types, including indoors, household compounds, termite mounds within villages, termite mounds in natural habitats, and natural habitats with mixed forest cover. Traps were operating from dusk to dawn. A total of 48 STs were deployed per night, amounting to a cumulative total of 384 trap-nights. Captured sand flies were collected the following morning using fine-tipped forceps, transferred into labeled vials containing 96% ethanol, and preserved for subsequent morphological identification (Fig. 13).



Figure 13: Field sampling methods and habitat characterization for sand fly collection in refugee camps of Gambella and Benishangul-Gumuz Regional States (A & B: savanna woodland and shrubland vegetation in Kule and Terkidi refugee camps; C & D: mixed vegetation habitats, mainly bamboo, in Sherkole and Tsore camps; E: sand fly collection in natural habitats at Kule camp; F: collection at the periphery of compounds in Terkidi camp G: collection within Kule camp compounds using CDC light trap)

3.9. Laboratory based data collection

3.9.1. Clinical sample collection and processing

Five milliliters of venous blood were collected in EDTA tubes (Shenzhen Medicalet Technology Co. Ltd., Shenzhen City, China) from each study participant (n=1223) by trained laboratory technologists. These collected samples were transported from the field to the regional laboratory under cold-chain conditions using a cool box with ice packs. The whole blood samples were centrifuged at 8000 rpm for 10 minutes to separate the plasma and buffy coat (Manomat et al., 2017). They were stored at -20°C until transported to EPHI

to preserve cellular components, ensuring the quality of the sample for *Leishmania* DNA extraction and PCR amplification (Huda et al., 2013) (Fig. 14).

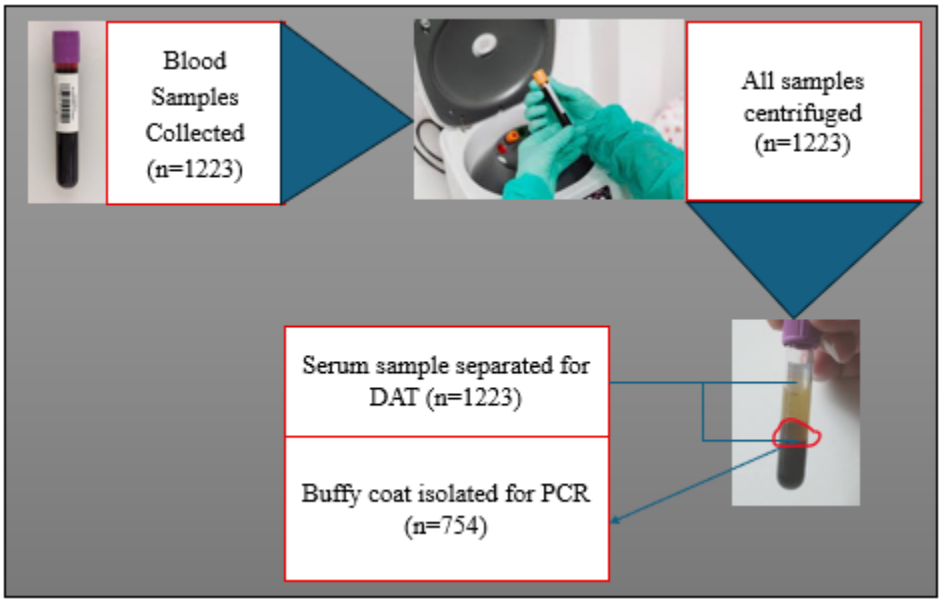


Figure 14: Blood sample collection and processing

3.9.2. Direct agglutination test (DAT)

The direct agglutination test (DAT) was carried out as previously described (Khanal et al., 2010). Briefly, 2 µl of plasma samples was mixed with 100 µl of diluent. The sample was diluted from 1:50 to 1:51200 in two-fold. *Leishmania* negative and positive controls prepared in an academic medical center (Amsterdam) were used. Freeze-dried antigen (FDA) containing *L. donovani* promastigotes was added to all 96 wells of the V-shaped microtiter plate (Thermo Fisher Scientific, USA). The plates were incubated at room temperature for 12-18 hours and the results were determined by visual examination of the agglutination pattern. In samples without anti-Leishmania antibodies, the antigen particles sedimented to the bottom of the well, forming a compact, sharply defined blue dot. Whereas, samples containing anti-Leishmania antibodies showed visible agglutination, characterized by a diffuse mat-like appearance, a dot with irregular or frayed edges, or an enlarged, less-defined dot.

The DAT was carried out as previously described, with titers ≥ 1:1600 considered positive (Bekele et al., 2018; Belay et al., 2025c). The test demonstrated an overall sensitivity (95.3%) for diagnosing VL in Ethiopia (Diro et al., 2007). The KDNA-based PCR was conducted as described elsewhere (Van Griensven et al., 2019). The diagnostic sensitivity of rtPCR for the diagnosis of VL using peripheral blood as specimens was measured at 98.1% and 100.0% specificity (Khatun et al., 2017) (Fig. 15).

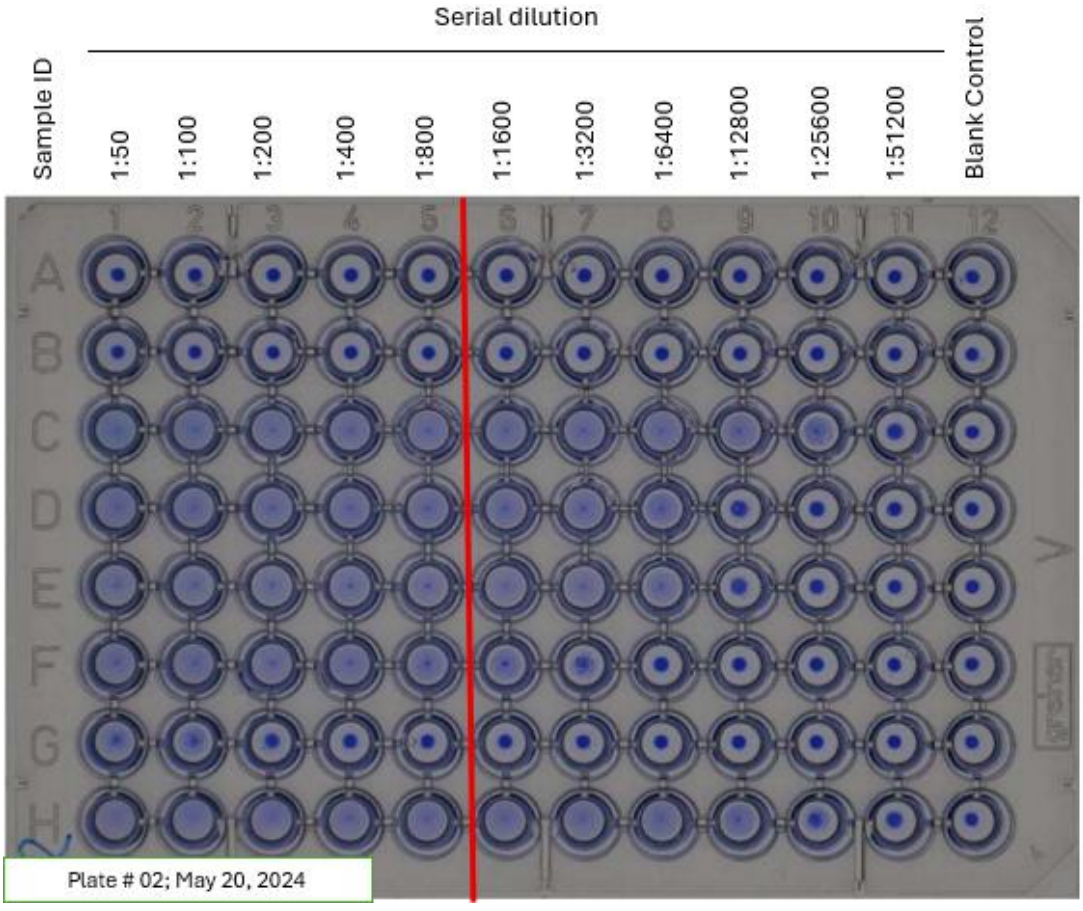


Figure 15: Direct agglutination test (DAT) results showing serial serum dilutions from 1:50 to 1:51200 (columns 1-11) incubated with DAT antigen overnight; column 12 represents the blank control without patient serum and red lines indicate the cutoff threshold.

3.9.3. Mounting and identification of sand flies

All collected sand flies were sex segregated based on the morphology of their reproductive organs observed under a stereomicroscope. Female sand flies were dissected by carefully removing the head and the last three abdominal segments using fine dissecting pins. These

parts were mounted on properly labeled glass slides in a drop of gum chloral mounting medium with the head ventral-side up under a single coverslip. Prepared slides were allowed to clear and dry at room temperature for one to two weeks before examination under a compound optical microscope for species identification. The remaining body parts (thorax and anterior abdominal segments) were preserved in sterile 1.5 mL Eppendorf tubes containing 95% ethanol, labeled identically to the corresponding slide, and stored for subsequent DNA extraction (Fig. 16). Species identification was carried out using well-established taxonomic keys (Abonnenc & Minter, 1965) and additionally (Benallal et al., 2022), with diagnostic morphological features including the cibarium, cibarial teeth, pharynx, and spermatheca.

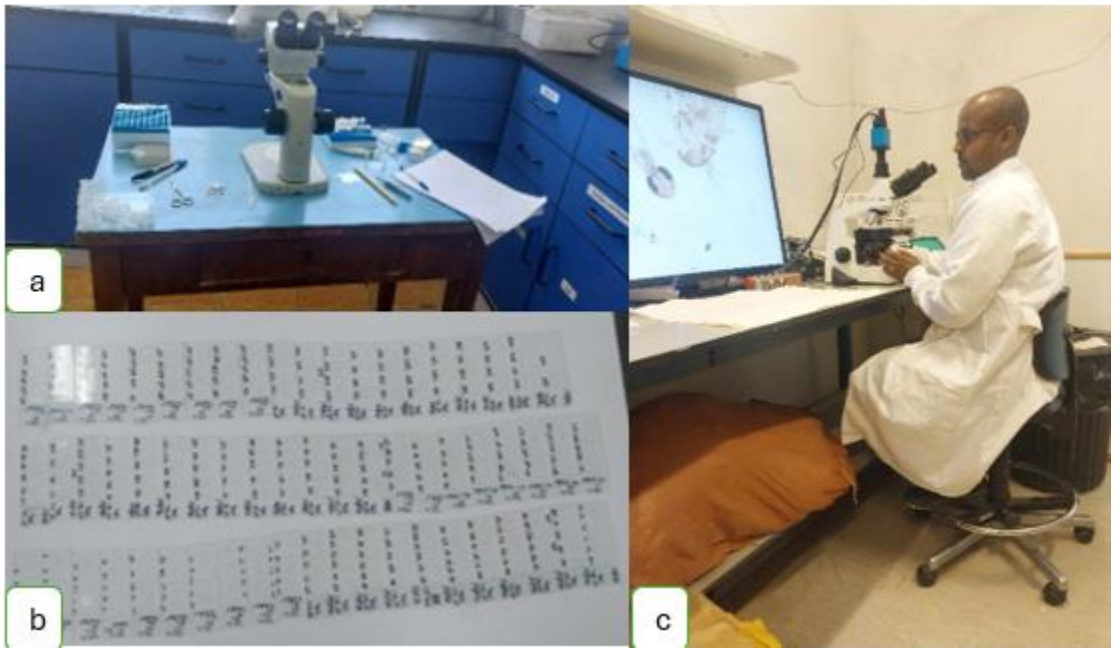


Figure 16: Laboratory activities related to sand fly processing: (a) dissection and mounting of sand flies at the ALIHR Entomology Laboratory; (b) air-drying of mounted sand flies at the ALIHR Entomology Laboratory; and (c) microscopic identification of sand fly species at the EPHI laboratory.

3.9.4. DNA extraction from buffy coat of centrifuged human blood

DNA extraction was performed on 754 randomly selected samples using the Geneaid DNA isolation kit (GMB300, Geneaid Biotech Ltd; New Taipei city, Taiwan) per the manufacturer's protocol. The extracted DNA was eluted in 100 µl of elution buffer. A

negative extraction control (NEC) or nuclease-free water was used to identify contamination and confirm the integrity of the extraction reagents and the procedural steps involved and treated as a sample.

3.9.5. Leishmania species DNA extraction from sand flies

We individually screened the only *Phlebotomus* species encountered during our study, *Ph. rodhaini*, while *Sergentomyia* spp. were pooled in sets of 1-22 female sand fly specimens based on species, trap type, collection site, and habitat. The head and abdominal parts of the individually prepared and pooled female specimens were soaked in DNA shield solution with beads and homogenized twice at high speed (6.5 m/s for 3 min) using a FastPrep®-24 bead beating homogenizer (MP Biomedicals), followed by centrifugation at 21,000 g for 1 minute. From the clarified homogenate, 180 µl was transferred into a 1.5 ml tube for DNA extraction. DNA was isolated using the DNeasy Blood & Tissue kit (QIAGEN) following the standard manufacturer's protocol for isolation of insect genomic DNA (Fig. 17).

3.9.6. *Leishmania* kDNA detection by real-time polymerase chain reaction

An rtPCR targeting minicircle kinetoplast DNA (kDNA) was employed for the detection of *Leishmania* parasites from the buffy coat and sand fly extracted samples. The primers used for the study were kDNA-CMF (CTTTTCTGGTCCTCCGGGTAGG) and kDNA-CMR (CCACCCGGCCCTATTTTACAC CAA) (Belay et al., 2024). The kDNA rtPCR was conducted as previously described (Van Griensven et al., 2019b). Briefly, a 25 µl reaction volume was prepared with 1 × HotStarTaq Master Mix (Qiagen, Venlo, The Netherlands), 0.6 µM of reverse and forward primers and 0.4 µM of the probe (Integrated DNA Technologies, Leuven, Belgium), 0.1 mg/mL bovine serum albumin (Roche, Vilvoorde, Belgium), and 5 µl of DNA template. The rtPCR was performed using the QuantStudio 5 real-time PCR system (Catalogue: A28568, Thermo Fisher). During each run, the genomic DNA extracted from the *Leishmania*-positive culture and the non-template control (NTC) were tested and used as a positive and negative control, respectively. Samples with cycle threshold (Ct) values between 12 and 40 with a sigmoidal

amplification curve were considered positive for *Leishmania* infection (Hossain et al., 2017).

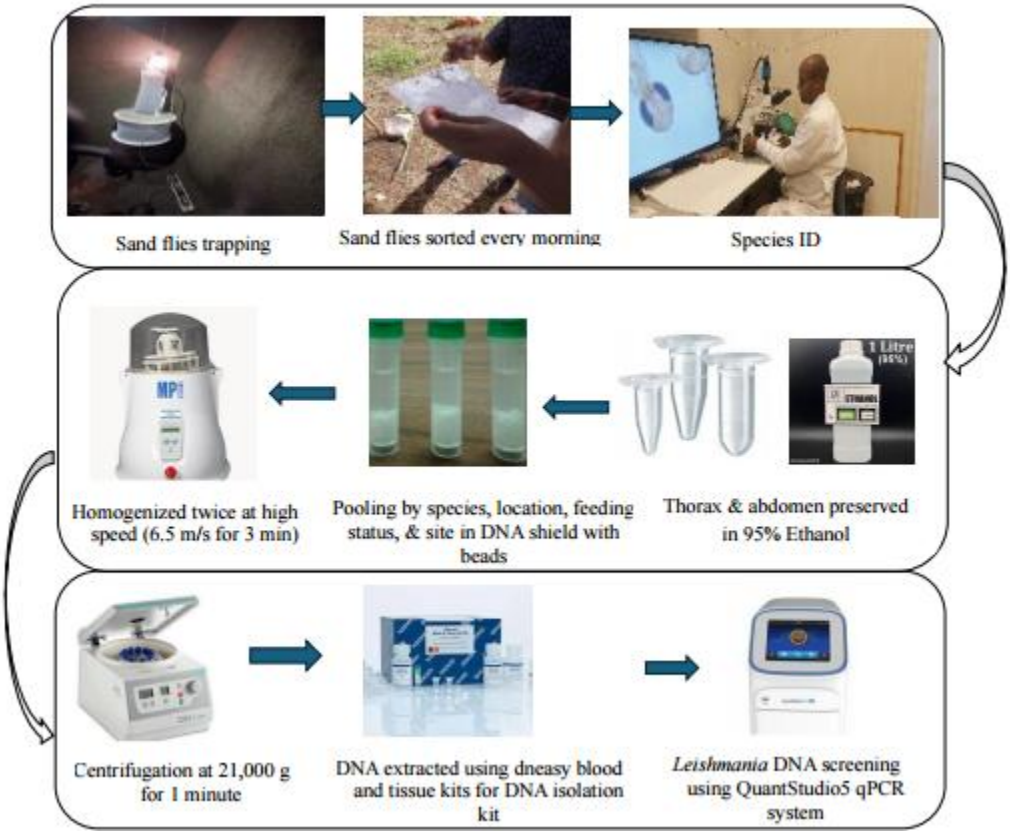


Figure 17: Workflow diagram illustrating the process from field collection of sand flies through laboratory processing to molecular screening for *Leishmania* DNA

3.10. Data quality assurance

To ensure data quality, several measures were implemented, including the development of appropriate data collection tools and the provision of training for data collectors, laboratory professionals, clinicians, and local guides before commencement of data collection. During the data collection process, questionnaires were reviewed daily for completeness, accuracy, and consistency. Blood samples are collected, processed, and diagnosed in laboratories by well-experienced laboratory experts. Quality control measures were applied for both DAT and rtPCR tests, utilizing positive and negative controls, and strict adherence to standard operating procedures was maintained throughout.

3.11. Ethical Statement

The study received approval from the Aklilu Lemma Institute of Pathobiology (presently, Aklilu Lemma Institute of Health Research)-Institutional Research Ethics Review Committee (ALIPB-IRERC) prior to data collection (Ref. No.: ALIPB IRERC/112/2015/23). A permission letter was obtained from the Federal Democratic Republic of Ethiopia Refugees and Returnees Service (FDRE RRS) and subsequently from the RRS regional office and camp coordination offices. Written informed consent and/or assent was obtained from all participants and from parents or guardians, with trained personnel assisting in participants' native languages to ensure comprehension. Privacy and confidentiality were strictly maintained, with all identifying data anonymized and securely stored, accessible only to the research team. The study followed all relevant guidelines for research on human subjects, implementing additional safeguards to protect participants' rights, dignity, and welfare. Although, according to the MoH treatment guidelines (MoH, 2013), asymptomatic VL cases are not eligible for treatment, all participants were informed of their test results. They were also advised about the possibility that asymptomatic infection may progress to symptomatic VL over time and were advised to seek medical evaluation promptly if they develop signs or symptoms suggestive of VL in the future. Gambella and Assosa hospitals function as VL treatment centers, with anti-leishmanial drugs supplied by the Ministry of Health upon request.

3.12. Statistical analysis

Data processing and analysis for prevalence study: The data were initially entered into Epi Info™ version 7.2 (Centers for Disease Control and Prevention, Atlanta, GA, USA), exported to Excel for data cleaning, then integrated into SPSS version 24.0 (SPSS Inc., Chicago, IL, USA) for descriptive analysis. Continuous variables were summarized with median and interquartile range (IQR), and categorical variables with numbers and proportions. Chi-square was used to assess the proportion differences among categorical variables. A significance level of 0.05 was considered statistically significant for comparison.

Data processing and analysis for matched case-control study: Data was cleaned for completeness and consistency, coded, and entered into EpiInfo version 7.0 and exported into Microsoft Office Excel and then to Statistical Package for the Social Sciences (SPSS) version 24.0 (SPSS Inc., Chicago, IL, USA) for further analysis. The results were organized, summarized, and presented using texts, tables, and graphs. Descriptive statistics were used to summarize characteristics of cases and controls. Chi-square tests were used to compare the percentages of cases and controls in terms of selected categorical variables. Bivariate conditional logistic regression was analyzed to examine the association between each independent variable and the outcome and to calculate unadjusted odds ratios with 95% confidence intervals. Independent variables that were statistically significant at $p<0.20$ in the bivariate analysis were retained and included in the multivariable conditional logistic regression model. All the candidate variables were entered into a multivariable binary logistic regression model to identify the effects of independent variables on the outcome variable. The fitness of the model was checked by the Hosmer and Lemeshow goodness-of-fit test. Multi-collinearity effects among independent variables based on the assumption of the variance inflation factor (VIF) were checked. The level of significance was set at 0.05.

Data processing and analysis for entomological survey: Data were entered using Microsoft Excel 2016, simply calculating the relative proportion from the overall sand fly collected (Aklilu et al., 2017a), and exported to SPSS software version 24.0 (SPSS Inc., Chicago, IL, USA) for further analysis. Sand fly density was calculated as the number of sand flies collected per trap per night using LT and ST to compare differences between habitats. The normality of the data was assessed using the Shapiro-Wilk test. When the data did not meet the assumption of normality, the non-parametric Kruskal-Wallis test was employed. This test was used to compare the mean number of sand fly species collected across different sampling habitats using CDC light traps and sticky traps. The chi-square (χ^2) test was applied to evaluate associations between categorical variables. The Shannon-Wiener (H) diversity index was calculated in PAST v4.03 for each study site using the parameters of the proportion in which each species was collected. The permutation test was employed to assess the statistical significance of differences in Shannon diversity indexes between study sites (Hammer et al., 2001). Statistical significance was determined at $p < 0.05$.

CHAPTER 4: RESULTS

4.1. Prevalence of *Leishmania* Infection in Refugee Camps

4.1.1. Demographic characteristics of study participants

A total of 1223 participants were involved in the study, slightly exceeding the planned sample size of 1214 individuals. From refugee camps in Benishangul-Gumuz region, 69.1% of respondents were females, while in the Gambella region, 58.3% were males. The majority of respondents in refugee camps in Benishangul-Gumuz (61.8%) came from Sudan, while all participants in Gambella were from South Sudan. Regarding educational levels of the participants, 30.8% in Benishangul-Gumuz and 41.2% in Gambella have no formal education. Over 63% of the refugees in both regions were married. The majority of the study participants, 61.2% and 70.0%, had a family size of more than four per single household in both Benishangul-Gumuz and Gambella regions, respectively (Table 4).

Table 4: Socio-demographic characteristics of study participants in selected refugee camps in Gambella (N=563) and Benishangul-Gumuz (N=660) regions, Ethiopia, 2023

Categories		Camp location by region		Total N (%)
		Benishangul-Gumuz N (%)	Gambella N (%)	
Gender	Female	456 (69.1)	235 (41.7)	691 (56.5)
	Male	204 (30.9)	328 (58.3)	532 (43.5)
Age group (years)	5-9	64 (9.7)	49 (8.7)	113 (9.2)
	10-19	202 (30.6)	141 (25.0)	343 (28.0)
	20-29	264 (40.0)	113 (20.1)	377 (30.8)
	30-39	71 (10.8)	121 (21.5)	192 (15.7)
	40-49	35 (5.3)	68 (12.1)	103 (8.4)
	50+	24 (3.6)	71 (12.6)	95 (7.8)
Camp Name	Kule	-	288 (51.2)	288 (23.5)
	Sherkole	220 (33.3)	-	220 (18.0)
	Terkidi	-	275 (48.9)	275 (22.5)
	Tsore	440 (66.7)	-	440 (36.0)
Country of Origin	South Sudan	252 (38.2)	563 (100.0)	815 (66.6)
	Sudan	408 (61.8)	0 (0.0)	408 (33.4)
Level of education	No formal education	203 (30.8)	232 (41.2)	435 (35.6)
	Primary school	342 (51.8)	232 (41.2)	574 (46.9)
	Secondary school	80 (12.1)	78 (13.9)	158 (12.9)
	Higher education	35 (5.3)	21 (3.7)	56 (4.6)
Marital Status	Married	428 (64.9)	358 (63.6)	786 (64.3)
	Single	232 (35.2)	205 (36.4)	437 (35.7)
Family Size	≤4 individuals	256 (38.8)	169 (30.0)	425 (34.8)
	>4 individuals	404 (61.2)	394 (70.0)	798 (65.2)

Study participants from both regions included individuals aged between 5 and 75 years. The median age of the participants was 23 years (range: 17-29) for those from the Benishangul-Gumuz region and 30 years (range: 15-40) for those from the Gambella region. The median duration of stay in the camp was 5 years (range: 1-8) for participants in the Benishangul-Gumuz refugee camps and 9 years (range: 8-10) for those in the Gambella camps. Additionally, the median family size was 6 members (range: 3-8) for

refugees in Benishangul-Gumuz and 6 members (range: 4-8) for refugees hosted in Gambella Regional State of Ethiopia (Table 5).

Table 5: Baseline characteristics of study participants by refugee camp region, presented as median values with interquartile ranges (IQR)

Variable	Region of refugee camp located		Overall, Median (IQR)
	Benishangul Gumuz	Gambella	
	Median (IQR)	Median (IQR)	
Age (Years)	23 (17-29)	30 (15-40)	25 (17-35)
Duration in the camp (years)	5 (1-8)	9 (8-10)	8 (4-9)
Family size (individuals)	6 (3-8)	6 (4-8)	6 (4-8)

4.1.2. Clinical assessment of study participants

The distribution of various clinical symptoms among individuals from four refugee camps across two regions, Benishangul-Gumuz and Gambella, along with their overall prevalence is presented in Table 6. In general, symptoms such as self-reported fever, chills, headache, and weakness were more frequently reported among refugees in Gambella compared to Benishangul-Gumuz. Specifically, fever was observed in 27.7% (156/563) of individuals in Gambella and 9.4% (62/660) in Benishangul-Gumuz, while chills were reported by 14.6% (82/563) and 7.3% (48/660), respectively. Headache prevalence was higher in Gambella, 24.3% (137/563), than in Benishangul-Gumuz, 11.7% (77/660), and weakness followed a similar trend (17.6% vs. 8.9%). Weight loss was relatively uncommon but more pronounced in refugees in Gambella (7.8%) compared to Benishangul-Gumuz (0.3%). No cases of hepatosplenomegaly were recorded among the study participants. Swelling of lymph nodes was extremely rare, reported in only two individuals (0.4%) from Gambella, with no cases observed in Benishangul-Gumuz. Similarly, rash was reported by only eight refugees across both regions, with a prevalence of 0.6% in Benishangul-Gumuz and 0.7% in Gambella. Among those with rashes, 50% had rashes on the general body, 37.5% on the face, and 12.5% on the lower extremities.

Table 6: Distribution of clinical signs and symptoms among study participants in refugee camps in Benishangul-Gumuz and Gambella Regional States of Ethiopia, 2023

Categories		Camp location by region		Overall N (%)
		Benishangul-Gumuz N (%)	Gambella N (%)	
Fever	Yes	62 (9.4)	156 (27.7)	218 (17.8)
	No	598 (90.6)	407 (72.3)	1005 (82.2)
Chills	Yes	48 (7.3)	82 (14.6)	130 (10.6)
	No	612 (92.7)	481 (85.4)	1093 (89.4)
Headache	Yes	77 (11.7)	137 (24.3)	214 (17.5)
	No	583 (88.3)	426 (75.7)	1009 (82.5)
Weakness	Yes	59 (8.9)	99 (17.6)	158 (12.9)
	No	601 (91.1)	464 (82.4)	1065 (87.1)
Weight loss	Yes	2 (0.3)	44 (7.8)	46 (3.8)
	No	658 (99.7)	519 (92.2)	1177 (96.2)
Splenomegaly	Yes	0 (0.0)	0 (0.0)	0 (0.0)
	No	660 (100.0)	563 (100.0)	1223 (100.0)
Hepatomegaly	Yes	0 (0.0)	0 (0.0)	0 (0.0)
	No	660 (100.0)	563 (100.0)	1223 (100.0)
Lymphadenopathy	Yes	0	2 (0.4)	2 (0.2)
	No	660 (100.0)	561 (99.6)	1221 (99.8)
Rash	Yes	4 (0.6)	4 (0.7)	8 (0.7)
	No	556 (99.4)	559 (99.3)	1215 (99.3)
Site of rash	General body	1 (33.3)	3 (60.0)	4 (50.0)
	Face	2 (66.4)	1 (20.0)	3 (37.5)
	Lower extremities	0 (0.0)	1 (20.0)	1 (12.5)

The distribution of refugees in studied camps is presented in Table 7. Refugees from South Sudan represent eight states of their country of origin. Half of the participants originated from the Upper Nile states, followed by 10.8% from Jonglei and 4.3% from Lakes. Refugees from Central Equatoria, Eastern Equatoria, Pibor Administrative Area, Unity, and Western Equatoria collectively accounted for only 1.3%. In contrast, refugees of Sudanese origin came from 14 states within Sudan. Among them, 20.1% were from Blue Nile state, followed by 2.7% each from Khartoum and River Nile, and 2.4% from North Darfur. Refugees from other Sudanese states each constituted less than 1% of the total participants, as shown in Table 7.

Table 7: Distribution of participants by state and origin of country in refugee camps located in Gambella and Benishangul-Gumuz Regional States, 2023

Country of Origin	State	Frequency	Proportion
South Sudan	Central Equatoria	2	0.2%
	Eastern Equatoria	2	0.2%
	Jonglei	132	10.8%
	Lakes	53	4.3%
	Pibor Adm Area	1	0.1%
	Unity	10	0.8%
	Upper Nile	613	50.2%
	Western Equatoria	1	0.1%
Sudan	Gedaref	2	0.2%
	Gazira	1	0.1%
	Blue Nile	245	20.1%
	Kassala	2	0.2%
	Khartoum	33	2.7%
	North Darfur	29	2.4%
	North Kordofan	8	0.7%
	Northern	3	0.2%
	River Nile	33	2.7%
	Sennar	1	0.1%
	South Darfur	15	1.2%
	South Kordofan	3	0.2%
	West Darfur	27	2.2%
	White Nile	5	0.4%
Total		**1221**	**100.0%**

4.1.3. Prevalence of *Leishmania* infection

DAT positivity was observed in 4.0% of cases (95% CI: 3.0% to 5.3%), with 49 out of 1223 participants testing positive. Among the positive cases, most patients exhibited titers of 1:1600 or 1:3200. Interestingly, 275 of the negative participants still showed some degree of agglutination (Fig. 18). Real-time PCR positivity was 10.3% (78 out of 754) (95% CI: 8.3% to 12.7%), with cycle threshold (Ct) values ranging from 30.9 to 39.9 and an average Ct value of 36.6.

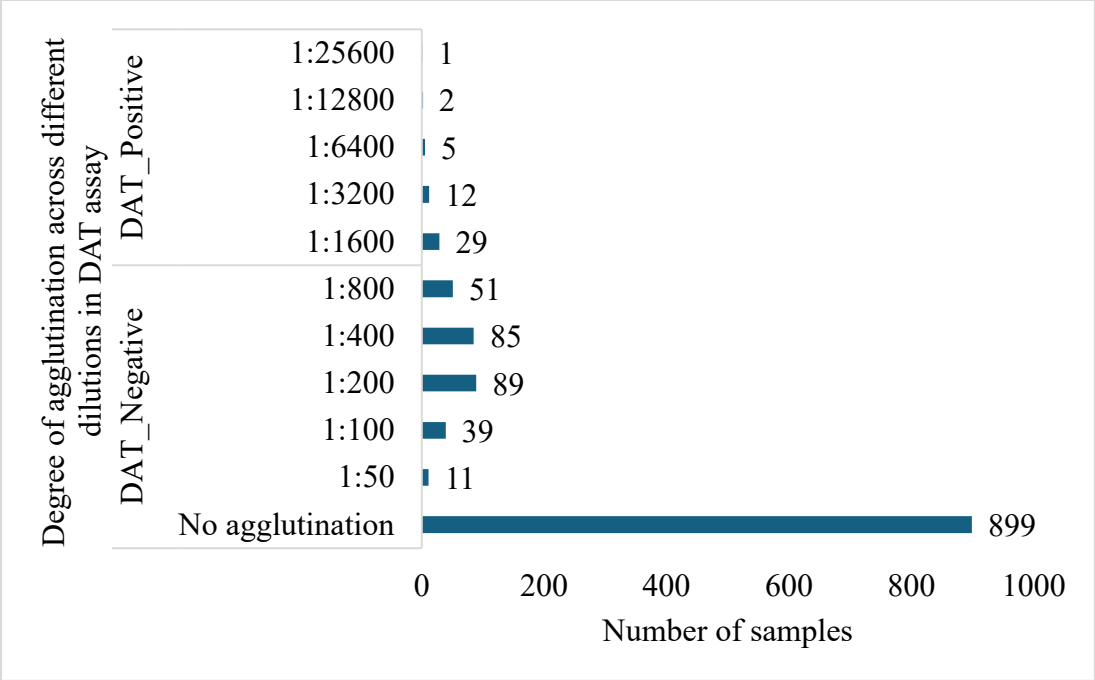


Figure 18: Distribution of anti-*Leishmania* antibody agglutination among selected refugee camp participants in Gambella and Benishangul-Gumuz regions, 2023.

A slightly higher DAT positivity rate was observed among males (4.7%) than females (3.5%). The DAT positivity rate showed that 7.8% of the participants were aged between 40 and 49 years, followed by 5-9 and greater than 50 years, each at 5.3%. However, the difference was not supported by a statistical test ($p>0.05$). The occurrence of *Leishmania* infection as tested by DAT in refugee camps in Benishangul-Gumuz region was higher than those in refugee camps in Gambella region (5.0% vs 2.8%), but not statistically significant ($p=0.055$). The distribution of DAT positivity by refugee camps revealed that it was 5.9%, 3.2%, 3.1%, and 2.5% in Tsore, Sherkole, Kule, and Terkidi camps, respectively. This distribution was also not statistically significant ($p=0.084$). The distribution of DAT positivity rate significantly varies among the educational levels of participants. A higher positivity rate (6.0%) was recorded for participants with no formal education than participants with the higher educational level ($p=0.024$) (Table 8).

Among individuals reporting specific symptoms, *Leishmania* DAT positivity rates varied as follows: those with fever had a DAT positivity rate of 6.0%, while individuals with chills and headache also showed rates at 4.6% and 4.7%, respectively. A feeling of weakness was

reported with a DAT positivity rate of 3.8%. Lymphadenopathy was reported by two participants; however, they were negative for DAT. Out of eight rash-presenting participants, two (25.0%) of them were positive for DAT, and the proportion was significantly higher than for participants without rash. Moreover, the rashes were observed on various body parts of the participants (Table 8). Among these, 66.7% (2/3) of the rashes on the face showed DAT positivity. In contrast, no DAT positivity was reported for rashes located on the lower extremities or other general body parts of the participants (Table 8).

Table 8: Distribution of *Leishmania* infection by sociodemographic and clinical characteristics of participants with DAT positivity in selected refugee camps in Gambella and Benishangul-Gumuz regions, Ethiopia, in 2023

Category		DAT Result		P-value
		Negative N (%)	Positive N (%)	
Gender	Male	508 (95.3)	25 (4.7)	0.430
	Female	666 (96.5)	24 (3.5)	
Age Group	5-9	107 (94.7)	6 (5.3)	0.211
	10-19	333 (97.1)	10 (2.9)	
	20-29	366 (97.1)	11 (2.9)	
	30-39	183 (95.3)	9 (4.7)	
	40-49	95 (92.2)	8 (7.8)	
	50+	90 (94.7)	5 (5.3)	
Region	Benishangul Gumuz	627 (95.0)	33 (5.0)	0.055
	Gambella	547 (97.2)	16 (2.8)	
Camp name	Kule	279 (96.9)	9 (3.1)	0.084
	Terkidi	268 (97.5)	7 (2.5)	
	Tsore	414 (94.1)	26 (5.9)	
	Sherkole	213 (96.8)	7 (3.2)	
Country of Origin	South Sudan	778 (95.5)	37 (4.5)	0.179
	Sudan	396 (97.1)	12 (2.9)	
Education level	No formal education	409 (94.0)	26 (6.0)	0.024
	Grades 1-8	561 (97.7)	13 (2.3)	
	Grades 9-12	150 (94.9)	8 (5.1)	
	College and above	54 (96.4)	2 (3.6)	
Marital Status	Married	753 (95.8)	33 (4.2)	0.646
	Single	421 (96.3)	16 (3.7)	
Family size Cat	≤4	407 (95.8)	18 (4.2)	0.766
	>4	767 (96.1)	31 (3.9)	
Fever	No	969 (96.4)	36 (3.6)	0.104
	Yes	205 (94.0)	13 (6.0)	
Chills	No	1050 (96.1)	43 (3.9)	0.708
	Yes	124 (95.4)	6 (4.6)	
Headache	No	970 (96.1)	39 (3.9)	0.584
	Yes	204 (95.3)	10 (4.7)	
Weakness	No	1022 (96.0)	43 (4.0)	0.886
	Yes	152 (96.2)	6 (3.8)	
Lymph node swelling	No	1172 (96.0)	49 (4.0)	0.772
	Yes	2 (100.0)	0 (0.0)	
Rash	No	1168 (96.1)	47 (3.9)	0.002
	Yes	6 (75.0)	2 (25.0)	
Site of rash	Face	1 (33.3)	2 (66.7)	0.001
	General body	4 (100.0)	0 (0.0)	
	Lower limb	1 (100.0)	0 (0.0)	

The distribution of *Leishmania* infection as determined by rtPCR was the highest in Tsore compared to other refugee camps in Gambella and Benishangul-Gumuz regional states ($P<0.001$). However, the distribution of rtPCR positivity rate by sex, age group, region where refugee camps are located, level of education, and marital status variables was similar. Similarly, rtPCR positivity rate variations were also observed among different clinical symptoms but not supported by statistical tests ($P>0.05$). Among individuals who reported fever, 11.7% tested rtPCR positive for *Leishmania*. Those with chills, headaches, and weakness had rtPCR positivity rates of 8.8%, 7.3%, and 6.2%, respectively (Table 9). On the other hand, the major clinical symptoms of VL were not reported by all participants, which is strongly suggestive of asymptomatic VL. The distribution of rtPCR positivity among gender, age group, regions, country of origin, educational variation, and marital status of the participants was similar ($P>0.05$).

Table 9: Distribution of *Leishmania* infection among sociodemographic and clinical characteristics of participants with rtPCR positivity in selected refugee camps in Gambella and Benishangul-Gumuz regions, Ethiopia, in 2023

Category		rtPCR result		P-value
		Negative N (%)	Positive N (%)	
Gender	Male	233 (90.3)	25 (9.7)	0.670
	Female	443 (89.3)	53 (10.7)	
Age Group in Years	5-9	63 (87.5)	9 (12.5)	0.561
	10-19	202 (90.2)	22 (9.8)	
	20-29	258 (90.8)	26 (9.2)	
	30-39	80 (90.9)	8 (9.1)	
	40-49	42 (87.5)	6 (12.5)	
	50+	31 (81.6)	7 (18.4)	
Region	Benishangul Gumuz	578 (89.2)	70 (10.8)	0.308
	Gambella	98 (92.5)	8 (7.5)	
Camp name	Kule	54 (91.5)	5 (8.5)	<0.001
	Terkidi	44 (93.6)	3 (6.4)	
	Sherkole	210 (96.8)	7 (3.2)	
	Tsore	368 (85.4)	63 (14.6)	
Country of Origin	South Sudan	313 (88.2)	42 (11.8)	0.412
	Sudan	361 (90.9)	36 (9.1)	
Education level	No formal education	211 (87.6)	30 (12.4)	0.281
	Grades 1-8	341 (90.2)	37 (9.8)	
	Grades 9-12	86 (89.6)	10 (10.4)	
	College and above	38 (97.4)	1 (2.6)	
Marital Status	Married	437 (89.4)	52 (10.6)	0.846
	Single	239 (90.1)	26 (9.8)	
Fever	No	593 (89.8)	67 (10.2)	0.644
	Yes	83 (88.3)	11 (11.7)	
Chills	No	614 (89.5)	72 (10.5)	0.666
	Yes	62 (91.2)	6 (8.8)	
Headache	No	574 (89.1)	70 (10.9)	0.252
	Yes	102 (92.7)	8 (7.3)	
Weakness	No	600 (89.2)	73 (10.8)	0.192
	Yes	76 (93.8)	5 (6.2)	
Lymph node swelling	No	676 (89.7)	49 (10.3)	ND
	Yes	0 (0.0)	0 (0.0)	
Rash	No	671 (89.7)	77 (10.3	0.610
	Yes	5 (83.3)	1 (16.7)	
Site of rash	Face	1 (50.0)	1 (50.0)	0.442
	General body	3 (100.0)	0 (0.0)	
	Lower limb	1 (100.0)	0 (0.0)	

Among the 754 individuals tested using both PCR and DAT, 90 were positive for *Leishmania* infection, resulting in an overall positivity rate of 11.9% (90/754) (95% CI: 9.7%-14.1%). Of these, 12 samples were positive only with DAT, 55 were positive only with rtPCR, and 23 were positive with both methods, resulting in a total of 90 positive cases. As depicted in the Venn diagram, although there was some overlap between the two tests, rtPCR detected substantially more positive cases compared to DAT (Fig. 19).



Figure 19: Venn diagram of DAT and rtPCR positivity (n=90) [DAT+ (Direct Agglutination Test Positive): represented by the yellow circle; rtPCR+ (Real-time PCR Positive): represented by the blue circle, and the intersection (23 cases) indicates samples that tested positive in both methods]

4.2. Determinants of *Leishmania* Infection in Refugee Camps

4.2.1. Socio-demographic characteristics

A total of 336 participants, 252 controls and 84 cases, participated in all selected refugee camps. Among the cases, 12 tested positive by DAT only, 53 tested positive by rtPCR only, and 19 tested positive by both DAT and rtPCR. The majority of individuals in both groups were from refugee camps located in the Benishangul-Gumuz regional state (89.3% control, 89.3% cases). Females predominate in both groups (65.5% control, 65.5% cases). The average age of participants in both case and control groups was similar. Most participants reside in Tsore camp (78.6% control, 74.6% cases). In terms of level of education, a

prominent portion in both groups had reported that they had completed grades one to eight (47.6% control, 53.6% cases). In marital status, married individuals were more common in both groups (64.3% control, 62.3% cases), followed by singles. The test of independence was performed to assess the distribution of proportions among the variables in the case and control groups. As a result, the distribution of matching variables (age group, sex, and region of camp location) showed that they were similarly distributed ($p > 0.05$). The mean number of family members per household was found to be six individuals for both controls and cases. Camp duration was also reported to have a mean year of duration of 5.6 and 6.8 for the control and case groups, respectively (Table 10).

Table 10: Socio demographic characteristics of participants in matched case-control study in refugee camps in Gambella and Benishangul-Gumuz regional states of Ethiopia, 2023

Variables		Case (%)	Control (%)	Total (%)	P-value
Region	Benishangul Gumuz	75 (89.3)	225 (89.3)	300 (89.3)	1,000
	Gambella	9 (10.7)	27 (10.7)	36 (10.7)	
Gender	Male	29 (34.5)	87 (34.5)	116 (34.5)	1,000
	Female	55 (65.5)	165 (65.5)	220 (65.5)	
Camp Name	Kule	6 (7.1)	21 (8.3)	27 (8.0)	0.729
	Terkidi	3 (3.6)	6 (2.4)	9 (2.7)	
	Tsore	66 (78.6)	188 (74.6)	254 (75.6)	
	Sherkole	9 (10.7)	37 (14.7)	46 (13.7)	
Country of Origin	South Sudan	44 (52.4)	118 (46.8)	160 (47.6)	0.313
	Sudan	40 (47.6)	134 (53.2)	176 (52.4)	
Level of education	No formal education	32 (38.1)	77 (30.6)	109 (32.4)	0.163
	Grades 1-8	40 (47.6)	135 (53.6)	175 (52.1)	
	Grades 9-12	11 (13.1)	25 (9.9)	36 (10.7)	
	College/university	1 (1.2)	15 (6.0)	16 (4.8)	
Marital Status	Married	54 (64.3)	157 (62.3)	211 (62.8)	0.745
	Single	30 (35.7)	95 (37.7)	125 (37.2)	
Family size; mean SD		6±3	6±3	6±3	-
Age, Years; median (IQR)		23 (13)	23 (12)	23 (13)	-
Camp duration, Y; mean SD		5.6±3.7	6.8±3.3	6±3.7	-

The distribution of case and control participants by country of origin (South Sudan vs. Sudan) and their respective states of origin is presented in Table 11. In South Sudan, most participants originated from Upper Nile (32.9% control, 45.2% cases), followed by Jonglei (11.5% control, 4.8% cases) and Lakes (0.8% control, 2.4% cases). In Sudan, the majority

of participants were from Blue Nile, with 30.2% in the control group and 40.5% in the case group. Smaller proportions were from Khartoum, comprising 4.8% of the control group and 3.6% of the case group, and from North Darfur, which accounted for 4.0% of the control group and 1.2% of the case group, as depicted in Table 11.

Table 11: Distribution of participants by country of origin and state

Country of Origin	State	Control (%)	Case (%)
South Sudan	Central Equatoria	1 (0.4)	0 (0.0)
	Eastern Equatoria	1 (0.4)	0 (0.0)
	Jonglei	29 (11.5)	4 (4.8)
	Lakes	2 (0.8)	2 (2.4)
	Unity	2 (0.8)	0 (0.0)
	Upper Nile	83 (32.9)	38 (45.2)
Sudan	Blue Nile	76 (30.2)	34 (40.5)
	Gedaref	1 (0.4)	0 (0.0)
	Kassala	2 (0.8)	0 (0.0)
	Khartoum	12 (4.8)	3 (3.6)
	North Darfur	10 (4.0)	1 (1.2)
	North Kordofan	3 (1.2)	1 (1.2)
	River Nile	9 (3.6)	0 (0.0)
	Sennar	1 (0.4)	0 (0.0)
	South Darfur	8 (3.2)	1 (1.2)
	West Darfur	10 (4.0)	0 (0.0)
	White Nile	2 (0.8)	0 (0.0)

4.2.2. Behavioral and environmental characteristics

Overall, the distribution of the possible behavioral and environmental exposure factors was observed more among cases than controls. A higher proportion of cases (31.0%) reported sleeping outside during the daytime compared with controls (9.5%), while the majority in both groups reported sleeping inside (69.0% among cases and 90.5% among controls). Similarly, a greater proportion of cases (45.2%) than controls (29.8%) reported sleeping

outside at night during the study period. Regarding bed net use, only 15.5% of cases reported always using a net while sleeping, compared to 25.4% of controls. The presence of bushes around the house was more common among cases (45.2%) than controls (27.4%), and house wall cracks were reported more frequently among cases (33.3%) than controls (18.3%). Likewise, termite hills within 5 meters of the sleeping area were reported by 23.8% of cases compared to 14.3% of controls. With respect to sleeping sites, cracked soil around the sleeping place was observed for 17.9% of cases and 12.7% of controls. When asked about the frequency of sleeping outside during the dry season, 46.4% of cases reported doing so always, compared to 25.8% of controls. Meanwhile, 23.8% of cases and 44.0% of controls reported sleeping outside sometimes, and about 30% in both groups reported never sleeping outside during the dry season. Finally, the presence of termite hills near the house was more frequently reported among cases (36.9%) than controls (27.8%) (Table 12).

Table 12: Distribution of behavioral and environmental characteristics of participants, case-control study in selected refugee camps, Gambella and Benishangul-Gumuz regional states of Ethiopia, 2023

Variables		Case N (%)	Control N (%)	Total N (%)
Sleep during the daytime?	Outside	26 (31.0)	24 (9.5)	50 (14.9)
	Inside	58 (69.0)	228 (90.5)	286 (85.1)
sleep during nighttime?	Outside	38 (45.2)	75 (29.8)	113 (33.6)
	Inside	46 (54.8)	177 (70.2)	223 (66.4)
Do you always use a net while sleeping?	Yes	13 (15.5)	64 (25.4)	77 (22.9)
	No	71 (84.5)	188 (74.6)	259 (77.1)
Are there bushes around the house?	Yes	38 (45.2)	69 (27.4)	107 (31.8)
	No	46 (54.8)	183 (72.6)	229 (68.2)
Is the house cracked?	Yes	28 (33.3)	46 (18.3)	74 (22.0)
	No	56 (66.7)	206 (81.7)	262 (78.0)
Are termite hills present 5 m from the sleeping place?	Yes	20 23.8)	36 (14.3)	56 (16.7)
	No	64 (76.2)	216 (85.7)	280 (83.3)
Is cracked soil a sleeping place?	Yes	15 (17.9)	32 (12.7)	47 (14.0)
	No	69 (82.1)	220 (87.3)	289 (86.0)
Frequency of sleeping outside the dry season	Always	39 (46.4)	65 (25.8)	104 (31.0)
	Sometimes	20 (23.8)	111 (44.0)	131 (39.0)
	Never	25 (29.8)	76 (30.2)	101 (30.1)
Termite hills are present near the house.	Yes	31 (36.9)	70 (27.8)	101 (30.1)
	No	53 (63.1)	182 (72.2)	235 (69.9)

4.2.3. Determinants of *Leishmania* infection

The bivariate analysis identified significant associations between sociodemographic, environmental, and housing conditions and behavioral risk factors with *Leishmania*-infected cases (Table 13). Sleeping outside during the daytime (COR=2.78; 95% CI=1.71-4.51, p<0.001) and nighttime (COR: 1.65; 95% CI: 1.07-2.56; p=0.024), bush presence around the house (COR: 1.81; 95% CI: 1.17-2.81; p=0.008), and cracked house walls (COR=1.82; 95% CI: 1.15-2.90; p=0.011) were associated with increased unadjusted odds of cases of *Leishmania* infection. In addition, among the sociodemographic factors, participants infected with *Leishmania* who lived less than five years in the camp were 1.91 times more exposed compared to those who lived more than five years in the camp (COR: 1.91; 95% CI: 1.16-3.15; p = 0.011). On the other hand, bed net use associated with lower odds of VL infection trend (COR = 0.59, p = 0.096), and termite hills near to sleeping places (COR = 1.60, p = 0.075) and always sleeping outside during the dry season (COR = 1.56, p = 0.093) had a higher risk of being infected with *Leishmania*, but the association was not statistically significant. Furthermore, factors including the presence of cracked floors and termite hills near the house were not significantly associated with cases of *Leishmania* infection among study participants (Table 13).

Table 13: The bivariate association between sociodemographic, environmental, and housing conditions and behavioral risk factors with Leishmania-infected cases: A Case-Control Study conducted in Gambella and Benishangul-Gumuz regional states of Ethiopia, 2023

Variables	Description	Case (%)	Control (%)	COR	P-value
Sociodemographic factors					
Camp Name	Kule	6 (7.1)	21 (8.3)	0.62 (0.14-2.85)	0.541
	Tsore	66 (78.6)	188 (74.6)	1.37 (0.66-2.83)	0.400
	Sherkole	9 (10.7)	37 (14.7)	1	
Country of Origin	South Sudan	44 (52.4)	116 (46.0)	1.26 (0.79-2.01)	0.336
	Sudan	40 (47.6)	136 (54.0)	1	
Level of education	No formal education	32 (38.1)	77 (30.6)	5.12 (0.68-38.65)	0.113
	Grades 1-8	40 (47.6)	135 (53.6)	3.90 (0.52-29.12)	0.184
	Grades 9-12	11 (13.1)	25 (9.9)	5.22 (0.66-41.22)	0.117
	College/university	1 (1.2)	15 (6.0)	1	
Marital Status	Married	54 (64.3)	157 (62.3)	1.09 (0.65-1.85)	0.741
	Single	30 (35.7)	95 (37.7)	1	
Length of stay in the camp	≤5 years	60 (71.4)	136 (54.0)	1.91 (1.16-3.15)	0.011
	>5 years	24 (28.6)	116 (46.0)	1	
Family Size (HH members)	≤4	57 (67.9)	151 (59.9)	1	
	>4	27 (32.1)	101 (40.1)	1.31 (0.82-2.10)	0.252
Behavioral practice factors					
Sleep during the daytime.	Outside	26 (31.0)	24 (9.5)	2.78 (1.71-4.51)	<0.001
	Inside	58 (69.0)	228 (90.5)	1	
sleep during nighttime	Outside	38 (45.2)	75 (29.8)	1.65 (1.07-2.56)	0.024
	Inside	46 (54.8)	177 (70.2)	1	
Always use a bed net while sleeping	Yes	13 (15.5)	64 (25.4)	0.59 (0.32-1.10)	0.096
	No	71 (84.5)	188 (74.6)	1	
Frequency of sleeping outside during the dry season	Always	39 (46.4)	65 (25.8)	1.56 (0.93-2.62)	0.093
	Sometimes	20 (23.8)	111 (44.0)	0.62 (0.34-1.12)	0.113
	Never	25 (29.8)	76 (30.2)	1	
Environmental and housing conditions					
Bushes are available around the house	Yes	38 (45.2)	69 (27.4)	1.81 (1.17-2.81)	0.008
	No	46 (54.8)	183 (72.6)	1	
Presence of cracked wall	Yes	28 (33.3)	46 (18.3)	1.82 (1.15-2.90)	0.011
	No	56 (66.7)	206 (81.7)	1	
Presence of termite hills near the house	Yes	31 (36.9)	70 (27.8)	1.38 (0.88-2.17)	0.164
	No	53 (63.1)	182 (72.2)	1	
Termite hills present near sleeping place <5 m	Yes	20 (23.8)	36 (14.3)	1.60 (0.95-2.67)	0.075
	No	64 (76.2)	216 (85.7)	1	
Presence of a cracked floor near the sleeping place	Yes	15 (17.9)	32 (12.7)	1.35 (0.77-2.39)	0.299
	No	69 (82.1)	220 (87.3)	1	

*<5m: less than five-meter distance; COR: Crude Odds Ratio; 95% CI: 95% Confidence Interval; Ref: Reference variable

The adjusted analysis examined various factors associated with the cases of *Leishmania* infection. Variables with <0.20 p values in the bivariate analysis were entered into the multivariate model and analyzed using the backward stepwise conditional logistic regression method. Durations in the camp lower than five years were significantly associated with cases of *Leishmania* infection (AOR=1.76, 95% CI: 1.06-2.93, P=0.028). Sleeping outside during the daytime (AOR=2.38, 95% CI: 1.46-3.89, P<0.001) and nighttime (AOR=1.68, 95% CI: 1.07-2.64, P=0.025) increased odds, and bushes available around the house were found to be an exposure factor to increase the odds of a case infected with *Leishmania* (AOR=1.76; 95% CI: 1.12-2.77, p=0.014). Conversely, other variables analyzed in the conditional logistic regression model showed no statistical significance. These included the level of education, the frequency of sleeping outdoors during the dry season, whether respondents always or sometimes slept outside during the dry season, whether respondents always or sometimes slept outside, and the presence of termite hills within 5 meters of the sleeping place <5 m (Table 14).

Table 14: Multivariable conditional logistic regression results showing adjusted odds ratios (AORs), with 95% confidence interval for factors associated with *Leishmania* infection in refugee camps in Gambella and Benishangul-Gumuz regional states, Ethiopia, 2023

Description	Case (%)	Control (%)	AOR (95% CI)	P-value
No formal education	32 (38.1)	77 (30.6)	4.30 (0.48-38.76)	0.190
Education level: Grades 1-8	40 (47.6)	135 (53.6)	2.59 (0.30-22.42)	0.390
Education level: Grades 9-12	11 (13.1)	25 (9.9)	4.06 (0.41-39.74)	0.230
Education level: College/university	1 (1.2)	15 (6.0)	1	
Sleep outside the house during the day.	26 (31.0)	24 (9.5)	2.38 (1.46-3.89)	<0.001
Sleep inside the house during the day (Ref).	58 (69.0)	228 (90.5)	1	
Sleep outside the house during the night.	38 (45.2)	75 (29.8)	1.68 (1.07-2.64)	0.025
Sleep inside the house during the night (Ref)	46 (54.8)	177 (70.2)	1	
Bushes are available around the house	38 (45.2)	69 (27.4)	1.76 (1.12-2.77)	0.014
Bushes are not available around the house (Ref)	46 (54.8)	183 (72.6)	1	
Always sleeping outside during the dry season	39 (46.4)	65 (25.8)	1.19 (0.69-2.04)	0.532
Sometimes sleeping outside during dry season	20 (23.8)	111 (44.0)	0.62 (0.34-1.14)	0.125
Never sleeping outside during dry season	25 (29.8)	76 (30.2)	1	
Termite hills present near to the house	31 (36.9)	70 (27.8)	1.26 (0.68-2.35)	0.468
Termite hills are not present near the house (Ref)	53 (63.1)	182 (72.2)	1	
Length of stay in the camp ≤5	60 (71.4)	136 (54.0)	1.76 (1.06-2.93)	0.028
Length of stay in the camp >5 (Ref)	24 (28.6)	116 (46.0)	1	

AOR: Adjusted odds ratio; 95% CI: 95% confidence interval

4.3. Sand fly survey in refugee camps

4.3.1. Preliminary assessments prior to actual sand fly collection

During the preliminary assessment of sand flies, a total of 474 specimens were collected in Terkidi and Kule refugee camps. However, no sand flies were collected in Tsore and Sherkole refugee camps. Morphological examination at the genus level revealed that all samples belonged to the genus *Sergentomyia*. However, species-level identification was not conducted at this time.

4.3.2. Sand fly species composition and relative abundance across sites

In the current study, a total of 2,196 adult phlebotomine sand flies (1,542 males and 654 females) belonging to eight species in two genera (*Phlebotomus* and *Sergentomyia*) were collected using CDC light traps (LTs) and sticky traps (STs). The genus *Phlebotomus* was represented by a single subgenus (*Anaphlebotomus),* while the genus *Sergentomyia* was represented by four subgenera (*Grassomyia, Sergentomyia, Sintonius,* and *Parrotomyia*) (Table 15). One species of *Phlebotomus* and seven species of *Sergentomyia* were identified from the four refugee camps in Gambella and Benishangul-Gumuz Regional States. The majority of the sand flies (85.1%, n=1,869) were collected from refugee camps in Gambella; specifically, Kule (39.0%, n=857) and Terkidi (46.1%, n=1012). In contrast, 14.9% (n=327) were collected from refugee camps in Benishangul-Gumuz Regional State: Sherkole (14.1%, n=310) and Tsore (0.8%, n=17). Among the refugee camps, Terkidi accounted for the largest proportion of specimens (46.1%), followed by Kule (39.0%), Sherkole (14.1%), and Tsore (0.8%) (Table 15).

Sergentomyia antennatus was the most abundant species (50.8%, n=1116) of the total collection, with dominance observed in Kule (60.9%) and Terkidi (54.5%) ($p<0.001$). *Sergentomyia africanus* ranked second in abundance (15.7%, n=345), with its highest distribution in Sherkole (39.4%) and Tsore (64.7%). *Sergentomyia schwetzi* (14.3%, n=315) and *Sergentomyia bedfordi* (10.9%, n=239) were also widely distributed, particularly in Terkidi and Sherkole. Less common species included *Sergentomyia clydei* (5.2%, n=115), *Phlebotomus rodhaini* (1.7%, n=38), and *Sergentomyia squamipleuris*

(1.2%, n=27), while *Sergentomyia adleri* was rare, represented by a single specimen (0.1%, n=1). Species richness was greatest in Terkidi, where all eight species were recorded, whereas Tsore exhibited both low abundance and diversity (Table 15).

Table 15: Distribution of sand fly species by refugee camp in Gambella and Benishangul-Gumuz Regional States of Ethiopia, April-May 2025

	Regional States				
	Gambella (n=1869)		**Benishangul-Gumuz (n=327)**		
Species (subgenus)	**Kule (%)**	**Terkidi (%)**	**Sherkole (%)**	**Tsore (%)**	**Total (%)**
Ph. rodhaini (*Anaphlebotomus)*	0 (0.0)	26 (2.6)	11 (3.5)	1 (5.6)	38 (1.7)
S. adleri (*Sintonius)*	0(0.0)	1 (0.1)	0 (0.0)	0 (0.0)	1 (0.1)
S. africanus (*Parrotomyia*)	78 (9.1)	134 (13.2)	122 (39.4)	11 (64.7)	345 (15.7)
S. antennatus (*Sergentomyia*)	522 (60.9)	552 (54.5)	41 (13.2)	1 (5.6)	1116 (50.8)
S. bedfordi group (*Sergentomyia)*	105 (12.3)	84 (8.3)	48 (15.5)	2 (11.8)	239 (10.9)
S. clydei (*Sintonius*)	84 (9.8)	28 (2.8)	2 (0.6)	1 (5.6)	115 (5.2)
S. schwetzi (*Sergentomyia*)	59 (6.9)	169 (16.7)	86 (27.7)	1 (5.6)	315 (14.3)
S. squamipleuris (*Grassomyia*)	9 (1.1)	18 (1.8)	0 (0.0)	0 (0.0)	27 (1.2)
Grand Total	**857 (39.0)**	**1012 (46.1)**	**310 (14.1)**	**17 (0.8)**	**2196 (100.0)**

4.3.3. Species composition relative to collection methods

The phlebotomine sand flies collected using LT and ST were presented in Table 16. The majority of sand flies (96.0%, n=2109) were captured using ST (X^2=22.1; p=0.016), with males representing 70.9% and females 29.1%. Sticky traps, deployed in household compounds, indoor and natural habitats, and termite hill habitats, proved highly effective in capturing all reported sand fly species. The majority of the *Ph. rodhaini* species (94.7%, 36/38) were obtained using the ST collection method. In contrast, LTs contributed only 4.0% of the total sand flies collected, with nearly equal proportions of males (52.9%) and females (47.1%). These findings demonstrate the superior efficiency of STs for capturing sand flies in diverse habitats, while LTs provided a more balanced representation of sexes and still collected a wide range of species.

Table 16: Phlebotomine sand flies collected using LT and ST by sex

	CDC			ST			Overall		
Species	M	F	total	M	F	total	M	F	total
Ph. rodhaini	1 (50.0)	150.0)	2 (2.3)	26 (72.2)	10 (27.8)	36 (1.7)	27 (71.1)	11 (28.9)	38 (1.7)
S. adleri	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	1 (0.1)	1 (100.0)	0 (0.0)	1 (0.1)
S. africanus	12 (48.0)	13 (52.0)	25 (28.7)	138 (43.1)	182 (56.9)	320 (15.2)	150 (43.5)	195 (56.5)	345 (15.7)
S. antennatus	21 (63.6)	12 (36.7)	33 (37.9)	867 (80.1)	216 (19.9)	1083 (51.4)	888 (79.6)	228 (20.4)	1116 (50.8)
S. bedfordi	4 (44.4)	5 (55.6)	9 (10.3)	188 (81.7)	42 (18.3)	230 (10.9)	192 (80.3)	47 (19.7)	239 (10.9)
S. clydei	2 (66.7)	1 (33.3)	3 (3.4)	56 (50.0)	56 (50.0)	112 (5.3)	58 (50.4)	57 (49.6)	115 (5.2)
S. schwetzi	5 (45.5)	6 (54.5)	11 (12.6)	205 (67.4)	99 (32.6)	304 (14.4)	210 (66.7)	105 (33.3)	315 (14.3)
S. squamipleuris	1 (25.0)	3 (75.0)	4 (4.6)	15 (65.2)	8 (34.8)	23 (1.1)	16 (59.3)	11 (40.7)	27 (1.2)
Grand Total	**46(52.9)**	**41(47.1)**	**87(4.0)**	**1496(70.9)**	**613(29.1)**	**2109 (96.0)**	**1541(70.2)**	**654(29.8)**	**2196(100.0)**

4.3.4. Indoor and outdoor collections of sand flies

Indoor collections across all sites were notably low, accounting for only 49 (2.2%) specimens out of 2,196 total specimens; however, this result was not statistically significant (p=0.209). Among the 38 *Ph. rodhaini* specimens, only 2 (5.3%) were collected from indoor habitats. *Sergentomyia africanus* 3.8% (n=13) and *S. antennatus* 2.0% (n=22) were among indoor collections, while other species, including *S. bedfordi, S. clydei, S. adleri,* and *S. schwetzi,* were rarely found in indoor habitats. This pattern highlights the predominantly outdoor behavior of these species in the study area (Table 17).

Table 17: Indoor and outdoor abundance of sand fly species across four study sites in Gambella and Benishangul-Gumuz Regional States, Ethiopia, April-May 2025

	Kule		**Sherkole**		**Terkidi**		**Tsore**		**Total (%)**	
Species	**Indoor**	**Outdoor**	**Indoor**	**Outdoor**	**Indoor**	**Outdoor**	**Indoor**	**Outdoor**	**Indoor**	**Outdoor**
Ph. rodhaini	0	0	0	11	1	25	1	0	2(5.3)	36 (94.7)
S. adleri	0	0	0	0	0	1	0	0	0 l(0.0)	1 (100.0)
S. africanus	8	70	0	122	4	130	1	10	13(3.8)	332(96.2)
S. antennatus	10	512	0	41	12	540	0	1	22(2.0)	1094(98.0)
S. bedfordi	5	100	0	48	2	82	0	2	7(2.9)	232 (97.1)
S. clydei	1	83	0	2	0	28	0	1	1(0.9)	114 (99.1)
S. schwetzi	2	57	0	86	1	168	0	1	3(1.0)	312 (99.0)
S. squamipleuris	0	9	0	0	1	17	0	0	1(3.7)	26(96.3)
Grand Total	**26(3.0)**	**831(97.0)**	**0(0.0)**	**310(100.0)**	**21(2.1)**	**991(97.9)**	**2(11.8)**	**15 (88.2)**	**49(2.2)**	**2147(97.8**

4.3.5. Sex ratio and abdominal status of sand flies

The overall male-to-female sex ratio of sand flies collected using LTs was 1.1:1, whereas a ratio of 2.4:1 was recorded for sand flies captured using STs. Across all sites and

collection methods, male sand flies significantly predominated over females ($p<0.001$). Male sand fly predominance was particularly pronounced in *Ph. rodhaini* (27/11), *S. antennatus* (888/228), and *S. bedfordi* (192/47) based on ST collections. In contrast, female-biased ratios were observed in *S. schwetzi, S. bedfordi*, and *S. squamipleuris,* while *S. clydei* showed a more balanced distribution at some sites (Table 18). Overall, STs captured significantly more females than LTs ($X^2=13.0$; $p<0.001$). Of the 654 female sand flies examined across all sites, 30 (4.6%) were blood-fed and 75 (11.5%) were gravid. In the current study, *S. schwetzi* and *S. clydei* showed a notable proportion of blood-fed and gravid female sand flies reported. Only one single gravid specimen of *Ph. rodhaini* was detected in Sherkole (Table 18).

Table 18: Male-to-female ratio of sand flies across refugee camps, stratified by collection methods

	Collection methods											
	CDC light trap				Sticky trap				Total			
	Kule	Sherkole	Terkidi	Tsore	Kule	Sherkole	Terkidi	Tsore	Kule	Sherkole	Terkidi	Tsore
Species	M/F	M/F	M/F	M/F	M/F	M/F	M/F	M/F	M/F	M/F	M/F	M/F
Ph. rodhaini	-	0/1	-	1/0	-	8/2	18/8	-	-	8/3	18/8	1/0
S. adleri	-	-	-	-	-	-	1/0	-	-	-	1/0	-
S. africanus	1/3	8/4	3/4	0/2	22/52	60/50	51/76	5/4	23/55	68/54	57/80	5/6
S. antennatus	11/0	2/0	8/12	-	415/96	30/9	421/111	1/0	426/96	32/9	429/123	1/0
S. bedfordi	2/0	1/4	1/1	-	97/6	37/6	53/29	1/1	99/6	38/10	54/30	1/1
S. clydei	-	0/1	2/0	-	42/42	0/1	14/12	0/1	42/42	0/2	16/12	0/1
S. schwetzi	1/0	2/0	2/6	-	47/11	61/23	97/64	0/1	48/11	63/23	99/70	0/1
S. squamipleuris	-	-	1/3	-	4/5	-	11/3	-	4/5	-	12/6	-
Grand Total	15/3	13/10	17/26	1/2	627/212	196/91	666/303	7/7	642/215	209/101	683/329	8/9

Of the 654 female sand flies examined across all sites, 30 (4.6%) were blood-fed and 75 (11.5%) were gravid. *Sergentomyia* species accounted for the majority of both categories, with notable proportions of *S. schwetzi*, *S. antennatus*, and *S. clydei*. In contrast, *Phlebotomus rodhaini* was detected only in Sherkole, represented by a single gravid specimen (Table 19).

Table 19: Distribution of blood-fed (BF) and gravid sand fly species across study sites in Gambella and Benishangul-Gumuz Regional States, Ethiopia, 2025

	Kule (n=215)		Sherkole (n=101)		Terkidi (n=329)		Tsore (n=9)		Total	
Species	**BF**	**Gravid**	**BF**	**Gravid**	**BF**	**Gravid**	**BF**	**Gravid**	**BF**	Gravid
Ph. rodhaini	0 (0.0)	0 (0.0)	0 (0.0)	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (9.1)
S. africanus	0 (0.0)	3 (5.5)	2 (3.7)	7 (13.0)	2 (2.5)	3 (3.8)	0 (0.0)	2 (33.3)	4 (2.1)	15 (7.7)
S. antennatus	7 (7.3)	14 (14.6)	0 (0.0)	0 (0.0)	3 (2.4)	11 (8.9)	0 (0.0)	0 (0.0)	10 (4.4)	25 (11.0)
S. bedfordi	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	3 (10.0)	4 (13.3)	0 (0.0)	0 (0.0)	3 (6.4)	5 (10.6)
S. clydei	3 (7.1)	8 (19.0)	1 (50.0)	1 (50.0)	0 (0.0)	1 (8.3)	0 (0.0)	0 (0.0)	4 (7.0)	10 (17.5)
S. schwetzi	2 (18.2)	4 (36.4)	1 (4.3)	4 (17.4)	6 (8.6)	10 (14.3)	0 (0.0)	0 (0.0)	9 (8.6)	18 (17.1)
S. squamipleuris	0 (0.0)	1 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (9.1)
Grand Total	12 (5.6)	31 (14.4)	4 (4.0)	13 (12.9)	14 (4.3)	29 (8.8)	0 (0.0)	2 (22.2)	30 (4.6)	75 (11.5)

4.3.6. Habitat preferences of the sand flies

Table 20 shows the mean number of sand flies collected for each trap, habitat, and night. *Ph. rodhaini, S. africanus, S. antennatus, S. bedfordi,* and *S. schwetzi* showed a strong association with habitat type according to the Kruskal-Wallis H test, indicating a preference for termite hills located in both village and natural habitat environments. However, *S. clydei* was associated with termite hills in natural habitats only. Additionally, *S. bedfordi* demonstrated a statistically significant preference for natural habitats, in addition to termite hills in both the village and natural habitats (Table 20).

Table 20: Mean sand fly density per sticky trap per night across habitats in refugee camps in Gambella and Benishangul-Gumuz regions, Ethiopia, 2025, presented with standard error (SE)

Species	Compound	Indoor	Natural Habitat	TH_in village	TH_ Natural Habitat
Ph. rodhaini	0.03 ± 0.02	0.07 ± 0.06	0.28 ± 0.25	0.42 ± 0.20*	0.23 ± 0.08*
S. africanus	0.52 ± 0.16	0.22 ± 0.12	0.59 ± 0.34	3.50 ± 1.96*	2.84 ± 0.97*
S. antennatus	1.09 ± 0.16	0.29 ± 0.12	5.19 ± 0.34	4.00 ± 1.96*	11.59 ± 0.97*
S. bedfordi	0.25 ± 0.61	0.09 ± 0.25	1.28 ± 1.34*	1.21 ± 1.26*	2.25 ± 4.04*
S. clydei	0.10 ± 0.04)	0.01 ± 0.01	0.16 ± 0.12	0.13 ± 0.13	1.61 ± 0.93*
S. schwetzi	0.23 ± 0.81	0.03 ± 0.08	1.16 ± 2.69	0.33 ± 3.30*	2.93 ± 3.48*

*Indicated statistically significant ($p<0.05$); TH natural habitat Habitat = termite hill located in the natural habitat of the study site; TH_in village = termite hills located in the village of the study areas

4.3.7. Diversity of sand flies among refugee camps

For the sand fly populations in four refugee camps, the Shannon-Wiener diversity index (H'), evenness (E), and species richness (S) were calculated (Table 21). Sherkole had the highest Shannon diversity index (H'=1.430), while Tsore had the lowest (H'=1.200). Compared to Kule, Sherkole had a varied sand fly population (p=0.001). Conversely, Kule had a greater diversity of sand fly species than Terkidi (H'=0.690 vs 0.662). On the other hand, Tsore had extremely low total abundance (n=17), which limits species richness and inflates the influence of small count changes on diversity metrics. The number of species reported in Terkidi (S=8) was higher than in Kule (S=6) (p=0.001).

Table 21: The Shannon-Wiener diversity index (H'), evenness (E), and richness (S) of the sand fly species from the study areas in Gambella and Benishangul-Gumuz Regional States, Ethiopia, April-May 2025

Refugee camp	H'	E	S
Kule	1.237	0.690	6
Sherkole	1,430	0.798	6
Terkidi	1.376	0.662	8
Tsore	1,200	0.670	6

4.4. PCR screening of sand flies for the detection of *Leishmania* DNA

PCR screening was performed on the 11 individually tested female *Ph. rodhaini* to detect *Leishmania* DNA. In contrast, female *Sergentomyia* specimens were pooled by species for PCR-based *Leishmania* screening as follows: 25 pools of *S. africanus* (n=195), 24 pools of *S. antennatus* (n=228), 13 pools of *S. bedfordi* (n=47), 10 pools of *S. clydei* (n=57), 16 pools of *S. schwetzi* (n=105), and 4 pools of *S. squamipleuris* (n=11), with each pool comprising from 1 to 22 sand flies. None of the 92 pools tested positive for *Leishmania*, and all individually analyzed *Ph. rodhaini* specimens were also negative for *Leishmania* kDNA-based molecular screening (Fig. 20).

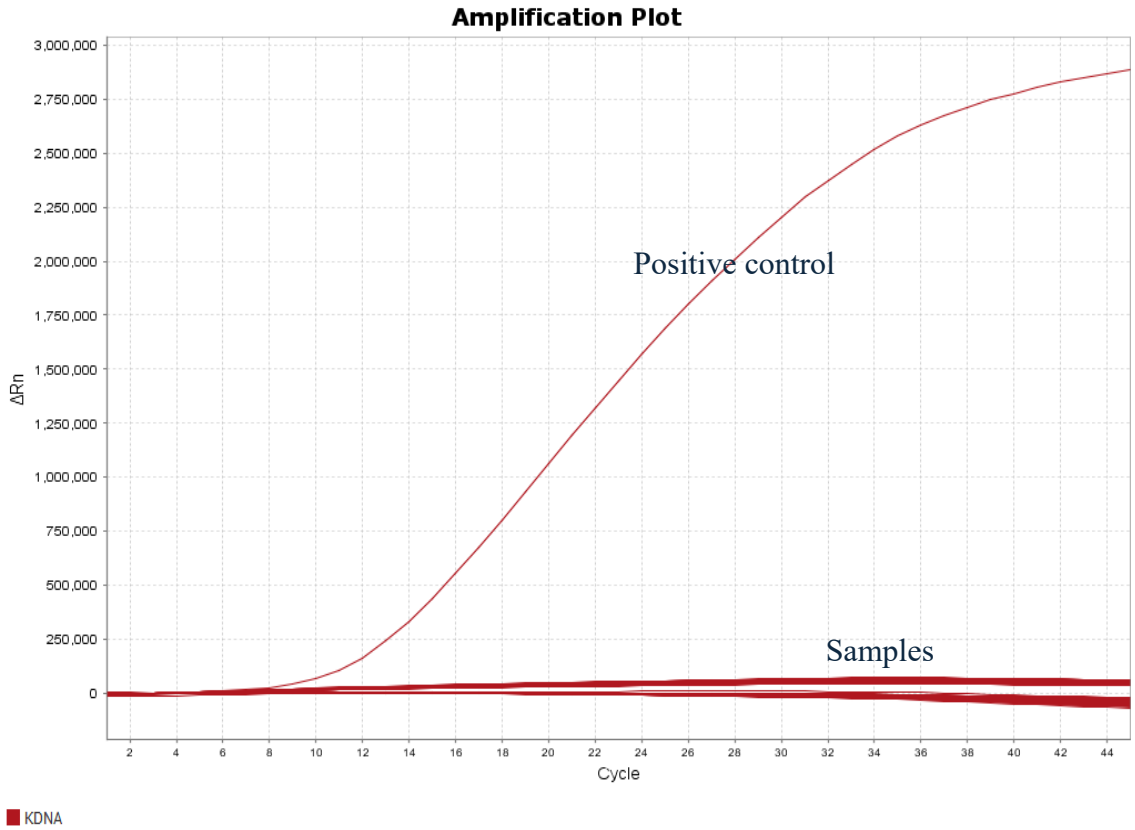


Figure 20: Real-time PCR amplification curve for kinetoplast DNA (kDNA). The figure describes the positive control of the assay, confirming successful target amplification and validating the assay's performance.

CHAPTER 5: DISCUSSION

This cross-sectional study assessed the distribution of visceral leishmaniasis, the factors influencing it, and the distribution of sand flies in four randomly selected refugee camps: Kule and Terkidi in the Gambella Regional State and Sherkole and Tsore in the Benishangul-Gumuz Regional State of Ethiopia. As one of the objectives of the study, the distribution of *Leishmania* infection in refugee camps was assessed to determine *Leishmania* infection prevalence in these vulnerable populations using serological (DAT) and molecular (rtPCR) methods. The prevalence of *Leishmania* infection was 4.0% and 10.3% as determined by DAT and rtPCR, respectively. The study participants did not report the typical VL symptoms during the data collection period. The prevalence and absence of clinical symptoms we reported highlights those asymptomatic individuals could be potentially sustaining VL transmission in the study area. This necessitates implementation of active case detection, community-wide screening, rigorous vector control measures, and targeted public awareness campaigns to effectively combat and halt the transmission and progression of VL.

This study provided evidence to guide *Leishmania* control in refugee camps, where populations are vulnerable due to overcrowding, limited resources, and environmental degradation. By integrating behavioral, environmental, and socio-demographic strategies into camp management protocols, policymakers and humanitarian actors can mitigate transmission risks and improve health equity in these high-risk settings. Sustained investment in prevention, community engagement, and context-specific research will be critical to achieving long-term impact. Moreover, the present findings reveal a diverse sand fly fauna dominated by species of the genus *Sergentomyia*, with clear ecological preferences for outdoor habitats, and importantly, there was no molecular evidence of *Leishmania* infection within the sampled sand fly populations.

The overall DAT positivity rate in the current study was 4.0%. A seroprevalence study conducted using Enzyme Immunosorbent Assay (ELISA) in refugee camps in Gambella region in 1990 reported that 18.1% (Hailu et al., 1996). This positivity rate is higher than what we found using DAT. These studies were conducted more than three decades apart

(1990 vs 2023). Changes in transmission dynamics over time environmental modification, population movement pattern since the current refugee camps newly established following the Sudan devastating conflict caused by armed force, housing condition, and access to healthcare could have substantially contribute for the lower infection rate. Moreover, differences in the sampling strategy, sample size, and population characteristics, and cut-off values used to define seropositivity rate could also contribute to this discrepancy. There are also studies that showed higher serological test positivity rates in other setting in Ethiopia, including 9.6% in southeastern Ethiopia (Ismail et al., 2023), and 12.5% among agricultural migrant laborers in northwest Ethiopia (Lemma et al., 2015). In addition, a household-based survey in eastern Sudan showed serological positivity rates of 8.9% (El-safi et al., 2002), and 13% in another study in southwest Sudan (Sharief et al., 2019). The lower DAT positivity rate in our study may be attributed to the refugees' prolonged stay in camps (average of nine years) in Benishangul-Gumuz and Gambella, displaced from high-risk areas in Sudan and South Sudan to relatively lower-risk regions in Ethiopia. Although these regions have ecological conditions favorable for VL transmission (Gadisa et al., 2015; Tsegaw et al., 2013), they are not known as endemic areas in Ethiopia. On the other hand, there are other studies that showed similar DAT positivity rate findings in Benishangul-Gumuz (Abera et al., 2016b; Bejano et al., 2021) and among pastoralist communities in the Borena zone of Oromia regional state of Ethiopia (Ketema et al., 2022), as well as in Gedarif state in Sudan (Mohamed et al., 2019). These reports suggest comparable levels of exposure to *Leishmania* in certain endemic settings but emphasize the sustained need for targeted interventions and surveillance in these areas.

There was no difference in the positivity rate between genders by DAT and rtPCR for *Leishmania* infection. This finding was in line with community-based reports from the Somali region, the South Omo zone (Alebie et al., 2019; Bekele et al., 2018), and Sudan (Mohammed et al., 2018). The lack of gender-based differences in *Leishmania* infection in our study is likely a reflection of the unique sociocultural and environmental conditions in refugee camps, which create a more uniform risk setting compared to resident populations. In contrast to our findings, previous reports in Ethiopia and Sudan showed that *Leishmania* infection was higher in males than females (Abera et al., 2016b; Bejano et al., 2021; Ahmed et al., 2022a). The age distribution of *Leishmania* infection among refugee camps as

determined by both DAT and rtPCR was similar among age groups in the current study. Similar findings were reported from the Somali region in Ethiopia (Alebie et al., 2019). However, a study conducted in Benishangul-Gumuz region reported that the prevalence of *Leishmania* infection gradually increased with age (Abera et al., 2016b). Susceptibility to *Leishmania* infection by sex and age may differ across societies based on prevailing patterns of household labor division (Alebie et al., 2019) and living conditions specific to individuals living in refugee camps.

In the current study, the rtPCR positivity rate varied significantly among refugee camps in the Benishangul-Gumuz and Gambella Regional States; Tsore refugee camp had the highest positivity rate (14.6%). Camp location may influence PCR positivity rates, with lower rates observed in Sherkole camp. Another study conducted among residents of Kumuruk district, which is adjacent to Homosha district, where these refugee camps are located, reported an LST prevalence of 14.1% (27/191) with one infected dog in the community out of 36 tested (2.8%) (Bejano et al., 2021). On the other hand, no *Leishmania* infection was reported from the same study in other districts (Sherkole and Bambasi). These districts are not in immediate proximity to the current study area. Variations in *Leishmania* infection rates between bordering and non-bordering districts near the camp indicated that proximity to these districts significantly influences leishmaniasis transmission risk. Moreover, further serological surveys should be conducted in neighboring local populations to better evaluate whether refugees represent a risk of *L. donovani* introduction in these regions and if the living conditions in refugee camps are facilitating *Leishmania* transmission within this specific population.

The current study showed no difference in risk of acquiring *Leishmania* infection among individuals who lived in the camp for varying durations. The lack of association between camp duration and *Leishmania* infection risk suggests continuous exposure to the parasite regardless of length of stay in the camp. Other possible factors like vector density, personal protection measures, or immunity levels of the participants may play a more significant role in infection risk than length of stay. Nationality-wise, a higher DAT positivity was observed in individuals from South Sudan compared to those from Sudan. This could be due to the fact that most of the study participants came from the Jonglei and Upper Nile

states of South Sudan. These regions are known for the highest percentage of VL cases among 10 states in South Sudan (Boateng et al., 2017).

Unlike the current finding, previous studies have shown that fever, splenomegaly, and hepatomegaly are major signs of VL in Sudan (Abass, 2020; Ahmed et al., 2022a), South Sudan (Pardal et al., 2020), and Ethiopia (Debash et al., 2023). However, in the current study, only the presence of a rash and its location on the face were associated with *Leishmania* infection. These earlier studies were conducted on diseased individuals, while the current report was based on community-level studies. Asymptomatic individuals tend to have a lower parasite load, which may not trigger noticeable clinical symptoms. Variations in immune responses to *Leishmania* infection can lead to different clinical outcomes, with some individuals effectively controlling the parasite and limiting the disease to the presence of a rash, while others may develop more extensive organ involvement. Furthermore, in this study, all *Leishmania*-infected participants were asymptomatic for VL.

The current results showed that rtPCR detected 78 positive *Leishmania* infections compared to the 35 identified by DAT. This marked discrepancy highlights important methodological and biological differences between molecular and serological diagnostic approaches, which should be carefully considered in interpreting epidemiological data. The observed discordance between the rtPCR and DAT therefore likely reflects differences in diagnostic targets (parasite DNA vs antibody), timing of detection (current infection vs immune response) and relative sensitivity in low parasite load or asymptomatic individuals. Moreover, the presence of 12 DAT-positive but rtPCR-negative cases could be attributed to persistent antibodies from past infections or false negatives in rtPCR due to low parasite DNA loads, as supported by studies indicating that serological tests can remain positive long after parasite clearance (Sundar & Rai, 2002; Belay et al., 2025c, b). The detection ability variation between these two testing methods has also been corroborated by other studies conducted in Ethiopia among asymptomatic individuals (Kaushal et al., 2017; Mohammed et al., 2023; Belay et al., 2025b). However, the current report contradicts the study of cohorts of HIV-infected subjects in known endemic areas from Ethiopia (Van Griensven et al., 2019). This variation could be the difference in study subjects (people

living with HIV vs. HIV status not mentioned). The discrepancy between the two test results suggests that combining both methods may enhance diagnostic accuracy. Public health strategies should incorporate rtPCR for confirmatory testing while using DAT for initial screening to improve case detection and disease control efforts (Gadisa et al., 2012; Belay et al., 2025b). Ultimately, integrating molecular diagnostics with serological methods is essential to strengthen VL surveillance and management programs. Even though the report indicated that detecting very low blood parasitemia is not a consistent parameter for determining *Leishmania* infections (Abbasi et al., 2013), its usefulness can be enhanced by optimizing laboratory conditions, such as strict contamination monitoring, the inclusion of known positive and negative controls, and the use of extraction controls.

The current study also assessed the behavioral, environmental, and socio-demographic factors associated with cases of *Leishmania* infection in refugee camp settings in Gambella and Benishangul-Gumuz regions of Ethiopia. The finding that sleeping outside during both daytime and nighttime increases the risk of *Leishmania* infection highlights a critical behavioral practice risk factor in refugee camp settings. This was supported by previous reports from other parts of Ethiopia showing that sleeping outside the house at night was significantly associated with VL (Bashaye et al., 2009; Argaw et al., 2013; Yared et al., 2014). When sleeping outside during the daytime, certain sand fly species may bite outdoors during the daytime, especially in hot climates or densely vegetated environments (Wijerathna & Gunathilaka, 2020). A case-control study conducted in West Pokot County, Kenya, supported the current report that VL transmission occurs both peri-domestically at night and outdoors during the day, particularly when sand fly resting sites are disturbed (van Dijk et al., 2024). This finding emphasizes daytime outdoor sleeping as a major modifiable risk factor for *Leishmania* infection in the camp settings. On the other hand, the strong association among sleeping outdoors, daytime, and cases of *Leishmania* infection suggests that behavioral interventions to reduce daytime outdoor rest could significantly lower transmission risk. Future studies should validate these results with longitudinal designs and explore interactions between sand fly activity patterns, environmental conditions, and human behavior, particularly in humanitarian settings like refugee camps. Sleeping outside during the nighttime is strongly associated with *Leishmania* infection in refugee camps in Gambella and Benishangul-Gumuz regions of Ethiopia. This was

supported by studies conducted in Sudan (Nackers et al., 2015) and Kenya (van Dijk et al., 2024). While sand flies are most active during crepuscular hours (dawn/dusk), nocturnal species or adaptations may bite at night, particularly in areas with high human activity or poor vector control (Alebie et al., 2019; Sales et al., 2019). However, in this study, the term 'nighttime' includes dusk and dawn periods rather than being strictly defined by specific hours. This broader timeframe accounts for the possibility that *Leishmania*-infected individuals may have been exposed to sand fly bites during these transitional periods. Cases who sleep outdoors at night may lack protection, increasing exposure to sand fly activity. Indoor sleeping environments typically offer structural barriers, including walls and roofs, and relatively better access to protective tools.

Staying in the camp for less than five years was significantly associated with cases of *Leishmania* infection. Longer stays may correlate with better adoption of protective measures or improved housing conditions. The practice and awareness of protective measures among cases and controls were not assessed in the current study; however, new residents might lack awareness of local risks or engage in high-exposure activities such as outdoor activities at dawn/dusk. Additionally, refugees may have acquired infection due to prior exposure in their areas of origin, such as Sudan or South Sudan, where visceral leishmaniasis is also endemic (Hammam & Hicks, 2021). Alternatively, this association may reflect new infections occurring shortly after arrival among individuals who had not previously been exposed and thus lacked immunity, regardless of protective measures. This finding is not in line with a study conducted in northern Ethiopia among farmland workers. The longer stay in the VL endemic area is a risk factor compared to staying for a shorter period of time, less than four years of stay in Humera (Fuller et al., 1976). This difference could be because the study area is well known to be VL endemic in the northern part of Ethiopia and contributes almost 60% of VL load in the country and to the large abundance of *Ph. orientalis,* the principal VL vector in the area (Gadisa et al., 2015).

In addition, the availability of bushes around the house was also found to be an exposure factor to increase the odds of a case infected with *Leishmania* infection in the current study. This finding was in line with reports from east African region, including Kenya, Sudan, Somalia, and Ethiopia (Marlet et al., 2003; Nackers et al., 2015; van Dijk et al., 2024).

Bushes near houses may increase human- sand fly contact, particularly during crepuscular/nocturnal biting periods. Vegetation may act as a bridge between natural sand fly habitats (such as forests) and human settlements. Other systematic reviews and meta-analyses revealed that living with domestic animals and being male were identified as associated with *Leishmania* infection in different regions of Ethiopia (Belay et al., 2025c). These exposure factors were not assessed in the current study because the target population's lifestyle differs. In this study, we focused on refugees with no gender-specific activities and without domestic animals.

For those who always use a bed net while sleeping, there was a decreased risk of *Leishmania* infection in the studied refugee camps in Gambella and Benishangul-Gumuz Regional States. This report is supported by a case-control study conducted in Ethiopia, Sudan and Kenya (Argaw et al., 2013; Yared et al., 2014Nackers et al., 2015; van Dijk et al., 2024), which found that using a bed net was associated with lower odds of asymptomatic VL infection.

Our analysis revealed no statistically significant association between *Leishmania* infection and exposure factors such as education level, always or sometimes sleeping outdoors during the dry season, and the presence of termite hills within 5 meters of sleeping areas. On the other hand, educational level was significantly associated with *Leishmania* infection in studies conducted in Gedaref, Sudan (Nackers et al., 2015) and Kafta-Humera, Ethiopia (Argaw et al., 2013). In the current study, lack of association with education may reflect limited socioeconomic variation in the refugee camp population or the dominance of environmental over behavioral risk factors in this setting. Moreover, education might correlate with protective behaviors (including bed net use) that were already adjusted for in the model. There was minimal proportion variability in the education levels among the individuals included in the study.

We assessed the seasonal variation in outdoor sleeping practices during the dry season. Since sand flies in the area are likely active during crepuscular hours (dawn and dusk), individuals may spend these peak biting times indoors, potentially reducing the relevance of their sleeping location. The null finding for termite hills, and always sleeping outside

during dry season is also supported by findings from Sudan (Nackers et al., 2015) but not by a study conducted in Ethiopia (Argaw et al., 2013). The termite hills, often cited as sand fly breeding sites, could suggest that proximity alone does not amplify risk in this context, possibly due to widespread environmental sand fly presence overwhelming localized effects, or termite mounds serving as poor proxies for actual vector habitats. Moreover, these discrepancies may be explained by differences in population characteristics as well as environmental factors, such as vegetation cover and the type and abundance of termite hills, which in the present study area are predominantly eroded. A comparative analysis across refugee camps that have not yet been studied would help to clarify variations in sand fly distribution. Such studies could also provide valuable insights into the habitat preferences and biting behaviors of the vector in these settings, which are important considerations for future research.

In the current study, the presence of cracked walls did not remain an independent predictor in the multivariable model. This was largely because its effect was adjusted for the habit of always sleeping outside during the dry season, which appears to be a stronger behavioral risk factor influencing exposure to sand flies. Previous studies have reported mixed findings regarding cracked walls: some have suggested that they may serve as potential resting or breeding sites for sand flies, thereby increasing transmission risk (Yared et al., 2014), while others have not found a significant association (Nackers et al., 2015). Our findings are therefore consistent with the latter, suggesting that structural housing conditions alone may not sufficiently explain transmission dynamics in this setting, and that behavioral and environmental factors, such as outdoor sleeping, may play a more dominant role.

Key factors such as proximity to vector breeding sites (availability of bushes around the house), limited access to protective measures (bed nets), and behavioral practices (day and nighttime outdoor sleeping) emerged as strong predictors of cases of *Leishmania* infection. These findings highlight targets for control strategies, including enhanced vector control, community education on risk-reducing behaviors, and distribution of protective materials. While unmeasured variables like housing construction materials or nutritional status may contribute to risk in broader contexts, the identified factors remain pivotal for designing

context-specific, feasible interventions in resource-constrained environments like refugee camps, where addressing modifiable exposures can yield immediate public health benefits.

In the present study, a total of 2,196 adult phlebotomine sand flies (1,542 males, 654 females) from eight species in two genera (*Phlebotomus* and *Sergentomyia*) were collected using LTs and STs. *Phlebotomus* was represented by one subgenus (*Anaphlebotomus*), while *Sergentomyia* included four subgenera; *Grassomyia, Sergentomyia, Sintonius,* and *Parrotomyia*. Most sand flies (85%) were collected from Gambella refugee camps (Kule: 357; Terkidi: 1,012), while 15% were from Benishangul-Gumuz (Sherkole: 310; Tsore: 17). One *Phlebotomus* and seven *Sergentomyia* species were identified from four refugee camps in Gambella and Benishangul-Gumuz Regional States. The current cross-sectional entomological survey revealed that 98.3% of the collected sand flies belong to the genus *Sergentomyia* and only 1.7% to the genus *Phlebotomus*. The report indicated that the genus *Sergentomyia* outnumbers the genus *Phlebotomus*, which is consistent with findings from endemic regions, including northern and southern Ethiopia (Hailu et al., 1995; Lemma et al., 2014; Yared et al., 2017).

The current study highlights the low abundance of *Ph. rodhaini* (1.7%), a finding that is consistent with previous reports from VL endemic regions of northern, southern, and eastern Ethiopia (Aklilu et al., 2023). This species was not reported in previous entomological assessment conducted in the Gambella refugee camps and surrounding areas in 1996 (Hailu et al., unpublished data). The presence of this sand fly species in the present study may indicate a shift in vector distribution due to climate change or environmental variation since the 1990s. Furthermore, the primary VL vectors reported elsewhere in Ethiopia appear to be absent in these refugee camps. The current finding may reflect the limitation of the current cross-sectional study design, as previous research documenting these vectors employed longitudinal and seasonal sampling methods (Gebre-Michael et al., 1993; Hailu et al., 1995; Gebresilassie et al., 2015; Aklilu et al., 2018). *Ph. rodhaini* is also distributed across parts of East Africa, including Sudan and South Sudan (Quate, 1964; Elnaiem et al., 1997; Lambert et al., 2002; Elnaiem, 2011).

To our knowledge, this entomological survey is the first to document its occurrence in refugee camps within the Gambella and Benishangul-Gumuz Regional States. Although reported at low abundance, its presence is epidemiologically relevant, as *Ph. rodhaini* has historically been implicated as a potential vector for *L. donovani* in Sudan (Elnaiem, 2011). A review by Al-Salem et al. on VL during the South Sudan conflict and its regional implications further highlighted the possible involvement of this species in zoonotic VL (ZVL) transmission in East Africa (Al-Salem et al., 2016). While its role in VL transmission has often been underestimated due to its low density (Aklilu et al., 2017b; Gebre-Michael et al., 2010) and preferential feeding on rodents rather than humans, its detection nevertheless signals a potential transmission risk, particularly in settings where primary vectors are rare or absent (Quate, 1964; Elnaiem, 2011). Ecologically, *Ph. rodhaini* is primarily associated with sylvatic and forest-derived environments, favoring humid, shaded microhabitats with dense vegetation (Elnaiem et al., 2011). It is commonly reported from forested habitats, rock crevices, tree roots, termite mounds, and natural soil fissures, which provide favorable conditions for resting and breeding (Aklilu et al., 2018; Elnaiem et al., 2011; Lambert et al., 2002).

In the present study, seven *Sergentomyia* species were identified, confirming the wide distribution of this genus across Ethiopia, including VL endemic regions where *Phlebotomus* species are recognized as the primary vectors of transmission (; Hailu et al., 1995; Gebresilassie et al., 2015; Aklilu et al., 2017b; Yared et al., 2017). The finding was the overwhelming dominance of *S. antennatus*, which accounted for more than 50% of all collected specimens, especially in the Gambella camps of Kule and Terkidi. Although *Sergentomyia* species are generally considered zoophilic (Polley et al. 2018) and of limited significance in VL transmission compared to primary vectors like *Ph. orientalis* and *Ph. martini*, their remarkable abundance needs to be further investigated. Large population size could increase human-vector contact rates, underscoring the need to investigate the potential role of specific *Sergentomyia* species in disease ecology.

The current study demonstrates a significant difference in sampling efficiency between trap types: ST captured significantly more sand flies than the LT. This variation may be partly attributable to the greater total sampling efforts (trap-nights) with STs. Furthermore, the

habitats sampled by each trap type differed: LTs were deployed exclusively in indoor habitats and peridomestic (household compound) settings, whereas STs were also placed in outdoor habitats, including termite hills (both within villages and in natural habitats) and mixed forests. Analysis of species diversity revealed a heterogeneous distribution across refugee camps. Sherkole camp exhibited the highest species diversity and evenness, likely driven by high sand fly densities in termite hills and a more equitable species distribution. Terkidi camp also showed high diversity, with all eight species present; however, the area was dominated by *S. antennatus.* In contrast, Tsore camp had exceptionally low abundance and diversity this could potentially reflect suboptimal local ecological conditions for the sand fly spread.

A key entomological finding was exophilic (outdoor resting) behavior of sand flies in the study area. Less than 2.3% of all specimens were collected indoors, a pattern consistent with the reports from other VL endemic regions in Ethiopia, Sudan, and South Sudan (Gebre-Michael et al., 2010; Gebresilassie et al., 2015; Amanya & Peng, 2018). This has major implications for vector control. While indoor residual spraying (IRS) remains a cornerstone of VL control, our data indicate that effective strategies must also incorporate targeted outdoor interventions. Significantly, the high densities of *S. antennatus* and *S. africanus* associated with termite mounds, particularly those near natural habitats and within villages, identify these structures as key ecological niches. The current report is supported by a study conducted in Kenya that showed these species more commonly collected in termite hills (Basimike et al., 1992). Environmental management, such as the modification or destruction of termite mounds in peridomestic areas, could be a highly effective strategy to reduce sand fly breeding and resting sites.

The negative result from the most sensitive kDNA PCR assay, applied to both *Sergentomyia* and individual *Ph. rodhaini* specimens, was a significant and valuable finding. These findings suggest several non-exclusive possibilities in the context of the refugee camps' asymptomatic *Leishmania* infection distribution (Belay et al., 2025a). First, parasite circulation may have been at an extremely low, undetectable level during the sampling period, which may not have coincided with seasonal transmission peaks. Second, the zoonotic transmission cycle may involve animal reservoirs that are not effectively bitten

by the sampled sand flies. Finally, known VL vectors might exist at such low densities that they were not captured in sufficient numbers to include an infected specimen. The absence of infection provides crucial baseline data, indicating a currently low transmission intensity within the local vector population, which is essential for monitoring future epidemiological trends. This finding contrasts with reports from other endemic regions where *Sergentomyia* and *Phlebotomus* spp. have tested positive for *Leishmania* DNA. For instance, in Iran, *S. dentata* (4/48, 8.3%) and *S. sintoni* (2/4, 50%) tested positive for *Leishmania major* (Nikookar et al. 2024), while in Portugal, *S. minuta* was reported to have tested positive for *Leishmania* DNA (2/1867) (Maia et al., 2015). This discrepancy may be partly explained by the absence of these specific *Sergentomyia* species in our study area. Furthermore, studies have challenged the paradigm of exclusive *Phlebotomus* transmission, implicating *S. squamipleuris* in Kenya as a potential vector (Owino et al., 2021) and detecting *L. donovani* in *Ph. rodhaini* from Sudan (Elnaiem et al., 2011). The absence of *Leishmania* DNA in *Ph. rodhaini* in the current study could therefore be explained by several factors, including ecological differences between sites, such as variations in host feeding preferences, local transmission intensity between sites, or differences in sand fly microbiota. Additionally, the predominance of asymptomatic human infection in our study areas (Belay et al., 2025a) may result in lower parasitemia, further reducing the probability of parasite detection in sand flies.

The current study provides insight that can guide targeted health interventions in leishmaniasis hotspot areas. However, there are several limitations to acknowledge. The significant disparity in sample sizes between the DAT and rtPCR presents a critical challenge to this study. DAT's typical limitations include its ability to differentiate between past and present infections. The underrepresentation of rtPCR results, a more sensitive method for detecting early-stage and subclinical infections, may limit the study's ability to accurately capture the true disease dynamics. This imbalance hinders the analysis using both testing tools, limiting conclusions regarding their diagnostic accuracy and potentially introducing a bias towards DAT outcomes. We did not study the genetic diversity or the presence of specific *Leishmania* species and cannot differentiate between past and current infections or track changes in infection status over time. Several potential predictors, such as occupation, income, domestic animal ownership, VL/HIV co-infection and nutritional

status, were not included in the analysis. This omission stemmed from the homogeneity of living conditions within the refugee population, which limited variability in these factors. Additionally, variables like nutritional status and domestic animal ownership are widely recognized as systemic challenges in refugee settings, where access to resources is often uniformly constrained. Findings from four camps may not be generalizable to all other VL endemic settings. The cross-sectional design provides only a snapshot, possibly missing seasonal transmission peaks. Moreover, some additional laboratory analyses, such as blood meal analysis that are not included in the current study may limit the understanding of host preferences and reservoir dynamics, as well as Cytochrome Oxidase I (COI) mitochondrial gene sequencing as a complementary molecular method, may hinder the confirmation of species identity. These constraints highlight the need for longitudinal, multi-site studies incorporating these additional laboratory techniques in future research.

Despite these limitations, the study provides valuable public health information for targeted disease control in vulnerable populations. Addressing these limitations in future research could further strengthen the findings and applicability of such studies in similar settings.

CHAPTER 6: CONCLUSION AND ECOMMENDATIONs

6.1. Conclusions

Leishmania infection was prevalent in refugee camps in Gambella and Benishangul-Gumuz regions, with detection rates of 4.0% using DAT and 10.7% using rtPCR. *Leishmania* infection was reported in all studied camps, although at varied proportions, with a noteworthy presence of asymptomatic cases and low parasite loads.

The current study identified critical behavioural and environmental factors associated with *Leishmania* infection risk in refugee camp settings. Sleeping outdoors during both daytime and nighttime significantly increased *Leishmania* infection. The presence of bushes near households and the shorter duration of residence in the camp (less than five years) were associated with *Leishmania* infection in refugee camps. This indicates that recent arrivals may not have developed adaptive behaviours or acquired immunity over time.

The study also showed a detailed picture of the sand fly fauna in southwestern and northwestern Ethiopian refugee camps for the first time. Moreover, the presence of *Phlebotomus* was represented by the only species, *Ph. rodhaini*.

The vector community was characterized by diversity, a strong predominance of outdoor-resting *Sergentomyia* species, and a notable absence of *Leishmania* DNA in the tested sand fly's specimen at the time of the study.

6.2. Recommendations

- The results highlight the need for tailored, region-specific interventions, especially in light of the higher positivity rates observed in the Benishangul-Gumuz region and among South Sudanese refugees.

- The results further emphasize the need for targeted approaches including active case detection, community-based screening, and vector control, to effectively manage and reduce VL transmission among these high-risk populations.

- Moreover, integrated surveillance and comprehensive healthcare support are essential to effectively manage and lesson the burden of VL in vulnerable population.

- The findings highlight the urgency need to prioritize vector control efforts, including spray insecticides on vegetation near households, distributing insecticide-treated nets, and promoting community awareness to reduce outdoor sleeping practices.

- Integrated vector management has been carried out in these areas, that includes environmental measures, that specifically target termite hills in peridomestic settings.

- Longitudinal entomological surveillance has to be in place across multiple seasons to more accurately capture temporal changes in sand fly density and *Leishmania* infection rates.

- Future research should include a larger sample of *Phlebotomus* species and consider analysing blood-fed females for host preferences to help identify potential reservoir hosts involved in the transmission cycle.

- Further investigation for potential animal reservoirs in and around the camps is also warranted to better understand the transmission cycle in the area.

- Future longitudinal cohort studies should be conducted among asymptomatic individuals in refugee camps to determine whether they subsequently develop the clinical hallmarks of VL and to elucidate the natural progression of infection
- In addition, further epidemiological and molecular studies are warranted to establish whether VL transmission in refugee camps and neighboring areas is locally established or primarily due to imported cases.

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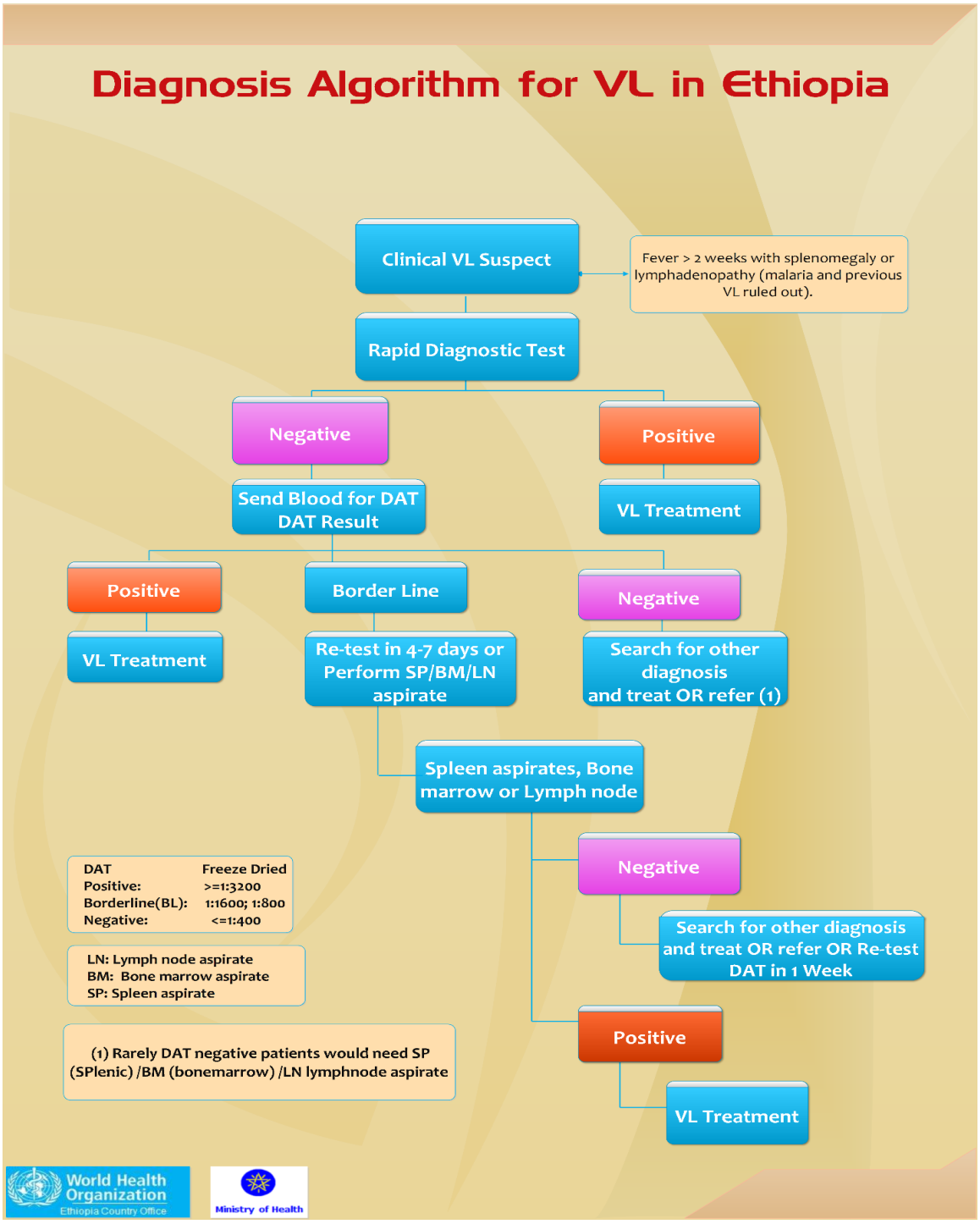
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Annexes

Annex 1: Diagnosis algorithm for VL in Ethiopia



Annex 2: Questionnaire for data collection for a study on epidemiology of VL infection in refugee camps Ethiopia (English Version)

Participant ID: _____ Date of interview: ___/_____/_____

I. Socio-demographic Information

Participant name: _____ Sex: Male [ ] Female [ ] Age: _____

Residence: region: Gambella [ ] Benishangul-Gumuz [ ]

Camp name: Kule [ ] Terkidi [ ] Tsore [ ] Sherkole [ ]

Household address in the camp: Zone_____ Block number_____ Community_____ House number_____

Duration in the camp: _____

Country of origin: _____ State_____

Village Name of specific area District/County_____

Level of education: No formal education [ ] Primary [ ] Secondary [ ] Tertiary [ ]

Marital status Married: Single [ ] Divorce [ ]

Family size: _____

II. Clinical Information

Signs and Symptoms

Fever	Yes [ ]	No [ ]
Chills	Yes [ ]	No [ ]

Severe headache	Yes [ ]	No [ ]	
Aching muscles	Yes [ ]	No [	
Weight loss	Yes [ ]	No [ ]	
Splenomegaly	Yes [ ]	No [ ]	
Spleen size _____			
Hepatomegaly	Yes [ ]	No [ ]	
Lymphadenopathy	Yes [ ]	No [ ]	
Anemia	Yes [ ]	No [ ]	
Malnutrition signs	Yes [ ]	No [ ]	
Rashes	Yes [ ]	No [ ]	
If yes Where?	Chest [ ]	Back [ ]	Arms [ ]
Dizziness resulting in fainting	Yes [ ]	No [ ]	
Family history of infection with VL	Yes [ ]	No [ ]	

Exposure information

Have you been in contact with anyone with similar illness 2 weeks prior? Yes [ ] No [ ]

Housing condition: Corrugated iron sheet [ ] Thatch [ ] Other: _____

Where do you usually sleep? In the house [ ] Outside [ ] Others_____

Do you always use a net while sleeping? Yes[ ] No [ ]

If yes, is it a treated net? Yes [ ] N [ ]

Do you have animals? Yes [ ] No [ ] if yes, list them: _____

_____

_____

_____

Do you sleep in the same house/share sleeping space with the animals? Yes [ ] No [ ]

Did you visit forested areas in the last 2 weeks? Yes [ ] No [ ] if yes where?_____

Are there bushes around the house? Yes [ ] No [ ]

Travel history: Have you travelled in the last 6 month? Yes [ ] No [ ] If yes, Where? Specify _____ When was this travel? Specify_____

Is house wall cracked? Yes [ ] No [ ]

Is crack soil present near sleeping place? Yes [ ] No [ ]

Is termites' hills present near house? Yes [ ] No [ ]

Laboratory Tests

Malaria Blood Smear or RDT Positive [ ] Negative [ ]

Kala Azar Tests:

DAT: _____

PCR confirmation.....

Other(specify): _____

Annex 3: Questionnaire for a study on epidemiology of Visceral Leishmaniasis infection in refugee camps Ethiopia (Arabic Version)

الاستبيان المحدث

معرّف المشارك : _____ تاريخ المقابلة:

___/_____/_____

المعلومات الاجتماعية والديموغرافية

_____ :اسم المشارك: _____ الجنس: ذكر [ ] أنثى [ ] العمر

مكان الإقامة:

[ ] المنطقة: غامبيلا [ ] بني شنقول-قمز

[ ] اسم المخيم: كولي [ ] تيركيدي [ ] تسوري [ ] شيركولي

عنوان الأسرة في المخيم:

_____المنطقة_____ رقم البلوك_____ المجتمع_____ رقم المنزل

مدة الإقامة في المخيم: _____

_____ :بلد المنشأ: _____ الولاية

القرية / اسم المنطقة المحددة / المقاطعة: _____

المستوى التعليمي:

[ ] بدون تعليم رسمي [ ] ابتدائي [ ] ثانوي [ ] جامعي

الحالة الاجتماعية:

[ ] أعزب [ ] متزوج [ ] مطلق

عدد أفراد الأسرة: _____

المعلومات السريرية

العلامات والأعراض

[ ] الحمى نعم [ ] لا

[ ] القشعريرة نعم [ ] لا

[ ] صداع شديد نعم [ ] لا

[ ] آلام في العضلات نعم [ ] لا

[ ] فقدان الوزن نعم [ ] لا

[ ] تضخم الطحال نعم [ ] لا

حجم الطحال: _____

[ ] تضخم الكبد نعم [ ] لا

[ ] تضخم العقد اللمفاوية نعم [ ] لا

[ ] فقر الدم نعم [ ] لا

[ ] علامات سوء التغذية نعم [ ] لا

[ ] طفح جلدي نعم [ ] لا

[ ] إذا كانت الإجابة نعم، أين؟ الصدر [ ] الظهر [ ] الذراعين

[ ] الدوار المؤدي إلى الإغماء نعم [ ] لا

[ ] تاريخ عائلي لعدوى الكالاآزار نعم [ ] لا

معلومات عن التعرض

[ ] هل كنت على اتصال بشخص مصاب بمرض مشابه خلال الأسبوعين الماضيين؟ نعم [ ] لا

نوع السكن:

ألواح حديدية مموجة [ ] قش [ ] أخرى: _____

أين تنام عادة؟ داخل المنزل [ ] خارج المنزل [ ] أخرى_____

[ ] هل تستخدم الناموسية أثناء النوم دائماً؟ نعم [ ] لا

[ ] إذا كانت الإجابة نعم، هل هي ناموسية معالجة؟ نعم [ ] لا

[ ] هل تملك حيوانات؟ نعم [ ] لا

إذا كانت الإجابة نعم، اذكرها: _____

[ ] هل تنام في نفس المنزل أو تشارك مكان النوم مع الحيوانات؟ نعم [ ] لا

هل زرت مناطق غابية خلال الأسبوعين الماضيين؟ نعم [ ] لا [ ] إذا كانت الإجابة نعم، أين؟

_____

[ ] هل توجد شجيرات حول المنزل؟ نعم [ ] لا

[ ] تاريخ السفر: هل سافرت خلال الأشهر الستة الماضية؟ نعم [ ] لا

إذا كانت الإجابة نعم، حدد المكان: _____ متى تم السفر؟

_____

[ ] هل جدران المنزل متشققة؟ نعم [ ] لا

[ ] هل توجد تشققات في التربة بالقرب من مكان النوم؟ نعم [ ] لا

[ ] هل توجد تلال نمل أبيض بالقرب من المنزل؟ نعم [ ] لا

الفحوصات المخبرية

[ ] فحص الملاريا (لطاخة دم أو اختبار سريع): موجب [ ] سلبي

اختبارات الكالاآزار:

اختبار DAT: _____

تأكيد بواسطة PCR: ..... أخرى (حدد): _____

Annex 4: Research Participant Information sheet (English version)

Name of the principal investigator (PI): Habtamu Belay

Name of institution: Addis Ababa University; Aklilu Lemma Institute of Health Research

You are/your child is invited to take part in the epidemiology of VL in refugee camps study, which is being conducted by Addis Ababa University and Ethiopian Public Health Institute. Before you decide whether to participate or not, it is important that you understand the information in this form, which explains your rights, our responsibilities to you, the purpose, procedures, possible benefits, risks and inconveniences related to this study, and the right to refuse or stop your (child's) participation at any time.

Please, feel free to ask any questions at any time, for example about the possible benefits and possible risks. Your participation is completely voluntary. You/your child can talk to anyone you trust about the research and you can take your time to think about whether you want to participate or not. In case you decide to participate, make sure you keep this background information and the signed consent form with you throughout the entire study period.

The purpose and description of the study

This research study is done to learn more about visceral leishmaniasis (VL) or kala-azar. Kala-azar is a fatal disease caused by parasites that are transmitted by small biting insects, called sand flies. We see that there are ongoing VL case reports to ministry of health every year, both the relapse and new cases. The Gambella and Benishangul-Gumuz regions, of course, not known endemic for VL, but previous work revealed that the regions are risk areas for VL transmission. Moreover, VL is currently associated with conflict, population displacement, immunity and malnutrition, unfortunately these situations frequently reported from refugee population. Although, there is one report a couple of years ago before establishment of the refugee camps in Gambella, there is little to no evidence on the current burden of VL, which factors influence who gets infected and who does not, and distribution of sand flies can also be assessed. With this information we can inform national programs and RRS to better coordinate, monitor and control the disease.

The study will be performed in in four refugee camps; Kule and Terkidi from Gambella region whereas, Tsore and Sherkole from Benishangul-Gumuz regions. About 607 households have been selected by chance to participate in this study.

The study is done in two parts:

1. A questionnaire, and sampling and testing of human participants
2. Sand fly (insect) collected and screened

How the Study is Done

If you agree (for your child) to take part in part 1 of the study, we will start by describing what your house and the surroundings look like (what type of walls, roof etc). We will also use a device that allows us to know the location of your house on a map. After that, we will ask you/your child more specific questions about your age, sex, and your (child's) behaviour. These questions will be asked to see whether we can identify why some people get infected and others do not in the Afar region. To see if some factors are related to infection, we need to check whether you/your child infected with *Leishmania* parasite. We plan to take your (child's) 5ml blood from the arm. This will not be painful, but it can sting a little bit while pricking. If it is positive, we will try inform you through a phone call.

We will check if you/your child may have any symptoms that may point to kala-azar or malaria (fever, malaise, enlarged belly, travel history, etc.). If you have/your child has symptoms, we will immediately check at your home if you have / your child has the disease, using the blood from your (child's) venous blood with RDT. If the test shows you / your child infected with the parasite, you/your child will be sent to a nearby treatment facility for treatment.

Taking part in this research will take about 15-20 minutes.

RISKS AND INCONVENIENCES

The risks for this study are very minor. If in the questionnaire there are questions you or your child do not want to respond to or if you / your child feel(s) uncomfortable, you can just indicate this and you / your child can skip questions. For the blood collection, there is

no major risk either. We will only take a few volumes of blood, so it will not affect you / your child. It may only give a little sting when they use the small needle.

BENEFITS

You/your child will have no direct benefits of this study, only that, if you/your child test positive for malaria or kala-azar, there is an early diagnosis of the disease and you/your child will be sent early to the treatment center, which often results in better resolution of the infection.

The main benefit of this research is for future patients. The data that will be collected will be the first ever in Ethiopia to document that this disease occurs in the refugee camps, the extent of it, how it is transmitted and how we can potentially control it. This information will be provided to policy makers to implement their monitoring and control programs in the region.

DIAGNOSTIC FINDINGS

In the laboratory, we will only look at the parasite that causes kala-azar, no other diseases or conditions. If in the laboratory at EPHI it appears that you or your child were positive for kala-azar but had no symptoms at the time of the sample collection, we will call you to inform you about this result and we might advise you to go to a treatment center for further diagnostics and potentially treatment.

STORAGE OF BODY SAMPLES

We would like to store the genetic material of your (child's) blood drops collected in the field. Also, if you are sent to the health center because your (child's) test for kala-azar or malaria is positive, routinely they might collect blood and other tissue (which is not part of the study). If these samples are collected, we would also like to store them. If you (and your child) agree with this, we will store the materials for 10 years after study completion to be used for future scientific research. Such future studies will be separately approved by the Ethics Committee of AAU in advance. Even if you decide that you do not want us to store your samples, you can still participate in the study.

Your (child's) samples will be stored at EPHI only, and will not be exported out of Ethiopia.

Your (child's) samples will not be sold or used for any commercial purposes.

PRIVACY AND DATA PROTECTION

We will do everything we can to protect your (child's) privacy. Information about you/your child will be treated as strictly confidential; it will be stored in an electronic database and identified by a code and not by your (child's) name. The documents where your (child's) name is mentioned will not be shared with anyone, except the study researchers and a few other people who must keep it confidential. One when that is needed, for example when your (child's) test is positive in the laboratory, we might link the code back to your name and phone number to inform you about the result. The document that has the link between your (child's) code and personal information will be stored in a secure place and is only accessible for very few people in the study team.

All study data will be retained for a period of 10 years. The findings of the study will be published in journals. Your (child's) data can be shared with other researchers for future research, only upon condition that they are either anonymized (meaning that they cannot be reasonably linked anymore to your name: nobody will know anymore that these data came from you/your child).

The data that we have about the location of your house will not directly be linked to your name to protect your (child's) privacy. There will be a code that will be used and different databases of the households, and who is part of which household.

Your (child's) name will not appear in any database, report or publication resulting from this study.

ETHICS COMMITTEE

Before the start, this study was reviewed and approved by the Ethics Committee(s) the Addis Ababa University; Aklilu lemma Institute of Health Research.

VOLUNTARY PARTICIPATION

Your (child's) participation in this study is entirely voluntary. It is your choice whether you want to take part in it or not. There will be no disadvantage if you/your child do(es) not participate. You also have the right to stop your (child's) participation in the study at any time, even after you have signed this Informed Consent Form. You do not have to give a reason for stopping. Also, the researcher can decide to stop your (child's) participation in this study at any moment as well, without your permission, if he/she judges this in your best interest, or if you do not follow the requirements for participation. Should this happen, the researcher will explain this to you and discuss what will happen afterwards.

Contact address for further information:

For any questions related to the study or you experienced any adverse event before/during participation in the study use the following contact address.

Name of PI: Habtamu Belay; Phone: 0911006034; email: hbtmbelay652@gmail.com

Annex 5: Research Participant Information sheet (Arabic version)

الملحق-3. ورقة معلومات المشارك في البحث (النسخة الإنجليزية) عنوان مشروع البحث: دراسات حول وبائيات داء الليشمانيات الحشوي في مخيمات اللاجئين بغرب إثيوبيا **اسم الباحث الرئيسي:** هبتامو بلاي **اسم المؤسسة:** جامعة أديس أبابا؛ معهد أكليلو لما لأبحاث الصحة تُدعى أنت / يُدعى طفلك للمشاركة في دراسة حول وبائيات داء الليشمانيات الحشوي في مخيمات اللاجئين، والتي تُجرى من قبل جامعة أديس أبابا والمعهد الإثيوبي للصحة ،العامة. قبل أن تقرر المشاركة من عدمها، من المهم أن تفهم المعلومات الواردة في هذه الورقة، والتي تشرح حقوقك ومسؤولياتنا تجاهك، والغرض من الدراسة، والإجراءات المتبعة، والفوائد المحتملة، والمخاطر والمضايقات المتعلقة بهذه الدراسة، وحقك في الرفض أو إيقاف مشاركتك (أو مشاركة طفلك) في أي وقت. يرجى عدم التردد في طرح أي أسئلة في أي وقت، على سبيل المثال حول الفوائد أو المخاطر المحتملة. مشاركتك اختيارية تمامًا. يمكنك / يمكن لطفلك .التحدث مع أي شخص تثق به حول البحث، ويمكنك أخذ الوقت الكافي للتفكير فيما إذا كنت ترغب في المشاركة أم لا .في حال قررت المشاركة، يُرجى الاحتفاظ بهذه المعلومات ونموذج الموافقة الموقّع معك طوال فترة الدراسة

الغرض من الدراسة ووصفها

تُجرى هذه الدراسة لمعرفة المزيد عن داء الليشمانيات الحشوي (الكالاآزار). الكالاآزار مرض قاتل تسببه طفيليات تُنقل بواسطة حشرات صغيرة تُعرف باسم ذباب الرمل. تشير التقارير إلى تسجيل حالات جديدة ومُتكررة من المرض ،لدى وزارة الصحة كل عام. منطقتا غامبيلا وبني شنقول-قمز ليستا معروفتين تقليديًا بكونهما مناطق متوطنة للمرض لكن دراسات سابقة أظهرت أنهما منطقتان معرضتان لخطر انتقال العدوى. علاوة على ذلك، يرتبط داء الليشمانيات الحشوي بالنزاعات، والنزوح السكاني، وضعف المناعة، وسوء التغذية، وهي ظروف شائعة بين اللاجئين. رغم وجود ،تقرير واحد قبل سنوات من إنشاء مخيمات اللاجئين في غامبيلا، إلا أن هناك أدلة قليلة جدًا حول العبء الحالي للمرض والعوامل المؤثرة على من يُصاب ومن لا يُصاب، وتوزيع ذباب الرمل. هذه المعلومات ستساعد البرامج الوطنية وهيئة شؤون اللاجئين على تحسين التنسيق والمراقبة والسيطرة على المرض. ستُجرى الدراسة في أربعة مخيمات للاجئين: كولي وتيركيدي في منطقة غامبيلا، وتسوري وشرقول في منطقة بني شنقول-قمز. تم اختيار حوالي 607 .أسر عشوائيًا للمشاركة

أجزاء الدراسة

1. .استبيان وأخذ عينات واختبار للمشاركين من البشر
2. .جمع وفحص ذباب الرمل (الحشرات)

كيفية إجراء الدراسة

إذا وافقت (عن نفسك أو عن طفلك) على المشاركة في الجزء الأول، سنبدأ بوصف منزلك والمناطق المحيطة به (نوع ،الجدران، السقف، إلخ). سنستخدم جهازًا لتحديد موقع منزلك على الخريطة. بعد ذلك، سنطرح أسئلة محددة عن عمرك .جنسك، وسلوكك (وسلوك طفلك). تهدف هذه الأسئلة إلى معرفة أسباب إصابة بعض الأشخاص وعدم إصابة آخرين للتحقق من الإصابة بطفيلي الليشمانيا، سنأخذ عينة دم مقدارها 5 مل من الذراع. هذا لن يكون مؤلمًا، ولكن قد تشعر بوخزة بسيطة. إذا كانت النتيجة إيجابية، سنبلغك عبر الهاتف. سنفحص ما إذا كانت لديك (أو لدى طفلك) أعراض تشير إلى الكالاآزار أو الملاريا (حمى، تعب، تضخم البطن، تاريخ سفر، إلخ). إذا وُجدت الأعراض، سنجري فحصًا في المنزل باستخدام اختبار سريع للدم. وإذا كانت النتيجة إيجابية، فسيُحال المريض إلى أقرب مركز علاج. تستغرق .المشاركة حوالي 15–20 دقيقة

المخاطر والمضايقات

المخاطر طفيفة جدًا. إذا كانت هناك أسئلة في الاستبيان لا ترغب أنت أو طفلك بالإجابة عليها، يمكنك تخطيها. بالنسبة .لأخذ عينة الدم، لا توجد مخاطر كبيرة، فقط وخزة بسيطة بالإبرة

الفوائد

لن تحصل أنت أو طفلك على فوائد مباشرة من هذه الدراسة، ولكن في حال كانت نتيجة اختبارك إيجابية للكالاآزار أو الملاريا، فسيتم تشخيص المرض مبكرًا، مما يُحسن فرص العلاج. لفائدة الرئيسية من البحث هي للمرضى في المستقبل. ستكون هذه أول بيانات توثّق وجود المرض في مخيمات اللاجئين في إثيوبيا، ومدى انتشاره، وكيفية انتقاله، وطرق السيطرة عليه. سيتم استخدام هذه المعلومات لمساعدة صُنّاع القرار على تنفيذ برامج المراقبة .والمكافحة

النتائج التشخيصية

في المختبر، سيتم فحص الطفيلي المسبب للكالاآزار فقط، ولن تُجرى فحوص لأمراض أخرى. إذا أظهرت النتائج في المعهد الإثيوبي للصحة العامة إصابتك أو إصابة طفلك دون ظهور أعراض، سنتصل بك لإبلاغك وقد ننصحك .بزيارة مركز العلاج

تخزين العينات

،نرغب في تخزين المادة الوراثية من قطرات الدم التي تم جمعها. إذا أُرسلت إلى المركز الصحي بسبب نتيجة إيجابية فقد تُجمع عينات إضافية كجزء من الإجراءات الروتينية. إذا وافقت، سنحتفظ بالعينات لمدة عشر سنوات بعد انتهاء ،الدراسة لاستخدامها في بحوث مستقبلية، بموافقة لجنة أخلاقيات جامعة أديس أبابا. حتى إذا لم توافق على التخزين يمكنك المشاركة في الدراسة. ستُخزن العينات في المعهد الإثيوبي للصحة العامة فقط ولن تُصدر خارج إثيوبيا، ولن .تُباع أو تُستخدم لأغراض تجارية

الخصوصية وحماية البيانات

سنبذل كل جهد ممكن لحماية خصوصيتك وخصوصية طفلك. ستُعامل جميع المعلومات بسرية تامة وتُحفظ في قاعدة بيانات إلكترونية برمز وليس بالاسم. لن يُكشف عن المستندات التي تحتوي على اسمك إلا للباحثين المخولين فقط. في حال كانت نتيجة الاختبار إيجابية، قد نربط الرمز باسمك ورقم هاتفك لإبلاغك بالنتيجة. سيُحفظ هذا الرابط في مكان آمن لا يمكن الوصول إليه إلا لأعضاء محددين من الفريق البحثي. ستُحتفظ بالبيانات لمدة عشر سنوات. قد تُنشر نتائج الدراسة في المجلات العلمية، وقد تُستخدم بياناتك لأبحاث مستقبلية بعد إخفاء الهوية بحيث لا يمكن ربطها بك أو بطفلك. لن يُذكر اسمك في أي قاعدة بيانات أو تقرير أو منشور ناتج عن هذه الدراسة.

لجنة الأخلاقيات

قبل البدء، تمت مراجعة هذه الدراسة والموافقة عليها من قبل لجنة الأخلاقيات بجامعة أديس أبابا ومعهد أكليلو لما لأبحاث الصحة.

المشاركة الطوعية

مشاركتك (أو مشاركة طفلك) طوعية بالكامل. لك مطلق الحرية في القبول أو الرفض. لن تتعرض لأي ضرر إذا قررت عدم المشاركة، ويمكنك الانسحاب في أي وقت حتى بعد توقيع نموذج الموافقة دون الحاجة إلى تبرير. كما يحق للباحث إيقاف مشاركتك في أي وقت إذا رأى أن ذلك في مصلحتك أو في حال عدم الالتزام بشروط الدراسة. سيتم شرح ذلك لك في حال حدوثه.

معلومات الاتصال للاستفسارات:

لأي استفسار يتعلق بالدراسة أو في حال تعرضك لأي آثار سلبية قبل أو أثناء المشاركة، استخدم بيانات الاتصال التالية:

اسم الباحث الرئيسي: هبتامو بلاي

الهاتف: 0911006034

البريد الإلكتروني: hbtmbelay652@gmail.com

Annex 6: Consent form for adults (> 18 years old) (English version)

Code No._____

I agree to participate in the research project titled: Studies on the Epidemiology of Visceral leishmaniasis and infection rates of Phlebotomus sand flies in Refugee camps of Western Ethiopia, conducted by Mr Habtamu Belay who have discussed the research project with me. I have received, read and kept a copy of the information letter. I have had the opportunity to ask questions about this research and I have received satisfactory answers. I understand the general purposes, risks and methods of this research.

I consent to participate in the research project and the following has been explained to me: the research may not be of direct benefit to me, my participation is completely voluntary, my right to withdraw from the study at any time without any implications to me, what I am expected and required to do, whom I should contact for any complaints with the research or the conduct of the research, I am able to request a copy of the research findings and reports and security and confidentiality of my personal information.

In addition, I consent to: publication of results from this study on the condition that my identify will not be revealed and store the leftover blood samples for future research tests.

Signature of adult participant: _____Date: _____

Annex 7: Consent form for adults (> 18 years old) (Arabic version)

Code No._____

أوافق على المشاركة في مشروع البحث بعنوان: دراسات حول وبائيات داء اللشمانيا الحشوي ومعدلات الإصابة بذباب الرمل (فليبوتوموس) في مخيمات اللاجئين في غرب إثيوبيا، والذي يجريه السيد حبتمو بيلاي، والذي ناقش معي تفاصيل هذا المشروع البحثي. لقد استلمت وقرأت واحتفظت بنسخة من رسالة المعلومات. وقد أُتيحت لي الفرصة لطرح الأسئلة حول هذا البحث وتلقيت إجابات مرضية. أفهم الأهداف العامة والمخاطر والأساليب المستخدمة في هذا البحث.

أوافق على المشاركة في هذا المشروع البحثي بعد أن تم توضيح ما يلي لي: أن هذا البحث قد لا يقدم فائدة مباشرة لي، وأن مشاركتي طوعية تمامًا، وأن لي الحق في الانسحاب من الدراسة في أي وقت دون أي تبعات، وأنني على علم بما يُتوقع مني القيام به، ومع من أتواصل في حال وجود أي شكوى تتعلق بالبحث أو بتنفيذه، وأنه يمكنني طلب نسخة من نتائج وتقارير البحث، وأن معلوماتي الشخصية ستظل آمنة وسرية.

بالإضافة إلى ذلك، أوافق على نشر نتائج هذه الدراسة بشرط عدم الكشف عن هويتي، وكذلك على حفظ عينات الدم المتبقية لإجراء اختبارات بحثية مستقبلية.

توقيع المشارك البالغ: _____

التاريخ: _____

Annex 8: Assent form for children (8-18 years old) (English version)

Code No._____

I agree to participate in the research project titled as “Studies on the Epidemiology of Visceral leishmaniasis and infection rates of sand flies in Refugee camps of Western Ethiopia”, conducted by Mr Habtamu Belay who have discussed the research project with me. I have received, read and kept a copy of the information letter. I have had the opportunity to ask questions about this research and I have received satisfactory answers. I understand the general purposes, risks and methods of this research.

I consent to participate in the research project and the following has been explained to me: the research may not be of direct benefit to me, my participation is completely voluntary, my right to withdraw from the study at any time without any implications to me, what I am expected and required to do, whom I should contact for any complaints with the research or the conduct of the research, I am able to request a copy of the research findings and reports and security and confidentiality of my personal information. In addition, I consent to: publication of results from this study on the condition that my identify will not be revealed and store the leftover blood samples for future research tests.

I voluntarily assent that I would participate in this study provided my parents/guardians give their consent.

Signature of child participant: _____Date: _____

Signature of data collector: _____ Date: _____

Annex 9: Assent form for children (8-18 years old) (Arabic version)

Code No._____

أوافق على المشاركة في مشروع البحث بعنوان "دراسات حول وبائيات داء الليشمانيات الحشوي ومعدلات إصابة ذباب الرمل في مخيمات اللاجئين في غرب إثيوبيا"، الذي يجريه السيد حبتمو بلاي والذي ناقش معي تفاصيل هذا المشروع البحثي. لقد تلقيت وقرأت واحتفظت بنسخة من رسالة المعلومات. كما أتيحت لي الفرصة لطرح الأسئلة حول هذا البحث وتلقيت إجابات مرضية. أفهم الأهداف العامة والمخاطر والأساليب المتبعة في هذا البحث.

أوافق على المشاركة في هذا المشروع البحثي وقد تم توضيح ما يلي لي: أن هذا البحث قد لا يكون ذا فائدة مباشرة لي، وأن مشاركتي فيه اختيارية تمامًا، ولي الحق في الانسحاب من الدراسة في أي وقت دون أي تبعات، ومعرفة ما هو متوقع مني القيام به، ومن يمكنني الاتصال به في حال وجود أي شكوى تتعلق بالبحث أو بتنفيذه، وأنه يمكنني طلب نسخة من نتائج وتقرير البحث، وضمان أمن وسرية معلوماتي الشخصية. بالإضافة إلى ذلك، أوافق على نشر نتائج هذه الدراسة بشرط عدم الكشف عن هويتي، وعلى حفظ عينات الدم المتبقية لاستخدامها في اختبارات بحثية مستقبلية.

أوافق طوعًا على المشاركة في هذه الدراسة شريطة أن يقدم والداي/أوصيائي موافقتهم.

_____ :توقيع المشارك الطفل: _____ التاريخ

_____ :توقيع جامع البيانات: _____ التاريخ

Annex 10: Consent for parents/guardians (English version)

Code No._____

I agree to participation of my child in the research project titled as “Studies on the Epidemiology of Visceral leishmaniasis and infection rates of sand flies in Refugee camps of Western Ethiopia”, conducted by Mr Habtamu Belay who have discussed the research project with me. I have received, read and kept a copy of the information letter. I have had the opportunity to ask questions about this research and I have received satisfactory answers. I understand the general purposes, risks and methods of this research.

I consent to participation of my child in the research project and the following has been explained to me: the research may not be of direct benefit to my child, participation is completely voluntary, whom I should contact for any complaints with the research or the conduct of the research and security and confidentiality of my child personal information. In addition, I consent to: publication of results from this study on the condition that my child identify will not be revealed and store the leftover blood samples for future research tests.

Child name _____

Signature of child participant: _____Date: _____

Signature of data collector: _____ date: _____

Annex 11: Consent for parents/guardians (Arabic version)

Code No._____

أوافق على مشاركة طفلي في مشروع البحث بعنوان "دراسات حول وبائيات داء الليشمانيات الحشوي ومعدلات إصابة ذباب الرمل في مخيمات اللاجئين في غرب إثيوبيا"، الذي يجريه السيد حبتمو بلاي والذي ناقش معي تفاصيل هذا المشروع البحثي. لقد تلقيت وقرأت واحتفظت بنسخة من رسالة المعلومات. كما أتيحت لي الفرصة لطرح الأسئلة حول هذا البحث وتلقيت إجابات مرضية. أفهم الأهداف العامة والمخاطر والأساليب المتبعة في هذا البحث.

أوافق على مشاركة طفلي في هذا المشروع البحثي وقد تم توضيح ما يلي لي: أن هذا البحث قد لا يكون ذا فائدة مباشرة لطفلي، وأن المشاركة فيه اختيارية تمامًا، ومن يمكنني الاتصال به في حال وجود أي شكوى تتعلق بالبحث أو بتنفيذه، وضمان أمن وسرية المعلومات الشخصية الخاصة بطفلي. بالإضافة إلى ذلك، أوافق على نشر نتائج هذه الدراسة بشرط عدم الكشف عن هوية طفلي، وعلى حفظ عينات الدم المتبقية لاستخدامها في اختبارات بحثية مستقبلية.

اسم الطفل: _____

توقيع الطفل المشارك: _____ التاريخ _____

توقيع جامع البيانات: _____ التاريخ _____

Annex 12: Direct Agglutination Test Standard Operational Procedure

Direct Agglutination Test Procedure

Principle: Infections with *L. donovani* yield the production of antibodies against the parasite. These antibodies can be demonstrated in blood or serum by the agglutination test. The DAT antigen is a whole, killed, promastigotes from cultures of *L. donovani* which have been stained blue for visibility. These are suspended in solution. When left in a V-shaped well, they will slowly fall to the tip of the V giving a dense blue dot. If anti-Leishmania antibodies are present in the blood or serum, the blue-stained parasites (DAT antigen) will become crosslinked by the antibodies (directly agglutinated) and settle to the bottom of the well as a hazy blue mat or a cloud, but NOT as a dot. By diluting the serum 2-fold in each well starting at 100 x dilutions, the titer (quantity of antibody) can be measured.

Collection of blood specimen for DAT: Collect capillary blood from the finger or the toe or heel in infants

Materials for sample collection: DAT request form, DAT registration book, Whatman No. 3 filter paper, Sterile lancet, Disinfectant, Cotton wool, Scissors, Plastic bag, Pen (ball point), Paper clips

Procedure for sample collection:

- ✓ Cut the circular filter paper into 8–16 segments depending on the size
- ✓ Each segment can be used for one patient's test only
- ✓ Draw a circle of approximately 1cm in radius on the segment of the filter paper
- ✓ Record the patient's details in the laboratory registration book
- ✓ Write the DAT number on the patient's request form and also on the segment
- ✓ Soak a piece of cotton wool in iodine or alcohol and disinfect finger, toe or heel thoroughly
- ✓ Allow the skin to dry
- ✓ Using the sterile lancet, prick the finger firmly so that blood flows freely
- ✓ Wipe the first drop of blood with a plug of dry cotton wool.

- ✓ Squeeze the finger gently and collect the next drop of blood into the circle on the segment of the filter paper. Check the DAT number on the filter paper before collecting the blood.
- ✓ Make sure the blood soaks through both sides of the filter paper and fills the circle
- ✓ Allow the filter paper to dry.
- ✓ Apply pressure to the finger prick with a dry cotton wool.
- ✓ Discard the used cotton wool into the waste bucket and the lancet into the sharps' container.
- ✓ When dry, very dry, clip the filter paper with the request form and put into a plastic bag.
- ✓ Store in a refrigerator or cool box until ready to send for testing or until ready for performing the test.

Elution of samples – Day 1

Requirements: Laboratory register, Microtitre plates (V-shaped), Sample (dry in filter paper), Micropipette to measure 125 µl, Normal saline, Paper punch (5mm), Scissors, Marker (permanent), Forceps and Refrigerator

Procedure

1. Write the number of the samples in order in the laboratory register. The list should be labeled to show which row of which microtitre plate corresponds to which sample, e.g. the list can be labeled A, B, C, D, E, F, G, and H in order.
2. The samples need be grouped by 8 samples in each group or less. Group one is called Plate I, group two is called Plate II, and so on.
3. Label the microtitre plates to correspond with the laboratory register, i.e., get out the micro-titer plates and write on the plate itself the plate number. The first one is plate I.
4. Using the paper punch, punch out a sample of filter-paper blood.
5. Using the forceps, put the punched sample of filter-paper blood into the well of the first column of the microtitre plates corresponding to the position recorded on the patient laboratory register. Ensure that the punched filter-paper blood is properly

inserted in the well, e.g. the sample listed as A should go in the first row labeled A. The sample listed as B should go in the second row labeled B, and so on.

6. Take the micropipette and adjust it to measure 125µl, fix the pipette tip firmly and pipette 125µl of normal saline. Add the 125µl of normal saline to each well with a sample paper. Make sure that the punched filter paper blood is completely immersed in the saline.
7. Cover the plates with another microtitre plate; incubate in the refridgerator at 4^0C overnight for at least 8 hours.

Dilution and titration – Day 2

Requirements: Measuring cylinder, 50ml container (plastic or glass bottle or conical tubes), Research (repetitive) pipette 100–1000µl, adjustable; brand Handy-Step or Eppendorf with volume display, Combitip ejection, Multipipette 100–1000µl Eppendorf or Handy-Step adjustable with volume display and tip ejection. Multichannel pipette, 8 channels, 5–50µl Eppendorf, adjustable volume and tip ejection, Micropipette tips (yellow tips for 100µl Multipette and the Multichannel pipette, blue tips for up to 1000µl research pipette, combitips standard 1.25ml and combitips plus 2.5 ml), 5–10ml syringe, 2-mercaptoethanol (2-ME), Normal saline, DAT antigen (freeze dried antigen OR liquid antigen), Freeze dried control sera.

Preparation of diluents: To be used with freeze-dried antigen (FDA);

1. Measure 50ml of normal saline and put it into the container
2. Adjust the multipipette to measure 390µl
3. Pipette 390µl of 2-ME and add to the normal saline then mix

Reconstitution of the freeze-dried antigen (FDA)

1. Add 5ml of fresh normal saline to the vial of the antigen
2. Mix gently by rotating and tilting the vial. Do not shake
3. Let it stand for about 10 minutes before use. Freeze-dried antigen is kept at room temperature!

Reconstitution of the freeze-dried control

- Use a new set of control sera with every new batch of DAT antigen. Make sure that all the freeze-dried powder is on the bottom of the vial. (The control kits made in Amsterdam contain 2µl of serum each)
- Strictly follow the manufacturer's instructions on the procedure for reconstitution especially the amount of normal saline or diluents to be added to the vial.
- Either add 100µl or 200µl of normal saline or diluent to the vial of the control sera depending on the manufacturer's instructions.
- Mix gently by rotating.
- Let it stand for at least 10 minutes before use.

Dilution of samples

- **Filter paper blood:** Take the microtitre plates with the eluted blood out of the refrigerator and allow it to come to room temperature (Serum dilution in column 1 is 1:50).
- Reconstituted control sera

Fill the control well(s) in column 1 with 100µl of reconstituted control serum. This is a 1:50 dilution.

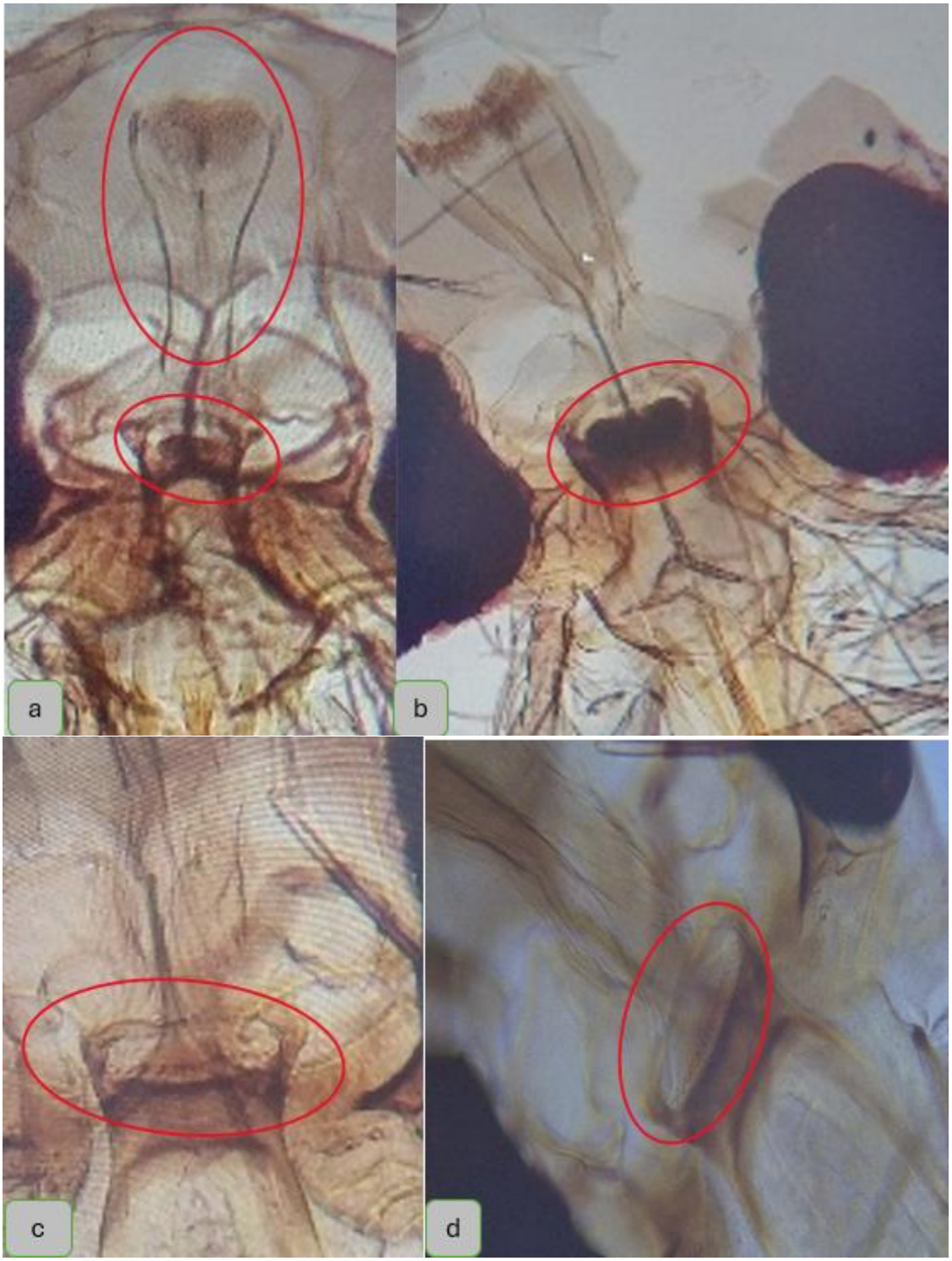
1. Adjust the pipette to measure 50µl.
2. Fill the wells from columns 2 to 12 with 50µl diluent.
3. Adjust the multichannel pipette to measure 50µl.
4. Place 8 standard tips (yellow tips) on the multichannel pipette and make sure they are firmly fixed to avoid pipetting errors. If the multichannel pipette is not available, you may use a single channel pipette. Pipette each row separately.
5. Mix the contents in column 1 by pipetting in and out at least 5 times. Avoid forming bubbles by expelling air prior to inserting the pipette tips into the wells and using a slow action.

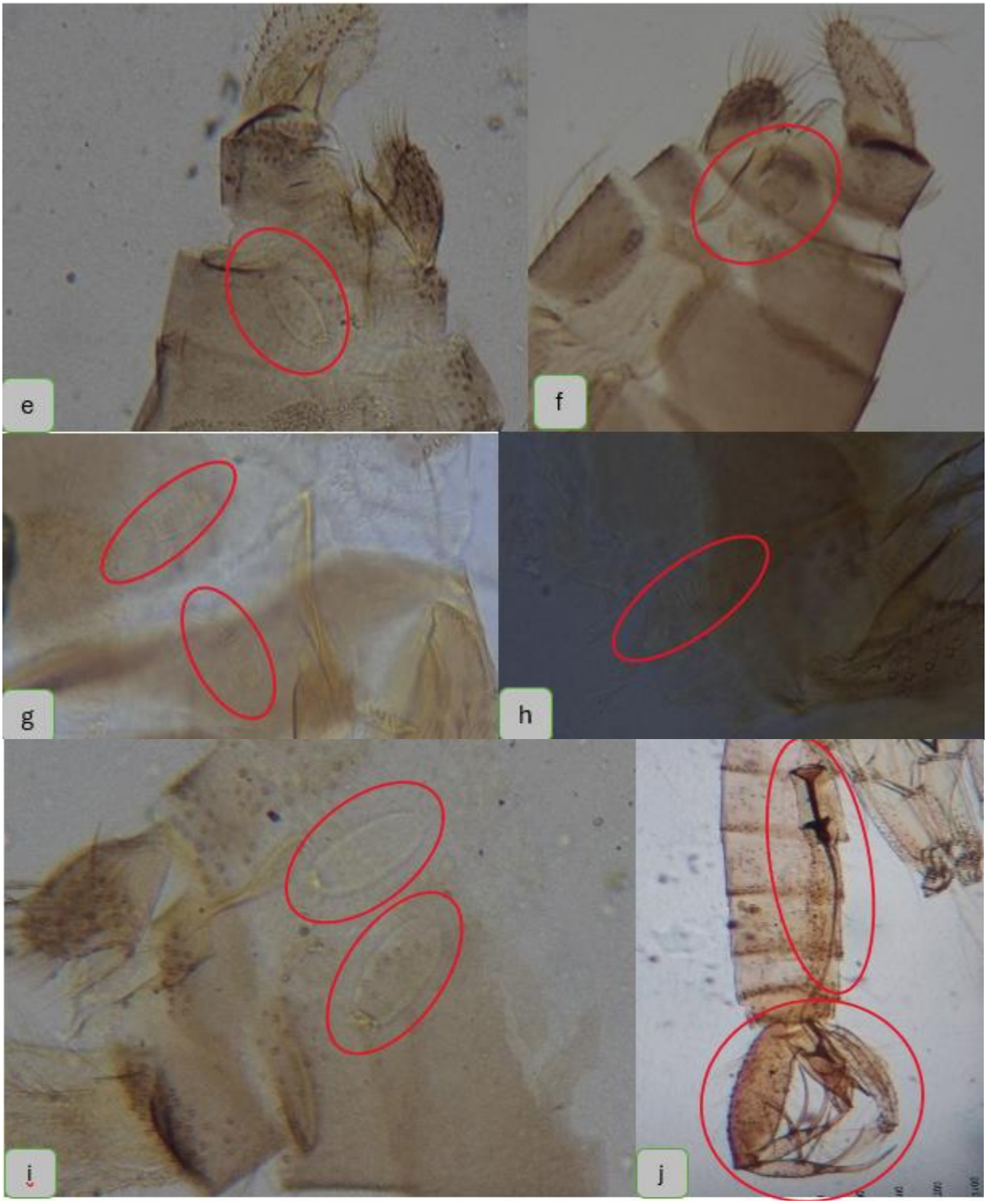
6. Pipette 50µl from column 1 and transfer to column 2. Continue this mixing and transferring until column 11. Discard the last 50µl from column 11. Do not add to column 12. (Serial dilution). Column 12 is the negative control. Adding antigen
7. Gently rotate the antigen bottle to mix it. Do not shake the bottle as this may destroy the antigen.
8. Adjust the pipette to measure 50µl and fit the pipette tips.
9. Add 50µl antigen to every well except wells in column 1. It is advisable to start with wells in column 12 (negative control) to avoid contamination, and then to add row by row. You should also change the pipette tip every time antigen is taken out of the bottle.
10. Rotate the plates gently clockwise and anticlockwise.
11. Cover the plates with a spare microtitre plate, leave them on a level surface at room temperature for about 12 to 18 hours.

Reading the DAT results – Day 3

- Put the plates against a white background.
- Estimate the titre by comparing the dots in column 12 (negative control) to those of the samples in the other columns.
- The titre is expressed as the last dilution that shows a difference compared to the negative control. A dark blue dot indicates that the result is negative and no reaction took place whereas a hazy blue mat or cloudy appearance indicates that reaction took place. The highest titre will be last dilution that still shows a hazy blue mat or cloud.
- Record the result in the lab book by titre and meaning. For example, record “well 9, 12800 positives”. If you are not sure of the meaning (positive, negative or borderline) simply record the titre. Currently, the positive titre is well 7 or 3200 with the FD antigen.
- Report the results in the patient’s request form. The titres are as follows: Column: 1 2 3 4 5 6 7 8 9 10 11 Dilution: 1:50 1:100 1:200 1:400 1:800 1:1600 1:3200 1:6400 1:12800 1:25600 1:51200

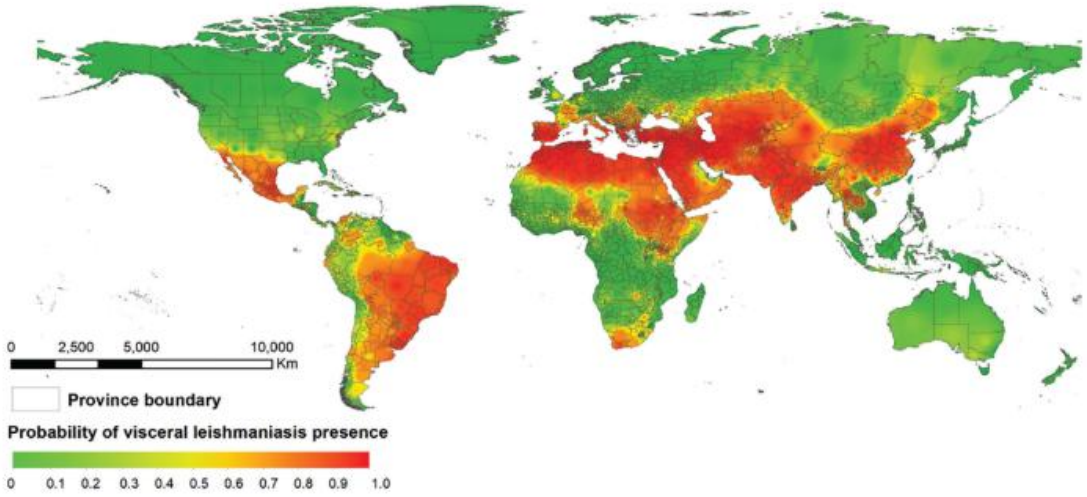
Annex 13: Additional pictures





Annex 13: Some of the diagnostic features for the identification of sand flies collected during the study period in Gambella and Benishangul-Gumuz regional states. The variation in pharynx and cibarium teeth (a-d); the genitalia of female sand flies for species differentiation (e-i); and the genitalia, spines, and their arrangement for male sand fly species identification (j).

Annex 14: Potential global distribution of human visceral leishmaniasis (Wu et al., 2024)



Annex 15: Published Articles

PLOS Neglected Tropical Diseases

RESEARCH ARTICLE

Prevalence of *Leishmania* infection in refugee camps: A serological and molecular study in Gambella and Benishangul-Gumuz, Ethiopia

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Check for updates

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Abstract

Background

Leishmaniasis, transmitted by sandflies, causes a severe health threat in East African refugee camps. High mobility, poor conditions and limited healthcare access heighten the risk of visceral leishmaniasis (VL) among refugees. Though, data on infection prevalence is remains limited. This study aims to determine the prevalence of *Leishmania* infection in refugee camps in Benishangul Gumuz and Gambella regions to improve VL detection and guide effective control strategies in humanitarian settings.

Methods

A cross-sectional study was conducted from May to August 2023 in four refugee camps to determine *Leishmania* infection using DAT and rtPCR on blood samples. Sociodemographic and clinical data were collected using structured questionnaires. Ethical approval was granted, and informed consent was obtained. Data were analyzed using SPSS v23, with associations assessed using logistic regression and Chi-square tests at a 0.05 significance level. Continuous variables summarized by median and interquartile range (IQR).

Result

The study included 1,223 participants (440 from Tsore camp and 220 from Sherkole in Benishangul Gumuz Region; 288 from Kule camp and 275 from Terkidi in Gambella Region), most of whom were from South Sudan (66.7%) and the majority were females (56.5%). 17.8% of the participants reported fever, with no spleen or liver

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enlargement and 0.2% lymph node swelling. Real-time PCR positivity was significantly higher in Tsore (14.6%, $\chi^2 = 21.4$, $p < 0.001$), no significant difference in DAT positivity was observed across refugee camps ($\chi^2 = 6.6$, $p = 0.084$). *Leishmania* DAT positivity rates were 6.0%, 4.6% and 4.7% in those with fever, chills and headache, respectively. *Leishmania* kDNA based rtPCR positivity rate were 11.7%, 8.8%, 7.3% and 6.2% in those with fever, chills, headache and weakness, respectively. Participants from Benishangul Gumuz region [AOR: 3.67 (95%CI: 1.57-8.59); $p = 0.003$]; South Sudanese [AOR: 2.87 (95%CI: 1.26-6.50); $p = 0.012$ and those with fever [AOR: 2.08 (95%CI: 1.01-4.28); $p = 0.047$] had a higher odds of DAT positivity. On the other hand, lower rtPCR positivity rates were seen in the Sherkole refugee camp compared to Tsore camps in Benishangul Gumuz region [AOR: 0.19 (95%CI: 0.08-0.45); $p < 0.0001$].

Conclusion

Leishmania infection was prevalent in refugee camps in Gambella and Benishangul Gumuz regions. Asymptomatic cases and low parasite loads were common, highlighting the need for active case detection, intervention including treatment and vector control to manage VL transmission effectively.

Author summary

Leishmaniasis poses a significant health threat in East African refugee camps, particularly among populations with high mobility, poor living conditions, and limited healthcare access. This cross-sectional study, conducted from May to August 2023, aimed to assess the prevalence of *Leishmania* infection in four refugee camps in the Benishangul Gumuz and Gambella regions. Using direct agglutination tests (DAT) and RT-PCR on blood samples from 1,223 participants, the study revealed a *Leishmania* infection prevalence of 4.0% by DAT and 10.3% by RT-PCR. Notably, RT-PCR positivity was significantly higher in Tsore camp (14.6%) compared to Sherkole camp (3.2%), where residents were 81% less likely to be tested positive. Fever, chills, and headache were common symptoms reported by the participants in varying proportions. Sociodemographic factors, such as being from the Benishangul Gumuz region or South Sudan, increased the odds of DAT positivity. These findings underscore the need for active case detection, intervention including treatment tailored interventions, including active case detection and vector control to manage visceral leishmaniasis (VL) in these vulnerable populations effectively.

Introduction

Leishmaniasis is a parasitic disease caused by protozoan parasites of the genus *Leishmania*, transmitted through the bites of infected female Phlebotomine sandflies

[1]. The disease has three clinical forms: Visceral leishmaniasis (VL) also known as kala-azar, which affects internal organs and is fatal if untreated; cutaneous leishmaniasis (CL), the most common form, causes skin ulcers; and mucocutaneous leishmaniasis (MCL) manifesting with both skin and mucosal lesions [2]. Leishmaniasis is recognized by the World Health Organization (WHO) as one of the neglected tropical diseases (NTDs) that significantly affects health in poor, in remote regions with limited access to healthcare [3].

VL poses a significant public health challenge in East Africa, where the region experiences some of the highest case rates globally, leading to outbreaks [4–7]. Factors believed to contribute to the occurrence of *Leishmania* infection include environmental changes, population movements between endemic and non-endemic areas, the presence of therapy-resistant strains, and immune suppression, which is often linked to malnutrition and co-infection with the human immunodeficiency virus (HIV) [4,8].

Population displacement plays a central role in the spread and transmission of infectious diseases, such as VL. The United Nations High Commissioner for Refugees (UNHCR) reported that there are more than 122 million displaced populations of these 37.4 million people are refugees across the globe in 2023 [9]. Refugees facing high mobility, poor living conditions and barriers to accessing health care are particularly susceptible to infectious diseases, with VL occasionally being identified in refugee camps [10]. In Kenya, an outbreak of VL was investigated in refugee camps in 2017. Thirty-four probable and confirmed VL cases were identified, with a case-fatality of 29.4%; in one case, molecular typing confirmed *Leishmania donovani* [5]. Moreover, reports revealed a strong relationship between civil unrest and VL [11].

The government of Ethiopia opened its borders for refugees, the majority originating from South Sudan, Somalia, Eritrea and Sudan. Ethiopia hosts a large number of refugees, many of whom are located in camps in the Gambella and Benishangul Gumuz regions, situated in the western part of the country. Large number of refugees in these camps came from areas where VL is endemic. Moreover, Gambella and Benishangul Gumuz regions have been identified as at high risk of VL based on geographical and climatological features [12,13]. Consequently, refugees could be at high risk of contracting and transmitting *Leishmania* parasites to the indigenous community and vice versa [14]. As *Leishmania* infections are often asymptomatic and the disease is difficult to diagnose, transmission can remain undetected for long periods of time.

VL is a public health concern in Ethiopia, particularly in vulnerable populations such as refugees. However, data on VL distribution in these populations is extremely limited. Refugee camps often lack the infrastructure needed for effective disease detection and control, and infections can spread rapidly in such settings. Timely identification of *Leishmania* cases can improve response efforts and reduce the disease burden. There is also a need for more global knowledge on managing VL in humanitarian and crisis settings. Thus, this study aimed to determine the prevalence of *Leishmania* infection among residents of the four refugee camps selected for the present study within Gambella and Benishangul Gumuz regions using serological and molecular analysis.

Methods and materials

Ethical statement

The study was conducted in accordance with ethical guidelines and received approval from the Aklilu Lemma Health Research Institute-Institutional Research Ethics Review Committee (ALIPB-IRERC) prior to data collection (Ref. No.: ALIPB IRERC/112/2015/23). A permission letter was obtained from Federal Democratic Republic of Ethiopia Refugees and Returnees Service (FDRE RRS) and subsequently from RRS regional office and camp coordination offices. Written informed consent or assent was obtained from all participants or, when necessary, from parents or guardians, with trained personnel assisting in participants' native languages to ensure comprehension. Privacy and confidentiality were strictly maintained, with all identifying data anonymized and securely stored, accessible only to the research team. The study followed all relevant guidelines for research on human subjects, implementing additional safeguards to protect participants' rights, dignity, and welfare.

Study sites

There are a total of seven refugee camps in Gambella [15] and five in Benishangul Gumuz Regional States [16]. The four refugee camps selected for the present study are located in Itang and Homosha districts of Gambella and Benishangul Gumuz regional states, respectively (Fig 1). Itang, west of Gambella city, accommodates the Terkidi and Kule camps, sheltering thousands of South Sudanese refugees who fled violence since 2013 [15]. Similarly, Homosha, located near Assosa in Benishangul-Gumuz, is home to the Tsore and Sherkole camps [16] (Fig 1). Healthcare services are provided by international NGOs, UN agencies, and local authorities, focusing on primary care, maternal health, and communicable diseases. Despite these efforts, the service has faced some challenges, such as resource limitations and high demand, may still affect access to comprehensive care to detect and treat VL. These camps have ecological conditions conducive to sandfly survival, increasing the risk of VL transmission [13] with prior cases reported to the Ethiopian Ministry of Health [17].

Study design and period

A cross-sectional study was conducted to determine the prevalence of *Leishmania* infection in selected refugee camps in the Gambella and Benishangul Gumuz regional states of Ethiopia. The study was conducted from May to August 2023 in refugee camps located in these regions.

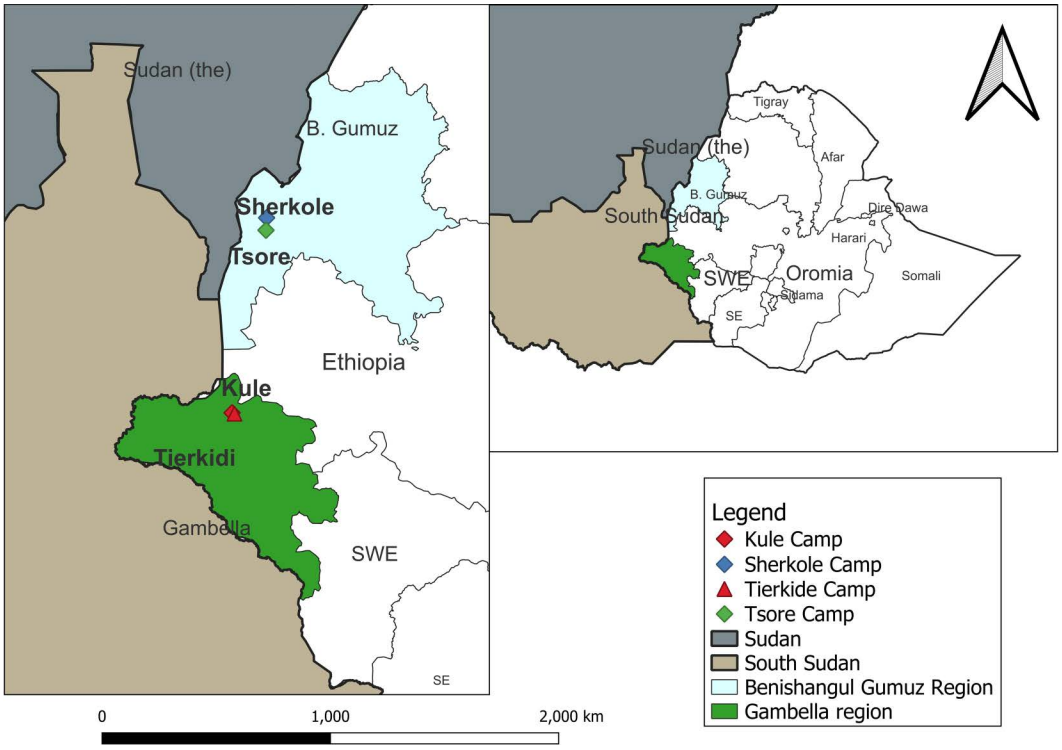


Fig 1. Map showing study refugee camps in Gambella and Benishangul Gumuz regions, Ethiopia [Kule Camp: represented by a blue diamond; Sherkole Camp: represented by a red diamond; Terkide Camp: represented by a red triangle; Tsore Camp: represented by a green square; Sudan: represented by a light gray color; South Sudan: represented by a dark gray color; Benishangul Gumuz Region: represented by a light blue color and Gambella Region: represented by a dark green color]. The maps were built using the free and open source QGIS software version 3.36.3 (QGIS Development Team (2024). QGIS Geographic Information System, version 3.36.3. Open-Source Geospatial Foundation Project. https://qgis.org) and shapefiles were obtained from the free and open-source site https://data.humdata.org.

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Study population

The study participants were refugees (five years of age and older) living in the selected camps. Individuals residing in the camp for at least six months and willing to provide assent and/or written informed consent were included in the study. Individuals who were unable to speak, listen or assent and/or provide written informed consent and who declined to participate or provide blood samples were excluded from the study.

Sample size determination

The sample size was estimated using a standard formula for household based cross-sectional studies, $Nh = \frac{(Z^2)2(p)(1-p)(DE)(k)}{(P)(n)(e^2)}$, where Z = 1.96 (95% confidence), p = 0.35 (proportion with VL) [18], DE = 1.5 (design effect), k = 1.1 (non-response multiplier), P = 0.95 (target population proportion), n = 4.0 (household size), and e = 0.025 (margin of error). This yielded the minimum sample size of 607 households from selected refugee camps in each regional states. Data were collected from 563 households in Gambella and 660 households in Benishangul-Gumuz, total 1,223 participants, with one participant selected per household.

Sampling procedure

The refugee camps in Gambella and Benishangul-Gumuz regions were purposely selected because of high concentration of refugee camps. Within each region, the refugee camps chosen were Kule and Terkidi in Gambella, and Tsore and Sherkole in Benishangul-Gumuz. A simple random sampling technique was applied at the camp level, minimize an unbiased selection process for identifying camps that represent the refugee population adequately. The sampling interval (k = 24) was calculated by dividing the total number of households by the desired sample size. The random starting point between 1 and 24 was determined by a lottery method and number was 9 chosen as starting point. From this point, every 24th household was systematically selected to ensure a representative and unbiased sample across all camps. This ensures that each camp contributes a sample size proportionate to its household population. The number of households allocated to each camp was based on its population size (S1 Fig).

Data collection tools

A structured questionnaire was prepared based on the existing forms [19–23]. Characteristics of each participant's sociodemographic status (i.e., age, sex, place of residence, number of family members) and clinical information (e.g., the presence of fever, headache, hepatomegaly or splenomegaly) were gathered.

Clinical sample collection and processing

Five milliliter blood was collected in EDTA tube (Shenzhen medicalet technology Co. Ltd, Shenzhen City, China) from each study participant (n = 1223). The whole blood samples were centrifuged at 5,000 X g for 10 minutes to separate the plasma and buffy coat [24] and was stored at −20°C to preserve cellular components, ensuring the quality of the sample for *Leishmania* DNA extraction and PCR amplification [25].

Direct agglutination test (DAT)

The direct agglutination test (DAT) was carried out as previously described [26]. Briefly, two µl of plasma samples was mixed with 100 µl diluent. The sample was diluted from 1:50–1:51200. *Leishmania* negative and positive controls prepared in an Academic Medical Center (Amsterdam) were used. Freeze-dried antigen (FDA) containing *L. donovani* promastigotes were added in all 96 wells of the V-shaped microtiter plate (Thermo Fisher Scientific, USA). The results were read after overnight incubation. A DAT agglutination of ≥1:1600 was considered positive for *Leishmania* infection, while samples of ≤1:800 were consider negative [19].

DNA extraction

DNA extraction was performed from 754 randomly selected samples due to logistical constraints, using Geneaid DNA isolation kit (GMB300, Geneaid Biotech Ltd) per the manufacturer's protocol. The extracted DNA was eluted in 100 µl of elusion buffer. A negative extraction control (NEC) or nuclease free water was used to identify contamination and confirm the integrity of the extraction reagents and the procedural steps involved and treated as sample.

Leishmania kDNA detection by real-time polymerase chain reaction

A rtPCR targeting minicircle kinetoplast DNA (kDNA) was employed for the detection of *Leishmania* parasites from the buffy coat samples. The primers used for the study were kDNA-CMF (CTTTTCTGGTCCTCCGGGTAGG) and kDNA-CMR (CCACCCGGCCCTATTTTACAC CAA) [27]. The kDNA rtPCR was conducted as previously described [28]. Briefly, a 25 µl reaction volume was prepared with 1 × HotStarTaq Master mix (Qiagen, Venlo, The Netherlands), 0.6 µM of reverse and forward primers and 0.4 µM of the probe (Integrated DNA Technologies, Leuven, Belgium), 0.1 mg/mL bovine serum albumin (Roche, Vilvoorde, Belgium) and 5 µl of DNA template. The rtPCR was performed using QuantStudio 5 real-time PCR system (Catalogue: A28568, Thermo Fisher). During each run, the genomic DNA extracted from the *Leishmania* positive culture and the non-template control (NTC) were tested and used as a positive and negative control, respectively. Samples with cycle threshold (Ct) values between 12 and 40 with a sigmoidal amplification curve were considered positive for *Leishmania* infection [29].

Data quality assurance

To ensure data quality, several measures were implemented, including the development of appropriate data collection tools and the provision of training for data collectors, laboratory professionals, clinicians and local guide before commencement of data collection. During the data collection process, questionnaires were reviewed daily for completeness, accuracy, and consistency. Blood sample collected, processed and diagnosed in laboratories and well experience laboratory experts. Quality control measures were applied for both DAT and rtPCR tests, utilizing positive and negative controls, and strict adherence to standard operating procedures was maintained throughout.

Statistical analysis

The data were initially entered into Epi Info version 7.2 (Centers for Disease Control and Prevention, Atlanta, GA, USA), exported to Excel for data cleaning, then integrated into SPSS version 23.0 (SPSS Inc., Chicago, IL, USA) for analysis. Continuous variables were summarized with median and inter-quartile range (IQR) and categorical variables with numbers and proportions. Chi-square was used to assess the proportion differences among categorical variables. Association between the socio demography and clinical symptoms of the study participants with *Leishmania* positive participants were assessed by binary logistic regression. A multivariate logistic regression model was applied to variables with $p < 0.20$ in bivariate analysis. Adjusted ORs with a significance level of 0.05 were considered statistically significant for comparison.

Results

Sociodemographic characteristics

A total of 1223 participants were involved in the study from the planned 1214 individuals. In the Benishangul Gumuz region, 69.1% of respondents were females, while in the Gambella region, 58.26% were males. The majority of respondents in Benishangul Gumuz (61.8%) came from Sudan, while all participants in Gambella were from South Sudan. Education levels were 30.8% in Benishangul Gumuz and 41.2% in Gambella have no formal education. Over 63% of the refugees in both regions were married (Table 1). The median age of participants was 25 (IQR = 16 years) and 27 years (IQR = 25 years) for participants from Benishangul Gumuz and Gambella regions, respectively. The median length of stay

Table 1. Socio demographic characteristics of study participants of selected refugee camps located in Gambella (N = 563) and Benishangul Gumuz (N = 660) regions, Ethiopia, 2023.

Categories		Camp location by region	
		Benishangul Gumuz N (%)	Gambella N (%)
Sex	Female	456 (69.1)	235 (41.7)
	Male	204 (30.9)	328 (58.3)
Age group in years	5-9	64 (9.7)	49 (8.7)
	10-19	202 (30.6)	141 (25.0)
	20-29	264 (40.0)	113 (20.1)
	30-39	71 (10.8)	121 (21.5)
	40-49	35 (5.3)	68 (12.1)
	50+	24 (3.6)	71 (12.6)
Camp Name	Kule	–	288 (51.2)
	Sherkole	220 (33.3)	–
	Terkidi	–	275 (48.9)
	Tsore	440 (66.7)	–
Country of Origin	South Sudan	252 (38.2)	563 (100.0)
	Sudan	408 (61.8)	0 (0.0)
Level of education	No formal education	203 (30.8)	232 (41.2)
	Primary school	342 (51.8)	232 (41.2)
	Secondary school	80 (12.1)	78 (13.9)
	Higher education	35 (5.3)	21 (3.7)
Marital Status	Married	428 (64.9)	358 (63.6)
	Single	232 (35.2)	205 (36.4)
Family Size	≤4	256 (38.8)	169 (30.0)
	>4	404 (61.2)	394 (70.0)

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in the camp was 8 years (IQR = 7 years) and 9 years (IQR = 4 years) participants from Benishangul Gumuz and Gambella refugee camps. The median family size of participants was 6 (IQR = 4) and 6 (IQR = 5) in refugee camps located in Benishangul Gumuz and Gambella regions, respectively.

Prevalence of *Leishmania* infection

Overall DAT positivity was 4.0% (95% CI: 3.0% to 5.3%) (49/1223). Most positive patients had a titer of 1:1600 or 1:3200; amongst the negative participants 275 still showed some agglutination (S2 Fig). Real-time PCR positivity was 10.3% (78/754) (95% CI: 8.3% to 12.7%). The cycle threshold (Ct) values were ranged from 30.9 to 39.9, with an average Ct value of 36.6.

The DAT positivity rates in the Benishangul Gumuz region were 5.9% in Tsore and 3.2% in Sherkole camps, while in the Gambella region, rates were 3.1% in Kule and 2.5% in Terkidi camps. For rtPCR, the highest positivity was observed in Tsore (14.6%) compared to Sherkole (3.2%, $p < 0.05$), whereas in Gambella, almost similar positivity rates were recorded in Kule (8.5%) and Terkidi (6.4%). Among the four refugee camps, rtPCR positivity was significantly higher for Tsore (14.6%, $\chi^2 = 21.4$, $p < 0.001$), while no significant difference in DAT positivity was observed across camps in Benishangul Gumuz and Gambella ($\chi^2 = 6.6$, $p = 0.084$).

Among individuals reporting specific symptoms, *Leishmania* DAT positivity rates varied as follows: those with fever had a DAT positivity rate of 6.0%, while individuals with chills and headache had also showed rates at 4.6% and 4.7%, respectively. Weakness was reported with a DAT positivity rate of 3.8% (Table 2). Among individuals who reported fever, 11.7%

Table 2. Association between sociodemographic and clinical characteristics of participants and DAT positivity in selected refugee camps in Gambella and Benishangul Gumuz regions, Ethiopia, in 2023.

Category		Tested (%)	DAT_Pos (%)	OR (95%CI)	P value	AOR (95%CI)	p-value
Gender	Male	532 (43.5)	25 (4.7)	1.26 (0.71-2.23)	0.43	1.66 (0.90-3.07)	0.1
	Female	691 (56.5)	24 (3.5)	1		1	
Age Group	5-9	83 6.8)	6 (7.2)	1			
	10-19	310 (25.3)	10 (3.2)	0.536 (0.19-1.51)	0.24		
	20-29	377 (30.8)	11 (2.9)	0.536 (0.19-1.48)	0.23		
	30-39	219 (17.9)	9 (4.1)	0.877 (0.30-2.53)	0.81		
	40-49	122 (10.0)	8 (6.6)	1.50 (0.50-4.48)2	0.47		
	50+	112 (9.2)	5 (4.5)	0.991 (0.29-3.35)	0.99		
Region	Benishangul Gumuz	660 (54.0)	33 (5.0)	1.80 (0.98-3.31)	0.06	3.67 (1.57-8.59)	0.003
	Gambella	563 (46.0)	16 (2.8)	1		1	
Camp name	Kule	288 (23.5)	9 (3.1)	1		1	
	Terkidi	275 (22.5)	7 (2.5)	1.02 (0.37-2.78)	0.97	0.93 (0.35-2.48)	0.89
	Tsore	440 (36.0)	26 (5.9)	0.81 (0.30-2.21)	0.68	0.76 (0.27-2.13)	0.6
	Sherkole	220 (18.0)	7 (3.2)	1.95 (0.90-4.22)	0.09		
Country of Origin	South Sudan	815 (66.6)	37 (4.5)	1.57 (0.81-3.04)	0.18	2.87 (1.26-6.50)	0.012
	Sudan	408 (33.4)	12 (2.9)	1		1	
Education level	No formal education	435 (35.6)	26 (6.0)	1.72 (0.40-7.43)	0.47		
	Grade 1–8	574 (46.9)	13 (2.3)	0.63 (0.14-2.85)	0.54		
	Grade 9–12	158 (12.9)	8 (5.1)	1.44 (0.30-7.00)	0.65		
	College and above	56 (4.6)	2 (3.6)	1			
Marital Status	Married	786 (64.3)	33 (4.2)	1.15 (0.63-2.12)	0.65		
	Single	437 (35.7)	16 (3.7)	1			
Family size Cat	≤4	723 (59.1)	18 (2.5)	1	0.77		
	>4	500 (40.9)	31 (6.2)	0.91 (0.51-1.65)			
Fever	No	1005 (82.2)	36 (3.6)	1		1	
	Yes	218 (17.8)	13 (6.0)	1.71 (0.89-3.28)	0.11	2.08 (1.01-4.28)	0.047
Chills	No	1093 (89.4)	43 (3.9)	1			
	Yes	130 (10.6)	6 (4.6)	1.18 (0.49-2.83)	0.71		
Headache	No	1009 (82.5)	39 (3.9)	1			
	Yes	214 (17.5)	10 (4.7)	1.22 (0.60-2.48)	0.59		
Weakness	No	1065 (87.1)	43 (4.0)	1			
	Yes	158 (12.9)	6 (3.8)	0.94 (0.39-2.24)	0.87		

DAT: direct agglutination test, DAT_Pos = *Leishmania* positive by DAT, AOR: Adjusted Odds Ratio, CI: Confidence Interval

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tested rtPCR positive for *Leishmania*. Those with chills, headache, and weakness had rtPCR a positivity rate of 8.8%, 7.3%, and 6.2%, respectively (Table 3). On the other hand, clinical symptoms were reported with the all of participants did not exhibit symptoms that strongly suggestive of VL including splenomegaly and hepatomegaly.

Out of 754 individuals tested with both PCR and DAT, overall, 90 participants were positive for *Leishmania* infection. This represents an overall positivity rate of 11.9% (90/754) (95%CI: 9.7% -14.1%). Twelve samples tested positive only with DAT, 55 tested positive only with rtPCR, and 23 tested positives with both methods, for a total of 90 positive results overall reported. As illustrated in the Venn diagram that, while some samples showed agreement between the two tests, rtPCR identified substantially more positive cases than DAT (Fig 2).

Table 3. Association between sociodemographic and clinical characteristics and participants' rtPCR positivity in selected refugee camps in Gambella and Benishangul Gumuz regions, Ethiopia, in 2023.

Category		PCR Tested (%)	PCR Pos (%)	OR (95%CI)	p value	AOR (95%CI)	p value
Gender	Male	258 (34.2)	25 (9.7)	0.90 (0.54-1.48)	0.67		
	Female	496 (65.8)	53 (10.7)	1			
Age Group in years	5-9	72 (9.5)	9 (12.5)	1			
	10-19	224 (29.7)	22 (9.8)	0.76 (0.33-1.74)	0.52		
	20-29	284 (37.7)	26 (9.2)	0.71 (0.32-1.58)	0.40		
	30-39	88 (11.7)	8 (9.1)	0.70 (0.26-1.92)	0.49		
	40-49	48 (6.4)	6 (12.5)	1.00 (0.33-3.02)	1.00		
	50+	38 (5.0)	7 (18.4)	1.58 (0.54-4.64)	0.41		
Region	Benishangul Gumuz	648 (85.9)	70 (10.8)	1.48 (0.69-3.18)	0.31	1.84 (0.68-4.98)	0.23
	Gambella	106 (14.4)	8 (7.5)	1		1	
Camp name	Kule	59 (7.8)	5 (8.5)	0.54 (0.21-1.41)	0.21	0.54 (0.20-1.47)	0.23
	Terkidi	47 (6.2)	3 (6.4)	0.40 (0.12-1.32)	0.13	0.48 (0.14-1.70)	0.26
	Sherkole	217 (28.8)	7 (3.2)	0.20 (0.09-0.44)	<0.001	0.19 (0.08-0.45)	<0.001
	Tsore	431 (57.2)	63 (14.6)	1		1	
Country of Origin	South Sudan	357 (47.3)	42 (11.8)	1.35 (0.84-2.15)	0.22	0.98 (0.58-1.66)	0.94
	Sudan	397 (52.7)	36 (9.1)	1		1	
Education level	No formal education	241 (32.0)	30 (12.4)	5.40 (0.72-40.81)	0.10	2.49 (0.31-19.96)	0.39
	Grade 1–8	378 (50.1)	37 (9.8)	4.12 (0.55-30.91)	0.17	1.91 (0.24-15.03)	0.54
	Grade 9–12	96 (12.7)	10 (10.4)	4.42 (0.55-35.75)	0.16	2.44 (0.29-20.64)	0.41
	College and above	39 (5.2)	1 (2.6)	1		1	
Marital Status	Married	489 (64.9)	52 (10.6)	1.09 (0.66-1.78)	0.75		
	Single	265 (35.1)	26 (9.8)	1			
Fever	No	660 (87.5)	67 (10.2)	1			
	Yes	94 (12.5)	11 (11.7)	1.17 (0.60-2.31)	0.64		
Chills	No	686 (91.0)	72 (10.5)	1			
	Yes	68 (9.0)	6 (8.8)	0.83 (0.35-1.98)	0.67		
Headache	No	644 (85.4)	70 (10.9)	1		1	
	Yes	110 (14.6)	8 (7.3)	0.64 (0.30-1.38)	0.26	0.63 (0.23-1.73)	0.37
Weakness	No	673 (89.3)	73 (10.8)	1		1	
	Yes	81 (10.7)	5 (6.2)	0.54 (0.21-1.38)	0.19	0.56 (0.23-1.73)	0.35

rtPCR: real time polymerase chain reaction, AOR: Adjusted Odds Ratio, CI: Confidence Interval

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Factors related to *Leishmania* positivity

Sociodemographic and clinical factors related to *Leishmania* DAT positivity are shown in Table 2. In multivariate analysis, region was significantly related to DAT positivity, with participants from Benishangul Gumuz that had 3.67 higher odds of being positive [AOR: 3.67 (95%CI; 1.57-8.59); p = 0.003]. Highest odds of DAT positivity were found in South Sudanese participants, with an AOR of 2.87 (1.26-6.50) compared to Sudanese participants (p = 0.012). Moreover, participants with fever had 2.08 more times higher DAT positivity rate than with no fever [AOR: 2.08 (1.01-4.28); p = 0.047].

Sociodemographic and clinical assessment factors related to *Leishmania rtPCR* positivity with characteristics are shown in Table 3. In adjusted analysis, refugee camp was significantly related to *Leishmania* rtPCR positivity, with participants from Sherkole refugee camp showed that 81% more protective than living in Tsore camps in Benishangul Gumuz region [AOR: 0.19 (95%CI; 0.08-0.45); p < 0.0001].



Fig 2. Venn diagram of DAT and rtPCR Positivity(n = 90) [DAT+ (Direct Agglutination Test Positive): represented by the yellow circle; rtPCR+ (Real-time PCR Positive): represented by the blue circle and the intersection (23 cases) indicates samples that tested positive in both methods].

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Discussion

Visceral leishmaniasis is a major public health concern in East Africa, including refugee camps in the region. In Ethiopia; the Gambella and Benishangul Gumuz regional states host many refugees living in conditions that favor *Leishmania* transmission [12]. The burden of *Leishmania* infection remains uninvestigated in refugee camps. This study aimed to determine *Leishmania* infection prevalence in these vulnerable populations using serological (DAT) and molecular (rtPCR) methods.

The prevalence rate was 4.0% and 10.3% as determined by DAT and rtPCR, respectively. The study participants did not report the typical VL symptoms in the data collection period. The prevalence and absence of clinical symptoms we reported highlights the asymptomatic reservoirs could be sustaining VL transmission in the study area. This necessitates implementation of active case detection, community-wide screening, rigorous vector control measures, and targeted public awareness campaigns to effectively combat and halt the transmission and progression of VL.

The overall DAT positivity rate in the current study was 4.0%. There are studies that showed higher DAT positivity rates in other regions of Ethiopia, including 18.1% in Gambella region among refugees in 1996 [30], 9.6% in southeastern Ethiopia [31] and 12.5% among agricultural migrant laborers in northwest Ethiopia [32]. In addition, a household-based survey in eastern Sudan showed serological positivity rates of 8.9% [33], and 13% in another study in southwest Sudan [34]. The lower DAT positivity rate in our study may be attributed to the refugees' prolonged stay in camps (average of nine years) in Benishangul Gumuz and Gambella, with displaced from high-risk areas in Sudan and South Sudan to relatively lower-risk regions in Ethiopia. Although these regions have ecological conditions favorable for VL transmission [12,13], they are not known as endemic areas in Ethiopia. On the other hand there are other studies that showed similar DAT positivity rate findings; in Benishangul Gumuz [35,36] and among pastoralist communities in Borena zone Oromia regional states of Ethiopia [37], as well as in Gedarif state in Sudan [38]. These reports suggest comparable levels of exposure to *Leishmania* in certain endemic settings but emphasizing the sustained need for targeted interventions and surveillance in these areas.

There was no difference in the positivity rate between gender by DAT and rtPCR for *Leishmania* infection. In contrast to our findings, previous reports showed that L*eishmania* infection was higher in males in studies conducted in Ethiopia [35,36] and Sudan [39]. On the other hand, our data were in line with community based reports from the Somali region, South Omo zone [19,22] and Sudan [40]. The lack of gender-based differences in *Leishmania* infection in our study is

likely a reflection of the unique sociocultural and environmental conditions in refugee camps, which create a more uniform risk setting compared to resident populations. The distribution of *Leishmania* infection among refugee camps as determined by both DAT and rtPCR was similar among age groups in the current study. Similar findings were reported from the Somali region in Ethiopia [22]. However, Abera et al reported that the prevalence of *Leishmania* infection gradually increased with age [35]. Susceptibility of gender and age to *Leishmania* infection may differ across societies based on prevailing patterns of household labor division [22] and living conditions specific for individuals living in refugee camps.

In the current study, the region of residence did not significantly predict the higher proportion of rtPCR positivity. On the other hand, the rtPCR positivity rate varied significantly among refugee camps in Benishangul Gumuz and Gambella regional states; Sherkole camp had the lowest positivity rate (3.2%) while other camps did not show significant differences. The DAT positivity rate showed significant variation, with refugee camps in the Benishangul Gumuz regional state facing a higher risk compared to those in the Gambella regional state. While these regions are not well known VL endemic foci, there is only one report on assessment of VL from 1996 in the Gambella [30] and few from the Benishangul Gumuz regional states [35,36,41].

Camp location may influence DAT positivity rates, with lower rates observed in Sherkole camp. In another study conducted among residents of Kumuruk district, which is adjacent to Homosha district, where these refugee camps located; reported an LST prevalence of 14.1% (27/191) with one infected dog in the community out of 36 tested (2.8%) [36]. On the other hand, no *Leishmania* infection was reported from the same study in other districts (Sherkole and Bambasi). These districts are not in immediate proximity to the current study area. Variations in *Leishmania* infection rates between bordering and non-bordering districts near the camp indicated that proximity to these districts significantly influences leishmaniasis transmission risk. Moreover, further serological surveys should be conducted in neighboring local populations to better evaluate whether refugees represent a risk of *L. donovani* introduction in these regions and/or if the living conditions in refugee camps are facilitating *Leishmania* transmission within this specific population.

The current study showed no difference in risk of acquiring *Leishmania* infection among individuals who lived in the camp for varying durations. The lack of association between camp duration and *Leishmania* infection risk suggests continuous exposure to the parasite regardless of length of stay in the camp. Other possible factors like vector density, personal protection measures, or immunity levels of the participants may play a more significant role in infection risk than length of stay. Nationality wise, a higher DAT positivity was observed in individuals from South Sudan, compared to those from Sudan. This could be due to most of the study participants came from the Jonglei and Upper Nile states of South Sudan. These regions reported the highest percentage of VL cases among 10 states in South Sudan [42].

Unlike the current finding, previous studies have shown that fever, splenomegaly, and hepatomegaly are major signs of VL in Sudan [39,43], South Sudan [44] and Ethiopia [45]. However, in the current study, only fever was associated with *Leishmania* infection. These earlier studies were conducted on diseased individuals, while the current report was based on community-level studies. Asymptomatic individuals tend to have a lower parasite load, which may not trigger noticeable clinical symptoms. Variations in immune responses to *Leishmania* infection can lead to different clinical outcomes, with some individuals effectively controlling the parasite and limiting the disease to fever, while others may develop more extensive organ involvement. Furthermore, in this study, all *Leishmania*-infected participants were asymptomatic for VL. However, HIV infection is known factor to significantly increase the risk of progression from asymptomatic infection to VL [28]. Due to logistical constraints, we were unable to screen participants for HIV status. We recommend further research on the epidemiology of HIV-VL co-infection in this population.

Since the distribution of sand flies within the camps has not yet been studied, and no clinically suspected *Leishmania*-infected individuals have been identified, our ability to directly assess the potential for *Leishmania* transmission within the camps was limited. However, our study provides insight into the distribution of the disease in the refugee camps we studied. Additionally, previous reports [30,36,46] and VL risk assessments in Ethiopia have classified the Gambella and Benishangul Gumuz regions as high-risk areas for VL transmission [12], suggesting that the camps may also be at risk for VL transmission.

The current results suggest that rtPCR detected 78 positive *Leishmania* infections compared to the 35 identified by DAT. The presence of 12 DAT-positive but rtPCR-negative cases could be attributed to persistent antibodies from past infections or false negatives in rtPCR due to low parasite DNA loads, as supported by studies indicating that serological tests can remain positive long after parasite clearance [27,47]. The detection ability variation among these two testing methods has also been corroborated by other studies conducted in Ethiopia among asymptomatic individuals [27,48,49]. However, the current report contradict with the study of cohorts of HIV infected subjects in known endemic areas from Ethiopia [28]. This variation could be the difference in study subjects (people living with HIV vs HIV status not mentioned). The discrepancy between the two test results suggests that combining both methods may enhance diagnostic accuracy. Public health strategies should incorporate rtPCR for confirmatory testing while using DAT for initial screening to improve case detection and disease control efforts [27,50]. Ultimately, integrating molecular diagnostics with serological methods is essential to strengthen VL surveillance and management programs. Even though the report indicated that detecting very low blood parasitemia is not consistent parameter for determining *Leishmania* infections [51], its usefulness can be enhanced by optimizing laboratory conditions, such as strict contamination monitoring, the inclusion of known positive and negative controls, and the use of extraction controls. By implementing these measures, as we did in our laboratory, the reliability and accuracy of the final results can be improved.

Strengths and Limitations of the study

This study on the prevalence of *Leishmania* infection in refugee camps in Gambella and Benishangul-Gumuz, Ethiopia, offers significant strengths by utilizing both serological and molecular diagnostics to improve detection and by focusing on a high-risk, underserved refugee population. Our findings can potentially guide health interventions in leishmaniasis hotspots, providing critical insights for targeted interventions. There are several limitations to acknowledge. The significant disparity in sample sizes between the DAT and rtPCR presents a critical challenge to this study. DAT's typical limitations, including lower sensitivity and specificity, can lead to misclassification of cases, potentially skewing prevalence estimates and underestimating the true disease burden, particularly in asymptomatic individuals or those with low parasite loads. The underrepresentation of rtPCR results, a more sensitive method for detecting early-stage and subclinical infections, may limit the study's ability to accurately capture the true disease dynamics. This imbalance hinders the analysis using both testing tools, weakening conclusions regarding their diagnostic accuracy and potentially introducing a bias towards DAT outcomes. We did not study the genetic diversity or the presence of specific *Leishmania* species, and cannot differentiate between past and current infections or track changes in infection status over time. Despite these limitations, the study provides valuable public health information for targeted disease control in vulnerable populations. Addressing these limitations in future research could further strengthen the findings and applicability of such studies in similar settings.

Conclusion and recommendation

Leishmania infection was prevalent in refugee camps in Gambella and Benishangul Gumuz regions, with detection rates of 4.0% using DAT and 10.7% using rtPCR. *Leishmania* infection was reported in all studied camps, although at varied proportions, with a noteworthy presence of asymptomatic cases and low parasite loads. These findings underscore the necessity for region-specific interventions, particularly given the higher positivity rates in the Benishangul Gumuz region and among South Sudanese refugees. This highlights the importance of tailored strategies, including active case detection, community-based screening, vector control measures, to effectively manage and reduce VL transmission within these high-risk populations. We additionally recommend assessing the presence of the vector sand-fly in both regions around the camp area. Continuous monitoring and comprehensive healthcare support are vital for effectively managing and mitigating the impact of leishmaniasis among this vulnerable population.

Supporting information

S1 Fig. Schematic presentation of sampling procedure (n = 1223) in this cross-sectional study. HH household: This figure depicted the selection process of refugee camps and households in two regions, Gambella and Benishangul Gumuz, using different sampling techniques. Regions were purposely selected followed by a simple random sampling technique to select camps. Finally, a systematic sampling technique with household (HH) proportion allocation was applied to determine the number of surveyed households in each camp.
(TIF)

S2 Fig. Distribution of anti-*Leishmania* antibody agglutination among selected refugee camps participants in Gambella and Benishangul Gumuz regions, 2023: This figure presents the distribution of Direct Agglutination Test (DAT) results based on agglutination titers. The x-axis represents the number of samples, while the y-axis categorizes the results into DAT-positive and DAT-negative groups based on different dilution levels. Samples without agglutination are also indicated. Higher dilution ratios (e.g., 1:25600, 1:12800) represent stronger positive reactions, while lower dilution ratios and "No agglutination" indicate negative results.
(TIF)

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Determinants of *Leishmania* infection in refugee camps in Gambella and Benishangul Gumuz regional states of Ethiopia: a matched case-control study

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Abstract

Background Visceral leishmaniasis (VL) is a neglected tropical disease affecting vulnerable refugee populations, including refugees residing in camps in Ethiopia's Gambella and Benishangul Gumuz regions. With refugees from VL-endemic areas, identifying key environmental, behavioral, and demographic risk factors is essential for effective prevention. This study addresses knowledge gaps on *Leishmania* infection determinants, supporting targeted interventions to enhance disease control and inform public health efforts in crisis-affected regions.

Methods A matched case-control study was conducted from May-August 2023 included 336 participants from four refugee camps (Kule, Terkidi, Tsore, and Sherkole) in Ethiopia. Data on socio-demographic, behavioral, and environmental factors were collected through interviewer-administered questionnaires. Blood samples were tested for *Leishmania* detection using the direct agglutination test and real-time polymerase chain reaction. Chi-square tests and multivariable conditional logistic regression assessed associations between independent variables and *Leishmania* infection.

Results A total of 336 participants, 252 controls and 84 cases, were enrolled from all selected refugee camps. The distribution of matching variables among cases and controls by age group, sex, and region of camp location showed that they were similarly distributed ($p > 0.05$). Significant risk factors included residing in the camp for less than five years (AOR = 1.76, 95% CI: 1.06–2.93, $P = 0.028$), sleeping outside during the daytime (AOR = 2.38, 95% CI: 1.46–3.89, $P < 0.001$) or nighttime (AOR = 1.68, 95% CI: 1.07–2.64, $P = 0.025$), and the presence of bushes around the house (AOR = 1.76; 95% CI: 1.12–2.77, $P = 0.014$). Other analyzed factors, including educational level, seasonal sleeping habits, and proximity to termite hills, were not statistically significant.

Conclusion The study identifies key behavioral and environmental risk factors for *Leishmania* infection among refugees, emphasizing targeted vector control and community education. Urgent interventions are needed to reduce

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transmission, protect vulnerable populations, and improve health outcomes in refugee camps and local villages in Gambella and Benishangul Gumuz regions of Ethiopia.

Clinical trial Not applicable.

Keywords Behavioral factors, Environmental factors, Epidemiology, Infectious diseases, Risk factors, Visceral leishmaniasis, Ethiopia

Background

Leishmaniasis represents a complex of diseases caused by protozoan parasites of the genus *Leishmania* and is spread through the bites of infected female sandflies [1, 2]. The disease is considered as one of the neglected tropical diseases (NTDs) that significantly affects health in poor, remote regions with limited access to healthcare [3]. The clinical presentation is classified as visceral and cutaneous leishmaniasis, depending on the manifestation of the disease [4]. Visceral leishmaniasis (VL), also known as Kala-azar, is of higher priority than cutaneous leishmaniasis as it is a fatal disease in the absence of treatment [2]. VL is endemic in 79 countries, with a global estimated cases of 50,000 to 90,000, 20,000 to 30,000 deaths per year, and 1 billion people at risk [5]. In East Africa, VL is mainly caused by *Leishmania donovani* species, a major public health challenge, with some of the highest case rates in the world, often resulting in outbreaks [6–9].

Several factors including environmental changes and population migration between affected and unaffected regions are thought to influence the spread of *Leishmania* infection. Additionally, the presence of drug-resistant parasite strains and weakened immunity often due to malnutrition or HIV co-infection play a significant role [6, 10]. Among these factors, population migration is particularly significant given the global scale of displacement reported by the United Nations High Commissioner for Refugees (UNHCR), that there are more than 122 million displaced populations; of these 37.4 million people are refugees across the globe in 2023 [11]. Refugees, due to their frequent movement, inadequate living conditions, and limited access to healthcare, are highly vulnerable to infectious diseases. Visceral leishmaniasis has occasionally been reported in refugee camps [7, 12]. There is also a report that showed a strong relationship between civil unrest and VL [13].

In this regard, the government of Ethiopia opens its borders for refugees, the majority originating from South Sudan, Somalia, Eritrea, and Sudan. Ethiopia hosts a large number of refugees, many of whom are located in camps in the Gambella and Benishangul Gumuz regions, situated in the western part of Ethiopia. A large number of refugees in these camps came from areas where VL is endemic. Moreover, Gambella and Benishangul Gumuz regions have been identified as at high risk of VL based on geographical and climatological features [14, 15]. Consequently, refugees could be at high risk of contracting and transmitting *Leishmania* parasites to the indigenous community and vice versa [14]. Studies conducted in East Africa have identified various exposure factors associated with VL infection across different study locations and population groups. For instance, sleeping under an acacia tree at night [15, 16], the presence of cracked walls, and living in large households have been highlighted as risk factors [15, 17, 18]. Similarly, findings from Kafta-Humera, a region in northern Ethiopia, indicate that among migrant agricultural workers, a lower level of education significantly increases the risk of infection [15]. On the other hand, the use of bed nets has been observed to have a protective effect for VL infection [15]. Broadly speaking, social and political influences, such as military activities and agricultural development projects, are major contributors to VL endemicity and outbreaks in both Sudan and Ethiopia [13]. Despite few reports showing the exposure factors of *Leishmania* infections in these areas, localized and current evidence on specific risk factors such as environmental exposures, behavioral practices, and demographic factors remain scarce in refugee populations. Without a clear understanding of these factors, interventions remain generalized and may fail to reduce the infection rates effectively. Generating evidence to address this knowledge gap is important for designing effective *Leishmania* infection prevention strategies in refugee camps.

This may also help policymakers, health authorities, and humanitarian organizations implement targeted interventions. Beyond immediate public health benefits, the study also contributes to the broader understanding of infectious disease epidemiology in crisis settings, offering a framework for similar displacement-affected regions. Thus, this study aimed to assess the risk factor of *Leishmania* infection among selected refugee camps in Gambella and Benishangul Gumuz regions using a matched case-control study design.

Methods

Study setting

This study was conducted in four purposely selected refugee camps (with opened date) as described by Belay et al., 2025 (publication under review): Kule (February 2014) and Terkidi (June 2014) in Gambella, and Tsore (January 2015) and Sherkole (January 2005) in the Benishangul

Gumuz regional states of Ethiopia. These camps were chosen among a total of seven camps in Gambella and three in Benishangul Gumuz regional states. The refugee camps host large populations of South Sudanese and Sudanese refugees and are located bordering the countries of South Sudan and Sudan [19]. In addition, these refugee camps are located in Itang and Homosha districts of Gambella and Benishangul Gumuz regional states, respectively. Itang, west of the Gambella region, hosts the Terkidi and Kule camps, sheltering 125,396 refugees. Similarly, Homosha, located near Assosa, the capital city of the Benishangul Gumuz Gumuz region, is home to the Tsore and Sherkole camps, which hold a total of 58,681 refugees [19] (Fig. 1). The regions are characterized by hot, humid climates, proximity to rivers and forests. Healthcare services are provided by NGOs and the Ethiopian Ministry of Health, but diagnosis and treatment remain inadequate, leading to underreporting and delayed interventions. Moreover, these camps have ecological conditions conducive to sandfly survival, increasing the risk of VL transmission [20, 21].

Study design and population

A matched case-control study design was derived from cross-sectional data that was collected from May to August 2023. The cross-sectional study provided a snapshot of exposures and outcomes during data collection. Cases were matched to controls based on predefined criteria (age, sex, and region of camp location) to control for confounding variables. The data collection occurred within a defined timeframe, ensuring consistency in exposure assessment and minimizing recall bias. The study captured exposure and outcome information simultaneously, allowing for an efficient analysis of associations while maintaining the advantages of a case-control approach.

The source population included all individuals living in the refugee camps in Gambella and Benishangul Gumuz regional states. The study participants were refugees (five years of age and older) living in the selected camps. Individuals living in the camp for at least six months and willing to provide assent and/or written informed consent were included in the study. Moreover, the study

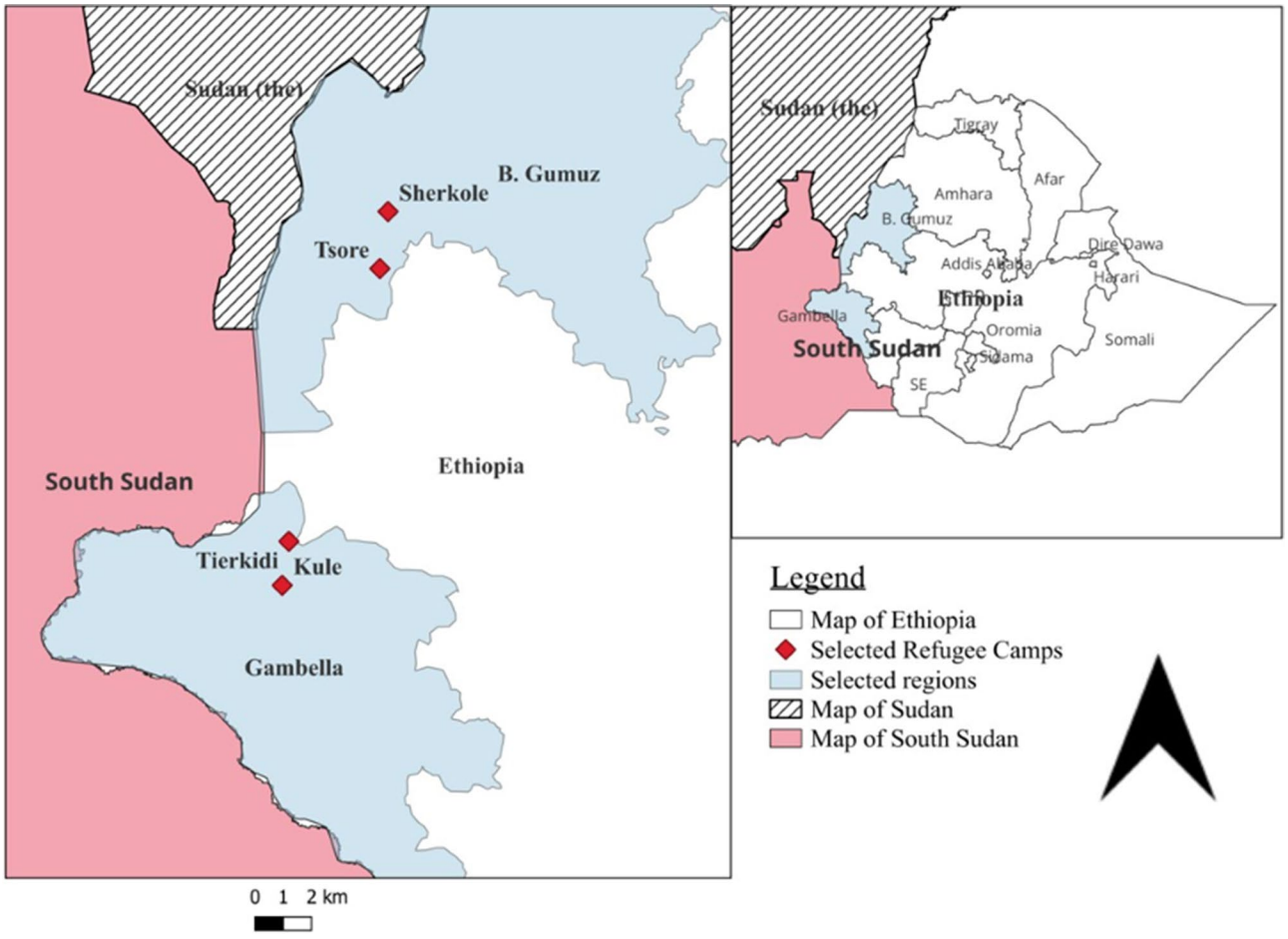


Fig. 1 Geographical location of selected refugee camps in Benishangul-Gumuz and Gambella regions, Ethiopia, and bordering South Sudan and Sudan, 2023

population consisted of cases and matched controls selected from the source population based on predefined eligibility criteria. This matching process helped reduce confounding and enhanced the validity of comparisons. Individuals who were unable to speak, listen, or assent and/or provide written informed consent and who declined to participate or provide blood samples were excluded from the study.

Cases were defined as participants testing positive for *Leishmania* by either DAT and/or rtPCR. Whereas controls were defined as individuals who tested negative for *Leishmania* by both the DAT and rtPCR methods. The DAT was carried out as previously described, with titers ≥ 1: 1600 considered to be positive [22, 23]. The overall test performance of sensitivity of the DAT was 95.3% for the diagnosis of VL in Ethiopia [24]. The KDNA-based PCR was conducted as described elsewhere [25]. The diagnostic sensitivity of rtPCR for the diagnosis of VL using peripheral blood as specimens was measured at 98.08% and 100.00% specificity [26].

Sample size determination

The sample size was calculated using the freely available statistical tool "(https://www.openepi.com/SampleSize/SSCC.htm)" based on the proportion of exposure and the odds of availability of bushes around the house. In the previous study conducted in Gedaref State, Sudan [16], it was revealed that the presence of bushes, including some acacia trees, was found to be a risk factor for VL. So that we used the following parameters to estimate the minimum sample size for the current study: 43.5% of the controls reported bushes around the house, odds ratio of 2.2, with 80% power and 95% CI, and 1:3 cases to control ratio, then the sample size was estimated to be 76 cases and 226 controls. Adding 10% non-response rate, the final sample size was 84 cases and 252 controls, with a minimum total sample size of 336.

Selection of cases and controls

Cases were recruited from the previously conducted cross-sectional prevalence study. All participants tested positive using direct agglutination test (DAT) with titer 1:1600 concentration [22, 23] and/or rtPCR were eligible for recruitment for the study. The cases were distributed in the four refugee camps: Kule, Terkidi, Tsore, and Sherkole. Controls were selected from the same dataset, considering a distribution of age groups, sex, and region of camp location with match tolerance factors of 2, 1, and 1, respectively. This means that cases and controls could differ by up to two years in age, but had to be identical in sex and camp location. Controls were also selected based on the negative test to DAT (agglutination < 1:1600) and rtPCR. Case-control matching was performed using in SPSS, with three controls selected percase according to predefined matching criteria.

Study variables

Dependent variable: *Leishmania* infection in the refugee camps.

Independent variables: stratified as socio-demographic, behavioral, environmental, and housing condition factors (Fig. 2).

Data collection

A structured questionnaire (Supp_File), based on existing forms [15, 18, 22, 23, 27, 28], was designed and utilized

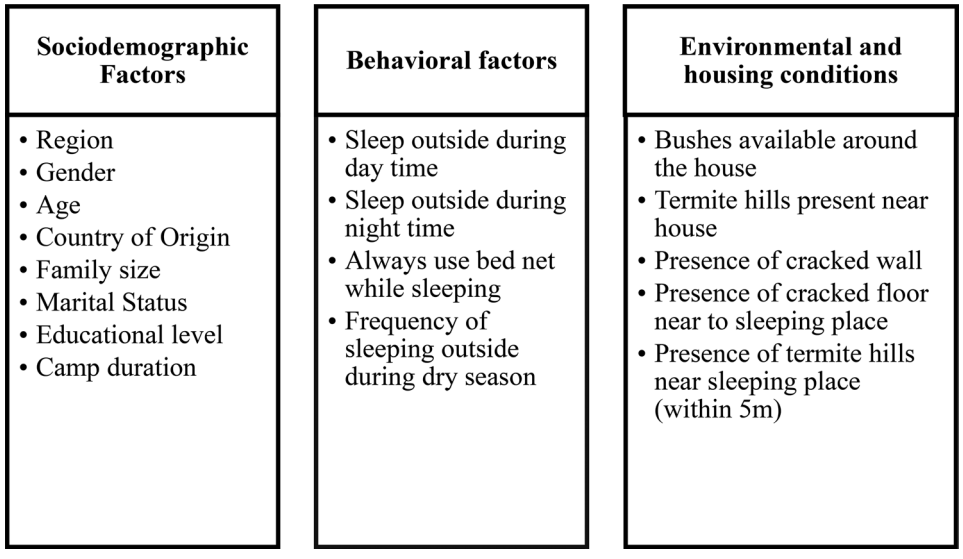


Fig. 2 Description of independent variables: stratified as socio-demographic, behavioral, environmental and housing condition factors in matched case-control study in refugee camps in Gambella and Benishangul-Gumuz regional states, Ethiopia, 2023

to collect demographics and potential risk factors, such as socio-demography, environmental and behavioral factors from both *Leishmania* infected individuals and healthy controls in the selected refugee camps. Then, blood samples were collected and tested for *Leishmania* antibody and kDNA presence to categorize study participants as cases or controls. Cases were selected based on operational definitions as stated above from the list of participants. After identification of the cases, controls who lived in the same location (region), age group (5–14, 15–39, and ≥ 40 years), and sex comparable to cases were included.

Data processing and analysis

Data was cleaned for completeness and consistency, coded and entered into EpiInfo version 7.0. and exported into Microsoft office excel then to Statistical Package for the Social Sciences (SPSS) version 24 for further analysis. The results were organized, summarized, and presented using texts, tables, and graphs. Descriptive statistics were used to summarize characteristics of cases and controls. Chi-square tests were used to compare the percentages of cases and controls in terms of selected categorical variables. Bivariate conditional logistic regression was analyzed to examine the association between each independent variable and the outcome and to calculate unadjusted odds ratios with 95% confidence intervals. Independent variables that were statistically significant at $p < 0.20$ in the bivariate analysis were retained and included in the multivariable conditional logistic regression model. All the candidate variables were entered into a multivariable binary logistic regression model to identify the effects of independent variables on the outcome variable. The fitness of the model was checked by the Hosmer and Lemeshow goodness-of-fit test. Multi-collinearity effects among independent variables based on the assumption of the variance inflation factor (VIF) were checked. The level of significance was set at 0.05.

Results

Socio-demographic characteristics

A total of 336 participants, 252 controls and 84 cases, participated in all selected refugee camps. Among the cases, 12 tested positive by DAT only, 53 tested positive by rtPCR only, and 19 tested positive by both DAT and rtPCR. The majority of individuals in both groups were from refugee camps located in Benishangul Gumuz regional state (89.3% control, 89.3% cases). Females predominate in both groups (65.5% control, 65.5% cases). The average age of participants in both case and control groups was similar. Most participants reside in Tsore camp (78.6% control, 74.6% cases). In terms of level of education, a prominent portion in both groups had reported that grade one to eight (47.6% control, 53.6% cases). In marital status, married individuals were more common in both groups (64.3% control, 62.3% cases), followed by singles. On the other hand, the test of independence was conducted to measure the proportion distribution among variables of cases and control. As a result, the distribution of matching variables age group, sex, and region of camp location showed that they were similarly distributed ($p > 0.05$) (Table 1). The mean number of family members per household was found to be six individuals for both controls and cases. Camp duration was also reported to have a mean year of duration of 5.6 and 6.8 for the control and case groups, respectively (Supp_Table_1). See Supplementary Table 2 for the distribution of environmental and behavioral risk factors of *Leishmania* infection among participants.

The distribution of case and control participants in terms of country of origin (South Sudan vs. Sudan) and their respective states where the refugees come from is depicted in supplementary Table 3. In South Sudan, the majority of the participants were from Upper Nile (32.9% control, 45.2% cases), followed by Jonglei (11.5% control, 4.8% cases) and Lakes (0.8% control, 2.4% cases). In Sudan, Blue Nile dominated (30.2% control, 40.5% cases), Khartoum (4.8% control, 3.6% cases), and North Darfur (4.0% control, 1.2% cases) states (Supp_Table_3).

Determinants of *Leishmania* infection

The bivariate analysis identified significant associated factors between sociodemographic, environmental, and housing conditions and behavioral risk factors with *Leishmania* infected cases as indicated in Table 2. Sleeping outside during daytime (COR = 2.78; 95%CI = 1.71–4.51, $p < 0.001$) and nighttime (COR: 1.65; 95%CI: 1.07–2.56; $p = 0.024$), bush presence around the house (COR: 1.81; 95%CI: 1.17–2.81; $p = 0.008$), and cracked house walls (COR = 1.82; 95%CI: 1.15–2.90; $p = 0.011$) were associated with increased unadjusted odds of cases of *Leishmania* infection. In addition, among the sociodemographic factors, *Leishmania* infected participants who lived less than five years in the camp were 1.91 times more exposed compared to those who lived more than five years in the camp (COR: 1.91; 95%CI: 1.16–3.15; $p = 0.011$). On the other hand, bed net use showed a protective trend (COR = 0.59, $p = 0.096$), termite hills near to sleeping place (COR = 1.60, $p = 0.075$), and always sleeping outside during the dry season (COR = 1.56, $p = 0.093$) had a higher risk of being infected with *Leishmania*, but the association was not statistically significant. Furthermore, factors including presence of cracked floors, and termite hills near the house were not statistically significantly associated with cases of *Leishmania* infection among study participants (Table 2).

The adjusted analysis examined various factors associated with the cases of *Leishmania* infection. Variables

Table 1 Socio demographic characteristics of participants in matched case-control study in refugee camps in Gambella and Benishangul Gumuz regional States of Ethiopia, 2023

Variables	Description	Case (%)	Control (%)	Total (%)	*P* value
Region	Benishangul Gumuz	75 (89.3)	225 (89.3)	300 (98.3)	1.000
	Gambella	9 (10.7)	27 (10.7)	36 (10.7)	
Gender	Male	29 (34.5)	87 (34.5)	116 (34.5)	1.000
	Female	55 (65.5)	165 (65.5)	220 (65.5)	
Camp Name	Kule	6 (7.1)	21 (8.3)	27 (8.0)	0.729
	Terkidi	3 (3.6)	6 (2.4)	9 (2.7)	
	Tsore	66 (78.6)	188 (74.6)	254 (75.6)	
	Sherkole	9 (10.7)	37 (14.7)	46 (13.7)	
Age Group in years	5–14	18 (21.4)	53 (21.0)	71 (21.1)	0.807
	15–39	56 (66.7)	175 (69.4)	231 (68.8)	
	≥ 40	10 (11.9)	24 (9.5)	34 (10.1)	
Country of Origin	South Sudan	44 (52.4)	116 (46.0)	160 (47.6)	0.313
	Sudan	40 (47.6)	136 (54.0)	176 (52.4)	
Level of education	No formal education	32 (38.1)	77 (30.6)	109 (32.4)	0.163
	Grade 1–8	40 (47.6)	135 (53.6)	175 (52.1)	
	Grade 9–12	11 (13.1)	25 (9.9)	36 (10.7)	
	College/university	1 (1.2)	15 (6.0)	16 (4.8)	
Marital Status	Married	54 (64.3)	157 (62.3)	211 (62.8)	0.745
	Single	30 (35.7)	95 (37.7)	125 (37.2)	
Length of stay in the camp	≤ 5 years	60 (71.4)	136 (54.0)	196 (58.3)	0.005
	> 5 years	24 (28.6)	116 (46.0)	140 (41.7)	
Family Size (HH members)	≤ 4	57 (67.9)	151 (59.9)	208 (61.9)	0.195
	> 4	27 (32.1)	101 (40.1)	128 (38.1)	

with < 0.20 p values in the bivariate analysis were entered into the multivariate model and analyzed using the backward step-wise conditional logistic regression method. Duration in the camp lower than five years were significantly associated with cases of *Leishmania* infection (AOR = 1.76, 95% CI: 1.06–2.93, $P = 0.028$). Sleeping outside during the daytime (AOR = 2.38, 95% CI: 1.46–3.89, $P < 0.001$) and nighttime (AOR = 1.68, 95% CI: 1.07–2.64, $P = 0.025$) increased odds, and bushes available around the house was found to be an exposure factor to increase the odds of a case infected with *Leishmania* (AOR = 1.76; 95% CI: 1.12–2.77, $p = 0.014$). On the other hand, other variables we analyzed in the conditional logistic regression model were not statistically significant: level of education, frequency of sleeping outside during dry season, either always or sometimes respondents, and presence of termite hills near sleeping place < 5 m (Table 3).

Discussion

The current study assessed the behavioral, environmental, and socio-demographic factors associated with cases of *Leishmania* infection in refugee camp settings in Gambella and Benishangul Gumuz regions of Ethiopia. This study provided evidence to guide *Leishmania* control in refugee camps, where populations are vulnerable due to overcrowding, limited resources, and environmental degradation. By integrating behavioral, environmental, and socio-demographic strategies into camp management protocols, policymakers and humanitarian actors can mitigate transmission risks and improve health equity in these high-risk settings. Sustained investment in prevention, community engagement, and context-specific research will be critical to achieving long-term impact.

The finding that sleeping outside during both daytime and nighttime increases the risk of *Leishmania* infection highlights a critical behavioral practice risk factor in refugee camp settings. This was supported by previous reports from other parts of Ethiopia showing that sleeping outside the house at night was significantly associated with VL [15, 18, 29]. When sleeping outside during the daytime, certain sandfly species (*Phlebotomus* spp.) may bite outdoors during the daytime, especially in hot climates or densely vegetated environments [30]. A case-control study conducted in West Pokot County, Kenya, supported the current report that VL transmission occurs both peri-domestically at night and outdoors during the day, particularly when sandfly resting sites are disturbed [31]. This finding emphasizes daytime outdoor sleeping as a major modifiable risk factor for *Leishmania* infection in the camp settings. On the other hand, the strong association among sleeping outdoors, daytime and cases of *Leishmania* infection suggest that behavioral interventions to reduce daytime outdoor rest could significantly lower transmission risk. Future studies should validate these results with longitudinal designs and explore interactions between sandfly activity patterns,

Table 2 The bivariate association between sociodemographic, environmental, and housing conditions and behavioral risk factors with *Leishmania* infected cases: a case-control study conducted in Gambella and Benishangul Gumuz regional states of Ethiopia, 2023

Variables	Description	Case (%)	Control (%)	COR	*P* value
Sociodemographic factors					
Camp Name	Kule	6 (7.1)	21 (8.3)	0.62 (0.14–2.85)	0.541
	Tsore	66 (78.6)	188 (74.6)	1.37 (0.66–2.83)	0.400
	Sherkole	9 (10.7)	37 (14.7)	Ref	
Country of Origin	South Sudan	44 (52.4)	116 (46.0)	1.26 (0.79–2.01	0.336
	Sudan	40 (47.6)	136 (54.0)	Ref	
Level of education	No formal education	32 (38.1)	77 (30.6)	5.12 (0.68–38.65)	0.113
	Grade 1–8	40 (47.6)	135 (53.6)	3.90 (0.52–29.12)	0.184
	Grade 9–12	11 (13.1)	25 (9.9)	5.22 (0.66–41.22)	0.117
	College/university	1 (1.2)	15 (6.0)	Ref	
Marital Status	Married	54 (64.3)	157 (62.3)	1.09 (0.65–1.85)	0.741
	Single	30 (35.7)	95 (37.7)	Ref	
Length of stay in the camp	≤ 5 years	60 (71.4)	136 (54.0)	1.91 (1.16–3.15)*	0.011
	> 5 years	24 (28.6)	116 (46.0)	Ref	
Family Size (HH members)	≤ 4	57 (67.9)	151 (59.9)	Ref	
	> 4	27 (32.1)	101 (40.1)	1.31 (0.82–2.10)	0.252
Behavioral practice factors					
Sleep during day time	Outside	26 (31.0)	24 (9.5)	2.78 (1.71–4.51)**	< 0.001
	Inside	58 (69.0)	228 (90.5)	Ref	
sleep during night time	Outside	38 (45.2)	75 (29.8)	1.65 (1.07–2.56)*	0.024
	Inside	46 (54.8)	177 (70.2)	Ref	
Always use bed net while sleeping	Yes	13 (15.5)	64 (25.4)	0.59 (0.32–1.10)	0.096
	No	71 (84.5)	188 (74.6)	Ref	
Frequency of sleeping outside during dry season	Always	39 (46.4)	65 (25.8)	1.56 (0.93–2.62)	0.093
	Sometimes	20 (23.8)	111 (44.0)	0.62 (0.34–1.12)	0.113
	Never	25 (29.8)	76 (30.2)	Ref	
Environmental and housing conditions					
Bushes available around the house	Yes	38 (45.2)	69 (27.4)	1.81 (1.17–2.81)*	0.008
	No	46 (54.8)	183 (72.6)	Ref	
Presence of cracked wall	Yes	28 (33.3)	46 (18.3)	1.82 (1.15–2.90)*	0.011
	No	56 (66.7)	206 (81.7)	Ref	
Presence of termite hills near house	Yes	31 (36.9)	70 (27.8)	1.38 (0.88–2.17)	0.164
	No	53 (63.1)	182 (72.2)	Ref	
Termite hills present near sleeping place < 5 m	Yes	20 (23.8)	36 (14.3)	1.60 (0.95–2.67)	0.075
	No	64 (76.2)	216 (85.7)	Ref	
Presence of cracked floor near to sleeping place	Yes	15 (17.9)	32 (12.7)	1.35 (0.77–2.39)	0.299
	No	69 (82.1)	220 (87.3)	Ref	

*<5 m: less than five-meter distance; COR: Crude Odds Ratio; 95% CI: 95% Confidence Interval; Ref: Reference variable; *p-values less than 0.05; **p-values less than 0.001

environmental conditions, and human behavior, particularly in humanitarian settings like refugee camps. Sleeping outside during the nighttime is strongly associated with *Leishmania* infection in refugee camps in Gambella and Benishangul Gumuz regions of Ethiopia. This was supported by studies conducted in Sudan [16] and Kenya [31]. While sandflies are most active during crepuscular hours (dawn/dusk), nocturnal species or adaptations may bite at night, particularly in areas with high human activity or poor vector control [32–34]. However, in this study, the term 'nighttime' includes dusk and dawn periods rather than being strictly defined by specific hours. This broader timeframe accounts for the possibility that *Leishmania* infected individuals may have been exposed to sandfly bites during these transitional periods. Cases who sleep outdoors at night may lack protection, increasing exposure to sandfly activity. Indoor sleeping environments typically offer structural barriers, including walls and roofs, and relatively better access to protective tools. Moreover, outdoor sleeping areas near vegetation or cracked floors could also serve as sandfly breeding sites.

Staying in the camp less than five years was significantly associated with cases of *Leishmania* infection. Longer stays may correlate with better adoption of protective

Table 3 Results from multivariable conditional logistic regression model of the adjusted odds ratios, together with 95% CI, for the risk of *Leishmania* infection in refugee camps in Gambella and Benishangul Gumuz regional states in Ethiopia, 2023

Description	Case (%)	Control (%)	AOR (95%CI)	*P* value
No formal education	32 (38.1)	77 (30.6)	4.30 (0.48–38.76)	0.190
Education level: Grade 1–8	40 (47.6)	135 (53.6)	2.59 (0.30-22.42)	0.390
Education level: Grade 9–12	11 (13.1)	25 (9.9)	4.06 (0.41–39.74)	0.230
Education level: College/university	1 (1.2)	15 (6.0)	1	
Sleep outside the house during day	26 (31.0)	24 (9.5)	2.38 (1.46–3.89)**	< 0.001
Sleep inside the house during day (Ref)	58 (69.0)	228 (90.5)	1	
Sleep outside the house during night	38 (45.2)	75 (29.8)	1.68 (1.07–2.64)*	0.025
Sleep inside the house during night (Ref)	46 (54.8)	177 (70.2)	1	
Bushes available around the house	38 (45.2)	69 (27.4)	1.76 (1.12–2.77)*	0.014
Bushes not available around the house (Ref)	46 (54.8)	183 (72.6)	1	
Always sleeping outside during dry season	39 (46.4)	65 (25.8)	1.19 (0.69–2.04)	0.532
Sometimes sleeping outside during dry season	20 (23.8)	111 (44.0)	0.62 (0.34–1.14)	0.125
Never sleeping outside during dry season	25 (29.8)	76 (30.2)	1	
Termite hills present near to house	31 (36.9)	70 (27.8)	1.26 (0.68–2.35)	0.468
Termite hills not present near to house (Ref)	53 (63.1)	182 (72.2)	1	
Length of stay in the camp ≤ 5	60 (71.4)	136 (54.0)	1.76 (1.06–2.93)*	0.028
Length of stay in the camp > 5 (Ref)	24 (28.6)	116 (46.0)	1	

AOR: Adjusted odds ratio; 95%CI: 95% confidence interval; *p-values less than 0.05; **p-values less than 0.001

measures or improved housing conditions. The practice and awareness of protective measures among cases and controls were not assessed in the current study; however, new residents might lack awareness of local risks or engage in high-exposure activities such as outdoor activities at dawn/dusk. Additionally, refugees may have acquired infection due to prior exposure in their areas of origin, such as Sudan or South Sudan, where visceral leishmaniasis is also endemic [35]. Alternatively, this association may reflect new infections occurring shortly after arrival among individuals who had not previously been exposed and thus lacked immunity, regardless of protective measures.

This finding is not supported by a study conducted in northern Ethiopia among farmland workers. The longer stay in the VL endemic area is a risk factor compared to the staying in shorter period of time, less than four years of stay in Humera [36]. This difference could be the study area well known to be VL endemic in the northern part of Ethiopia and contribute almost 60% of VL load in the country [21].

In addition, the availability of bushes around the house was also found to be an exposure factor to increase the odds of a case infected with *Leishmania* infection in the current study. This finding was in line with reports from East African region, including Kenya, Sudan, Somalia, and Ethiopia [16, 31, 37]. Bushes near houses may increase human-sandfly contact, particularly during crepuscular/nocturnal biting periods. Vegetation may act as a bridge between natural sandfly habitats (such as forests) and human settlements. Other systematic reviews and meta-analyses revealed that living with domestic animals and being male were identified as associated with *Leishmania* infection in different regions of Ethiopia [38]. These exposure factors were not assessed in the current study because the target population's lifestyle differs. In this study, we focused on refugees with no gender-specific activities and without domestic animals.

For those who always use a bed net while sleeping, there was a decreased risk of *Leishmania* infection in the studied refugee camps in Gambella and Benishangul Gumuz regional states. This report is supported by a case control study conducted in Sudan and Kenya [16, 31]. On the other hand, this was in contrast with previous studies in Ethiopia [15, 18], which found that using a bed net was protective for VL infection. This difference may be due to the fact that refugee camps often have temporary shelters with gaps in walls/roofs, allowing sandfly entry even when nets are used. Contrast this with studies in solid housing structures where nets complement other barriers.

Our analysis revealed no statistically significant association between *Leishmania* infection and exposure factors such as education level, always or sometimes outdoor sleeping during the dry season, and the presence of termite hills within 5 m of sleeping areas. On the other hand, educational level significantly associated with *Leishmania* infection in studies conducted in Gedaref Sudan [16] and Kafta-Humera, Ethiopia [15]. In the current study, lack of association with education may reflect limited socioeconomic variation in the refugee camp population or the dominance of environmental over behavioral risk factors in this setting. Moreover, education might correlate with protective behaviors (bed net use) that were already adjusted for in the model. There was minimal proportion variability in the education

levels among the individuals included in the study. We assessed the seasonal variation in outdoor sleeping practices during the dry season. Since sandflies in the area are likely active during crepuscular hours (dawn and dusk), individuals may spend these peak biting times indoors, potentially reducing the relevance of their sleeping location. The null finding for termite hills, and always sleeping outside during dry season also supported by findings from Sudan [16] but not study conducted in Ethiopia [15]. The termite hills often cited as sandfly breeding sites, could suggest that proximity alone does not amplify risk in this context, possibly due to widespread environmental sandfly presence overwhelming localized effects, or termite mounds serving as poor proxies for actual vector habitats. Moreover, these discrepancies may be explained by differences in population characteristics as well as environmental factors, such as vegetation cover and the type and abundance of termite hills, which in the present study area are predominantly eroded. A comparative analysis across refugee camps that have not yet been studied would help to clarify variations in sand fly distribution. Such studies could also provide valuable insights into the habitat preferences and biting behaviors of the vector in these settings, which are important considerations for future research.

In the current study, the presence of cracked walls did not remain an independent predictor in the multivariable model. This was largely because its effect was adjusted for the habit of always sleeping outside during the dry season, which appears to be a stronger behavioral risk factor influencing exposure to sand flies. Previous studies have reported mixed findings regarding cracked walls: some have suggested that they may serve as potential resting or breeding sites for sand flies, thereby increasing transmission risk [18], while others have not found a significant association [16]. Our findings are therefore consistent with the latter, suggesting that structural housing conditions alone may not sufficiently explain transmission dynamics in this setting, and that behavioral and environmental factors, such as outdoor sleeping, may play a more dominant role.

Key factors such as proximity to vector breeding sites (availability of bushes around the house), limited access to protective measures (e.g., bed nets), and behavioral practices (day and nighttime outdoor sleeping) emerged as strong predictors of cases of *Leishmania* infection. These findings highlight targets for control strategies, including enhanced vector control, community education on risk-reducing behaviors, and distribution of protective materials. While unmeasured variables like housing construction materials or nutritional status may contribute to risk in broader contexts, the identified factors remain pivotal for designing context-specific, feasible interventions in resource-constrained environments like refugee camps, where addressing modifiable exposures can yield immediate public health benefits.

Strengths and limitations of the study

The current study has several strengths when investigating risk factors for *Leishmania* infection in refugee camps, particularly when cases and controls were recruited prospectively, this would help to minimize recall and selection biases. Moreover, matching cases and controls on variables of age group, sex, and region of location of the camps helps to control confounding factors, improving the validity of comparisons. Several potential predictors, such as occupation, income, domestic animal ownership, VL/HIV co-infection and nutritional status, were not included in the analysis. This omission stemmed from the homogeneity of living conditions within the refugee population, which limited variability in these factors. Additionally, variables like nutritional status and domestic animal ownership are widely recognized as systemic challenges in refugee settings, where access to resources is often uniformly constrained. Nevertheless, the limitations outlined in our analysis identified robust and statistically significant associations between exposure variables and *Leishmania* infection risk, underscoring their critical role in informing targeted interventions in refugee camp settings.

Conclusion and recommendation

In conclusion, this study identified critical behavioral and environmental factors associated with *Leishmania* infection risk in refugee camp settings. Sleeping outdoors during both daytime and nighttime significantly increased *Leishmania* infection. The presence of bushes near households and the shorter duration of residence in the camp (less than five years) were associated with *Leishmania* infection in refugee camps. This indicates that recent arrivals may not have developed adaptive behaviors or acquired immunity over time. These findings underscore the urgency of prioritizing vector control measures, such as clearing vegetation near shelters, distributing insecticide-treated nets, and promoting community awareness about minimizing outdoor sleeping behaviors. Targeted interventions for newer residents, coupled with environmental management, could considerably reduce transmission and mitigate the burden of leishmaniasis in similar resource-constrained, high-risk settings.

Abbreviations

DAT	Direct agglutination test
HH	Household
NTD	Neglected tropical diseases
PCR	Polymerase chain reaction
RRS	Refugees and returnees service
UNHCR	United nations high commissioner for refugees
VL	Visceral leishmaniasis
WHO	World Health Organization

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions

HB, EA, AA, BA and BE designed the proposal. HB, BA, MB, and GT acquired and analyzed the data, and drafted the manuscript. All authors discussed the results and implications, contributed to the work, commented on the manuscript throughout, and approved the final version.

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Data availability

Data is provided within the manuscript or supplementary information files.

Declarations

Ethical approval and consent to participate

The study was conducted in accordance with the national ethical guidelines and received approval from the Aklilu Lemma Institute of Health Research-Institutional Research Ethics Review Committee (ALIPB-IRERC) prior to data collection (Ref. No.: ALIPB IRERC/112/2015/23). A permission letter was obtained from the Federal Democratic Republic of Ethiopia Refugees and Returnees Service (RRS) and subsequently from the RRS regional office and camp coordination offices. Written informed consent or assent was secured from all adult participants, as well as from parents or guardians for minors and illiterate participants, with trained personnel assisting in participants' native languages (including Nuer, Arabic and Dinka) to ensure comprehension. Privacy and confidentiality were strictly maintained, with all identifying data anonymized and securely stored, accessible only to the research team. The study followed all relevant guidelines for research on human subjects, implementing additional safeguards to protect participants' rights, dignity, and welfare.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Entomological survey of sand fly vectors and molecular screening for *Leishmania* parasite in refugee camps in Ethiopia

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Visceral leishmaniasis is transmitted by infected female sand flies. However, entomological data from refugee camps remained limited. This study aimed to describe sand fly fauna and assess natural *Leishmania* infection in selected refugee camps in Ethiopia. The study was conducted in four refugee camps. Sand flies were collected using a CDC light trap and sticky trap. Both male and female sand flies were dissected, and morphologically identified. The female sand flies were preserved in 95% ethyl alcohol for *Leishmania* DNA screening. A total of 2196 sand flies were collected. *Phlebotomus rodhaini*, the only *Phlebotomus* species identified, accounted for 1.7% of the collections. *Sergentomyia antennatus* was the most abundant species (50.8%), followed by *S. africanus* (15.8%), *S. schwetzi* (14.3%), *S. bedfordi* (10.9%), *S. clydei* (5.2%), *S. squamipleuris* (1.2%), and *S. adleri* (0.1%). Species richness was highest in Terkidi camp, whereas overall diversity was greatest in Sherkole. Indoor collections were limited (< 2.3%), confirming predominantly exophilic resting behavior. Termite hills and peridomestic habitats harbored the highest sand fly densities. Polymerase chain reaction screening showed no evidence of *Leishmania* infection. This first entomological survey conducted in refugee camps in Ethiopia documents a sand fly fauna dominated by outdoor collections, a very low abundance of *Ph. rodhaini*, and no detectable *Leishmania* infection. These findings suggest the need for longitudinal monitoring to capture seasonal variation.

Keywords Ethiopia, Phlebotomus rodhaini, Refugee camps, *Sergentomyia* spp., Vector surveillance, Visceral leishmaniasis

Abbreviations

CDC LT	Centers for Diseases control and prevention light trap
CPD	Compounds that contains households
DNA	Deoxyribonucleic acid
MoH	Ministry of health of Ethiopia
NH	Natural habitat
PCR	Polymerase chain reaction
ST	Sticky trap
TH	Termite hill
VL	Visceral Leishmaniasis

Visceral leishmaniasis (VL), also known as kala-azar, is a neglected tropical disease caused by protozoan parasites of the genus *Leishmania*. These parasites are transmitted through the bites of infected female *Phlebotomus* sand flies[1]. The disease is a significant cause of global morbidity and mortality, with East Africa bearing a substantial burden of cases[2]. Among the East African countries, Ethiopia is particularly affected, with six of its regions classified as endemic for VL[3]. The disease primarily impacts the lowland areas of the country. Annually, Ethiopia

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records an incidence of 3700–7400 VL cases[4]. Furthermore, an estimated 3.2 million people in Ethiopia live in areas at risk for contracting VL[3].

Sand flies of the genus *Phlebotomus* are confirmed vectors responsible for transmitting leishmaniasis in the Old World[6]. In Ethiopia, the principal vectors for VL are *Phlebotomus orientalis*, predominantly found in the lowland and highland regions of the northwest, and *Phlebotomus martini* and *Phlebotomus celiae*, prevalent in the southern and southwestern areas[3,7–10]. The distribution and abundance of these vectors are strongly influenced by ecological factors. *Phlebotomus orientalis* is closely associated with black cotton clay soils (vertisols) and *Acacia seyal* woodlands, while *Ph. martini and Ph. celiae* are typically linked to the presence of termite hills[3,11–13]. Conversely, sand fly species within the genus *Sergentomyia* are generally considered as non-vectors of VL[14]. However, the detection of *Leishmania* DNA in these species suggests the need for further investigation into their potential role in VL parasite transmission. Studies from Kenya[15], West Africa[14,16], and other regions[17,18] have reported the presence of *Leishmania* DNA in *Sergentomyia* spp., which often outnumber *Phlebotomus* populations. Similarly, research in Ethiopia has indicated that *Sergentomyia* is the predominant genus in VL endemic areas[11,19,20].

A strong association has been documented between civil unrest and the transmission of VL[21,22]. The VL infection often arises when immunologically naïve migrants are displaced into VL endemic areas or when *Leishmania* infected individuals move into new areas and establish additional foci of transmission. In East Africa, the situation is further intensified by limited access to timely diagnosis and effective treatment, HIV co-infection, food insecurity, and malnutrition[4,21] and presence of arid areas with acacias and termite mounds[23]. Additionally, a meta-analysis from northern Ethiopia indicated that the pooled prevalence of HIV infection among VL infected individuals was 24% (95%CI: 17–30%)[24]. Moreover, migration is also documented as a factor to increase the burden of VL[5].

Ethiopia ranks as the third largest refugee hosting country in Africa, sheltering over one million refugees and asylum seekers, mainly from South Sudan, Sudan, Somalia, and Eritrea. Nearly half of these refugees (48%) are hosted in the Gambella and Benishangul Gumuz Regional States[25]. Environmental risk assessments for VL transmission have identified these regions as high-risk areas[2]. Many refugees, particularly those from Sudan and South Sudan, originate from VL endemic regions[21]. Previous studies in these areas have revealed a high prevalence of asymptomatic *Leishmania* infections through serological and molecular methods[22,26,27], while confirmed VL cases continue to be reported annually to the Ethiopian Ministry of Health (MoH unpublished data). Despite these findings, systematic entomological surveillance in refugee settings within the Gambella and Benishangul Gumuz Regional States remains limited. This lack of comprehensive data hampers the development and implementation of evidence-based vector control measures, a critical element of the WHO's strategy to eliminate VL as a public health issue in East Africa. Ethiopia, together with other East African countries, has committed to achieving this goal by 2030[28].

To address this gap, we investigated the species composition, relative abundance, habitat preference, and natural infections of *Leishmania* DNA in sand flies from refugee camps in Gambella and Benishangul Gumuz Regional States, Ethiopia. This study provides essential evidence to guide targeted vector control strategies by enhancing the understanding of sand fly behavior and resting sites, strengthening surveillance systems, and supporting public health efforts aimed at reducing VL transmission among vulnerable populations. Additionally, these findings contribute to better epidemic prevention, more efficient resource allocation, and progress toward Ethiopia's and WHO's goals for VL elimination.

Results

Preliminary assessments prior to actual sand fly collection

During the preliminary assessment of sand flies, a total of 474 specimens were collected in Terkidi and Kule refugee camps. However, no sand flies collected in Tsore and Sherkole refugee camps. Morphological examination at the genus level revealed that all samples belonged to the genus *Sergentomyia*. However, species-level identification was not conducted at this time.

Sand fly species composition and relative abundance across sites

In the current study, a total of 2,196 adult phlebotomine sand flies (1,542 males and 654 females) belonging to eight species in two genera (*Phlebotomus* and *Sergentomyia*) were collected using CDC light traps (LTs) and sticky traps (STs). The genus *Phlebotomus* was represented by a single subgenus (*Anaphlebotomus)*, while the genus *Sergentomyia* was represented by four subgenera (*Grassomyia*, *Sergentomyia*, *Sintonius*, and *Parrotomyia*) (Table 1). One species of *Phlebotomus* and seven species of *Sergentomyia* were identified from the four refugee camps in Gambella and Benishangul Gumuz Regional States. The majority of the sand flies (85.1%, $n = 1{,}869$) were collected from refugee camps in Gambella; specifically, Kule (39.0%, $n = 857$) and Terkidi (46.1%, $n = 1012$). In contrast, 14.9% ($n = 327$) were collected from refugee camps in Benishangul Gumuz Regional State: Sherkole (14.1%, $n = 310$) and Tsore (0.8%, $n = 17$). Among the refugee camps, Terkidi accounted for the largest proportion of specimens (46.1%), followed by Kule (39.0%), Sherkole (14.1%), and Tsore (0.8%) (Table 1).

Sergentomyia antennatus was the most abundant species (50.8%, $n = 1116$) of the total collection, with dominance observed in Kule (60.9%) and Terkidi (54.5%) ($p < 0.001$). *Sergentomyia africanus* ranked second in abundance (15.7%, $n = 345$), with its highest distribution in Sherkole (39.4%) and Tsore (64.7%). *Sergentomyia schwetzi* (14.3%, $n = 315$) and *Sergentomyia bedfordi* (10.9%, $n = 239$) were also widely distributed, particularly in Terkidi and Sherkole. Less common species included *Sergentomyia clydei* (5.2%, $n = 115$), *Phlebotomus rodhaini* (1.7%, $n = 38$), and *Sergentomyia squamipleuris* (1.2%, $n = 27$), while *Sergentomyia adleri* was rare, represented by a single specimen (0.1%, $n = 1$). Species richness was greatest in Terkidi, where all eight species were recorded, whereas Tsore exhibited both low abundance and diversity (Table 1).

Species (subgenus)	Regional States				Total (%)
	Gambella ($n=1869$)		Benishangul Gumuz ($n=327$)		
	Kule (%)	Terkidi (%)	Sherkole (%)	Tsore (%)	
Ph. rodhaini (*Anaphlebotomus*)	0 (0.0)	26 (2.6)	11 (3.5)	1 (5.6)	38 (1.7)
S. adleri (*Sintonius*)	0(0.0)	1 (0.1)	0 (0.0)	0 (0.0)	1 (0.1)
S. africanus (*Parrotomyia*)	78 (9.1)	134 (13.2)	122 (39.4)	11 (64.7)	345 (15.7)
S. antennatus (*Sergentomyia*)	522 (60.9)	552 (54.5)	41 (13.2)	1 (5.6)	1116 (50.8)
S. bedfordi group (*Sergentomyia*)	105 (12.3)	84 (8.3)	48 (15.5)	2 (11.8)	239 (10.9)
S. clydei (*Sintonius*)	84 (9.8)	28 (2.8)	2 (0.6)	1 (5.6)	115 (5.2)
S. schwetzi (*Sergentomyia*)	59 (6.9)	169 (16.7)	86 (27.7)	1 (5.6)	315 (14.3)
S. squamipleuris (*Grassomyia*)	9 (1.1)	18 (1.8)	0 (0.0)	0 (0.0)	27 (1.2)
Grand Total	**857 (39.0)**	**1012 (46.1)**	**310 (14.1)**	**17 (0.8)**	**2196 (100.00)**

Table 1. Distribution of sand fly species by refugee camp in Gambella and Benishangul Gumuz regional States of Ethiopia, April-May 2025.

Species	Kule		Sherkole		Terkidi		Tsore		Total (%)	
	Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor	Indoor	Outdoor
Ph. rodhaini	0	0	0	11	1	25	1	0	2(5.3)	36(94.7)
S. adleri	0	0	0	0	0	1	0	0	0(0.0)	1(100.0)
S. africanus	8	70	0	122	4	130	1	10	13(3.8)	332(96.2)
S. antennatus	10	512	0	41	12	540	0	1	22(2.0)	1094(98.0)
S. bedfordi	5	100	0	48	2	82	0	2	7(2.9)	232(97.1)
S. clydei	1	83	0	2	0	28	0	1	1(0.9)	114(99.1)
S. schwetzi	2	57	0	86	1	168	0	1	3(1.0)	312(99.0)
S. squamipleuris	0	9	0	0	1	17	0	0	1(3.7)	26(96.3)
Total (%)	**26(3.0)**	**831(97.0)**	**0(0.0)**	**310(100.0)**	**21(2.1)**	**991(97.9)**	**2(11.8)**	**15(88.2)**	**49(2.2)**	**2147(97.8)**

Table 2. Indoor and outdoor abundance of sand fly species across the four study sites in Gambella and Benishangul Gumuz regional States, Ethiopia, April-May 2025.

Species composition relative to collection methods

The distribution of phlebotomine sand flies by collection method is presented in Supplementary File, Table 2. The majority of sand flies (96.0%, $n=2109$) were captured using ST ($X^2=22.1$; $p=0.016$), with males representing 70.9% and females 29.1%. Sticky traps, deployed in household compounds, indoor, natural habitats, and termite hill habitats, proved highly effective in capturing all reported sand fly species. Majority of the *Ph. rodhaini* species (94.7% 36/38) was obtained using ST collection method. In contrast, LTs contributed only 4.0% of the total sand flies collected, with nearly equal proportions of males (52.9%) and females (47.1%). These findings demonstrate the superior efficiency of STs for capturing sand flies in diverse habitats, while LTs provided a more balanced representation of sexes and still collected a wide range of species.

Indoor and outdoor collections of sand flies

Indoor collections across all sites were notably low, accounting for only 49 (2.2%) specimens out of 2,196 total specimens; however, this result was not statistically significant ($p=0.209$). Among the 38 *Ph. rodhaini* specimens, only 2 (5.3%) were collected from indoor habitats. *Sergentomyia africanus* 3.8% ($n=13$) and *S. antennatus* 2.0% ($n=22$) were among indoor collections, while other species, including *S. bedfordi*, *S. clydei*, *S. adleri*, and *S. schwetzi*, were rarely found in indoor habitats. This pattern highlights the predominantly outdoor behavior of these species in the study area (Table 2).

Sex ratio and abdominal status of sand flies

The overall male-to-female sex ratio of sand flies collected using LT was 1.1:1, whereas a ratio of 2.4:1 was recorded for sand flies captured using ST. Across all sites and collection methods, male sand flies significantly predominated over females ($p<0.001$). Male sand fly predominance was particularly pronounced in *Ph. rodhaini* (27/11), *S. antennatus* (888/228), and *S. bedfordi* (192/47) based on ST collections. In contrast, female-biased ratios were observed in *S. schwetzi*, *S. bedfordi*, and *S. squamipleuris*, while *S. clydei* showed a more balanced distribution at some sites (Table 3). Overall, STs captured significantly more females than LTs ($X^2=13.0$; $p<0.001$), highlighting their efficiency for sampling host-seeking females. Of the 654 female sand flies examined across all sites, 30 (4.6%) were blood-fed and 75 (11.5%) were gravid. In the current study *S. schwetzi*, and *S. clydei* showed notable proportion of blood feed and gravid female sand flies reported. Only one single gravid specimen of *Ph. rodhaini* was detected in Sherkole (Supplementary Table 1).

Species	Collection methods											
	LT				ST				Total			
	Kule	Sherkole	Terkidi	Tsore	Kule	Sherkole	Terkidi	Tsore	Kule	Sherkole	Terkidi	Tsore
	M/F	M/F	M/F	M/F	M/F	M/F	M/F	M/F	M/F	M/F	M/F	M/F
Ph. rodhaini	-	0/1	-	1/0	-	8/2	18/8	-	-	8/3	18/8	1/0
S. adleri	-	-	-	-	-	-	1/0	-	-	-	1/0	-
S. africanus	1/3	8/4	3/4	0/2	22/52	60/50	51/76	5/4	23/55	68/54	57/80	5/6
S. antennatus	11/0	2/0	8/12	-	415/96	30/9	421/111	1/0	426/96	32/9	429/123	1/0
S. bedfordi	2/0	1/4	1/1	-	97/6	37/6	53/29	1/1	99/6	38/10	54/30	1/1
S. clydei	-	0/1	2/0	-	42/42	0/1	14/12	0/1	42/42	0/2	16/12	0/1
S. schwetzi	1/0	2/0	2/6	-	47/11	61/23	97/64	0/1	48/11	63/23	99/70	0/1
S. squamipleuris	-	-	1/3	-	4/5	-	11/3	-	4/5	-	12/6	-
Grand Total	15/3	13/10	17/26	1/2	627/212	196/91	666/303	7/7	642/215	209/101	683/329	8/9

Table 3. Male to female ratio of sand flies among refugee camps by collection methods in Gambella and Benishangul Gumuz regional States, Ethiopia, April-May 2025.

Species	Compound	Indoor	Natural Habitat	TH_in village	TH_ Natural Habitat
Ph. rodhaini	0.03 ± 0.02	0.07 ± 0.06	0.28 ± 0.25	0.42 ± 0.20*	0.23 ± 0.08*
S. africanus	0.52 ± 0.16	0.22 ± 0.12	0.59 ± 0.34	3.50 ± 1.96*	2.84 ± 0.97*
S. antennatus	1.09 ± 0.16	0.29 ± 0.12	5.19 ± 0.34	4.00 ± 1.96*	11.59 ± 0.97*
S. bedfordi	0.25 ± 0.61	0.09 ± 0.25	1.28 ± 1.34*	1.21 ± 1.26*	2.25 ± 4.04*
S. clydei	0.10 ± 0.04)	0.01 ± 0.01	0.16 ± 0.12	0.13 ± 0.13	1.61 ± 0.93*
S. schwetzi	0.23 ± 0.81	0.03 ± 0.08	1.16 ± 2.69	0.33 ± 3.30*	2.93 ± 3.48*

Table 4. Mean density with standard error (± SE) of sand fly species per ST per night across different habitats in refugee camps located in Gambella and Benishangul Gumuz regions, Ethiopia, April-May 2025. *Indicated statistically significant ($p < 0.05$). TH natural Habitat= termite hill located in the natural habitat of the study site; TH_in village= termite hills located in the village of the study areas.

Refugee camp	H'	E	S
Kule	1.237	0.690	6
Sherkole	1.430	0.798	6
Terkidi	1.376	0.662	8
Tsore	1.200	0.670	6

Table 5. The Shannon-Wiener diversity index (H'), evenness (E) and richness (S) of the sand fly species from the study areas in Gambella and Benishangul Gumuz regional States, Ethiopia, April-May 2025.

Habitat preferences of the sand flies

Table 4 shows the mean number of sand flies collected for each trap, habitat, and night. *Ph. rodhaini*, *S. africanus*, *S. antennatus*, *S. bedfordi*, and *S. schwetzi* showed a strong association with habitat type according to the Kruskal-Wallis H test, indicating a preference for termite hills located in both village and natural habitat environments. However, *S. clydei* was associated with termite hills in natural habitats only. Additionally, *S. bedfordi* demonstrated a statistically significant preference for natural habitats, in addition to termite hills in both the village and natural habitats (Table 4).

Diversity of sand flies among refugee camps

For the sand fly populations in four refugee camps, the Shannon-Wiener diversity index (H'), evenness (E), and species richness (S) were calculated (Table 5). Sherkole had the highest Shannon diversity index (H'=1.430), while Tsore had the lowest (H'=1.200). Compared to Kule, Sherkole had a varied sand fly population ($p = 0.001$). Conversely, Kule had a greater diversity of sand fly species than Terkidi (H'=0.690 vs. 0.662). On the other hand, Tsore had extremely low total abundance ($n = 17$), which limits species richness and inflates the influence of small count changes on diversity metrics. The number of species reported in Terkidi (S = 8) was higher than in Kule (S = 6) ($p = 0.001$).

PCR screening of sand flies for the detection of *Leishmania* DNA

PCR screening was performed on the 11 individually tested female *Ph. rodhaini* to detect *Leishmania* DNA. In contrast, female *Sergentomyia* specimens were pooled by species for PCR-based *Leishmania* screening as follows: 25 pools of *S. africanus* ($n = 195$), 24 pools of *S. antennatus* ($n = 228$), 13 pools of *S. bedfordi* ($n = 47$), 10 pools of *S. clydei* ($n = 57$), 16 pools of *S. schwetzi* ($n = 105$), and 4 pools of *S. squamipleuris* ($n = 11$), with each pool comprising from 1 to 22 sand flies. None of the pools tested positive for *Leishmania*, and all individually analyzed *Ph. rodhaini* specimens were also negative for *Leishmania* kDNA-based molecular screening (Supplementary Fig. 1).

Discussion

This study provides an entomological assessment of sand fly vectors in refugee camps in the Gambella and Benishangul-Gumuz Regional States of Ethiopia, regions not known to be endemic but at high risk for VL[2,27]. The present findings reveal a diverse sand fly fauna dominated by species of the genus *Sergentomyia*, with clear ecological preferences for outdoor habitats, and importantly, no molecular evidence of *Leishmania* infection within the sampled sand fly populations.

A total of 2,196 adult phlebotomine sand flies (1,542 males and 654 females) from eight species in two genera (*Phlebotomus* and *Sergentomyia*) were collected using LT and ST collection methods. *Phlebotomus* was represented by one subgenus (*Anaphlebotomus*), while *Sergentomyia* included four subgenera: *Grassomyia*, *Sergentomyia*, *Sintonius*, and *Parrotomyia*. Most sand flies (85%) were collected from Gambella refugee camps (Kule: 357; Terkidi: 1,012), while 15% were from Benishangul Gumuz (Sherkole: 310; Tsore: 17). One *Phlebotomus* and seven *Sergentomyia* species were identified from four refugee camps in Gambella and Benishangul Gumuz Regional States. The current cross sectional entomological survey revealed that 98.3% of the collected sand flies belong to the genus *Sergentomyia* and only 1.7% to the genus *Phlebotomus*. The report indicated that *Sergentomyia* outnumbers other species, which is consistent with findings from endemic regions, including northern and southern Ethiopia[11,19,20].

The current study highlights the low abundance of *Ph. rodhaini* (1.73%), a finding that is consistent with previous reports from VL endemic regions of northern, southern, and eastern Ethiopia[9]. An entomological assessment conducted in the Gambella refugee camps and surrounding areas in 1996 by Hailu et al. (unpublished data); however, this species was not reported. The presence of this sand fly species in the present study may indicate a shift in vector distribution due to climate change or environmental variation since the 1990s. Furthermore, the primary VL vectors reported elsewhere in Ethiopia appear to be absent in these refugee camps. The current finding may reflect the limitation of the current cross sectional study design, as previous research documenting these vectors employed longitudinal and seasonal sampling methods[8,20,29,30]. *Ph. rodhaini* is also distributed across parts of East Africa, including Sudan and South Sudan[31–34]. To our knowledge, this entomological survey is the first to document its occurrence in refugee camps within the Gambella and Benishangul Gumuz Regional States. Although reported at low abundance, its presence is epidemiologically relevant, as *Ph. rodhaini* has historically been implicated as a potential vector for *L. donovani* in Sudan[34]. A review by Al-Salam et al. on VL during the South Sudan conflict and its regional implications further highlighted the possible involvement of this species in zoonotic VL (ZVL) transmission in East Africa[21]. While its role in VL transmission has often been underestimated due to its low density[7,35] and preferential feeding on rodents rather than humans, its detection nevertheless signals a potential transmission risk, particularly in settings where primary vectors are rare or absent[31,34]. Ecologically, *Ph. rodhaini* is primarily associated with sylvatic and forest-derived environments, favoring humid, shaded microhabitats with dense vegetation[34]. It is commonly reported in forested habitats, rock crevices, tree roots, termite mounds, and natural soil cracks, which provide favorable conditions for resting and breeding[8,33,34].

In the present study, seven *Sergentomyia* species were identified, confirming the wide distribution of this genus across Ethiopia, including VL endemic regions where *Phlebotomus* species are recognized as the primary vectors of transmission[6,19,20,30]. The striking finding was the overwhelming dominance of *S. antennatus*, which accounted for more than 50% of all collected specimens, especially in the Gambella camps of Kule and Terkidi. Although *Sergentomyia* species are generally considered zoophilic and of limited significance in VL transmission compared to primary vectors like *Ph. orientalis* and *Ph. martini*, their remarkable abundance needs to be further investigated. Large population size could increase human-vector contact rates, underscoring the need to investigate the potential role of specific *Sergentomyia* species in disease ecology.

The current study demonstrates a significant difference in sampling efficiency between trap types: ST captured significantly more sand flies than the LT. This variation may be partly attributable to the greater total sampling efforts (trap-nights) with STs. Furthermore, the habitats sampled by each trap type differed: LTs were deployed exclusively in indoor habitats and peridomestic (household compound) settings, whereas STs were also placed in outdoor habitats, including termite hills (both within villages and in natural habitats) and mixed forests.

Analysis of species diversity revealed a heterogeneous distribution across refugee camps. Sherkole camp exhibited the highest species diversity and evenness, likely driven by high sand fly densities in termite hills and a more equitable species distribution. Terkidi camp also showed high diversity, with all eight species present; however, the area was dominated by *S. antennatus*. In contrast, Tsore camp had exceptionally low abundance and diversity this could potentially reflecting suboptimal local ecological conditions for the sand fly spread.

A key entomological finding was exophilic (outdoor resting) behavior of sand flies in the study area. Less than 2.3% of all specimens were collected indoors, a pattern consistent with the reports from other VL endemic regions in Ethiopia, Sudan, and South Sudan[35–37]. This has major implications for vector control. While indoor residual spraying (IRS) remains a cornerstone of VL control, our data indicate that effective strategies must also

incorporate targeted outdoor interventions. Significantly, the high densities of *S. antennatus* and *S. africanus* associated with termite mounds, particularly those near natural habitats and within villages, identify these structures as key ecological niches. The current report is supported by a study conducted in Kenya that showed these species more commonly collected in termite hills[38]. Environmental management, such as the modification or destruction of termite mounds in peridomestic areas, could be a highly effective strategy to reduce sand fly breeding and resting sites.

The negative result from the most sensitive kDNA PCR assay, applied to both *Sergentomyia* and individual *Ph. rodhaini* specimens, was a significant and valuable finding. These findings suggest several non-exclusive possibilities in the context of the refugee camps' asymptomatic *Leishmania* infection distribution[22]. First, parasite circulation may have been at an extremely low, undetectable level during the sampling period, which may not have coincided with seasonal transmission peaks. Second, the zoonotic transmission cycle may involve animal reservoirs that are not effectively bitten by the sampled sand flies. Finally, known VL vectors might exist at such low densities that they were not captured in sufficient numbers to include an infected specimen. The absence of infection provides crucial baseline data, indicating a currently low transmission intensity within the local vector population, which is essential for monitoring future epidemiological trends. This finding contrasts with reports from other endemic regions where *Sergentomyia* and *Phlebotomus* spp. have tested positive for *Leishmania* DNA. For instance, in Iran, *S. dentata* (4/48, 8.33%) and *S. sintoni* (2/4, 50%) tested positive for *Leishmania major*[18], while in Portugal, *S. minuta* was reported to have tested positive for *Leishmania* DNA (2/1867)[17]. This discrepancy may be partly explained by the absence of these specific *Sergentomyia* species in our study area. Furthermore, studies have challenged the paradigm of exclusive *Phlebotomus* transmission, implicating *S. squamipleuris* in Kenya as a potential vector[15] and detecting *L. donovani* in *Ph. rodhaini* from Sudan[34]. The absence of *Leishmania* DNA in *Ph. rodhaini* in the current study could therefore be explained by several factors, including ecological differences between sites, such as variations in host feeding preferences, local transmission intensity between sites, or differences in sand fly microbiota. Additionally, the predominance of asymptomatic human infection in our study areas[22] may result in lower parasitemia, further reducing the probability of parasite detection in sand flies.

The strength of this study lies in its rigorous methodological design. The parallel use of LTs and STs across diverse habitats enabled a comprehensive assessment of sand fly ecology in previously under-surveilled refugee camps in Ethiopia. The application of highly sensitive PCR assays provides reliable data on *Leishmania* infection status within the vector populations. Furthermore, focusing on a vulnerable population and inclusion of ecological diversity indices generate crucial baseline data essential for informing targeted public health interventions against VL. On the other hand, the study also has several limitations that should be considered when interpreting these findings. First, while the study encompassed four refugee camps, its results may not be fully generalizable to all refugee camps in Ethiopia. Second, the cross sectional design provides only a temporal snapshot of sand fly ecology and infection, which may have missed seasonal variations in vector density and transmission peaks. Third, the specific laboratory analyses were not performed, limiting insights into host feeding preferences and potential reservoir dynamics. Additionally, the reliance on morphological identification alone, without complementary molecular confirmation, e.g., Cytochrome Oxidase I (COI) mitochondrial gene sequencing, may affect species-level certainty. To address these gaps and build upon this work, future studies should prioritize longitudinal sampling to capture seasonal dynamics. Laboratory analysis should include performing blood meal analysis to improve our understanding of host preferences and reservoir dynamics. Cytochrome Oxidase I (COI) mitochondrial gene sequencing should complement morphological species identification and resolve cryptic species complexes.

Conclusion

Our study showed a detailed picture of the sand fly fauna in southwestern and northwestern Ethiopian refugee camps for the first time. The vector community was characterized by high diversity, a strong predominance of outdoor-resting *Sergentomyia* species, and a notable absence of *Leishmania* DNA in the tested sand fly specimens at the time of the study. Integrated vector management has been implemented in these areas that includes environmental management, specifically targeting termite mounds in peridomestic areas. It is recommended to establish longitudinal entomological surveillance across different seasons to better capture temporal variations in sand fly density and *Leishmania* infection rates. Future studies should employ a larger sample size for *Phlebotomus* species and consider screening blood-fed females for host preferences to identify potential reservoir hosts involved in the transmission cycle. It is also desirable to investigate potential animal reservoirs in and around the camps to understand the complete transmission cycle.

Materials and methods

Study areas

The study was conducted in four refugee camps of Terkidi and Kule (Itang district, Gambella Regional State in southwestern Ethiopia) and Sherkole and Tsore (Homosha district, Benishangul-Gumuz Regional State in northwestern Ethiopia). These regions border South Sudan and Sudan. As of 31 May 2025, Gambella hosted 395,203 refugees, and Benishangul-Gumuz hosted 110,606 refugees[25]. These camps were randomly selected among a total of seven and five refugee camps in Gambella and Benishangul Gumuz Regional States, respectively[39,40] (Fig. 1).

Itang is a special district that lies in the low-lying floodplain area of Gambella, characterized by a flat to gently undulating landscape. The district is bordered to the south and southeast by the Anuak Zone, to the west by the Nuer Zone, to the north by the Oromia region, and to the northwest by South Sudan, with the Alwero River demarcating part of its southern boundary. The altitude of this district ranges from 350 to 480 m above sea level[41].

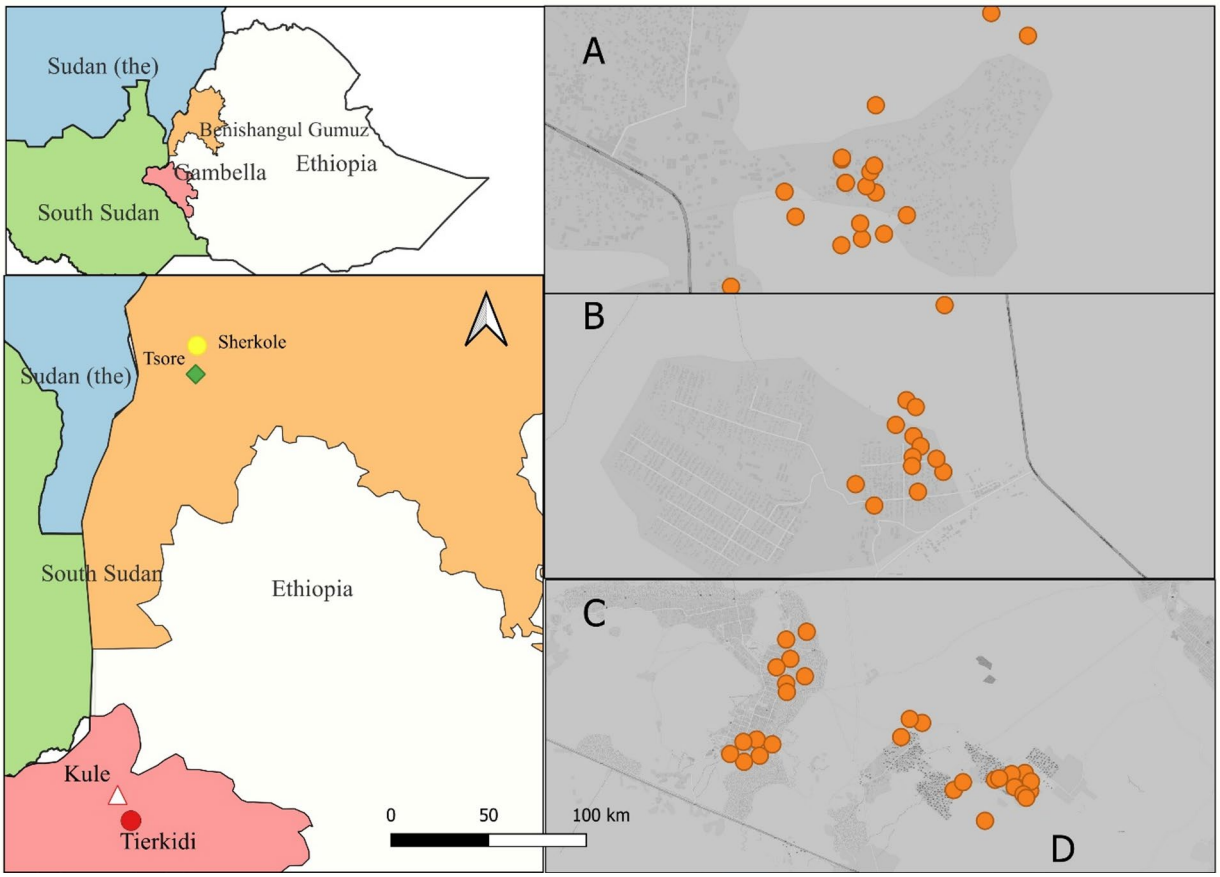


Fig. 1. Map showing sand fly collection sites in refugee camps in Gambella and Benishangul Gumuz regions, Ethiopia [Kule Camp: represented by a white triangle; Terkidi Camp: represented by a red circle; Sherkole Camp: represented by a yellow circle; Tsore Camp: represented by a green diamond]. The sand fly collection sites are represented as orange dots where; **A**: Sherkole, **B**: Tsore, **C**: Kule and **D**: Terkidi refugee camp sand fly collection sites. The maps were built using the free and open source QGIS software version 3.36.3 (QGIS Development Team (2024). QGIS Geographic Information System, version 3.36.3. Open-Source Geospatial Foundation Project. https://qgis.org) and shapefiles were obtained from the free and open-source site https://data.humdata.org.

The district's average annual temperature and total rainfall are 29 °C and 1000 mm, respectively. Temperatures remain high year-round with peaks in March–May. Vegetation is dominated by savanna woodland, *Acacia seyal*, and extensive grasslands[42]. Moreover, there are scattered distributions of small-sized termite hills in the village and natural habitats of the refugee camps.

The Homosha district is found in the eastern parts of the Benishangul Gumuz Regional State, which is bordered by Kurmuk in the northwest, Menge in the northeast, and Assosa in the south. The study area receives 588-1,549 mm of rainfall annually, with maximum temperatures of 21.4–31.5 °C during the dry season and minimum temperatures of 7.4–17.6 °C. The soil is deep reddish-brown sandy clay, and the landscape is largely covered by natural vegetation, including shrub grassland, dense woodland, bamboo thickets, and open grassland[43]. Termite mounds are common in the district, particularly in woodland and grassland habitats, but rarely distributed in the refugee camp.

Collection of sand flies

The collection of adult sand flies was conducted by employing two standard techniques: LTs and STs, as detailed elsewhere[6,10,30]. Each method was deployed individually, without the presence of the other methods.

Before initiating the main sand fly collection, preliminary field assessments were conducted in order to assess the abundance, distribution, and diversity of sand flies in potential collection sites. The first assessment took place during the fourth week of May 2024 in refugee camps in Gambella Regional State. LTs were deployed indoors and in household compounds. We used 32 trap-nights (4 LT per night for 8 nights). Whereas, for ST, we deployed four traps for each of the five collection habitats (indoors, household compounds, termite mounds within villages, termite mounds in natural habitats, and natural habitats) per night. Sticky traps were deployed for eight nights each in all refugee camps, accumulating to a total number of trap nights of 160 (four ST in each of five habitats over eight nights). A second assessment was conducted

in Benishangul-Gumuz Regional State in August 2024 using the same trapping procedures; however, no sand flies were captured during this assessment.

CDC light trap (LT) Two fixed sampling points (indoor and household compounds) were established per refugee camp. A single LT was deployed at each point per night, positioned 30–50 cm above ground level and operating from dusk to dawn. A total of 5 LTs were deployed per night, amounting to a cumulative total of 40 trap-nights. This LT sampling was performed over two consecutive nights across all collection sites in the refugee camps of the Gambella (April 6–10, 2025) and Benishangul Gumuz (May 5–9, 2025) Regional States. Specimens were traced and aspirated each morning from the collection LT, inactivated using a combination of cold and mechanical vibration, and subsequently preserved in 95% ethyl alcohol for future morphological and molecular analysis (Fig. 2).

Sticky traps (STs) White transparent polypropylene sheets (A4 size) coated with a thin layer of castor oil were used as STs for sand fly collection. In each survey site, four traps were deployed in five habitat types, including indoors, household compounds, termite mounds within villages, termite mounds in natural habitats, and natural habitats with mixed forest cover. A total of 48 STs were deployed per night, amounting to a cumulative total of 384 trap-nights. Traps were operated from dusk to dawn. Captured sand flies were collected the following morning using fine-tipped forceps, transferred into labeled vials containing 95% ethyl alcohol, and preserved for subsequent morphological identification (Fig. 2).

Mounting and identification of sand flies

All collected sand flies were sex segregated based on the morphology of their reproductive organs observed under a stereomicroscope. Female sand flies were dissected by carefully removing the head and the last three abdominal segments using fine dissecting pins. Whereas, the male sand fly's genitalia were detached from the rest of its body parts. These parts were mounted on properly labeled glass slides in a drop of gum chloral



Fig. 2. Field sampling methods and habitat characterization for sand fly collection in selected refugee camps in Gambella and Benishangul Gumuz Regional States, 2025: (**A** & **B**: Landscape views of savanna woodland and shrubland vegetation in Kule and Terkidi refugee camps respectively; **C** & **D**: Natural habitat presentation with mixed vegetation plots mainly bamboo tree in Sherkole and Tsore refugee camps respectively; **E**: Sand fly collection in Kule camp natural habitats; **F**: Depicted that sand fly collection in the periphery of the compound in Terkidi refugee camps and **G**: Indicating sand fly collection in Kule refugee camp compound using LT).

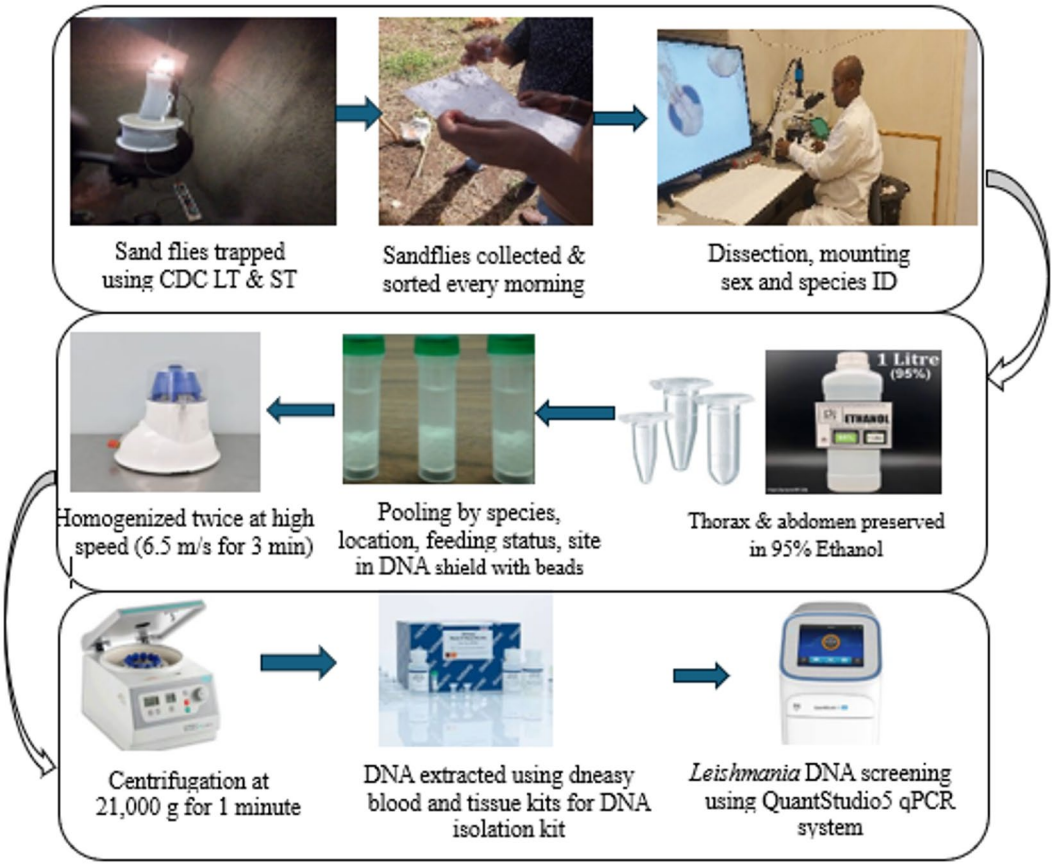


Fig. 3. A workflow diagram depicting the process from field collection of sand flies to molecular screening for *Leishmania* DNA.

mounting medium with the head ventral-side up under a single coverslip. Prepared slides were allowed to clear and dry at room temperature for one to two weeks before examination under a compound optical microscope for species identification. The remaining body parts (thorax and anterior abdominal segments) were preserved in sterile 1.5 mL Eppendorf tubes containing 95% ethyl alcohol, labeled identically to the corresponding slide, and stored for subsequent DNA extraction (Fig. 3). Species identification was carried out using well established taxonomic keys[44] and additionally[45] with diagnostic morphological features, including the cibarium, cibarial teeth, pharynx, and spermatheca.

DNA extraction and molecular screening of *Leishmania* DNA

We individually screened the only *Phlebotomus* species encountered during our study, *Ph. rodhaini*, while the *Sergentomyia* genus were pooled in sets of 1–22 female sand fly specimens based on species, trap type, collection site and habitat. The individually prepared and pooled female specimens were soaked in DNA shield solution with beads and homogenized twice at high speed (6.5 m/s for 3 min) using a FastPrep-24 bead beating homogenizer (MP Biomedicals), followed by centrifugation at 21,000 g for 1 min. From the clarified homogenate, 180 µl was transferred into a 1.5 ml tube for DNA extraction. DNA was isolated using the DNeasy Blood & Tissue kit (QIAGEN) following the standard manufacturer's protocol for isolation of insect genomic DNA (Fig. 3).

A real-time PCR (rtPCR) assay targeting the minicircle kinetoplast DNA (kDNA) was used to screen *Leishmania* DNA from sand fly specimens. The primers employed were kDNA-CMF (5′-CTTTTCTGGTCCT CCGGGTAGG-3′) and kDNA-CMR (5′-CACCCGG CCCTAT TTTACA CC AA-3′). The assay was performed as previously described[22]. In brief, each reaction was set up in a final volume of 25 µl containing 1× HotStarTaq Master Mix (Qiagen, Venlo, The Netherlands), 0.6 µM of each primer, 0.4 µM of the probe (Integrated DNA Technologies, Leuven, Belgium), 0.1 mg/ml bovine serum albumin (Roche, Vilvoorde, Belgium), and 5 µl of DNA template. Amplification was carried out on a QuantStudio 5 real-time PCR system (Thermo Fisher, Cat. No. A28568). Each run included genomic DNA from a *Leishmania*-positive culture as the positive control, a non-template control (NTC) as the negative control, and one used extraction control (ExC).

Data analysis

Data were entered using Microsoft Excel 2016, simply calculating the relative proportion from the overall sand fly collected[6] and exported to SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA) for further analysis. Sand fly density was calculated as the number of sand flies collected per trap per night using LT and ST to compare

differences between habitats. The normality of the data was assessed using the Shapiro–Wilk test. When the data did not meet the assumption of normality, the non-parametric Kruskal–Wallis test was employed. This test was used to compare the mean number of sand fly species collected across different sampling habitats using LTs and STs. The chi-square (χ^2) test was applied to evaluate associations between categorical variables. We calculated the Shannon-Wiener (H) diversity index in PAST v4.03 for each study site using the parameters of the proportion in which each species was collected. Shannon-Wiener index: H=−∑si = 1(Pi)ln(Pi), where S is the number of species and Pi is the proportion of the total samples belonging to the i^{th} species. H expresses the differences in the diversity of the sand fly fauna between refugee camps. Evenness: E = H/ln(S), where H is the value of Shannon-Wiener, and E expresses how evenly the individuals in the refugee camps are distributed over the different species. Species richness: S = the number of species in each refugee camp. The permutation test was employed to assess the statistical significance of differences in Shannon diversity indexes between study sites[46]. Statistical significance was determined at $p < 0.05$.

Data availability

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

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Author contributions

HB, AA, GT, BE and EA conceived and designed the study. All authors were involved in proposal writing and participated in field coordination, data collection, supervision and overall implementation of the study. HB analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

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Declarations

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

Consent was sought from each refugee camp coordinators including protection, security and health coordinators) and informed consent was also sought from head of households from where sand flies were collected in their compound and/or indoor. The study obtained ethical clearance from the Aklilu Lemma

Institute of Health Research-Institutional Research Ethics Review Committee (ALIHR-IRERC) prior to data collection (Ref. No.: ALIPB IRERC/112/2015/23). A permission letter was obtained from Federal Democratic Republic of Ethiopia Refugees and Returnees Service (FDRE RRS) and subsequently from RRS regional office and camp coordination offices.

Consent for publication

Not applicable.

Additional information

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