CUTANEOUS LEISHMANIASIS IN YEMEN: EPIDEMIOLOGY, KNOWLEDGE, ATTITUDE AND PRACTICES AMONG RURAL POPULATIONS, AND GENETIC DIVERSITY OF *LEISHMANIA TROPICA* IN UTMAH DISTRICT

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CUTANEOUS LEISHMANIASIS IN YEMEN: EPIDEMIOLOGY, KNOWLEDGE, ATTITUDE AND PRACTICES AMONG RURAL POPULATIONS, AND GENETIC DIVERSITY OF *LEISHMANIA TROPICA* IN UTMAH DISTRICT

ABSTRACT

Cutaneous leishmaniasis (CL) is a major public health problem in Yemen and is hyperendemic in many rural areas across the country. However, there is a dearth of research investigating the epidemiology of CL in the local communities where it is endemic. This study aimed to investigate the epidemiology (prevalence, risk factors and population's knowledge, attitude and practices towards CL), and genetic diversity of CL-causing *Leishmania* species among rural populations in Utmah district, western Yemen. A community-based cross-sectional survey, followed by unmatched case-control comparisons, was conducted among 612 participants from 289 randomly selected households in four rural sub-districts. A total of 223 participants were included in the case-control analysis. Skin scrapping/slits samples were collected from 81 individuals found with suspected active CL lesions while skin slits and/or blood samples were collected from 122 animals. The samples were examined by microscopy and internal transcribed spacer 1 (ITS-1) nested PCR. Genetic structure and haplotype diversity were analysed. Demographic, socioeconomic, environmental and KAP related data were collected using a pre-tested questionnaire. Overall, 8.7% and 16.4% of the human and animal (goats, cows, donkeys, bulls, rabbits, bats, dogs and rats) samples were found positive, with *Leishmania tropica* was the only causative agent identified. Multivariate hierarchical logistic regression analyses showed that being ≤ 10 years old, being female, living in poor housing conditions with cracked walls, living in the presence of other family members with typical ulcerating skin diseases, sleeping outside and keeping livestock on the ground floor of the house were factors significantly associated CL. A total of 51.2%, and 33.9% of the participants had good knowledge

about CL and sandflies, respectively. Moreover, only 38.1% and 16.3% had a positive attitude and good CL-related practices, respectively. The phylogenetic analysis of 54 human and animal sequences segregated six different *L. tropica* haplotypes, with low haplotype diversity (H_d = 0.242 ± 0.077) and no genetic differentiation (F_{ST} = 0.050). The genetic diversity analysis for 367 *L. tropica* sequences (54 obtained by this study plus 313 sequences were retrieved from GenBank) showed high haplotype diversity (0.605 ± 0.028) but low nucleotide diversity (0.005 ± 0.0004), with significant Tajima's *D* (-2.583) and Fu's *Fs* tests (-33.210). The median-joining haplotype network produced 54 haplotypes with one dominant haplotype. Overall, the study revealed an alarmingly high prevalence of CL and identified related key risk factors among the rural population in Utmah district. Also, the study demonstrated poor levels of KAP about CL and sandfly vector among studied population. Moreover, *L. tropica* was identified in different kinds of animal, suggesting potential important role of animal in the transmission of CL. These findings indicated that there is an urgent need for effective preventive and control measures to protect these vulnerable populations from this devastating disease.

Keywords: Cutaneous leishmaniasis, *Leishmania tropica*, Epidemiology, Genetic diversity, Yemen

LEISHMANIASIS CUTANEOUS DI YAMAN: EPIDEMIOLOGI, PENGETAHUAN, SIKAP DAN AMALAN DALAM KALANGAN PENDUDUK LUAR BANDAR DAN KEPELBAGAIAN GENETIK UNTUK *LEISHMANIA TROPICA* DI DAERAH UTMAH

ABSTRAK

Leishmaniasis cutaneous (CL) merupakan salah satu penyakit utama bagi masalah kesihatan awam yang hiperendemik di banyak kawasan luar bandar di Yemen. Namun begitu, hasil kajian epidemiologi CL di kawasan endemik komuniti tempatan adalah terhad. Kajian ini bertujuan untuk mengkaji epidemiologi (kelaziman, faktor risiko dan pengetahuan umum, sikap dan amalan penduduk terhadap CL), dan pengkelasan genetik spesies *Leishmania* yang menyebabkan CL dalam kalangan penduduk luar bandar di daerah Utmah, barat Yaman. Seramai 612 orang peserta daripada 289 isi rumah di empat dearah kecil luar bandar telah dipilih secara rawak bagi tinjauan keratan rentas berdasarkan komuniti dengan perbandingan kawalan kes yang tidak dapat ditandingi. Daripada jumlah tersebut, seramai 223 peserta telah dimasukkan dalam analisis kawalan kes. Sampel pengikisan/ celah kulit telah diperoleh daripada 81 individu yang disyaki mempunyai luka CL aktif manakala sampel celah kulit dan/atau sampel darah dikumpulkan daripada 122 haiwan. Sampel diperiksa menggunakan ujian mikroskop dan internal transcribed spacer 1 (ITS-1) nested PCR. Dari hasil PCR, struktur genetik dan kepelbagaian haplotaip telah dianalisis. Data demografi, sosioekonomi, alam sekitar dan berkaitan KAP dikumpul menggunakan soal selidik yang telah diuji. Secara keseluruhan, 8.7% dan 16.4% sampel manusia dan haiwan (kambing, lembu, keldai, lembu jantan, arnab, kelawar, anjing dan tikus) didapati positif dengan *Leishmania tropica* sebagai satu-satunya agen penyebab yang dikenal pasti. Analisis regresi logistik hierarki pelbagai variasi menunjukkan bahawa individu berumur ≤10 tahun, perempuan, tinggal dalam keadaan perumahan yang buruk dengan dinding retak, tinggal bersama

ahli keluarga lain dengan penyakit kulit ulser tipikal, tidur di luar dan memelihara ternakan di tingkat bawah. rumah adalah faktor yang berkait dengan CL. Sebanyak 51.2% dan 33.9% daripada peserta mempunyai pengetahuan yang baik tentang CL dan lalat pasir. Bagaimanapun, hanya 38.1% dan 16.3% bagi skala faktor mempunyai sikap positif dan amalan berkaitan CL yang baik. Analisis filogenetik daripada 54 jujukan manusia dan haiwan mengasingkan enam haplotip *L. tropica* yang berbeza. Hasil analisis menunjukkan nilai kepelbagaian haplotaip yang rendah (Hd = 0.242 ± 0.077) dan tiada pembezaan genetik (F_{ST} = 0.050). Analisis kepelbagaian genetik untuk 367 jujukan *L. tropica* (54 diperolehi dari kajian ini, 313 jujukan diambil daripada GenBank) menunjukkan kepelbagaian haplotaip yang tinggi (0.605 ± 0.028) tetapi kepelbagaian nukleotida yang rendah (0.005 ± 0.0004). Hasil ujian Tajima's D (-2.583) dan ujian Fu's *Fs* (-33.210) adalah signifikan. Rangkaian haplotaip penggabungan median menghasilkan 54 haplotaip dengan satu haplotaip yang dominan. Secara keseluruhannya, kajian ini menunjukkan kelaziman bagi penyakit CL yang sangat tinggi dan mengenal pasti faktor risiko utama yang berkaitan dalam kalangan penduduk luar bandar di daerah Utmah. Selain itu, kajian menunjukkan tahap KAP yang lemah mengenai CL dan vektor lalat pasir dalam kalangan populasi yang dikaji. Pengenalpastian *L. tropica* dalam pelbagai jenis haiwan, mencadangkan potensi peranan haiwan dalam transmisi CL. Kajian ini menekankan keperluan mendesak bagi langkah pencegahan dan kawalan yang berkesan untuk melindungi populasi yang terdedah, daripada penyakit ini.

Kata kunci: Leishmaniasis cutaneous, *Leishmania tropica*, Epidemiologi, Kepelbagaian genetik, Yaman

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LIST OF SYMBOLS AND ABBREVIATIONS

CL	Cutaneous leishmaniasis
VL	Visceral leishmaniasis
MCL	Mucocutaneous leishmaniasis
WHO	World Health Organization
SD	Standard deviation
χ^2	Chi-square
CI	Confidence interval
ºC	Degree centigrade
≥	Equals or larger than
≤	Equals or smaller than
P	P value
OR	Odd ratio
AOR	Adjusted odds ratio
%	Percent
k	Average no. of nucleotide differences
H_d	Haplotypes diversity
N_d	Nucleotide diversity
ITS-1	Internal transcribed spacer 1
PCR	Polymerase chain reaction
KAP	Knowledge, attitude and practices
NTDs	Neglected tropical diseases

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CHAPTER 1

INTRODUCTION

1.1 Background

Leishmaniasis, a neglected tropical disease (NTD), is a vector-borne disease caused by a protozoan parasite of the genus *Leishmania*. The disease is typically transmitted by the sandflies belonging to the genus *Phlebotomus* in the old world, and the genera *Lutzomyia*, *Psychodopygus*, and *Nyssomyia* in the new world (Akhoundi et al., 2016; Bhor et al., 2020). Over 20 *Leishmania* parasite species are pathogenic to humans and are transmitted by more than 90 sandfly species (Akhoundi et al., 2016; WHO, 2023). The disease has been described as the most ancient disease found in the mummies of the New and Old World countries (Akhoundi et al., 2016). It is currently endemic in more than 90 countries of the tropics, subtropics, and the Mediterranean basins (Du et al., 2016; Karami et al., 2023). The World Health Organisation (WHO) has estimated that about 1.1 million new cases of leishmaniasis and approximately 7,000 deaths occur each year, with more than 1 billion people live in areas endemic for leishmaniasis and are at risk of developing this disease (WHO, 2023). Moreover, it is estimated that disability-adjusted life years (DALYs) lost due to leishmaniasis are nearly 2.4 million (Scheufele et al., 2021).

There are different species of *Leishmania* parasites that have been reported to cause different forms of leishmaniasis in humans: *Leishmania tropica* and *L. major* commonly cause the cutaneous leishmaniasis (CL) in the old world, *L. braziliensis* and *L. panamensis* cause the mucocutaneous leishmaniasis (MCL) or "espundia" in the new world (Akhoundi et al., 2016; Karami et al., 2023). However, these species are also the major CL causative parasites in the new world along with other species such as *L. mexicana* and *L. amazoniensis* (de Vries & Schallig,

2022). Moreover, *L. donovani* and *L. infantum* are the major causative parasites of visceral leishmaniasis (VL), also known as kala-azar (Akhoundi et al., 2016). On the other hand, cutaneous skin lesion infections have been reported elswhere due to species of VL such as *L. infantum* in the Old World, particularly in the Mediterranean region (Alcover et al., 2023), *L. donovani* in some African countries and Sri Lanka (Bhor et al., 2021; Siriwardana et al., 2019), and *L. chagasi* (identical species to *L. infantum* but in New World) in Central and South America (Convit et al., 2005). Clinical presentation of the disease ranges from spontaneously healing skin lesions to overwhelming cutaneous and mucocutaneous ulcerative lesions and fatal VL, depending on the infecting species and the patient‟s immune status (Burza et al., 2018).

Cutaneous leishmaniasis is the most common form of leishmaniasis. It causes disfiguring skin lesions on exposed parts of the body, leaving life-long disfiguring scars, and leads to serious disability or severe social stigma, particularly for women and children (Bennis et al., 2017b; Bilgic-Temel et al., 2019). The disease is generally subdivided into two groups, Old World CL (occurs widely in the Eastern Hemisphere, including the Middle East, East Africa, the Indian subcontinent and Southern Europe) and New World CL (widespread in the Western Hemisphere, including Latin and Central America and the USA) (Kevric et al., 2015). The disease is also classified into two main forms, anthroponotic (*L. tropica*) and zoonotic CL (*L. major*, *L. aethiopica*, and all the New World species) (Akhoundi et al., 2016).

It is estimated that about 600,000 to 1 million new symptomatic cases of CL occur worldwide each year (Alvar et al., 2012b; WHO, 2023), and about 40 million people worldwide are currently living with inactive CL scarring (Alvar et al., 2012b; Bailey et al., 2017). About 95% of the cases occur in four eco-epidemiological regions, the Americas, the Mediterranean basin, the Middle East and Central Asia (WHO, 2023). Due to conflict and war situations in the

Middle East and North Africa (MENA) and East Africa regions, there has been an upsurge in new cases of leishmaniasis, with new foci established due to the migration of infected people and naïve populations moving into endemic areas due to internal displacement . For instance, new foci of transmission within endemic countries such as Syria, Libya and Yemen were reported due to huge internal displacement (Al-Salem et al., 2016a; Du et al., 2016; Warusavithana et al., 2022). Likewise, thousands of imported cases reported in many countries of the region, including Lebanon, Turkey and Jordan, as well as some European countries, such as Spain, Italy and Germany (Du et al., 2016; Rocha et al., 2022; Warusavithana et al., 2022).

Climate change, poverty, cultural age- and gender-specific occupations and activities (e.g. military, farming, mining and hunting), migration, deforestation, urbanisation, malnutrition, illiteracy and the lack of fully functional health care systems are among the significant predictors of CL (Burza et al., 2018; Mallawarachchi et al., 2021; Oryan & Akbari, 2016). Adherence to treatment and preventive measures is crucial to control CL; however, adherence is influenced by people‟s knowledge and attitude toward the disease and its vector (Tamiru et al., 2019).

A wide range of various vertebrate hosts, especially mammals, was reported to play a role in maintaining the *Leishmania* parasite circulation including domestic, peridomestic and wild animals (Montaner-Angoiti & Llobat, 2023). The most investigated and well-researched animals as reservoir hosts are dogs and rodents (Maroli et al., 2013). However, many previous *Leishmania* animal research has pointed to several probable sylvatic reservoir hosts in endemic leishmaniasis foci such as hares (Jimenez et al., 2013), marsupials (Roque & Jansen, 2014), wild canids as bush dogs (*Speothos venaticus*) (Figueiredo et al., 2008; Luppi et al., 2008), bats (Castro et al., 2020; Kassahun et al., 2015a; Medkour et al., 2019), primates, mongooses (*Herpestes ichneumon*), livestock animals such as sheep, horses and goats (Gao et al., 2015), and

other mammals (Bruschi & Gradoni, 2018; Montaner-Angoiti & Llobat, 2023; Otranto et al., 2015; Paiz et al., 2019; Roque & Jansen, 2014). In addition, rock hyrax (*Procavia capensis*) has been incriminated as a reservoir host of *L. tropica* in different African and Middle Eastern countries including Kenya, Namibia, Israel, Jordan, and Saudi Arabia (Sang et al., 1994; Sawalha et al., 2022; Talmi-Frank et al., 2010a). Environmental changes, particularly climate change and deforestation, are affecting the density and dispersion of sand flies, resulting in the emergence of leishmaniasis in new areas, and making transmission to new animal hosts or reservoirs possible. Therefore, a One Health approach incorporating environmental, entomological, animal and human health becomes imperative for the successful control and elimination of leishmaniasis worldwide.

The diagnosis of leishmaniasis is based on clinical presentations supported by epidemiological information and laboratory testing that achieved by microscopic examination for the detection of *Leishmania* parasites in clinical specimens. The leishmanin skin test (LST) (also known as the Montenegro skin test) is performed through intradermal injection of *Leishmania* antigens to monitor past exposure and immunity to *Leishmania* (Carstens-Kass et al., 2021). The LST has been a useful test in epidemiological studies to monitor exposure and immunity to *Leishmania* and also in vaccine studies as a proxy marker of immunity; however, the use of the LST has recently declined. Moreover, multilocus enzyme electrophoresis (MLEE) or polymerase chain reaction (PCR) assays followed by DNA sequencing or restriction enzyme digestion are widely used to detect and distinguish the parasite at a species level (de Vries & Schallig, 2022). The most common gene of interest for the molecular detection of *Leishmania* parasites is the internal transcribed spacer 1 (*ITS-1*) (Nasereddin et al., 2008). In addition, differentiating *Leishmania* parasites at the strain level and related phylogenetic analysis are achieved by MLEE

and different DNA-based assays including PCR-RFLP and multilocus sequence typing (MLST) (Schonian et al., 2008).

In Yemen, leishmaniasis (both CL and VL) is widespread particularly in rural areas where people live in poverty and with a partial or total lack of proper sanitation. The CL is the most common form of the disease which is disfiguring and sometimes disabling particularly in cases with multiple lesions (Al-Kamel, 2015; Khatri et al., 2016; Mahdy et al., 2010; Mogalli et al., 2016). The clinical pattern of the disease showed variation in the duration and severity of the disease and was characterized by a low response to treatment and with more than one parasite species or genotype being possibly isolated from an infected individual (Al-Kamel, 2015; Mahdy et al., 2010).

1.2 Problem statement of the study

Yemen is one of the world‟s poorest countries, with over 60% of the population living on less than USD2 a day (WB, 2010). Leishmaniasis (both CL and VL) is a public health problem with a nationwide distribution and is responsible for 60% of disability-adjusted life years due to prevalent tropical-cluster diseases (Hotez et al., 2012; WHO, 2023). The first description of CL in Yemen was in 1933 (Sarnelli, 1933). CL is the most common form of leishmaniasis in Yemen, with 9,120 cases reported in 2016 and more than 12,000 cases reported in 2019 (MOPHP, 2019; WHO, 2022). However, it is believed that the disease is largely underreported, and official data may only indicate the peak of an iceberg of underreported cases of CL, particularly among women and children in rural areas (Al-Kamel, 2016b; Ali, 2009; Du et al., 2016).

Several previous studies demonstrated the occurrence of CL in all regions across Yemen, with the highest rates reported in Dhamar, Al-Bayda and Hajjah governorates (Al-Kamel, 2016b;

Khatri et al., 2016; Khatri et al., 2009; Mahdy et al., 2010; Mogalli et al., 2016). The majority of available reports on CL in Yemen are health facility-based retrospective studies; therefore, community-based studies that can better ascertain the real prevalence in target populations are required. The ongoing civil war in Yemen since 2015 plays a major role in exacerbating the spread of parasitic infections, including leishmaniasis due to interruption of primary health services, insufficient surveillance in addition to the huge internal displacement escaping from cities of armed conflict to more safe areas, mostly rural (Al-Mekhlafi, 2018; Mogalli et al., 2016). However, recent information on the prevalence and distribution of CL in Yemen that could reveal the impact of the ongoing war is lacking. Similarly, there is a dearth of studies assessing the risk factors of CL and the level of populations‟ awareness towards the disease and its vector. Nonetheless, molecular analysis of cutaneous *Leishmania* parasite populations is limited, and studies to explore potential roles of domestic or wild animals in the disease transmission in Yemen have not yet been done.

Within these contexts, the present study aimed to investigate the epidemiology of CL by investigating the prevalence, risk factors associated with the disease, and participants‟ level of knowledge, attitude and practices towards CL and its vector. Moreover, molecular characterization of cutaneous *Leishmania* parasites in both suspected humans and the surrounding animals in Utmah district, Yemen was also examined for the identification of the *Leishmania* species. In addition, all *L. tropica* ITS-1 sequences obtained from the studied isolates and previous sequences available from GenBank were used to investigate the genetic diversity and haplotype networking for *L. tropica* across many endemic countries. Understanding the parasite species, its reservoir hosts and the associated risk factors are crucial to designing effective strategies to eliminate this devastating disease.

1.3 Objectives of the study

1.3.1 General objective

This study was carried out to investigate the molecular epidemiology of cutaneous leishmaniasis (CL) among rural communities in Utmah District of Dhamar governorate, Yemen, and to assess the knowledge, attitude, and practices towards CL among the study populations. It is also aimed to investigate the genetic structure and haplotype diversity of CL-causing *Leishmania* species in Yemen using internal transcribed spacer -1 (*ITS-1*) gene and to investigate the relationship with other geographical populations.

1.3.2 Specific objectives

1. To determine the prevalence and distribution of CL among participants in rural Yemen.
2. To identify the associated risk factors of CL among the study population in rural Yemen.
3. To assess the knowledge, attitude and practices (KAP) of the study population towards CL and its vector.
4. To explore the occurrence of *Leishmania* species infections in animal hosts in the study area.
5. To genetically analyze human and animal *Leishmania* parasite‟s populations isolated from the study area in rural Yemen.
6. To investigate the genetic structure and haplotype diversity of *Leishmania* species haplotypes based on the internal transcribed spacer -1 (*ITS-1*) gene of *Leishmania* species isolated from the study area, and after combining them with *Leishmania* species sequences, available from GenBank, across many endemic countries.

1.4 Research questions

From the objectives of the study, research questions have been formulated as follow:

1. What is the prevalence of CL among the study population in Utmah district?
2. What are the risk factors (predictors) associated with the prevalence of CL among the study population in Utmah district?
3. What is the level of knowledge, attitude and practices towards CL and sand fly vector among the study population in Utmah district?
4. What type of domestic or wild animals that can be infected with cutaneous *Leishmania* species parasites in the study area?
5. How many *Leishmania* species haplotypes, based on *ITS-1* gene, circulating in the study area? And what is the level of genetic diversity of those haplotypes?
6. What is the relationship of cutaneous *Leishmania* species haplotypes isolated from Utmah district with other geographical populations from other endemic countries?

1.5 Hypothesis of the study

1. Cutaneous leishmaniasis is highly prevalent in the Utmah district of Yemen.
2. There are significant associations between prevalence of CL and participant's demographic, socioeconomic, environmental and health factors.
3. Knowledge, attitude and practices of the participants towards CL and its vector are insufficient and significantly affect the burden of CL in Utmah district, Yemen.
4. Domestic and wild animals surrounding the population habitat in Utmah district, Yemen are infected with cutaneous *Leishmania* parasites.

5. Human and animal *Leishmania* genotypes are genetically inter-related, belonging to the same parasites cluster.
6. There is a high genetic diversity within and between *Leishmania* species populations in isolates from the study area, and there are significant associations between the genetic diversity of Yemeni isolates and other isolates from other endemic countries.

1.6 Significance of the study

The purpose of this study is to investigate the molecular epidemiology of CL in rural areas of the northwestern highlands of Yemen (Utmah district). Therefore, the prevalence and key risk factors significantly associated with the CL among the study population were identified. Participants‟ KAP towards the disease and its vector were also assessed. Moreover, the present study is the first to explore the occurrence of *Leishmania* parasites in animal hosts in Yemen, proposing their capability to transmit the disease as reservoir hosts. Besides, the study aimed to identify the different species causing CL using PCR amplification and sequencing of the *ITS-1* gene of *Leishmania* isolates. The new sequences were deposited at the NCBI GeneBank and will be available for future comparative research studies.

It is believed that the findings of the present study will contribute to a better understanding of the burden of the disease and its determinants in the study area. Therefore, the findings will assist the public health authorities and policy-makers to identify and implement integrated effective control strategies to reduce the burden of CL significantly in rural Yemen and to promote community mobilisation to achieve and sustain disease elimination. Also, it is hoped that the findings will be useful in designing and implementing a One Health approach incorporated entomological, environmental and epidemiological studies against the disease.

1.7 Organization of the thesis

According to the Universiti Malaya guidelines for the preparation of research reports dissertations and theses (2021), this thesis consists of six chapters and the format is "Conventional Style Format". According to the guidelines, the main body of a thesis should contains the following chapters: "Introduction", "Literature Review", "Methodology", "Results", "Discussion", and finally "Conclusion and Recommendations". Therefore, the main body of this thesis consists of the following chapters:

Chapter 1 (Introduction) provides a general background of the topic and the rationale for this study. It also includes the problem statement, objectives, research questions, hypothesis and the significance of the study.

Chapter 2 (Literature Review) provides extensive background information on past studies and current knowledge pertaining to CL. This chapter provides basic knowledge about the history, biology, epidemiology, diagnosis, treatment, prevention and control, and genetics of CL.

Chapter 3 (Methodology) provides specific details about the stages of the study and the methods employed. It includes information on the study settings, study population selection and recruitment, description of data collection and the development and introduction of the questionnaire, procedures and protocols of parasitological and molecular diagnostic methods utalised, data management and analysis and the ethical considerations.

Chapter 4 (Results) provides the results of the study that are presented in text, tables and figures. It involves the descriptive and inferential statistical analyses results and the related molecular and genetic results.

Chapter 5 (Discussion) interprets the results obtained from the study and offers an in-depth analysis and critical discussion of the main findings and where applicable, links the literature to the study outcomes.

Chapter 6 (Conclusion and Recommendation) presents the important results and findings of the study and lists a number of conclusions that can be drawn from the results as well as the implications of the study and provides recommendations for related health authorities and for the future studies. This chapter also involves the strengths and limitations of the study.

CHAPTER 2

LITERATURE REVIEW

2.1 Leishmaniasis

Leishmaniasis is a vector-borne NTD caused by a kinetoplastid protozoan parasite of the genus *Leishmania* and typically transmitted by the bite of infected sandflies. There are three different forms of leishmaniasis in humans: cutaneous leishmaniasis (CL), visceral leishmaniasis (VL) and mucocutaneous leishmaniasis (MCL). Leishmaniasis is endemic in many tropics, subtropics, and Mediterranean basins. The disease is zoonotic with a wide range of domestic and wild animals as reservoir hosts, but it can be anthroponotic as well (Maurício, 2018). While substantial progress has been achieved in improving the diagnosis and treatment of leishmaniasis over the last 20 years, this has encouraged the introduction of sustainable national and regional leishmaniasis management programmes. However, the world still has a long way to go through to limit disease morbidity and mortality (Bruschi & Gradoni, 2018). The disease is responsible for public health and economic problems in the affected regions (Alvar et al., 2006).

2.2 Historical background of leishmaniasis

According to the ancient description, the disease has been thought to have a long history stretching back more than 4500 years (Maurício, 2018), yet to extract the *Leishmania* parasites from archaeological contexts. Paleoparasitological research has made important contributions to showing that leishmaniasis has a long history dating at least to 2.500 BC (Frias et al., 2013). The first descriptions of conspicuous lesions identical to the current CL lesions have been found in mummies from Egypt, Sudan and Peru aged between 1500- 700 BC (Akhoundi et al., 2016). Leishmanial mitochondrial DNA of *L. donovani* was successfully extracted from four specimens

in a paleoparasitological study of 42 Egyptian mummies from the Middle Kingdom tomb in West Thebes (2050–1650 BCE), indicating VL existence in ancient Egypt (Zink et al., 2006). Moreover, leishmaniasis was also listed in the Ebers Papyrus, a compilation of ancient Egyptian medical documents dating back to 1500 BC in which a skin disorder known in English as "Nile Pimple," which is thought to be a CL scar was described (Steverding, 2017).

Leishmaniasis has been reported from various locations in the Middle East, including Baghdad, Aleppo and Jericho, and the conditions have been given local names according to these locations (e.g. Baghdad sore or Baghdad boil, Aleppo boil and Jericho boil). The oriental sores have been documented by the Arab physicians and scientists such as Al-Razi (Abu-Bakr Muhammad Ibn Zakariya Al-Razi, 854–935), who reported the occurrence cutaneous sores in the Baghdad region in the year 930 (Steverding, 2017). Avicenna (Ibn Senna) was the first to provide an accurate description of oriental sore in the 10th century AD in Balkh “Balkh sore” in north Afghanistan (Bray, 1987). On the other hand, kala-azar (a Sanskrit term means 'black fever') was first reported in India in 1824 and later defined as VL (Kumar, 2013).

In 1901, William Boog Leishman, the Scottish pathologist, identified ovoid single-celled organisms in a spleen biopsy of a soldier who had died from emaciation and splenomegaly symptoms of an illness called 'dum-dum fever". Initially, the identified organisms were considered to be trypanosomes and were suggested as the causative agent of dum-dum fever. In 1903, Charles Donovan reported similar ovoid bodies in biopsies of live patients and post-mortem as well (Donovan, 1994). Later, Ronald Ross proposed a new protozoan parasite genus and gave the name *Leishmania donovani* to the parasites (Kumar, 2013; Ross, 1903).

2.3 *Leishmania* taxonomy

The parasites of the genus *Leishmania* are belong to the *Trypanosomatidae* family (order Kinetoplastida); several genera in this family are pathogenic to humans and animals. More than 50 *Leishmania* species have been recorded to date; 30 species were documented as pathogenic species, and about 21 species were significant in terms of medical and veterinary importance (Guizani et al., 2019).

There have been several attempts at the classification of *Leishmania*. In 1908, Nicolle proposed an early leishmanial classification that distinguished *L. infantum* from *L. donovani* (Akhoundi et al., 2016). Various *Leishmania* species from the New World and the species that infected animals were included in the *Leishmania* classification by Biagi in 1953. In 1964, Adler discussed the difficulties of embracing clinically-based taxonomy. After the molecular data revolution, the *Leishmania* classification was updated, and it was proposed that *Leishmania* species be divided into two main phylogenetic lineages: Euleishmania and Paraleishmania (Akhoundi et al., 2016). The Euleishmania consisted of three subgenera: *Leishmania*, *Viannia* and *Sauroleishmania* (Cupolillo et al., 2000; Maurício, 2018). These groups of *Leishmania* parasites taxonomy depend on which parasites colonise portions of the sandfly's intestine (Akhoundi et al., 2016; Bates, 2007). In addition, some new species were found associated with leishmaniasis in humans and animals; however, after more careful characterisation and description they were either re-classified as other genus or as synonym of existed species (Espinosa et al., 2018; Steverding, 2017).

In 2002, Cavalier-Smith placed *Leishmania* under the protozoa Kingdom, and is nowadays belonging to the following classification (Maurício, 2018):

Kingdom: Protozoa

Phylum: Sarcomastigophora

Superclass: Mastigophora

Class: Flagellatea

Subclass: Zoomastigophora

Order: Kinetoplastida

Suborder: Trypanosomatina

Family: Trypanosomatidae

Genus: *Leishmania*

2.4 *Leishmania* species and clinical forms of leishmaniasis

Types of leishmaniasis vary according to the three main principles: geographical distribution, clinical diagnosis, and source of infection (Burza et al., 2018). So far, there are two main types of leishmaniasis that exist which are classified according to the site of infection and the tissue affected; these are CL and VL. The causative *Leishmania* species for each form also vary.

VL is the most severe form of the disease, and it is the world‟s second most lethal tropical parasite infection after malaria with a 10% mortality rate. The disease occurs as a result of the spread of *Leishmania* parasites in macrophage-rich tissues of internal organ including liver, spleen and bone marrow caused mainly by *L. donovani* and *L. infantum* (Alvar et al., 2012a; Handler et al., 2015; Mathers et al., 2007). The second form of the disease CL is a benign but frequently disfiguring skin condition that resolves spontaneously, also well-known for being the second most common arthropod-borne parasitic disease after malaria (Mohammadi Azni et

al., 2010; Norouzinezhad et al., 2016). Several species are known to be responsible for producing CL illness which markedly depends on the geographical locations and the type of the vector.

Different acute and chronic clinical types of cutaneous leishmaniasis are also known but less common or unrecognised. They include localised leishmanial lymphadenopathy, localised mucosal leishmaniasis (such as laryngeal or lingual leishmaniasis), mucocutaneous leishmaniasis (MCL), diffuse and disseminated CL and post-kala-azar dermal leishmaniasis (PKDL), the last occurs in the chronic stage of VL (Akhoundi et al., 2016; Burza et al., 2018). The MCL typically develops after the apparent resolution of cutaneous infection, though it may coexist with skin involvement. It may lead to partial or total destruction of mucous membranes of the nose, mouth and throat. Lesions usually appear within two years of cutaneous infection, but it can take up to 30 years (Daneshbod et al., 2011; Handler et al., 2015). MCL is thought to be a destructive form of leishmaniasis, which is only seen in the Latin American countries, and the main agent of MCL is *L. braziliensis* of (*Leishmania viannia subspecies*) (Gonzalez et al., 2009)**.** Nevertheless, several cases of MCL due to *L. aethiopica* and *L. tropica* have been described (Baneth et al., 2014; Sabzevari et al., 2020). Recently, over 90% of MCL cases occur in Bolivia, Brazil, Ethiopia and Peru (WHO, 2023).

In addition to the pathological burden of leishmaniasis, cutaneous and mucocutaneous forms for instance, like any other skin diseases, have psychological impacts due to the lesion scars that take months or years to heal (Khan et al., 2016; Yanik et al., 2004). Asymptomatic human infections are also increasingly reported, however, their epidemiological role in various endemic settings has not been determined (Maurício, 2018). Therefore, identifying *Leishmania* species based on their morphology or geographical distribution could be very difficult while identification based on clinical symptoms is regarded as untrustworthy. After all, some

Leishmania species can cause both cutaneous and visceral diseases at the same time (Khatri et al., 2009; Schönian et al., 2003). The main causal agents of CL are *L. major* and *L. tropica*, whose lesion presentation differs based on the species. However, since the middle of the last century, sporadic CL cases caused by *L. donovani* were reported in the East African VL foci, particularly in Sudan and Kenya. Later, many CL cases of *L. donovani* continued to be discovered outside the VL endemic areas, such as Yemen (Pratlong et al., 1995a). Moreover, in some countries such as Sri Lanka, *L. donovani* is the main species causing CL (Siriwardana et al., 2019).

In regard to the geographical distribution, leishmaniasis has been classified into Old World leishmaniasis (Asia, Africa and Europe) and New World leishmaniasis (South and North America, Australia and Antarctica). In the „Old World", it is caused by five *Leishmania* species that can also be divided into Old World CL including, *L. major, L. tropica* and *L. aethiopica* (being the main causative parasites), as well as Old World VL: *L. infantum* and *L. donovani*. In the New World CL is caused by *L. amazonensis, L. braziliensis, L. guyanensis, L. panamensis, L. Mexicana, L. venezuelensis, and L. peruviana*, wheareas New World VL is caused by *L. infantum (*syn. *L. chagasi*) (Burza et al., 2018).

Geographical distribution plays a significant role in classifying leishmaniasis because each location has its own environmental characteristics that determine the type of leishmaniasis. The CL form is common with 1.5 million new cases, most of which were reported annually from Iran, Afghanistan, Algeria, Iraq, Saudi Arabia, and Syria in the Old World; and Bolivia, Brazil, Colombia, and Peru in the New World (Desjeux, 2004; Herwaldt & Magill, 2012; WHO, 2010a). Table 2.1 presents the *Leishmania* parasite species causing leishmaniasis in humans in the Old and New Worlds and the clinical forms of disease they cause.

Table 2.1: Clinical forms of leishmaniasis caused by *Leishmania* species infecting humans

Leishmania species	Main form of disease	Other forms and unique clinical presentations	References
Old World:			
L. tropica	CL	Leishmaniasis recidivans & Mild VL	(Alabaz et al., 2022; Alborzi et al., 2008)
L. major	CL	-	(Karami et al., 2023)
L. aethiopica	CL	DCL	(Aberra et al., 2019)
L. donovani	VL	CL & PKDL	(Gelanew et al., 2011; Siriwardana et al., 2010)
L. infantum	VL	CL	(Burza et al., 2018; Echchakery et al., 2020)
New World:			
L. infantum/ chagasi	VL	Non-ulcerated CL	(Araujo Flores et al., 2020)
L. amazonensis	CL	DCL &VL	(Coelho et al., 2016; Porto et al., 2022)
L. braziliensis	CL	MCL	(Guimarães et al., 2016)
L. guyanensis	CL	MCL	(de Almeida et al., 2021; Goto & Lauletta Lindoso, 2012)
L. panamensis	CL	MCL	(Olivo Freites et al., 2018; Osorio et al., 1998)
L. mexicana	CL	DCL	(Burza et al., 2018)
L. venezuelensis	CL	-	(Bonfante-Garrido et al., 1992)
L. peruviana	CL	MCL	(Hashiguchi et al., 2018)
L. lainsoni	CL	-	(Ducharme et al., 2020)
L. utingensis	CL	-	(de Almeida et al., 2021)
L. lindenbergi	CL	-	(de Almeida et al., 2021; Silveira et al., 2002)
L. naiffi	CL	DCL	(Ducharme et al., 2020)
L. shawi	CL	-	(Oliveira et al., 2022)
L. martiniquensis	CL	VL	(Liautaud et al., 2015)

CL: cutaneous leishmaniasis; VL: visceral leishmaniasis; MCL: mucocutaneous leishmaniasis; DCL: diffuse cutaneous leishmaniasis; PKDL: post-kala-azar dermal leishmaniasis.

Specifically, cutaneous and visceral types of leishmaniasis are the common forms of the diseases in the Eastern Mediterranean Region (McDowell et al., 2011). *L. donovani*, *L. infantum* and occasionally *L. tropica* are the causes of visceral leishmaniasis in the region (Alabaz et al., 2022; Sarkari et al., 2016). While in the New World, VL is often caused by *L. infantum* (also called *L. chagasi*). However, *L. amazonensis* has also been reported (Medkour et al., 2019).

VL can infect various types of mammals including humans and a wide range of domestic and wild animals. Dogs, for instance, are the most common reservoir hosts for *L. infantum* causing VL in dogs known as canine leishmaniasis (CanL). This severe systemic disease has been reported in over 70 countries and is particularly prevalent in the Mediterranean and South America. In the Mediterranean basin only, it is estimated that 2.5 million dogs are infected.

According to the source of infection, leishmaniasis is classified into two broad epidemiological categories: zoonotic leishmaniases and anthroponotic leishmaniasis. The zoonotic leishmaniasis is transmitted from wild or domestic animals, while humans serve as an unintentional host. The transmission of zoonotic leishmaniasis occurs through a wide range of non-human reservoirs such as rodents, hyraxes, and canids. Animal reservoirs for zoonotic leishmaniasis vary depending on the foci' environment and parasite type. In contrast, humans in the anthroponotic leishmaniasis acts as sole reservoir host and a source of infection to the vector and consequently to other humans (Desjeux, 2004; Gramiccia & Gradoni, 2005; WHO, 2010a). Therefore, both anthroponotic and zoonotic epidemiological patterns of transmission need to be determined to become more comprehensible.

Two species are exclusively contributing to anthroponotic transmission patterns of leishmaniasis: those are *L. donovani* (including *L. archibaldi*) and *L. tropica* (including *L. killicki*). However, animal reservoirs have recently been discovered in several endemic settings, including Eastern Sudan (Dereure et al., 2003) and Ethiopia (Kassahun et al., 2015b) for *L. donovani*. *L. tropica* was detected in reptiles and animals in China (Zhang et al., 2016), Morocco (Rhajaoui et al., 2004), Turkey (Pasa et al., 2015), Ethiopia (Kassahun et al., 2015a; Kassahun et al., 2015b) and Iran (Akhtardanesh et al., 2020; Mohebali et al., 2005). Nevertheless, data registration systems usually do not distinguish between ACL and ZCL (Postigo, 2010)

2.5 Leishmaniasis vector

The sandfly is the vector of leishmaniasis. The phenetic approach has historically been used as the criteria to classify both the Old and New World sandflies. Therefore, the result was establishing overall similarity links between genera and subgenera rather than ancestor-descendant relationships. Previously, studies on phlebotomine sandfly classification relied solely on morphological characteristics of dead samples (Desjeux, 2004). Hence, this method has set off a proliferation of taxa, notably at the subgeneric level, as well as the simplification and incorporation of higher taxonomic groups into species. Therefore, the knowledge of phlebotomine sandflies" systematic classification has increased after introducing several new methods, such as chromosome analysis, molecular and phylogenetic analysis, multivariate morphometrics, isoenzyme, laboratory rearing and colonisation and lately, mass spectrometry (Akhoundi et al., 2016).

Overall, there are more than 800 sandfly species; around 464 species are found in the New World, while 375 in the Old World. These species belong to the order Diptera, suborder Nematocera, family Psychodidae, and subfamily Phlebotominae. A new taxonomy of *Phlebtominae* sandflies has been proposed (Bates et al., 2015). The Phlebtomini tribe, according to the proposed classification, consists of 931 species (916 legitimate species and 15 with uncertain taxonomic status) divided into six subtribes: Phlebotomina, Australophlebotomina, Brumptomyiina, Sergentomyiina, Lutzomyiina, and Psychodopygina. Besides, a few more species have been named or are due to be named, but their medical significance is unknown. The Old World sandflies are divided into three genera: Phlebotomus, Sergentomya, and Chinius, which are exclusively found in the Old World's warm zones, deserts or semi-arid areas in vast regions ranging from the Mediterranean, Afrotropical, and Oriental areas to central Asia and the Pacific regions.

In addition, the New World sandflies include three genera: Lutzomyia, Warileya, and Brumptomyia. These genera are found in forests in the Nearctic and Neotropical regions (Maroli et al., 2013). However, they are absent from the Pacific islands and New Zealand. Morphologically, both the New World and Old World sandflies are highly similar to each other. However, only 10% of them are vectors, and only 30 species have significant medical implications (Bates et al., 2015). Table 2.2 shows the main sandfly vector for different *Leishmania* parasite species.

With regards to feeding habits, certain species, such as *P. arabicus*, *P, bergeroti* and *P. papatasi*, prefer indoor habitats, whereas others, like *P. alexandri*, are found in outside environments. In addition, *P. ariasi* were found in humid or sub-humid areas with cold winters, making it the only potential vector of leishmaniosis in cold zones, while *P. perniciosus* prefers

semi-arid or sub-humid zones with warm winters and mild summers. According to Khan *et al*. (2017), *P. major* was found in mud houses, while *P. sergenti* were the dominant species in mud and stone houses.

ZCL caused by *L. major* is transmitted mainly by *P. papatasi P. bergertori, P. salehi* and *P. sammomys*, prevalent in Central and North Africa, Middle East and Central Asia. They feed on Meriones rodents‟ blood and other mammals, while the genus *L. tropica* transmitted via *P. sergenti* and *P. gundi* (Tabbabi, 2019).

Table 2.2: *Leishmania* parasite species infecting humans and their reservoirs, distribution, and potential or proven vectors of Old and New World leishmaniases

Subgenus	Species	World	Main reservoir hosts	Sandfly vector	Geographical distribution (region/country)	References
Leishmania	*L. tropica (syn of L. killicki)*	OW	Human, various mammals	*P. aculeatus, P. arabicus, P. rossi, P. chabaudi, P. guggisbergi, P. saevus, P. sergenti*	Central and North Africa, Middle East, Central Asia, India	(Akhoundi et al., 2016; Gramiccia & Gradoni, 2005)
Leishmania	*L. major*	OW	Human, various rodents	*P. papatasi, P. ansarii, P. bergeroti, P. salehi, P. caucasicus, P. duboscqi, P. mongolensis,*	Middle East, Central Asia, North Africa, Subsaharian Africa	(Azami-Conesa et al., 2021; Gramiccia & Gradoni, 2005)
Leishmania	*L. aethiopica*	OW	Human, rock hyraxes	*P. longipes, P. pedifer, P. sergenti*	East Africa (Eithiopia, Kenya)	(Akhoundi et al., 2016)
Leishmania	*L. donovani (syn of L. archibaldi)*	OW	Human, mammals	*P. alexandri, P. argentipes, P. celiae, P.chinensis, P. longiductus, P. martini, P. orientalis, P. schuanensis, P. vansomerenae*	Central Africa, South Asia, Middle East, India, China	(Azami-Conesa et al., 2021; Gelanew et al., 2011)
Leishmania	*L. infantum* (syn. of *L. chagasi*)	OW, NW	Human, canids and wild rodents	*P. alexandri, Lu. almerioi, P. ariasi, Lu. antunesi, P. brevis, P. balcanicus, P. chinensis, Lu. cruzi, Lu. evansi, Lu. foratteni, P. major, P. halepensis, P. kandelaki, P. kyreniae, P. simici, P. langironi, P. tobbi, P. longicuspis, P. longiductus, Lu. longipalpis, P. smimovi, P. wui, P. perfiliewi, P. turanicus, Lu. migonei, Lu. olmeca olmeca, Lu. ovallesi, P. pemiciosus, Lu. sallesi, Lu. pseudolongipalpis*	North Africa, Mediterranean countries, Southeast Europe, Middle East, Central Asia, Central and South America (Brazil, Bolivia, Mexico)	(Akhoundi et al., 2016; Azami-Conesa et al., 2021)
Leishmania	*L. amazonensis*	NW	Human, various forest rodents	*Lu. diabolica, Lu. flaviscutellata, Lu. longipalpis, Lu. nuneztovari englesi, Lu. olmeca novica, Lu. townsendi, Lu. ylephiletor, Lu. youngi*	South America (Bolivia, Venezuela)	(Akhoundi et al., 2016)

Table 2.2: Continued

Subgenus	Species	World	Main reservoir hosts	Sandfly vector	Geographical distribution (region/country)	References
Leishmania	*L. mexicana (syn of L. pifanoi)*	NW	Human, various forest rodents	*Lu. anthophora, Lu. ayacuchenisis, lu. christophie, Lu. columbiana, Lu. cruciate, Lu. diabolica, Lu. gomezi, Lu. flaviscutellata, Lu. longipalpis, Lu. olmeca olmeca, Lu. ovallesi, Lu. panamensis, Lu. migonei, Lu. shannoni, Lu. ylephiletor*	United States, Ecuador, Peru, Venezuela	(Azami-Conesa et al., 2021)
Leishmania	*L. venezuelensis*	NW	Human, mammals	*Lu. lichyi, Lu. olmeca bicolor, Lu. panamnsis, Lu. spincrassa*	Northern South America, Venezuela	(Akhoundi et al., 2016)
Viannia	*L. braziliensis*	NW	Human, numerous rain forest mammals	*Lu. anduzei, Lu. ayrozai, Lu. carreai, Lu. columbiana, Lu. complexa, Lu. cruciata, Lu. neivai, Lu. edwardsi, Lu. fischeri, Lu. gomezi, Lu. lichyi, Lu. pia, Lu. intermedia, Lu. llanosmartinsi, Lu. longipalpsi, Lu. migonei, Lu. ovallesi, Lu. nuneztovari, Lu. trindadensis, Lu. pessoai, Lu. panamensis, Lu. shawi, Lu. spinicrassa, Lu. paraensis, Lu. youngi, Lu. tejadai, Lu. townsendi, Lu. yucumensis, Lu. wellcomei, Lu. trapiodi, Lu. umbralitis, Lu. whitmani, Lu. yelphiletor*	Western Amazon basin, South America, Brazil, Bolivia, Peru, Guatemala, Venezuela	(Akhoundi et al., 2016; Azami-Conesa et al., 2021)
Viannia	*L. guyanensis*	NW	Human, sloths	*Lu. anduzei, Lu. ayacuchensis, Lu. ovallesi, Lu. flaviscutellata, Lu. longiflocosa, Lu. shawi, Lu. llanosmartinsi, Lu. migonei, Lu. whitmani, Lu. umbratilis*	Northern South America, Bolivia, Brazil, French Guiana, Suriname	(de Almeida et al., 2021)
Viannia	*L. lainsoni*	NW	Human, rodents	*Lu. nuneztovari anglesi, Lu. olmeca bicolor, Lu. ubiquitalis, Lu. whitmani*	Brazil, Bolivia, Peru, Ecuador	(Ducharme et al., 2020)

Table 2.2: Continued

Subgenus	Species	World	Main reservoir hosts	Sandfly vector	Geographical distribution (region/country)	References
Viannia	*L. utingensis*	NW	Human	*Lu. tuberculata*	Brazil	(Braga et al., 2003)
Viannia	*L. lindenbergi*	NW	Human	*Lu. antunesi, Lu. tuberculata*	Brazil	(de Almeida et al., 2021; Silveira et al., 2002)
Viannia	*L. naiffi*	NW	Human, armadillos	*Lu. amazonensis, Lu. ayrozai, Lu. paraensis, Lu. gomezi, Lu. squamiventris, Lu. trapiodi*	Brazil, French Guyana, Ecuador, Peru	(Ducharme et al., 2020)
Viannia	*L. panamensis*	NW	Human, sloths	*Lu. (T). cruciata, Lu.(N). flaviscutellata, Lu. gomezi, Lu. hartmanni, Lu. migonei, Lu. ovallesi, Lu. Panamensis, Lu. Sanguinaria, Lu. Spinicrassa, Lu. trapiodi, Lu. umbratills, Lu. ylephiletor, Lu. yuili*	Central and South America, Brazil, Panama, Venzuella, Colombia	(Akhoundi et al., 2016; Azami-Conesa et al., 2021)
Viannia	*L. peruviana*	NW	Human, dogs	*Lu. ayacuchensis, Lu. noguchii, Lu. peruensis, Lu. tejadi, Lu. verrucarum*	Peru, Bolivia	(Hashiguchi et al., 2018)
Viannia	*L. shawi*	NW	Human, arboreal mammals (e.g. monkeys, sloths, procyonid)	*Lu. whitmani*	Brazil	(de Almeida et al., 2021)
Mundinia	*L. martiniquensis*	OW, NW	Human, mammals	*S. (Ne.) gemmea, Culicoides sonorensis*	Martinique Island, Central Europe, Thailand, USA	(Liautaud et al., 2015; Mendes Junior et al., 2023)

OW: Old World; NW: New World; CL: cutaneous leishmaniasis; VL: visceral leishmaniasis; MCL: mucocutaneous leishmaniasis; PKDL: post-kala-azar dermal leishmaniasis; P: Phlebotomus; S: Sergentomya; Lu: Lutzomyia; Ne: Neophlebotomus; Psy: Psychodopygus; N: Nyssomyia; T: Tricholateralis.

2.6 The hosts of *Leishmania* parasites

A natural host of *Leishmania* parasites is important for the natural life cycle of the parasite to complete. Animal reservoirs are required to maintain the life cycle and the transmission of zoonotic infections of many *Leishmania* species. Infected rodents, domestic animals and small wild animals infected with *L. donovani* were reported in several countries of the old world, such as Ethiopia (Kassahun et al., 2015b), Sudan (Mukhtar et al., 2000), Egypt (Morsy et al., 1994), India (Jambulingam et al., 2017; Singh et al., 2013) and Saudi Arabia (Ibrahim et al., 1992; Morsy et al., 1999). Other species of mammals, such as *Crycetomys gambianus*, *Heterohyrax brucei* and *Dendrohyrax arboreus*, were reported to be infected with *L. aethiopica* (Ashford, 1996).

Moreover, more than 40 mammalian species are recognised in the New World as *Leishmania* parasites hosts. However, few species in the natural transmission cycle are considered necessary (Motazedian et al., 2006). In the Old World, rodents are the most well-known hosts of *Leishmania* species, playing an essential role as potential reservoir host for leishmaniasis (Hertig, 1957). However, rodent species differ from one country to another. For example, the black rat (*Rattus rattus*) was infected with different species of *Leishmania,* such as *L. martiniquensis* in Thailand, *L. donovani* in Saudi Arabia (Ibrahim et al., 1992) and *L. (Viannia)* spp of the New World countries (Caldart et al., 2017; Pereira et al., 2017). Besides, other species of rodents were reported as reservoir hosts of different species of *Leishmania*, such as *L. turanica* from China (Guan Li-Ren et al., 1995), Spain (Galan-Puchades et al., 2022), Central Asia and Iran (Mirzaei et al., 2011; Vojtkova et al., 2020), as well as the New World countries.

The Rock hyraxes are the main ZCL reservoir hosts in the Middle East's *Leishmania* epidemic foci besides the rodents (Postigo, 2010). The CL reservoir hosts a map of the Middle East consisting of Iran, Pakistan and Afghanistan to the east, passing

through the Arabian Peninsula and continuing towards the North African countries. The rodents of great gerbil are dominant in Afghanistan and Iran (Ahmad, 2002; Nadim et al., 1979; van Thiel et al., 2010), while rock hyrax is in Jordan, Palestine and Yemen. However, the fat sand rat (*Psammomys obesus*) is more common in Egypt, Jordan, Libya, Saudi Arabia, Syria and Tunisia. Neither reservoir hosts of rodents have yet been reported in Iraq, nor have studies been conducted on Yemen's CL reservoir animals. Table 2.2 shows the main reservoir hosts for zoonotic *Leishmania* parasite species.

Several kinds of domestic animals such as livestock, cats, dogs and other canines are also established as reservoir hosts for the disease and natural parasite survival (Rasheed et al., 2023). On the other hand, *Leishmania* species can cause similar symptoms in animals as in humans, particularly animals such as canids causing canine leishmaniasis (CanL). Canids include such as dogs (*Canis lupus* familiaris), Jackals (*Canis aureus*), wolves (*Canis lupus*) and red fox (*Vulpes vulpes*) are the main reservoir hosts of ZVL in the countries of the Mediterranean Basin (Alvar et al., 2012a; Amro et al., 2009; Gramiccia & Gradoni, 2005) and the Middle East countries (Khatri et al., 2009; Postigo, 2010). Although distribution of CanL and human VL overlaps in the Mediterranean Basin region, the incidence in dogs is much higher, with several thousand cases being diagnosed annually in dogs from all endemic zones in this region.

Furthermore, in comparison to humans, dogs appear to be more susceptible to *L. infantum* infection and develop severe forms of CanL at a higher rate. However, according to Abdeen et al. (2002), 5.5% of the surveyed domestic dogs in West Bank, Palestine, were seropositive for *L. major*. Domestic dogs are the main reservoir hosts of *L. infantum*, besides a range of carnivores and rodents. However, a single case was reported from a Mediterranian monk seal (*Monachus monachus)* (Toplu et al., 2007). Among the recent reports of the uncommon newly identified animal hosts, the

involvement of domestic cats deserves much attention due to the obvious implications to public health. The feline leishmaniasis is commonly non-specific in terms of clinical symptoms. However, several studies from different countries worldwide reported the occurrence of leishmaniasis in cats due to various species of *Leishmania* parasites (Gramiccia & Gradoni, 2005). Similarly, the increased reporting of *Leishmania* lesions in domestic equines poses questions about the possible role of these animals in the transmission of zoonotic leishmaniases.

On the other hand, several species of *Leishmania* have been recovered from rodents and other animals but not found in humans, such as *L. gerbilli*, *L.turanica* (Guan Li-Ren et al., 1995), and *L. arabica* from Old World rodents (Ashford, 2000); *L. equatoriensis* from arboreal mammals in Ecuador (Grimaldi Júnior & Tesh, 1993)*; Leishmania* sp. from red kangaroo, *Macropus rufus* (Rose et al., 2004).

2.7 Life cycle of *leishmania* parasites

Leishmania parasites were initially thought to have a simple heteroxenous and digenetic life cycle involving only two asexual stages (Alemayehu & Alemayehu, 2017; Sharma & Singh, 2008). However, studies have suggested the existence of meiosis-like sexual reproduction in *Leishmania*. The cross-species genetic exchange between cutaneous and visceral *Leishmania* species has also been experimentally demonstrated.

The *Leishmania* life cycle (Figure 2.1) involves two physiologically and morphologically different stages, non-flagellated intracellular amastigote (also called Leishman form) in the mammalian hosts and flagellated extracellular promastigote in the female sandfly vectors (Alcolea et al., 2010; Ashutosh et al., 2007; Molyneux & Killick-Kendrick, 1987). The promastigote (Figure 2.2a) is the fusiform motile stage with a polar flagellum that protrudes from the parasite‟s body, while the amastigote stage is non-flagellated, non-motile ellipsoidal body. The most striking difference

between the amastigote and promastigote forms is the change in the flagellum from a long motile flagellum with a 9+2 axoneme in the promastigotes to a short non-motile flagellum with a 9+0 (9v) axoneme in the amastigotes. Furthermore, amastigote has an ovoid or ellipsoidal cell body with a small bulbous tip extending beyond the cell, whilst the promastigote has an elongated cell shape. This change in the parasite shape within the sandfly and the mammalian host minimises cell surface to volume ratio, thus reducing the area over which the cell is exposed to the harsh environment of the parasitophorous vacuole reformatting flagellum use.

In hematophagous female sandflies, there are four developmental forms of promastigote which is known as metacyclogenesis. These forms occur within the gut of the vectors (Diptera: Psychodidae, Phlebotominae) and differ based on the cell body and flagellum length and/or width (Bates, 2007) (Figure 2.2b):

i. Procyclic promastigote: the cell body length ranges between 6.5 and 11.5 mm, with the flagellum shorter than the cell body.

ii. Nectomonad promastigote: the cell body is longer than 12 mm.

iii. Leptomonad promastigote: the cell body length is between 6.5 and 11.5 mm, with the flagellum longer than the cell body.

iv. Metacyclic promastigote: the cell body is less than 8 mm long and 1mm wide, with a flagellum that is longer than the cell body.

These four cell types are thought to represent a developmental sequence with specific precursor-product relationships (Figure 2.2b).

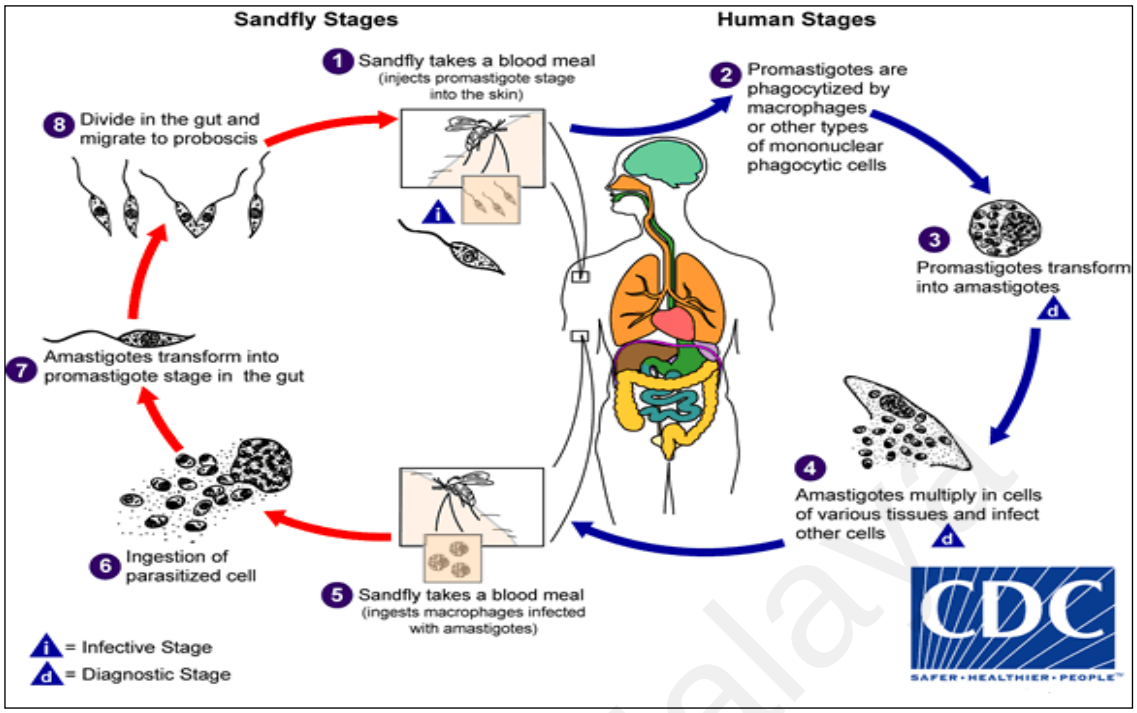

Figure 2.1: The life cycle of *Leishmania* species.

Source: CDC at http://www.dpd.cdc. gov/dpdx/HTML/Leishmaniasis.htm

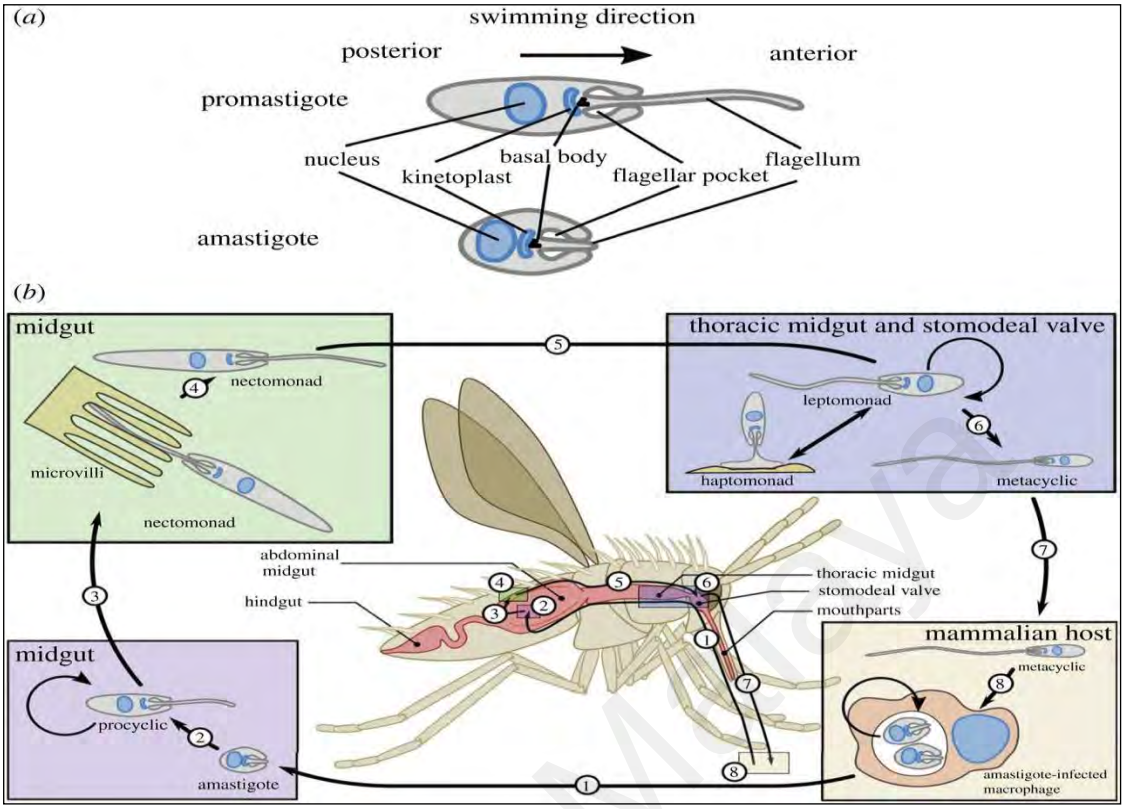

Figure 2.2: Life cycle of *Leishmania* and parasite's developmental stages.

(**a**) Promastigote and amastigote morphologies aligned along the posterior-anterior axis, with essential features in the cells marked. (**b**) The current understanding of the life cycle of *Leishmania* showing important events (Sunter & Gull, 2017).

When sandflies ingest intracellular amastigotes with their blood meal of the infected host (Figure 2.1), the amastigotes transform immediately into procyclic promastigotes (Figure 2.2b) characterized by short flagellum, then rapidly divide by binary fission. During the blood meal digestion the parasites are surrounded by a peritrophic matrix which later broken by sandfly‟s enzymes. On the third day, procyclic promastigotes transform into long slender nictomanods (Figure 2.2b) and escape through the broken peritrophic matrix. The next step of parasite development inside the insect vector depends on the parasite‟s species. Species of the subgenus *Leishmania* develop in the abdominal midgut of the insect, where they stick to the epithelium, avoiding expulsion during defecation.

While species of subgenus *Viannia* move to the hindgut, where they transform into round flagellated promastigotes or short nectomonads called leptomonad promastigote (Figure 2.2b) and attach to the wall by hemidesmosomes. After defecation, actively multiplying leptomonads migrate to the thoracic midgut and produce the promastigote secretory gel (PSG), which plugs the anterior part of the sandfly gut, in which forcing the vector multiple feeding attempts, thereby regurgitating parasites into the biting site (Alcolea et al., 2019; Sunter & Gull, 2017). In the late stage, two stages are observed at the stomodeal valve in the foregut, non-motile haptomonad, which attach to the chitinous lining of the stomodeal valve (SV), and highly motile metacyclic (the infective stage), which found behind the stomodeal valve and are ready for successful transmission to the mammalian host. Each of these stages is characterised by morphological and functional changes to ensure its survival in the sandfly. In subgenera *Viannia* and *Sauroleishmania*, haptomonads also attach to chitin lining of the pylorus region (Akhoundi et al., 2016; Alcolea et al., 2019).

Depending on the sandfly species, a heavy pharyngeal infection of the sandfly is usually observed between the 6^{th} and 9^{th} day after its infected blood meal. When infected sandflies bite mammals to feed, the metacyclic forms of the promastigote stage are injected into the bloodstream of the vertebrate host. Together with *Leishmania*, *Leishmania*-derived exosomes, PSG, sandfly saliva, and gut bacteria are introduced to the host skin and modulate its immune response. Then, an acute inflammatory response develops, neutrophils, monocytes and macrophages migrate into the feeding site and take up the metacyclic promastigotes that transform into amastigotes. Initially, Neutrophils internalise the metacyclic promastigotes before being phagocytosed by macrophages delivered to the mature phagolysosome compartment and differentiated into small, round non-flagellate amastigotes. Amastigotes multiple inside the phagolysosome of the host cells, then rupture the cell and infect next macrophages, which also marks the starting point for an onset of the disease (Maroli et al., 2013).

The sandfly bite attracts macrophage cells that immediately rush to the affected area. Macrophages are the most common cell type within lesions since they can be infected with a few to several hundred amastigotes depending on the species (Khan & Zakai, 2014). The metacyclic promastigotes are highly motile cells that are capable of migrating through a collagen matrix. Therefore, the parasite‟s phagocytosis may occur at locations far from the bite site. Moreover, the spectrum of disease caused by *Leishmania* from cutaneous to visceral is perhaps reflected in the ability of the parasite to invade the host beyond the bite site either directly or via infected macrophage movement (Bates, 2007). The promastigotes transform into an amastigote stage in the phagolysosome of reticuloendothelial cells within 12-24 hours, where they multiply in the macrophage's hostile environment until the host cell dies, releasing the parasites to enter other cells and repeat the cycle (Bates, 2007; Gossage et al., 2003).

2.8 Epidemiology of cutaneous leishmaniasis

The main epidemiological determinants are the intrinsic tropism including the *Leishmania* species and their virulence, the sandflies vector, the ecological characteristics of the transmission sites, and the different human-associated risk factors (Desjeux, 2004). Almost 70 animal species, including humans, are reported as natural reservoir hosts of *Leishmania* parasites in over 98 tropical and subtropical countries worldwide, most of which are developed countries. Rainforests and deserts are among the ecological settings.

In addition to the classical transmission mode through female sandfly bites, disease transmission through biting midges (Diptera: Ceratopogonidae) and blood transfusion have been recently confirmed. According to (Jimenez-Marco et al., 2016), blood from healthy asymptomatic donors was significantly found positive for *L. infantum* parasite in Balearic Islands, Spain. However, there is no evidence on the infectivity of these parasites and their capability to initiate visceral leishmaniasis in blood recipients. On the other hand, blood transfusion-transmitted visceral leishmaniasis has been reported among infants and children from endemic and non-endemic areas (Dey & Singh, 2006; Mansueto et al., 2014).

2.8.1 Risk factors of leishmaniasis

The risk factors for leishmaniasis are divided into biological risk factors and external risk factors. Biological factors include the vulnerable host‟s nutrition and immune status, vectors and parasite species, and the reservoir hosts. External factors or human-associated risk factors (demographic, socioeconomic, spatial and environmental factors) include age-specific duties, gender, urban-night activities, population growth, displacement, altitude, climate, poverty, living or travelling to endemic regions,

ecotourism activity, military operations, and immigration (Cizmeci et al., 2019; Di Muccio et al., 2015; Du et al., 2016; Maurício, 2018).

The disease is usually more prevalent in rural areas than in urban areas. It is predominantly an occupational disease due to its relation to outdoor activities, such as military operations, road construction, livestock grazing, and agricultural activities in enzootic areas and forests, such as harvesting crops and guarding farms at night. In addition, those who spend their holidays in nature reserves without appropriate protection against sandfly bites, such as campers, travellers, reserves rangers and ecotourists (Maurício, 2018). On the other hand, urban and peri-urban foci of leishmaniasis in medium- and large-size cities were also recorded (Baghad et al., 2020; Firouraghi et al., 2022). Some researchers believe that the type of parasite that causes the disease and the region are linked; for example, *L. tropica* is found in rural areas, whereas *L. infantum* is found in urban areas. This association may be attributed to other factors related to the reservoir host and the insect vector. Furthermore, new foci of leishmaniasis may emerge due to the increasing human mobility from rural to urban areas, for instance, *L. tropica.*

Leishmaniasis infections have been recorded in all age groups. However, according to several studies, children are the most affected age group for both CL and VL worldwide. It can be explained by the fact that children have less immunity than adults. Nevertheless, American CL is more important in the age group of economically active adults in comparison with visceral leishmaniasis, which affects higher percentages of children.

Theoretically, males of the working age group are most likely to be affected by leishmaniasis because of their outdoor activities in agriculture and forestry and other works in areas closer to sandflies' habitats, which would increase their chances of

becoming infected. However, some studies found that females are more affected to leishmaniasis than males, particularly in areas with a low male population, for a variety of reasons, such as wars, migration to towns or other countries or societal customs that impose women to work in fields, agriculture, fetching water and harvesting, making them more likely to be exposed to sandfly bites.

Migration, displacement and travelling are all important risk factors for the rise in leishmaniasis cases. Several cases of leishmaniasis have been reported in non-endemic areas, either from locals who visited endemic areas or from migrants and refugees who carried the disease with them from their home countries (Ab Rahman & Abdullah, 2011; Shin et al., 2013; Tan et al., 2022; Viroj, 2012). The term "imported leishmaniasis" refers to cases discovered in foreigners who brought the disease from their home countries, as well as cases discovered in locals who returned from endemic areas carrying the disease (Demers et al., 2013; Tan et al., 2022). During 2008-2018, Canada recorded a significant increase in cutaneous leishmaniasis cases among the locals who travel to endemic regions compared to immigrants from endemic areas. The reason for this is the rise in Canadian visitors to endemic areas, particularly South American countries (Lemieux et al., 2022).

Until the year 2004, Australia and Southeast Asia were thought to be the only large areas with suitable climates where the leishmaniasis is absent (Ashford, 2000; Conlan et al., 2011; Rose et al., 2004). However, the first autochthonous *Leishmania* infection was reported in the red kangaroo of Australia (*Macropus rufus*), and the parasite was characterised as a unique species (Rose et al., 2004). Furthermore, Australian cutaneous leishmaniasis was later registered in captive wallaroos in Australia but the transmitting vector remains unknown (Dougall et al., 2009). In the recent years, new leishmaniasis cases have been notified in Australia due to the increase in the

international travel, immigration, and defence personnel deployment to the endemic areas (Khairnar et al., 2013; Roberts et al., 2015). The increasing number of travellers, immigrants and refugees, as well as foreign workers, has boosted the spread of the disease to non-endemic countries, creating new foci for imported *Leishmania* species (Handler et al., 2015). The presence of the insect vector is a key factor in forming these foci. However, several cases were recorded in Malaysia and Singapore after detecting imported cases of migrant workers, despite the fact that the insect vector of VL is thought to be absent in those countries (Tan et al., 1997; Viroj, 2012).

It is well established that vector-borne diseases are linked to the environment. As a result, leishmaniasis, like other vector-borne infections, is considered a climate-sensitive disease that can be affected by humidity, rainfall and temperature changes. Since the sandflies are poikilothermic invertebrates, temperature and humidity changes can directly and severely impact their environment. These effects are likely to be lesser for the reservoirs which are homeothermic vertebrates by altering their geographical distribution and life cycle and influencing their survival and breeding time. For instance, canine leishmaniasis in Europe was thought to be confined only to the Mediterranean region due to climatic conditions unique to the sandfly's life cycle. However, recently northern countries such as Poland, Germany and the Netherlands have reported a high number of canine leishmaniasis. Moreover, Indirect impacts such as drought, famine and flood resulting from changes in climate conditions could lead to massive displacement and migration of people to areas with the transmission of leishmaniasis, and poor nutrition could compromise their immune resistance to parasites.

Leishmaniasis is known as a poverty-related disease because most of the VL cases emerged in poorest countries such as India, Sudan and Pakistan. However, VL is not only a disease of the poor; it is also a source of poverty. Many days of productive life are lost due to this severely debilitating disease. This indirectly accounts for up to 60% of the total household cost (Meheus et al., 2006). Many studies in India (Sarnoff et al., 2010; Sundar et al., 2010), Nepal (Adhikari & Maskay, 2003; Adhikari et al., 2009; Rijal et al., 2006), and Bangladesh (Ozaki et al., 2011) have reported a significant economic burden on the cases" households imposed by VL. The resulting expenditure is always disastrous, necessitating the sale of assets and livestock or the loans.

According to epidemiological studies conducted in endemic areas with VL by *L. infantum*, asymptomatic human infections are common. Age, malnutrition, HIV coinfection, and other immunosuppressive conditions are also risk factors for leishmaniasis progression (Boelaert et al., 2019; Gramiccia & Gradoni, 2005). It has also been observed that the parasite can be transmitted through blood transfusion, even though these cases are still very rare. As a result, the low parasitaemia level, at least in immunocompetent asymptomatic carriers, as well as their possible role as reservoir hosts, must be addressed.

The presence of domestic animals is also important risk factor associated with leishmaniasis. However, VL was described as anthroponotic disease in India, and the role of domestic animals in transmitting the disease is limited (Singh et al., 2010).

2.8.2 Prevalenc of cutaneous leishmaniasis

2.8.2.1 Global situation

The WHO has classified leishmaniasis endemic countries into five regions based on their geographical location (Figure 2.3). These regions are the African, the Americas, the Eastern Mediterranean, European, and Southeast Asia regions (WHO, 2020c).

According to the WHO, a country is classified as endemic if it has at least one autochthonous case that has been reported in it (WHO, 2021). In 2020, leishmaniasis was considered endemic in 98 countries (about 49% of the world's countries). About 71 countries were reported as endemic for both visceral and cutaneous types. Most of these countries are developing countries, which reinforces the link between this disease and poverty (Ghatee et al., 2020).

According to the WHO‟s recent estimates (WHO, 2023), CL and VL are endemic in 18 Eastern Mediterranean Region countries, and 80% of worldwide CL cases were accounted to this region. Moreover, CL is endemic in 21 countries, and VL in 13 countries of the Americas Region; however, 97% of VL cases in this region came from Brazil. CL and VL are endemic in 19 and 14 African countries, respectively. For the European Region, CL is endemic in 25 countries, and VL in 27 countries. Regarding the Southeast Asia Region, CL is endemic in 5 countries and VL in 6 countries (WHO, 2022).

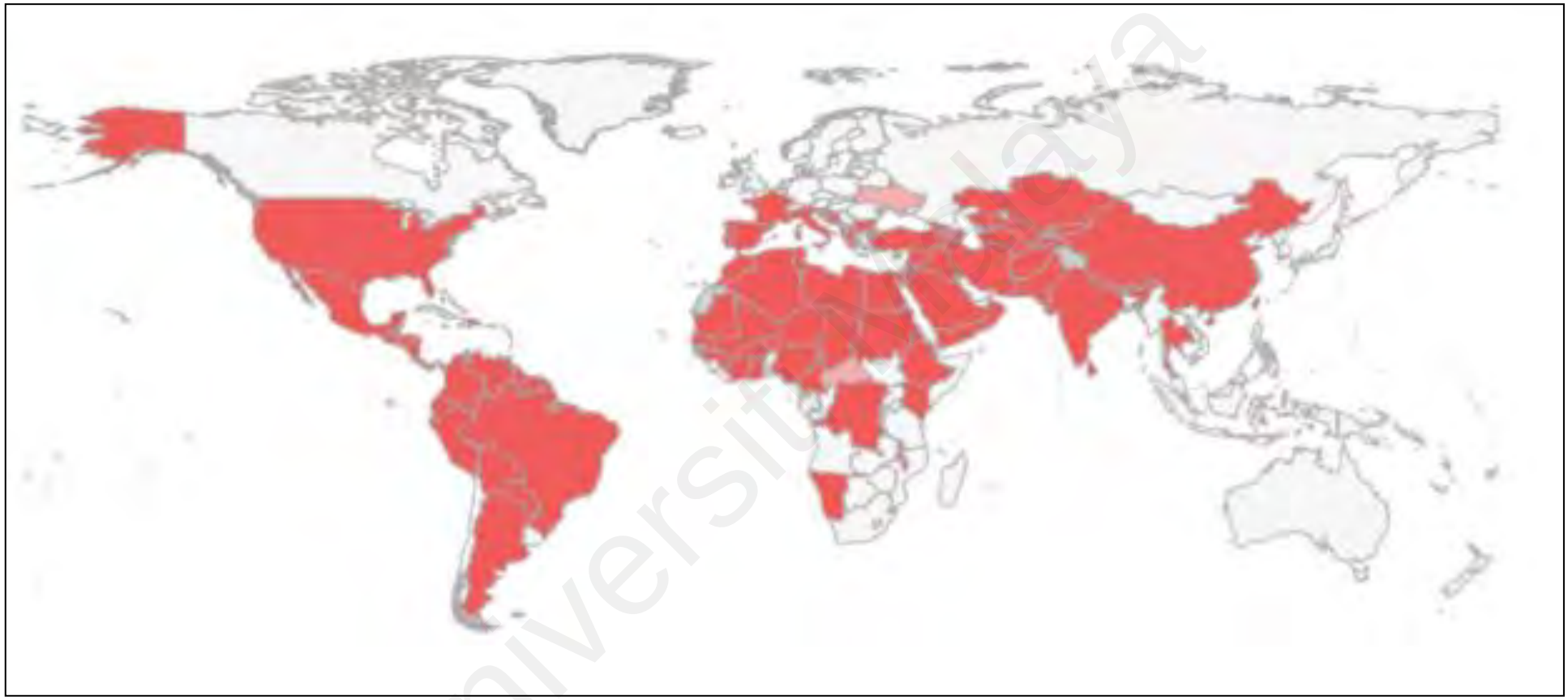

Figure 2.3: Status of endemicity of cutaneous leishmaniasis, 2022. *Source: WHO. (https://apps.who.int/neglected_diseases/ntddata/leishmaniasis/leishmaniasis.html)*

Based on the geographical distribution of the parasite species and vector's type, leishmaniasis is classified into two main groups: Old World and New World leishmaniasis (Akhoundi et al., 2016; Alvar et al., 2012a; Ghatee et al., 2020). The Old World leishmaniasis predominantly exists in the Eastern part of the globe and is found endemic mainly in Europe, Asian and African countries. The New World leishmaniasis is, on the contrary, found endemic to the Western hemisphere in the central and South Americas (except Chile and Uruguay). However, no indigenous cases are reported in Australia and the Pacific islands (Maurício, 2018).

The disease is responsible for public health and economic problems in the affected regions, with over one billion people at risk in the endemic areas. Besides, almost 1.5 to 2 million symptomatic children and adults (CL 600,000 - 1 million; VL 0.07 million) and 7,000 deaths have been reported annually (WHO, 2021). Moreover, Disability-adjusted life years (DALYs) lost due to leishmaniasis is nearly 2.4 million, and the highest number of DALYs was in the Middle East and North Africa region (MENA) and South Asia respectively (Scheufele et al., 2021).

2.8.2.2 Regional situation (WHO Middle East and North Africa, MENA, Region)

The Middle East and North Africa region is considered one of the most important leishmaniasis endemic areas globally. Both cutaneous and visceral forms are endemic in this region. According to WHO, The MENA includes the Eastern Mediterranean region and the Middle East and North Africa countries (WHO, 2021). However, no standardized list of countries is included in the MENA and Mediterranean regions. The term typically consists of Morocco in northwest Africa to Iran in southwest Asia, and down to Somalia in Africa. The following countries generally are included in the MENA region: Algeria, Bahrain, Djibouti, Egypt, Iran, Iraq, Jordan, Kuwait, Lebanon, Libya, Morocco, Oman, Qatar, Saudi Arabia, Sudan, Syria, Tunisia, Turkey, United

Arab Emirates, Palestinian territory, and Yemen (Tabbabi, 2019). Moreover, Pakistan, Afghanistan, Somalia and Ethiopia are sometimes included (Alvar et al., 2012a; McDowell et al., 2011).

According to the reports of the World Bank, the MENA region accounts for approximately 6% of the world's population, 60% of the world's oil reserves, and 45% of the world's natural gas reserves, making it one of the most attractive region for migrant workers. However, MENA stands out as the only region to experience an absolute decline in its data transparency index. In fact, since the beginning of the 21st century, the growth of output per capita across MENA has been lower than typical for economies with the same levels of development (WorldBank, 2020, 2021). However, the region is still one of the most vulnerable areas to civil conflicts and wars, and most of the world's refugees belong to this region. Although the region has been endemic for centuries, migration, Poverty, war, and the unhealthy environment, especially in refugee camps, are all essential risk factors associated with leishmaniasis (Al-Salem et al., 2016b; WHO, 2015a).

The MENA region accounts for 83% of all leishmaniasis cases worldwide. According to WHO, 18 of 22 countries are endemic for CL and VL. However, CL is more prevalent in the region (82%), and all the MENA countries are endemic except Qatar, Bahrain and United Arab of Emirates (UAE). In 2020, only 15 countries have reported cases, with 7 of them having high burden which are Afghanistan, Algeria, Iraq, Iran, Yemen, Pakistan and Syria. Algeria accounted for the majority of cases. A total of 150,805 new autochthonous CL cases were reported, while “high-burden” countries reported 132,177 cases (WHO, 2021). In addition, 349 out of 800 imported cases were reported in the region, with 32 imported cases recorded in high-burden countries. UAE recorded 42 imported cases, Saudi Arabia 32, Egypt 19, Qatar 15 and Kuwait 3 cases.

Regarding VL cases, a total of 3,709 (29%) new cases were reported in the region, and 3034 were reported from the 4 of high burden countries. These countries are Iraq, Somalia, Sudan and Yemen. Only two imported VL cases have been reported in the region, one in Qatar and the other in Djibouti. Furthermore, fatality VL cases were reported from Somalia (2.3%, 11/472), Djibouti (2/10, 20%) and Sudan (2.8%, 23/827). Post-Kala-Azar Dermal leishmaniasis (PKDL) also has been reported in the region, however, only 496 cases (6%) were reported in MENA region, mostly from Sudan (57 cases), South Sudan (4 cases) and Yemen (2 cases).

There are 5 *Leishmania* species reported in the MENA region (Bruschi & Gradoni, 2018), including *L. major*, *L. tropica*, *L. aethiopica*, *L. infantum* and *L.donovani* (Akhoundi et al., 2016). In addition, *L. killicki* was mostly circulated in Tunisia, Libya and Algeria, and no reservoir host or vector has so far been identified (Chaara et al., 2015; Du et al., 2016). However, *L. major* is currently the only clinical form endemic in Algeria (Beniklef et al., 2021). Although *L. aethiopica* is placed only in the Horn of Africa, especially in Ethiopia and Kenya (Gebre-Michael et al., 2004), it has been found in Rock hyrax in Saudi Arabia (Morsy et al., 1997; WHO, 2010b). In Saudi Arabia, although *L. major* is described as the main causative agent of CL in the country, *L. tropica* was identified in the southwestern regions (Al-Rashed et al., 2022). This region is considered highlands compared to the Central and East regions, where *L. major* is distributed (Abuzaid et al., 2017). In Morocco, *L. tropica* and *L. major* are both the causative agents of CL (Bennis et al., 2017a).

Regarding VL in the MENA region, the causative agents are *L. infantum*, *L. donovani* and occasionally *L. tropica*. *L. infantum* is distributed in the Mediterranean Basin and includes Syria, Iran, Lebanon, Palestine and North African countries bordering the Mediterranean, while *L. donovani* is distributed in East African countries

such as Sudan, Somalia and Djibouti. Saudi Arabia, Yemen and Egypt are among the countries where both species are present. In addition, human cases due to *L. infantum* were recorded in Yemen and Egypt. However, in Saudi Arabia, *L. infantum* cases are only reported in dogs. In contrast, CL cases due to *L. infantum* are mainly reported in VL endemic regions in Morocco (Baghad et al., 2020) and other countries of the MENA region, such as Tunisia, Iran, Syria, Palestine and Turkey. However, in Lebanon, *L. infantum* is only detected in CL cases (El Hajj et al., 2018).

The MENA region is characterized by a variety of sandfly species distributed in different areas representing their own environment. More than 50 Phlebotomus species were described in MENA region. However, only few species had been confirmed or suspected vectors of leishmaniasis in the region such as *P. sergenti* for *L. tropica* and *P. papatasi* for *L. major* (Akhoundi et al., 2016). Moreover, several animal species including dogs, rodents, hyraxes, jackals and foxes have been implicated as reservoirs in the region (al-Zahrani et al., 1989; Dantas-Torres et al., 2012; Lima et al., 2019). For instance, different rodents species were reported infected naturally with *Leishmania* spp from Tunisia (Bousslimi et al., 2012), Algeria (Chaara et al., 2014), Iran (Foroutan et al., 2017), and Morocco (Echchakery et al., 2017). However, no data are available from the east-central part of the Arabian Peninsula on leishmaniasis animal reservoirs.

According to WHO (2018) report, the MENA region faces many challenges to achieve leishmaniasis control, such as uncontrolled urbanization, limited funding, poor monitoring, emergencies, and crises that have led to deteriorating sanitation. Additional challenges include the water supply, collapse and destruction of health system infrastructure and internal displacement due to civil wars and internal conflicts in some countries of the region. These factors make the implementation of a comprehensive leishmaniasis control program difficult. Nevertheless, Saudi Arabia has achieved zero

cases of visceral leishmaniasis in 2019 through the National Leishmaniasis Research Program, which was established in 1982 to eliminate both types of leishmaniasis (Abuzaid et al., 2020).

Although Ethiopia doesn't share any borders with Yemen, many ties existed between the country and Yemen, mainly marine trade routes that extended to the Indian Ocean through Eritrea and Djibouti, in addition to the ancient human migration between the two countries. Ethiopia is highly endemic in leishmaniasis with CL being the most familiar form of leishmaniasis in Ethiopia since 1913, and is endemic in most regions, especially the Ethiopian highlands. The disease exists in three clinical types: localized CL, diffuse CL and mucocutaneous leishmaniasis caused mainly by *L. aethiopica* zoonotically (Assefa, 2018). The Rock hyrax‟s species of *Procavia capensis* and *Heterohyrax brucei* have been incriminated as the only known reservoir hosts of *L. aethiopica* (Aberra et al., 2019; Assefa, 2018), while the vectors include *P. longipes* and *P. pedifer* sandflies. Rare cases of *L. tropica* and *L. major* have also been reported in the lowland regions, with the *Arvicanthis niloticus* rodent as a reservoir host for *L. major* (Assefa, 2018). According to WHO (2021), Ethiopia is among the top 10 VL-endemic countries, with 66 VL death cases reported between 2019 to 2020. The essential VL endemic areas are the northwest (60% of the total cases) and the southwest (Abbasi et al., 2013; Ayehu et al., 2018).

Another country which is contributing to the spread of disease in the region is Somalia. The country has been listed by WHO as among the most VL endemic countries (WHO, 2021). The disease has been known in southern and northern Somalia since 1930 (Aalto et al., 2020). Nevertheless, geographical distribution and reservoir hosts remain unknown due to the lack of a surveillance system. *L. donovani* parasite‟s species and the *P. orientalis* and *P. martini* sandflies are responsible for the VL in

Somalia (Elnaiem et al., 2011). Before the civil war (ongoing since 1991), sporadic cases of VL were reported from several regions of Somalia (Marlet et al., 2003). An endemic source of kala-azar was first described by (Baruffa, 1965), then other cases were reported from hospitals in Mogadishu and Kismayo. The cases decreased during the war period, which lasted for more than twenty years. Presently, the number of new cases has increased during the past ten years. Between 2013 and 2015, more than 3110 cases were reported from 2013 to 2015, and 471 cases were recorded in 2020 (WHO, 2021). This increase in cases can be attributed to the security stability and the relative calm after the wars, which allow the cases to reach the medical facilities seeking for treatment. Interestingly, no CL cases have been identified in the country.

VL cases have been also recorded in Djibouti and Eritrea (Alamin, 2020; Pratlong et al., 2005). In Djibouti, nine autochthonous cases of VL and one imported case were reported in 2020, with two fatalities (WHO, 2021). But the exact location of the transmission regions is unknown. On the other hand, Eritrea recorded 1,034 VL cases in 2020 (WHO, 2020b). However, no data has been documented for CL cases in both countries (Alamin, 2020; WHO, 2020b).

2.8.2.3 Yemen situation

Yemen is highly endemic to leishmaniasis and more than 10 neglected tropical diseases (NTDs) such as dengue fever, filariasis, malaria, ascariasis, trichuriasis, fascioliasis, trachoma, schistosomiasis, onchocerciasis, chikungunya, and leprosy (WHO, 2020a). Besides, Yemen is the only country in the Middle East that is endemic for lymphatic filariasis and onchocerciasis (Hotez et al., 2012). Due to the miserable situation of the country as a result of the ongoing civil war (since 2015) and, more recently, the Covid-19 pandemic, epidemic diseases of cholera and diphtheria have begun to occur and expanded catastrophically (Al-Mekhlafi, 2018; El Bcheraoui et al., 2018).

The first report of leishmaniasis in Yemen was documented in 1933 by Sarnelli (1933), who reported 5 cases of mucocutaneous leishmaniasis in Sanaa city. The *Leishmania* parasites have been then detected and isolated in skin lesions of 18 patients in Taiz city by Rioux et al. (1989). They are identified as *L. tropica*. A few years later, a French patient who has returned from a short trip to Yemen has been reported with VL (Pratlong et al., 1995a). In 2016, Yemen was classified as one of 9 endemic countries that are considered the source for 80% of the world‟s new CL cases (WHO, 2020a). Tracking the distribution of reported CL cases in Yemen revealed that the entire country is endemic, except for the highlands above 2300 meters.

According to Al-Kamel (2015), the country‟s highest endemicity was reported in Al-Bayda governorate. Rural children and the women represent the highest vulnerable groups. The transmission of CL is almost constant in most of the endemic areas; however, it was increasingly reported in the last few years in Hajjah governorate (El Sawaf et al., 2016). Generally, CL exists in many governorates of the country including Amran, Sadah, Sana‟a, Al-Hudeidah, Taiz, Ibb, Mahweet, Raimah, and Al-Jouf in northern, western, and southern Yemen. The disease is mainly caused by *L. tropica* and *L. major* and the thought to be of both zoonotic and Anthroponotic types (Al-Kamel, 2015; Khatri et al., 2016; Khatri et al., 2009; Mahdy et al., 2010; Mogalli et al., 2016).

Four species of *Leishmania* have been reported in Yemen, *L. major*, *L. tropica*, *L. infantum*, and *L. donovani* (Khatri et al., 2016; Khatri et al., 2009; Mahdy et al., 2016; Mahdy et al., 2010). *L. tropica* is the predominant species that is frequently reported across the country (Mogalli et al., 2016) while *L. major* was commonly found in the southwest and northwest regions (Rioux et al., 1986). Moreover, *L. donovani* was reported to cause CL (Mahdy et al., 2016; Pratlong et al., 1995a). *L. donovani* and *L.*

infantum parasites were isolated from dry and wet skin lesions of Yemeni patients from northern governorates (Khatri et al., 2016; Khatri et al., 2009).

The first identification of the *Leishmania* vector in Yemen was in 1953 when Theodor identified nine species of the genus *Phlebotomus* around Taiz (Theodor, 1953). Later *Sergentomyia taizi* sandfly was identified (Lewis, 1974). In a study that aimed at determining the potential vectors for CL and VL in Taiz city, nine *Phlebotomus* species and nine *Sergentomyia* sandflies were identified with four *Phlebotomus* isolates were found responsible for *Leishmania* transmission in the area (Daoud et al., 1989). In the same vein, the first systematic entomological survey was conducted in north-western Yemen and identified 16 species of sandflies from Hajjah governorate (six species of *Phlebotomus* and ten species of *Sergentomyia*) (El Sawaf et al., 2016). However, none of them were found infected with *Leishmania* parasites.

Recently, cases of mucocutaneous leishmaniasis have been reported sporadically. For instance, an endonasal mucocutaneous leishmaniasis with necrotizing granulomatous inflammation was reported in a 34-year-old Yemeni male patient (Ibrahim et al., 2023). This case was treated successfully with intravenous antimonial stibogluconate. However, molecular analysis to identify the *Leishmania* species causing these cases was not performed.

A summary of findings from previous studies on cutaneous leishmaniasis in Yemen is shown in Table 2.3.

Table 2.3: A summary of previous studies on cutaneous leishmaniasis in Yemen

Study/ Reference	Year/period	Study design	Study area/ setting	Study subject	Diagnostic methods	Positive cases	*Leishmania* species	Remarks
(Khatri & Haider, 1999a)	1997-1998	Hospital-based	Hajjah and Amran	n=42 suspected cases; 1–65 years	Microscopy	35	NA	
(Khatri et al., 2006)	1997-2001	Hospital-based	Hajjah, Amran, Sadah, Sanaʺa	n=136 suspected cases; 1–65 years	Microscopy	128	*L. tropica*	Specis was identified by isoenzyme characterization
(Khatri et al., 2009)	2001-2008	Hospital-based	10 provinces; majority from Hajjah & Amran	n=265 Suspected cases; 1–80 years	Microscopy and PCR-RFLP	255 (155 PCR+ve)	133 *L. tropica,* 17 *L. infantum* 5 *L. donovani*	-
(Mahdy et al., 2010)	2008-2010	Hospital-based	Sanaʺa	n=35 suspected cases	Microscopy and PCR-RFLP	22 (17 PCR+ve)	*L. tropica*	-
(Al-Kamel, 2015)	2013	Hospital-based	Al Bayda 94.1% rural	n = 152 confirmed cases	Microscopy	152	NA	49.3% had MCL & 47.4% had CL
(Alharazi et al., 2016)	2014	Hospital-based	Taiz 79.3% rural	n = 100 suspected cases	Microscopy	87	NA	-
(Khatri et al., 2016)	1997-2012	Hospital-based	Hajjah, Amran, Sadah, Sanaʺa	n=1343 positive cases; 1–80 years	Microscopy and PCR-RFLP	1315 (576 PCR+ve)	529 *L. tropica,* *20 L. infantum* 11 *L. donovani*	-
(Mogalli et al., 2016)	2013-2014	Hospital-based	Hajah	n=143 positive cases; all age groups	Microscopy and PCR-ITS1	106	*L. tropica*	-

Table 2.3: Continued

Study/ Reference	Year/period	Study design	Study area/ setting	Study subject	Diagnostic methods	Positive cases	Leishmania species	Remarks
(Asmaa et al., 2017)	2012-2013	Community -based, cross-sectional	Rural Shara'b district, Taiz	n=525; 1–60 years	Microscopy	99	NA	-
(Al-Kamel, 2018)	2016	Case report	Raimah	A 70-year-old, male	Microscopy	1	NA	MCL
(Alkulaibi et al., 2019)	2015	Community -based, cross-sectional	Rural Utmah district, Dhamar	n=1165; all age groups	LST, microscopy	237	NA	215 had scars & 26 had active lesions
(Nassar et al., 2021)	2017-2018	Case control	Rural areas in Hajah	n=30; 1–50 years	Microscopy	30	NA	-
(Muthanna et al., 2022)	2010-2015	Hospital-based	Rural areas in Taiz, Ibb, Lahj	n=145; 1–60 years	Microscopy	145	NA	-
(Ibrahim et al., 2023)	2022	Case study	Ibb	A 34-year-old patient	Microscopy	1	NA	Nasal MCL with necrotizing granulomatous inflammation

CL: cutaneous leishmaniasis; MCL: mucocutaneous leishmaniasis; LST: leishmanin skin test.

2.9 Diagnosis of leishmaniasis

Medical causes of human leishmaniasis are often mostly diverse. Two major clinical types are widespread worldwide: VL, a life-threatening disease that results from the proliferation of *Leishmania* in macrophage-rich tissues, and CL, a mild but frequently disfiguring skin condition that appears to cure spontaneously. Some acute or chronic clinical forms can be less common or may become unrecognised. They comprise of major disorders or sequelae of major clinical types, including localised leishmanial lymphadenopathy, localised mucosal leishmaniasis (such as laryngeal or lingual leishmaniasis), MCL, diffuse and disseminated CL and post-kala-azar dermal leishmaniasis (PKDL). Thus, despite *Leishmania*'s precise clinical diagnosis, some microbiological conditions appear similar to *Leishmania,* such as pyoderma gangrenosum (Aguilar & Reigosa, 1994; Handler et al., 2015).

Depending on leishmaniasis' types, the disease can generally cause fever, weight loss, enlargement of the spleen (splenomegaly) and liver (hepatomegaly), leukopenia, anaemia or pancytopenia, diarrhoea, rash and skin ulcer. Both CL and MCL can lead to disfiguring scars and associated stigma (WHO, 2014a). Besides, leishmaniasis has multiple clinical symptoms that tend to be related to a wide range of other disorders such as those with a history of malignancy that may manage clinically as cutaneous or visceral leishmaniasis. In general, the diagnosis is problematic due to a wide variety of clinical symptoms associated with the disease. For example, ulcerative skin lesions at the site of the sandfly bite (localised cutaneous leishmaniasis); numerous nonulcerative nodules (diffuse cutaneous leishmaniasis); and disruptive mucosal inflammation (mucosal leishmaniasis). These significant symptoms can themselves deviate lead to further complicating the conclusive clinical diagnosis. CL lesions, for example, can vary in severity (e.g., lesion size), clinical presentation (e.g., open ulcer versus flat plate

versus wart-like lesions) and length (e.g., in time of evolution or in time of spontaneous cure).

Detecting amastigotes in lymph nodes, bone marrow, and other tissue samples remains the perfect standard for diagnosing VL in humans. However, according to some reports, due to the damage in acute VL patients‟ kidneys, urine can be an excellent standard for diagnosing acute VL, especially in children, as sometimes obtaining blood and tissue samples (spleen biopsy or bone marrow) is challenging (Asfaram et al., 2018).

In comparison, MCL causes destructive changes similar to syphilis, yaws, rhinoscleroma, skin cancer and oral squamous cell carcinoma. However, unlike syphilis and yaws, MCL does not induce cartilage damage, and unlike rhinoscleroma, leishmaniasis does not cause nasal septum perforation (Handler et al., 2015). In addition, nasal swabs can be used to detect MCL and CL by PCR in endemic regions (Oliveira et al., 2022). Modern diagnostics are increasingly used to diagnose asymptomatic human infections, however, their epidemiological function in various endemic settings has not been elucidated (Maurício, 2018).

2.9.1 Microscopic examination

The light microscope is still considered to be the most effective tool used for parasitical diagnosis in endemic areas with leishmaniasis, particularly in poor and developing countries, due to its simplicity and availability that is relatively inexpensive (Goto & Lindoso, 2010). The Giemsa or Leishman stain is used for dyeing the samples; this process is cheap and straightforward and does not take a long time to obtain the result. Samples to be examined microscopically may be obtained as skin lesion scraping or biopsy or lymph nodes, spleen and bone marrow aspiration.

CL diagnosis depends on a small skin biopsy, or a smear was taken from the skin lesions then put on a glass slide (de Vries et al., 2015). This process is considered a painless procedure compared to the bone marrow samples or spleen biopsies to diagnose the VL. What is essential in the microscopic examination process to confirm the results is the technical expertise in collecting, preparing and examining the sample to obtain good results, making the microscopic examination less efficient and less sensitive than the other sophisticated techniques as polymerized chain reaction (PCR) amplification and DNA sequencing.

2.9.2 Parasite cultivation

At the beginning of the 19th century, artificial culture media started to be used by Robert Koch, who is the father of culture. Culture media use started at the beginning of growing bacteria outside the host. Many fungi species and other microbes were succeeded in growing in culture media. Nowadays, it is possible to cultivate most parasites, including *Leishmania* ones. One of the most common culture media in the 20th century to cultivate *Leishmania* parasites is the Novy, MacNeal-Nicolle medium (NNN) (Boggild et al., 2008). The biphasic medium contains a liquid and a solid phase comprising unique components like brain and heart infusions and the defibrinated rabbit blood.

To culture cutaneous form of leishmaniasis, the samples are taken using the fine-needle aspiration technique. The promastigote culture is incubated at 26° C to recover large numbers of parasites which is up to 10^{10} cells/ml. The key component in these circles is the rabbit blood, favoured over other blood types. However, currently, they adopt the use of AB negative blood group instead (de Vries et al., 2015).

Leishmania promastigotes can be also grown easily in various culture media such as RPMI 1640, Schneider, Grace and M199 medium, supplemented with Foetal

calf serum (FCS) at 26º C, but they are considered more expensive than NNN medium. However, it is necessary for some *Leishmania* stocks or WHO reference strains to use USMARU medium with PBSS plus antibiotics to recover and the promastigotes yields before transferring into the liquid medium such as RPMI or Schneider (Elmahallawy et al., 2014). Although culture has greater sensitivity than that of the light microscopy method, it is rarely used for leishmaniasis diagnosis. This is due to the requirement of technical expertise, expensive equipment, as well as time-consuming cultivation process (de Vries & Schallig, 2022). However, both microscopy and culture examinations have low sensitivity in diagnosing chronic stage of the disease due to low parasites count (Reithinger et al., 2007).

2.9.3 Serological examination

The majority of leishmaniasis serological tests are only suitable for VL, especially the acute type, due to higher levels of humoral immune response induced by VL parasites than those by CL. This differentiation in the antibody levels occurs due to the immune system's differentiation of reaction to different types of leishmaniasis. The most commonly sereolgical tests used for leishmaniasis diagnosis are: enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test (IFAT), western blot, direct agglutination test (DAT), immunochromatographic assay(IC) and leishmanin skin test(LST) (Akhoundi et al., 2017; Elmahallawy et al., 2014).

ELISA is among the most sensitive and specific tests, especially for diagnosing visceral leishmaniasis (Elmahallawy et al., 2014; Erber et al., 2022). However, the specificity depends upon the antigen used. For a few years, a particular recombinant antigen called rK39, which is part of a kinesin-related gene that contains 39 repetition amino acid residues, has been developed and is being widely used for the leishmaniasis diagnosis (Maurício, 2018). In addition, it was more sensitive and specific to human VL

(a lesser extent to canine VL) in India (98%) than in other countries(Elmahallawy et al., 2014). This antigen is higher sensitivity and specificity in HIV co-infection patients (Sundar & Rai, 2002; Sundar et al., 2006). Another antigen is rKE16 that showed the same sensitivity as rK39 in China, Pakistan and Turkey. In contrast, K28 is higher sensitivity in Sudan (96%) and Bangladesh (98%) compared with rK39(Elmahallawy et al., 2014).

IFAT is more sensitive to canine VL and is recommended by the World Organization for Animal Health (OIE). Unlike ELISA, this technique needs a laboratory with a fluorescence microscope. Besides, cross-reactivity with other trypanosomiasis sera was reported (Elmahallawy et al., 2014). Despite that, RDTs have been mainly developed for VL. In addition, few tests have also been developed for CL, such as CL Detect Rapid test (DRT), which was initially designed to detect *L.major* and used for detecting CL species in skin samples in Tunisia, Morocco, Afghanistan, Ethiopia and Suriname (de Vries & Schallig, 2022).

The other alternative is the Western blots analysis to identify the anti-leishmanial antibodies in response to *Leishmania* antigens. In this technique, they use different antibodies that can distinguish several antigenic products in a single assay. In India, the rk39 dipstick test has been used successfully to diagnose VL and PKDL with a sensitivity average of 95-100 % (Salotra et al., 2001; Singh et al., 2010; Sundar et al., 2007).

Compared with other serological tests, the DAT is a simple and low-cost test that can be used in fieldwork and for screening patient sera in the laboratory. The method is based on stained promastigotes' agglutination either as a suspension or freeze-dried form with *Leishmania*-specific antibodies. Furthermore, the test's sensitivity is almost 100 %, and the specificity for leishmaniasis is equally high except for countries

with Chagas disease (The New World countries), where cross-reactivity with *Trypanosoma cruzi* is commonly observed (Ali, 2009).

The Leishmanin Skin Test (LST) also known as the Montenegro test or Montenegro skin test, is the most important serological test for CL and MCL in humans and animals (Carstens-Kass et al., 2021). At the same time, this method is also used for VL diagnosis after treatment and cure (Sundar et al., 2006). This test is a diagnostic aid focused on eliciting delayed hypersensitivity reactions following exposure to *Leishmania* antigens (killed promastigotes) administered intradermally into the patient's forearm and assessed induration after 48-72 hours (Krolewiecki et al., 2017). However, this method and its reagents are not approved for use in the United States and Canada (Aronson et al., 2016). In addition, this method is very effective and is less sensitive than the microscopic examination. As a result, LST gives positive denotes for present or past leishmaniasis infections, and it is strongly positive when the lesions of cutaneous and mucosal leishmaniasis are present. However, the test may appear negative in active VL. Moreover, although *Leishmania* shares common antigens with mycobacteria and trypanosomes, the LST was found negative in pulmonary tuberculosis, leprosy, African trypanosomiasis or Chagas‟ disease. Besides, occasional false-positive reactions have been reported in patients with glandular tuberculosis and systemic fungal infections (Carstens-Kass et al., 2021).

2.9.4 Molecular diagnosis

Recent advancements in molecular biology have produced alternative approaches for diagnosing and profiling *Leishmania*. The polymerase chain reaction (PCR) has evolved as one of the most specific and sensitive method for detecting *Leishmania* DNA (Reithinger & Dujardin, 2006). It is considered a qualitative and quantitative approach to avoid traditional methods' drawbacks.

Several PCR protocols have been developed to detect *Leishmania* DNA in clinical samples. These protocols can differ in sensitivity and specificity depending on the *Leishmania* species, type of samples, the protocol of DNA extraction, the choice of sequences target of *Leishmania* genome, PCR methodology, type of primers used and sample storage. The most common methods used to detect *Leishmania* species are either on the conventional PCR or real-time PCR. Recently, the PCR methods helps in identifying the *Leishmania* parasites at species level through either species-specific amplification, restriction fragment length polymorphism (RFLP_PCR) using restriction enzymes or DNA sequencing (Salotra et al., 2001; Schönian et al., 2000; Schönian et al., 2003).

Typically screening genes are either ribosomal genes or mitochondrial genes, mostly because these genes are quite conserved within the species. Mitochondrial genes can also increase sensitivity because often times mitochondrial genes have multiple copy numbers. However, the highest sensitivity is achieved when these genes are targeted in real-time PCR. On the other hand, *ITS-1* and hsp70 are considered the best targets for *Leishmania* species identification in both the Old and New World (Maurício, 2018).

2.10 Treatment of leishmaniasis

Unlike fatal VL, which requires urgent treatment, CL can disappear spontaneously even without treatment, but this can take an extended period, from six months to two years (Maurício, 2018). Medical treatment with ointments, pills, or injections can shorten the disease's duration and reduce the size of the scars (Pradhan et al., 2022). However, cases of multiple lesions and lesions in the facial area almost always require treatment and those that cause skin disfigurement may require cosmetic procedures. Antiparasitic pentavalent antimonial agents such as sodium stibogluconate and meglumine

antimoniate are commonly used for CL treatment. In addition, oral miltefosine, amphotericin B, pentamidine and antibiotics, such as paromomycin ointment (Madusanka et al., 2022). For chronic CL and MCL cases, amphotericin B and paromomycin are often used to treat them (Pradhan et al., 2022).

Regarding VL treatment, sodium stibogluconate, amphotericin B, paromomycin, and miltefosine are the most commonly used drugs for the treatment of VL (Maurício, 2018). Currently, combination therapies are considered the best regimens for treating VL in many parts of the world as dosing and duration of therapy are decreased, thereby decreasing toxicity, costs and drug resistance. In general, the drug selection must be based on the *Leishmania* species involved, the immune situation of the patient and the prevalence of therapeutic failure rates in the geographic area of acquisition.

Sodium stibogluconate is a widely used antibiotic for leishmaniasis diseases. It is only available for administration by intravenous injection, intramuscular injection or intralesional injection (injection directly on the lesions). It has been the drug of choice for the treatment of CL and MCL in Yemen (Al-Ghazaly & Al-Dubai, 2016), the United States and other countries. This agent is also effective against VL and is often the first-line treatment in developed countries. It is also being investigated as an anti-tumour agent for cancer and solid tumours (Maurício, 2018).

There are other treatment methods than chemotherapy that can be used in treating CL, such as thermotherapy, cryotherapy and photodynamic therapy (Azim et al., 2021; Pradhan et al., 2022; Varzandeh et al., 2021). In recent years, dermal doctors have shown an increase in thermotherapy interest, which is an effective and safe treatment, but they are expensive compared to chemotherapy. However, treatment using heat is an ancient method, and some poor societies still practice primitive and harmful techniques. In this socities using caustic materials (powder, hot brown sugar, silver

nitrate, oil, battery) or ironing the lesions with hot metal objects (spoons and knives) are very common (Maurício, 2018).

2.11 Vaccine for leishmaniasis

The high toxicity of anti-leishmanial drugs, their side effects, the increase in relapse cases and the increasing cases of drug resistance put the world in front of a real dilemma. Hence, alternative drugs which provide long-term immune protection against disease, such as vaccines, are required urgently (Kedzierski, 2010). Unfortunately, there are still no effective vaccines against different types of human leishmaniasis to date, even with continuous attempts to develop a successful vaccine (Maurício, 2018; Volpedo et al., 2022a; Volpedo et al., 2021). The problem lies in the inability to make an effective vaccine because the parasite lives in several organisms, including humans, sandflies, and other animals (Maurício, 2018). Therefore, manufacturing a specific vaccine for human only is a difficult task because it will not completely eliminate the parasite and will not prevent its reproduction in insects or other animals. There is also a fundamental lack of understanding of the parasite's life cycle inside human cells and how the vaccine should generate and maintain an immunological memory during parasitic infection (Ghorbani & Farhoudi, 2018).

The first-generation vaccines for leishmaniasis appeared in the year 1940. Their action method depends on leishmanization, a practice of inoculation where a whole killed or live-attenuated parasite is injected into the skin and develops into a small pimple that self-heals after a short period (Pacheco-Fernandez et al., 2021; Volpedo et al., 2021). The Leishmanization method was well-known in some endemic countries especially Middle East countries and used before the vaccines developed (Pacheco-Fernandez et al., 2021). These vaccines were widely used in Palestinian-occupied territories and Russia until 1980 when large-scale clinical trials showed that this

practice has side effects that led to long-term skin lesions, exacerbation of psoriasis and immunosuppression in some people (Handman, 2001; Palatnik-de-Sousa, 2008). However, over two million people in Iran were vaccinated by leishmanization vaccines during the Iraq-Iran war (Azizi et al., 2016), and until the year 2006, these vaccines were still licensed for use in Uzbekistan (Gillespie et al., 2016). Moreover, small clinical trials continued with extensive clinical trials in Ecuador, Brazil, and Iran through the 1990s and 2000 (Handman, 2001).

Second generation vaccines focus on pathogen antigens including purified native protein fractions, synthetic or recombinant subunits, and recombinant viruses or bacteria possessing antigen DNA (Volpedo et al., 2021). Even though only a few second-generation vaccines have been successfully tested in experimental animals and clinical trials. However, due to vaccines' inability to interpret data in animal models for human application, as well as their inability to transfer knowledge from laboratory settings to field practice effectively, none of these vaccines were able to reach the market (Maurício, 2018). In March 2022, a development of a vaccine based on Th2 cytokines induction decreasing using CRISPR-Cas9 technology was published, and showed a complete inhibition of the typical cutaneous lesions in mouse models (Volpedo et al., 2022b).

In contrast to human vaccines, dog vaccines have proven to be effective in providing immunity (Moafi et al., 2019). The LeishTech vaccine is one of the most important vaccines used in Brazil. This vaccine is based on a recombinant protein (Bowman, 2014). In addition, another vaccine licenced in Europe is the CaniLeish vaccine, which contains antigens from *L.infantum* (Bowman, 2014). Recently, efforts have been made to approve newly developed vaccines for dogs that are still being tested (Ghorbani & Farhoudi, 2018).

2.12 Prevention and control of leishmaniasis

Civil unrest, forced migration, climate changes and famine are still the main reasons to lead to large epidemics in endemic countries (Inci et al., 2015; Maroli et al., 2013; Valero & Uriarte, 2020). Therefore, the elimination of these epidemics needs security stability and food security and subsequently, the control of the insect vector and reservoir hosts become more achievable. In addition, the increase in HIV-VL coinfection cases and the emergence of asymptomatic patients represent a further challenge for zero case achievement (Burza et al., 2018; WHO, 2015b, 2015c). Leishmaniasis prevention programs often include the provision of early diagnosis and treatment, elimination of the insect vector by spraying insecticides in their breeding places, culling infected dogs and rodents, dogs vaccination, health education messages and awareness programs (Lun et al., 2015; Makau-Barasa et al., 2022; Maurício, 2018; Zhao et al., 2016). Moreover, in Europe, insecticide-treated collars and spot-on insecticides are commonly used to protect individual dogs against sandfly bites in endemic areas (Berriatua et al., 2021; Maia & Cardoso, 2015).

Many countries have yet to succeed in completely eliminating leishmaniasis. Even though they are following a comprehensive or partial control program, some of the challenges mentioned above have impeded their success (Maurício, 2018). To date, Yemen has no leishmaniasis comprehensive control program. Although the treatment was provided free of charge, the diagnosis is still expensive, and most infected people cannot afford it. Furthermore, the lacks of awareness in affected areas, as well as the lack of diagnostic supplies and skills, pose significant challenges to control the disease.

On the other hand, since 2005, India, Nepal and Bangladesh have achieved a successful elimination program against kala-azar in South-East Asia. India and Bangladesh have reduced the number of reported cases by 67% and 60%, respectively,

while only 46% in Nepal. Another successful leishmaniasis control program was in China, where a nationwide control campaign had done against VL between 1950 and 1958. This program could potentially bring the disease under control in the plains where the anthroponotic form had previously reigned. However, transmission could not be stopped in the mountainous and desert regions, where sporadic cases of zoonotic transmission cases continue to appear intermittently to this day (Lun et al., 2015).

Similarly, Saudi Arabia reached zero cases of VL in 2019 after long years of the control program that extended over thirty years (Abuzaid et al., 2020). The success in these countries could be attributed to the AVL control programs focused on eliminating the insect vector in the endemic areas where the reservoir hosts are absent (Zhao et al., 2016). Contrariwise, other countries such as Brazil where dogs are the main source of infection have not been able to control it. In addition, the diversity of sandfly species, *Leishmania* species, vertebrate reservoirs, and ecological niches have been challenges for zoonotic CL and zoonotic VL control interventions compared with anthropnotic VL (Maurício, 2018; Rangel & Shaw, 2018).

CHAPTER 3

METHODOLOGY

3.1 Yemen profile

Yemen, officially known as the Republic of Yemen, is located in Western Asia, in the southern end of the Arabian Peninsula between longitudes 42 and 55 °E and latitudes 12 and 19 °N. It is the second biggest country in the peninsula, after Saudi Arabia, covering 530,000 km^2, with a total population of 33.70 million (WB, 2022). It is bordered by the Red Sea to the West, Oman to the East, the Gulf of Aden and the Arabian Sea to the South, and Saudi Arabia to the North (Figure 3.1). It has more than 200 islands; with Socotra Island in the Indian Ocean, and Kamaran Island in the Red Sea are the most populated. Yemen is consists of twenty governorates (called *muhafazat*). The governorates are subdivided into 333 districts (called *muderiah*), which are subdivided into 2,210 sub-districts. The capital city is Sana‟a (administratively known as Amanat Al-Asemah) and is located in the Sana‟a governorate northern the country.

Geographically, Yemen is divided into five main regions: the eastern highlands, the western highlands, the central highlands, the sand desert (known as al-Rub' al-Khali) in the east, and the coastal plains in the west. Accordingly, the climate in Yemen differs depending on the region. In the highland areas, the climate is moderate in summer and cold in winter with average rainfall of 800 mm/year, and annual temperature of 20 ºC and a relative humidity of between 20% and 50%. Whereas, the coastal plain areas that extend for approximately 2,000 km, receive much lower rainfall (average of 200 mm/year) during rainy season that extends from February to April and from July to September. The climate is a combination of tropical monsoon with occasional rains in the summer and dry weather in winter. The average temperature is 37.5 ºC in summer and 24 ºC in winter with a relative humidity ranging between 70% and 90%. On the

other hand, the desert region has a "hyper-arid" climate, with annual rainfall of less than 50 mm/year, and a relative humidity ranging between 15% in June–July and 52% in January. The daily minimum average temperature is 12 °C in January and February, and the maximum average temperature is 47 °C in July and August). Generally, the desert region is populated only by few groups of Bedouin people.

Economically, Yemen is considered as one of the least developed countries in the world, and ranked 158 out of 175 countries by the United Nation‟s Least Developed Countries 2019 Report (UN, 2019). According to the World Bank, over 60% of Yemen‟s population lives on less than USD2 a day (WB, 2010). Due to the ongoing civil war that started in 2015, Yemen has been designated as one of the world‟s worst humanitarian crises. In view of that, less than 50% of the country‟s health facilities are fully or partially functional, more than 3.1 million people internally displaced, and over 80% of the population faces significant challenges in reaching food, drinking water and access to health care services (WB, 2021). Indeed, these conditions put the entire population at great risk of many infectious diseases, and deadly outbreaks of cholera, diphtheria, dengue, malaria and leishmaniasis have been occurred since 2015 (Al-Mekhlafi, 2018; Alghazali et al., 2019; Badell et al., 2021).

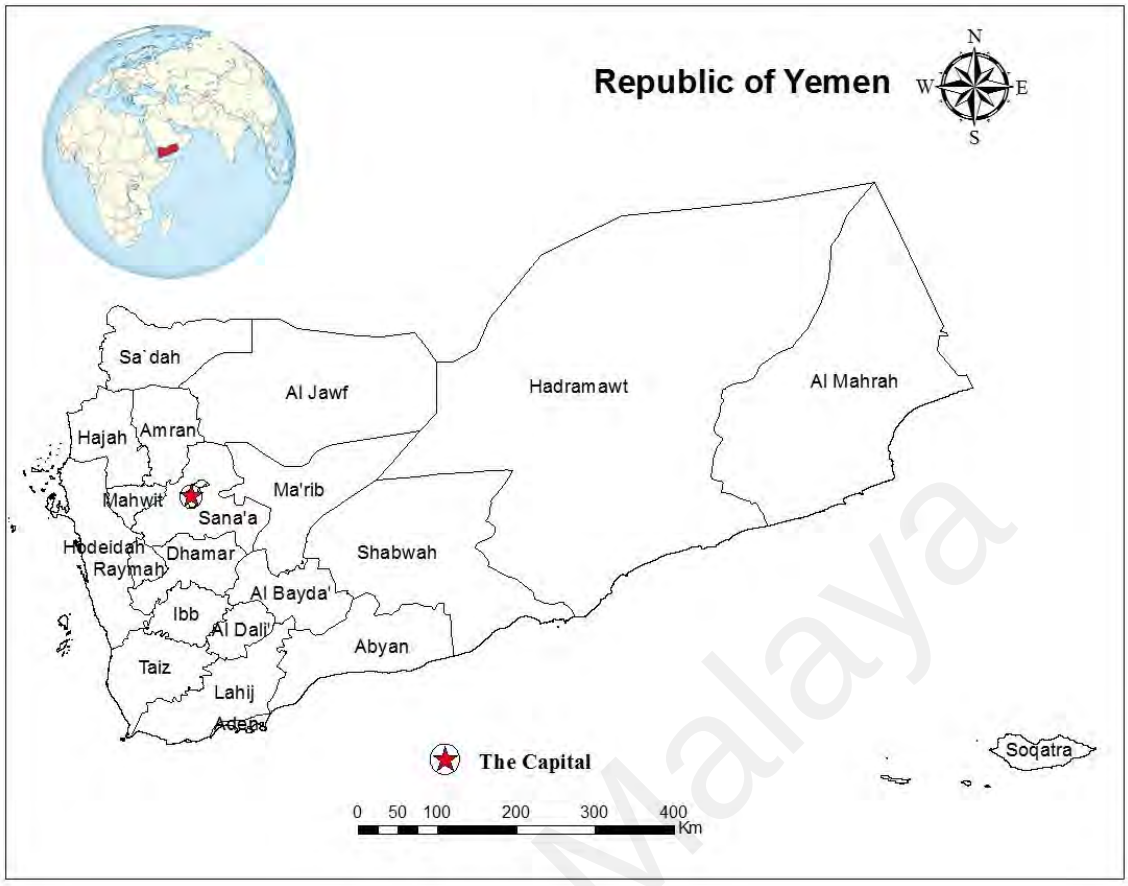

Figure 3.1: A geographic map of the Republic of Yemen.

The map was created using the Esri ArcGIS 10.7 software.

3.2 Study design and settings

A community-based cross-sectional study with unmatched case-control comparisons was carried out between January 2017 and May 2019 among rural communities in the Utmah district of Dhamar governorate, Yemen. This study involved four components, a questionnaire survey, physical examination, parasitological examination and genetic diversity analysis. The study was undertaken in three phases: 1) a cross-sectional household survey in order to determine the true prevalence of CL in the study area and the participants‟ knowledge, attitude and practices (KAP) towards CL; 2) a case-control analysis to identify the potential risk factors of the disease; and 3) a broad-scale analysis of genetic diversity for *L. tropica* based on the internal transcribed spacer -1 (*ITS-1*) gene across many endemic countries. The active case detection of suspected CL cases was conducted house-to-house in the targeted communities. Figure 3.2 shows the study flow chart.

All household members available during the survey period were assessed for CL infection status; however, for logistical reasons, only one participant from each household was selected for questionnaire administration and/or skin-scraping sampling. One individual with active skin lesions suspected to be CL was purposively selected from each household, where identified. In case a household has more than one individual with active skin lesions, a simple random sampling by lottery method was used to select one case for skin scrapping sampling and questionnaire survey. For households with no active skin lesion cases, one individual was randomly selected for questionnaire administration and was allocated to the control group if eligible.

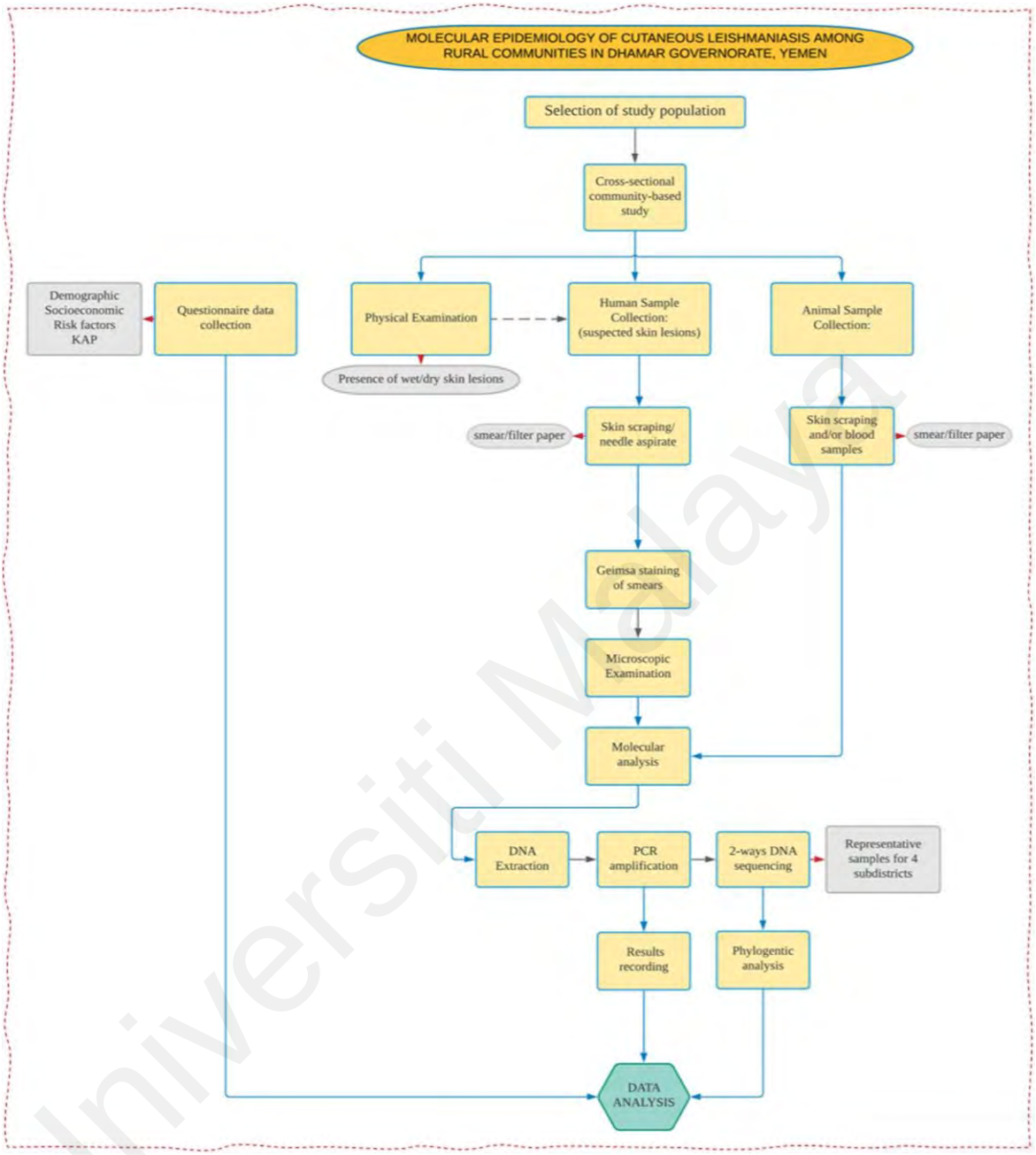

Figure 3.2: A flow chart outlines the steps and phases involved in the study.

3.3 Ethical considerations

The protocol of this study was approved by the Medical Ethics Committee of the Universiti Malaya Medical Centre, Kuala Lumpur, Malaysia (Ref. No. 201411-805) (Appendix A). Moreover, the protocol and involvement of animals including capturing and sampling were also approved by the Medical Ethics Committee of Thamar University, Yemen (Ref. No. TUMEC-17018) (Appendix B). Before the commencement of data collection, the household residents were informed about the project‟s nature, objectives and procedures. Written and signed or thumb-printed informed consent was obtained from adult participants, while the consent of children below 18 years of age to participate in this study was obtained from their parents or guardians (Appendix C). Permission was obtained from the owner of the domestic animals prior to the collection of samples from the animals.

3.4 Study area

Dhamar governorate is located about 100 km to the south of Sana‟a, the capital city. It is divided into 12 administrative districts, including Dhamar City. Utmah district is located between longitude 43.95 °E and latitude 14.66 °N, 151 km southwest of Sana‟a and about 60 km west of Dhamar City (Figure 3.3). The district is divided into five sub-districts called "*Mekhlaf*" and comprises 57 areas called "*Uzlat*", with a total land area of 460 km^2 and a total population of about 145,000 as reported by the National Census of 2004. Most of the population in Utmah, like the majority of people in Dhamar and other rural areas of Yemen, are working in farming and animal husbandry. Livestock is firmly integrated with the farming structure in Utmah; almost every farmer has animals on his farm as a source of food, cash, traction power, or means of transportation. However, people from the suburban/urban region of Utmah are involved in administration, trading, and small business.

Topographically, the Utmah district is a highland area representing the natural extension of the Sarawat mountain range at elevations ranging from 920 to 2800 meters above sea level (USAID, 2013). The climate varies between wet and arid, with cold winters and warm summers and an annual rainfall of approximately 750–800 mm, and a mean annual temperature of approximately 22°C.

The district is characterised by natural forests, woodlands, agroforestry and rangelands biodiversity that provide essential habitats and refuge for several wild mammal species such as wolves, hyenas, foxes, hares, hyraxes and hedgehogs (USAID, 2013). In the remote corners of this area, the Hamadryas baboon is still raiding sorghum and other crops and is therefore hunted. The mountain gazelle and the dorcas gazelle are not yet completely extinct but exist in only very small numbers. In the remote corners of this area, the hamadryas baboon is still raiding sorghum and other crops and is therefore hunted. The wild ibex, mountain gazelle and the dorcas gazelle are still exist in small numbers. Moreover, the Arabian hare, Asiatic jackal and foxes are the species mentioned as regularly observed. Indeed, very little ornithological and entomological data have been collected in this part of the country.

Moreover, it is rich in farming (mountainous terraced agriculture), and farming and livestock breeding represent the main sources of livelihood for the population (Appendix D). The district is famous for its coffee bean and cereals production (e.g. maize, sorghum, millet, wheat and barley). Utmah is extensively forested, especially by the wild olive (African Olive), *Olea europaea subspecies*, which is also known locally as the Utam tree (Dark tree). In 1999, the Utmah district was designated as a protected area (Utmah Protectorate) because of its relic tropical forests and wildlife ecosystems. Nonetheless, the area has been exposed to deforestation as a result of the population increase. Trees and the green cover are used for firewood and as rangeland for livestock.

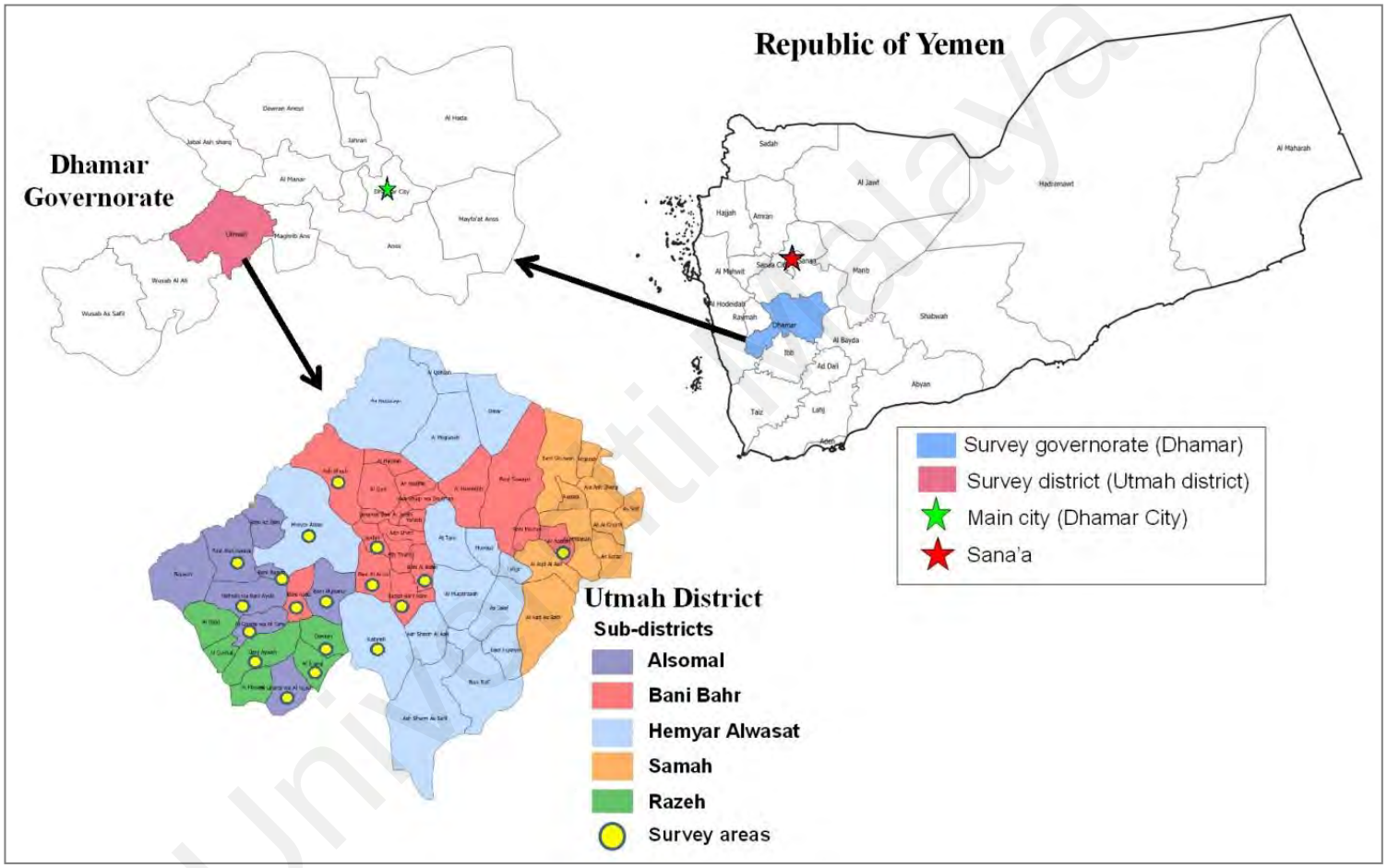

Figure 3.3: A geographic map showing the location of Utmah district (and the survey sub-districts and areas involved in the study), Dhamar governorate, western Yemen.

The Utmah district was selected for this study because of its endemicity to many infectious diseases, such as malaria, leishmaniasis, onchocerciasis, and schistosomiasis. (Alkulaibi et al., 2019; MOPHP, 2019; Sady et al., 2013). The hardship situation in Yemen, particularly in rural areas, has been influenced by the ongoing armed conflict that started in 2015 and spread throughout the country. As a result, more than 80% of the country‟s population is at risk of hunger; about 4.5 million people have been internally displaced, and only 50% of healthcare facilities are functional (Alsabri et al., 2022). These issues have been exacerbated by multiple and overlapping spreads of infectious diseases such as cholera, diphtheria, and COVID-19 (Al-Mekhlafi, 2018; Badell et al., 2021).

The present survey was conducted in 18 areas (*Uzlat*) that represent four sub-districts; Alsomal, Bani Bahr, Hemyar Alwasat and Razeh (Figure 3.3). The selected areas were Bani Abdulsamad, Bani Asad, Bani Algharib, Halma waBani Ayoub, Bani Aywah, Alsharqi, Hemyar Abzar, Alshaoob, Bani Buaith, Kabirah, Bani Albahri, Rabiaat Bani Bahr, Alrabiaah, Bani Alaras, Al Gharbi wa AtTafn, Al Gharbi wa Alfajrah, Jawfah, and Dawrah. The selection process was guided by the sub-districts‟ administrative and health officials based on the following criteria: 1) accessibility, 2) security and 3) high incidence of CL. Due to armed conflicts during the study period, Samah, the fifth sub-district with 10 areas, was not included (UNHCR, 2017). The required number of households from each selected area was calculated using a probability proportional to size approach based on an estimate provided by district officials of the total number of households. In each area, eligible households were selected by simple random sampling using a sorted list of households or a map compiled by local administrators.

3.5 Study population and sample size

3.5.1 Sample size estimation

The minimum sample size required for this study was calculated according to the practical manual for sample size determination in health studies as defined by the World Health Organisation (WHO) (Lwanga & Lemeshow, 1991) based on a) a 95% confidence level at a desired margin of 5%; b) the prevalence of people with a good level of knowledge about CL (Alharazi et al., 2021); and c) the average prevalence of infections in the Utmah district and neighbouring governorates in Yemen (Alkulaibi et al., 2019; Asmaa et al., 2017). Accordingly, 267 households/subjects were determined to be the minimum sample size required to achieve the objectives of this study. Overall, out of 320 households, 289 (90.3%) were included in the study, with 31 household heads refusing to participate. All individuals available in the households during the survey visits were screened for the presence of active lesions and/or scars of healed ulcers in the first phase of the study (universal sampling); 612 individuals were involved (This contributed towards the achievement of objective 1).

For the case-control comparison, the sample size was calculated by using the Kelsey formula for case-control studies (Kelsey et al., 1996) via an online calculator (http://www.openepi.com/SampleSize/SSCC.htm). At a confidence level of 95%, a power of 80% and a case-to-control ratio of 1:3, 61% of the control subjects were living in cracked-wall houses with an odds ratio of 3.0 (taken from the questionnaire pilot study and a previous study) (Nassar et al., 2021), the minimum sample size required was 204 (51 cases and 153 cases). This contributed towards the achievement of objective 2.

For the KAP part of this study, to ensure that the participants were the main decision-making individuals within the household, the study participants were preferably the household heads if available during the time of the home visit; otherwise, wives of heads of households were invited to participate in the survey (This contributed towards the achievement of objective 3).

3.5.2 Inclusion and exclusion criteria

A confirmed case of CL was defined as "a person showing clinical signs (skin or mucosal lesions) with parasitological confirmation of the diagnosis (positive smear or culture)", following the WHO"s operational definition (WHO, 2014b). Nevertheless, molecular methods were employed in this study instead of culture. Upon obtaining written informed consent, all individuals who had active lesions of CL confirmed by parasitological and/or molecular methods were included in the case group and individuals who had never been infected with CL were included in the control group. On the other hand, individuals who had suspected CL active lesions but screened negative for *Leishmania* parasites, CL remnant scar, critically ill, and those who refused to be screened were excluded from the case-control study.

3.5.3 Study population

Out of 320 households, 289 (90.3%) were included in the survey, while the heads of 31 households declined to participate. At the participant level, 82 individuals (mostly adult females) refused to participate and were, therefore, replaced by other subjects from the respective households.

For reporting the prevalence, distribution (according to age, sex and location) and clinical features of CL cases, 612 out of 685 individuals who were available during the study visits were involved. However, as only one participant from each household was interviewed, 289 participants were available for the unmatched case-control

comparisons; these included 53 laboratory-confirmed cases and 170 participants served as a control group. A total of 66 participants were excluded from the case-control comparisons (28 with active lesions but were not confirmed by laboratory examination and 38 individuals with scars of healed lesions indicating a history of past infections).

3.5.4 Animal sampling

With regards to animal samples, this was considered a pilot study based on convenience sample of different vertebrates available in the study area, considering households with active CL cases. Accordingly, animals were included for this study from seven areas, namely Halmah WaBni Ayoub, Bani Asad, Bani Alaras, Bani Aywah, Alshaoob, Dawrah and Hemyar Abzar (Figure 3.4). All domestic animals in the selected houses and some other wild animals in the surroundings were targeted for sample collection (Appendix E).

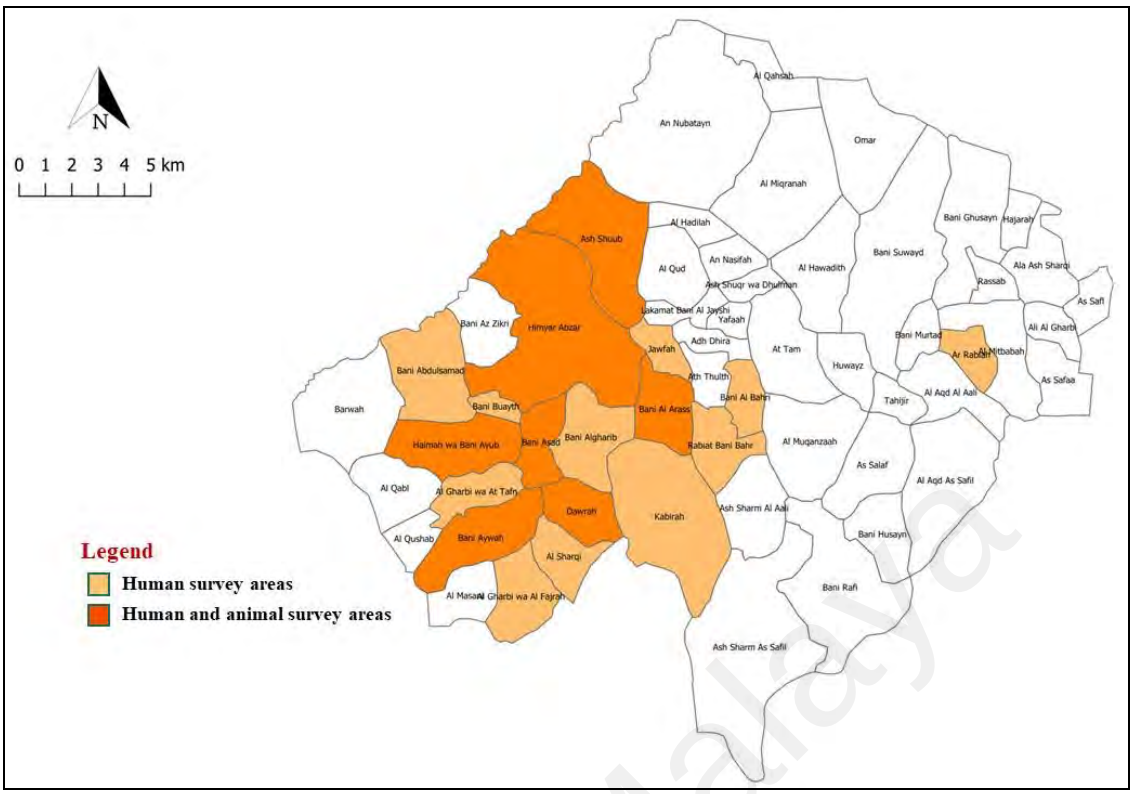

Figure 3.4: A map showing the areas involved in the sampling of human and animal samples in Utmah district, Dhamar governorate, western Yemen.

Livestock animals such as goats, sheep and cows were selected randomly after obtaining oral permission from the head of household. Domestic dogs and cats existed at the participating households and whose owner provided the consent were included in the study while stray animals were excluded. Likewise, rats were captured using locally purchased live traps with bread as bait. Traps were placed overnight in and around the participating households (in close proximity to store rooms and livestock shelters). Wild rabbits were captured alive on farmlands and surrounding areas using local wire box traps baited with vegetables and fruit. Bats were captured either by manual direct capture during daytime or by using mist nets at presumed flyways during the night.

3.6 Recruitment of participants and data collection

In each included area, the survey objectives were explained to the local leader (called *Sheikh* or *Aqil*) and verbal permission to conduct the survey in the area was obtained before any household invitation. Permission was sought from the head of the household for his/her household to be included in the survey using a door-to-door invitation approach. The subject recruitment strategy of house-to-house visits was carefully selected instead of health campaigns due to the characteristics of the study area and targeted populations. The mountainous, rugged terrain and the scattered distribution of the households made it difficult to gather people for health campaigns and sampling. Moreover, many adult villagers were often on their farms far from their households. The visits were conducted during the day, as night visits were not possible.

When permission was given, all the available individuals were assessed for CL infection. The study team asked every individual to disclose the presence of any skin lesion or scar on his/her body, and the team conducted physical examinations of the exposed parts of the body to identify any skin lesions and/or scars. As treatment for CL was not provided during the survey and due to prior information on skin scrapping

sampling procedures, household heads restricted participation to only one subject, regardless of the clinical status, while many refused to allow females to participate in this study. Accordingly, one person from each household was selected for the questionnaire administration and skin scrapping/slits sampling (where applicable) for laboratory examination (Appendix F). The selected adult subject, or the parent/guardian if the selected subject was a child, was given the opportunity to go through the informed consent process to decide whether he/she agreed to participate. If the selected household member did not wish to participate, another member was selected. Individuals who had mental or severe medical illnesses and individuals who did not wish to participate were excluded. In these areas, the houses are dispersed or scattered without any clear pattern (Appendix G); nonetheless, every second household was selected for the study. If the residents of the selected house were unavailable or if the head of the household declined to allow his/her house to take part in the study, the household was not involved in the survey. In this case, the next household was approached; if involved, then the selection of every second household continued until the sample size was achieved.

All the individuals who through the household survey were suspected of having CL were included in the wider case group. According to the WHO‟s operational definition, "a confirmed case of cutaneous leishmaniasis is a person showing clinical signs (skin or mucosal lesions) with parasitological confirmation of the diagnosis (positive smear or culture)" (WHO, 2014b). Participants who had never been infected with CL and had no active lesions were included in the control group. Controls were selected at random from the same villages as the potential cases (individuals with active skin lesions), with two controls recruited for each case from the same village and sub-district. Household visits in each area were repeated until the required number of controls or the proportional number of sampled households in each area had been

achieved. Based on a detection rate of 50–60% by microscopy and PCR (Kariyawasam et al., 2015; Mahdy et al., 2010), this recruitment approach allowed a case-to-control ratio of 1:3 for confirmed CL cases and a ratio of 1:2 for suspected CL cases (active lesions), as well as a ratio of 1:1 for total CL infections (active or past infection).

3.7 Questionnaire survey

A pretested questionnaire was utilised in this study. The questionnaire was designed in the English language and then translated into Arabic (the participants‟ native language). It comprised six sections (Appendix H). The first section included demographic (age, sex and number of household members) and socioeconomic (e.g. educational level, employment and monthly household income) variables. The second section covered the potential variables associated with CL, such as environmental (e.g. sanitation and living condition characteristics, such as the type of water supply, the latrine system, the garbage disposal system, the presence of open water sources near the house and the location of livestock [barn/shelter]), and behavioural (practices related to CL exposure, such as outdoor sleeping, wearing long sleeve clothes and owning and using bed nets) variables.

Moreover, the third to fifth sections involved questions about the participants‟ knowledge about the disease (e.g. mode of transmission, main clinical presentation, common treatment methods and preventive measures) and its sandfly vector (e.g. ability to identify sandflies, biting time, breeding sites, and control measures). In addition, the questionnaire also included questions to assess participants‟ attitude towards CL (e.g. disease severity, prevention, treatment, and stigmatisation) and their CL-related practices (e.g., working and sleeping outside during night, wearing long sleeve clothes, and keeping windows closed during night). The sixth section focused on the participants‟ histories of past CL infection and comorbidities with chronic diseases. The

questionnaire was pretested among 60 subjects from two villages in the targeted district; those villages were not involved in the main study. The consistency and reliability of the developed questionnaire were evaluated, and the results revealed good consistency and high test-retest reliability.

The adult participants and existing parents/guardians of participating children, who had voluntarily agreed to participate, were interviewed face-to-face in their household settings by two research assistants from the district‟s health centre and the Faculty of Medicine, Thamar University, who had received proper training on how to administer the questionnaire for the purpose of the present study. During the interviews, observations were made on household conditions (e.g., the types of walls and the presence of cracks, the existence and status of bed nets and windows screens, the availability of functioning toilets, the location of livestock shelters/barns and the presence of other family members with active skin lesions) and surroundings (e.g. the presence of open water sources near the house, plantations, forest remains, tree trunks, caves and waste disposal).

3.8 Clinical examination

The participants underwent physical examinations by a medical doctor (Appendix I). The skin surfaces of the face, hands, arms, feet, legs and mucous membranes of oral and nasal cavities were carefully examined for the presence of any scars and/or active lesions according to standard criteria: appearance, smooth surface, depressed scar, pigment change, duration of > 2 weeks and no history of trauma (Alkulaibi et al., 2019; Weigle et al., 1993). Data and samples were collected from all clinically suspected CL patients, and all patients were advised to seek treatment at their nearest healthcare centre. At the end of each field visit, the names and addresses of the individuals suspected of having CL were given to the district‟s health personnel.

3.9 Sampling and microscopic examination

3.9.1 Skin scrapping samples from human

After cleaning the lesion with 70% ethanol, the edges of the lesion were compressed between two fingers and a scalpel blade was used to collect slit skin smears from the active edge of the lesion. The samples were smeared directly onto filter papers and onto glass slides (Appendix F). Two slides for skin scrapping/slits samples were collected from each participant suspected of having CL; one was processed for Giemsa staining procedures and microscopic examination (Ramirez et al., 2000), while the other was air-dried and kept with the filter papers for molecular analysis. Briefly, 1-2 mL of the 10% Giemsa stain working solution was placed on the smear, in sufficient quantity to cover the entire surface, for 10 minutes. The stained smear was rinsed with water until the edges showed faintly pinkish red. The slide was allowed to air dry. The stained smears were then examined by light microscopy for the presence of the amastigotes (intra- and extracellular amastigotes) under a 100 × objective (oil immersion lens).

The parasite density was quantified using a PLUS scoring system from "+" to "++++" according to the number of parasites detected per high power fields (HPFs) (Mahdy et al., 2010; Ramirez et al., 2000) as shown in Table 3.1. The smear was considered negative if amastigotes were not detected after the examination of at least 100 high power field.

Table 3.1 PLUS scoring system for determining *Leishmania* parasite density

Parasite density score	Description
+	1 amastigote in the whole smear or in a total of at least 100 HPFs
++	2-10 amastigotes in a total of at least 50 HPFs
+++	11-20 parasites in a total of at least 50 HPFs
++++	> 20 parasites in a total of at least 10 HPFs

HPFs = high power fields.

3.9.2 Skin scraping and blood samples from animals

The selected animals were restrained using rope/halters for physical examination and blood sample collection by a veterinary specialist. Dogs were restrained with a muzzle and immobilized to avoid any biting accident during blood samples collection. The rats and rabbits were immobilized in plastic restraints. The skin of the animals was examined carefully for the presence of any suspicious skin lesions. Skin scraping specimens were collected on two microscopic slides as previously described in human sample collection (see 3.9.1). Moreover, about 0.5–1 mL whole blood samples was collected from each animal, having suspicious skin lesions or not. Directly after collection, blood spots on 5 mm Whatman filter papers were prepared according to recommended protocols and kept for pending molecular assays (Gao et al., 2015). No animals were sacrificed for the purpose of this study and all procedures were performed by veterinarians according to ethical standards of the relevant national and international regulations on the care and use of animals.

3.10 Molecular examination

3.10.1 DNA extraction

Parasite's genomic DNA was extracted from the skin scraping/slits smears and the filter paper spots using DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. A drop of phosphate buffer saline (PBS) (not included in the kit) was placed on the blood smear and the dried blood sample was removed from the smear by clean filter paper disc (6 mm cut using paper puncture) and transferred into a 1.5 mL microcentrifuge tube. For the filter paper sample spots, an alcohol-flamed puncher was used to cut 2-3 filter paper discs (6 mm) into a 1.5 mL microcentrifuge tube. The tube was then processed for DNA extraction accordingly.

Twenty µL of proteinase kinase was added into the microcentrifuge tube followed by 200 µL of buffer AL and 200 µL of PBS buffer. Then, the tube was mixed thoroughly by vortexing, incubated at 56 ºC for 30 minutes, vortexed shortly then incubated again for 30 minutes, 200 µL of absolute ethanol was added and mixed thoroughly by vortexing. Then, the mixture was pipetted into a DNeasy mini spin column, centrifuged at 8000 rpm for 1 min, and then the flow-through and collection tube were discarded. The DNeasy mini spin column was placed in a new 2 mL collection tube. Then, 500 µL of buffer AW1 was added, and the tube was centrifuged at 8000 rpm for 1 minute. The flow-through and collection tube were discarded, and the DNeasy mini spin column was placed again in a new 2 mL collection tube. Then, 500 µL of buffer AW2 was added into the tube, and then centrifuged at 14,000 rpm for 3 minute to dry the DNeasy membrane. The flow-through and collection tube were discarded, and the DNeasy mini spin column was placed into a new 1.5 mL microcentrifuge tube. The final DNA yield was eluted in 30-60 µL of Qiagen TE elution buffer and kept at -20 ºC until use for molecular analysis.

3.10.2 DNA amplification

The genomic DNA extracts were subjected to molecular genus identification using nested PCR amplification flanking mostly the Internal Transcribed spacer (*ITS-1*) of the *Leishmania* ssu rRNA gene according to established protocol by Schönian et al (Schönian et al., 2003). The primary PCR reaction was carried out in 25 µL reaction volume containing 1X of GoTaq® Flexi Buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP, 2 units of GoTaq® Flexi DNA polymerase (Promega Corp., USA), 0.2 µM of each of LITSR-F forward (5'-CTGGATCATTTTCCGATG-3‟) and LITSV-R (5'_ACACTCAGGTCTGTAAAC-3') reverse primers. 2 µL of primary PCR products were used as a template for the secondary PCR reaction using LITSR-F forward and

L5.8S-R (5'-TGATACCACTTATCGCACTT-3') reverse primer (Figure 3.5). A list of Oligonucleotide primers used is shown in Appendix J. Known DNA extracts from *Leishmania* clinical samples (confirmed by sequencing) were used as positive controls in all the PCR runs while DNase-free, RNase-free distilled water was used as a negative control. All PCR amplification reactions were performed in MyCycler™ Thermal Cycler (Bio-Rad, Hercules, CA, USA), with PCR conditions previously described (Table 3.3) (Schönian et al., 2003). The expected size for the amplicons was 300-350 bp which differs with different *Leishmania* spp.

3.10.3 Agarose gel electrophoresis

The PCR products of the nested PCR were subjected to agarose gel electrophoresis using 2.5% agarose in 1X Tris-Acetate-EDTA (TAE) buffer pre-stained with SYBR® safe DNA gel stain (Invitrogen™, USA). PCR products were loaded in the gel wells and electrophoresed at 90 V, 100 mA for 35 minutes. The gel bands were then visualized under UV using Molecular Imager® Gel Doc™ XR+ Imaging System (Bio-Rad, USA).

Table 3.2: Master-mix composition for the PCR amplification of *Leishmania* ITS-1 gene

Reagents	Stock conc.	Volume (µL)
ddH_2O	-	14.6
GoTaq® Flexi buffer	5X	5.0
$MgCl_2$	25 mM	1.5
dNTPs	10 mM	0.5
Primers: (Primary PCR)	10 µM	
Forward primer: LITSR-F		0.5
Reverse primer: LITSV-R		0.5
Primers: (Secondary PCR)	10 µM	
Forward primer: LITSR-F		0.5
Reverse primer: L5.8S-R		0.5
GoTaq® DNA polymerase	(5 U/µL)	0.4
DNA template/ddH_2O*	-	2.0
Total reaction		25.0

* ddH_2O for negative control only, N1 = primary reaction, N2 = secondary reaction.

Table 3.3 Thermal cycling conditions of PCR amplification of *Leishmania ITS-1* gene

Cycle/Step	Temperature	Time	Cycle
Initial denaturation	94°C	3 min	1
Denaturation	94°C	30 sec	30*
Annealing	53°C	30 sec	
Extension	72°C	1 min	
Final extension	72°C	6 min	1
Hold	16°C	∞	1

* 25 cycles for secondary PCR.

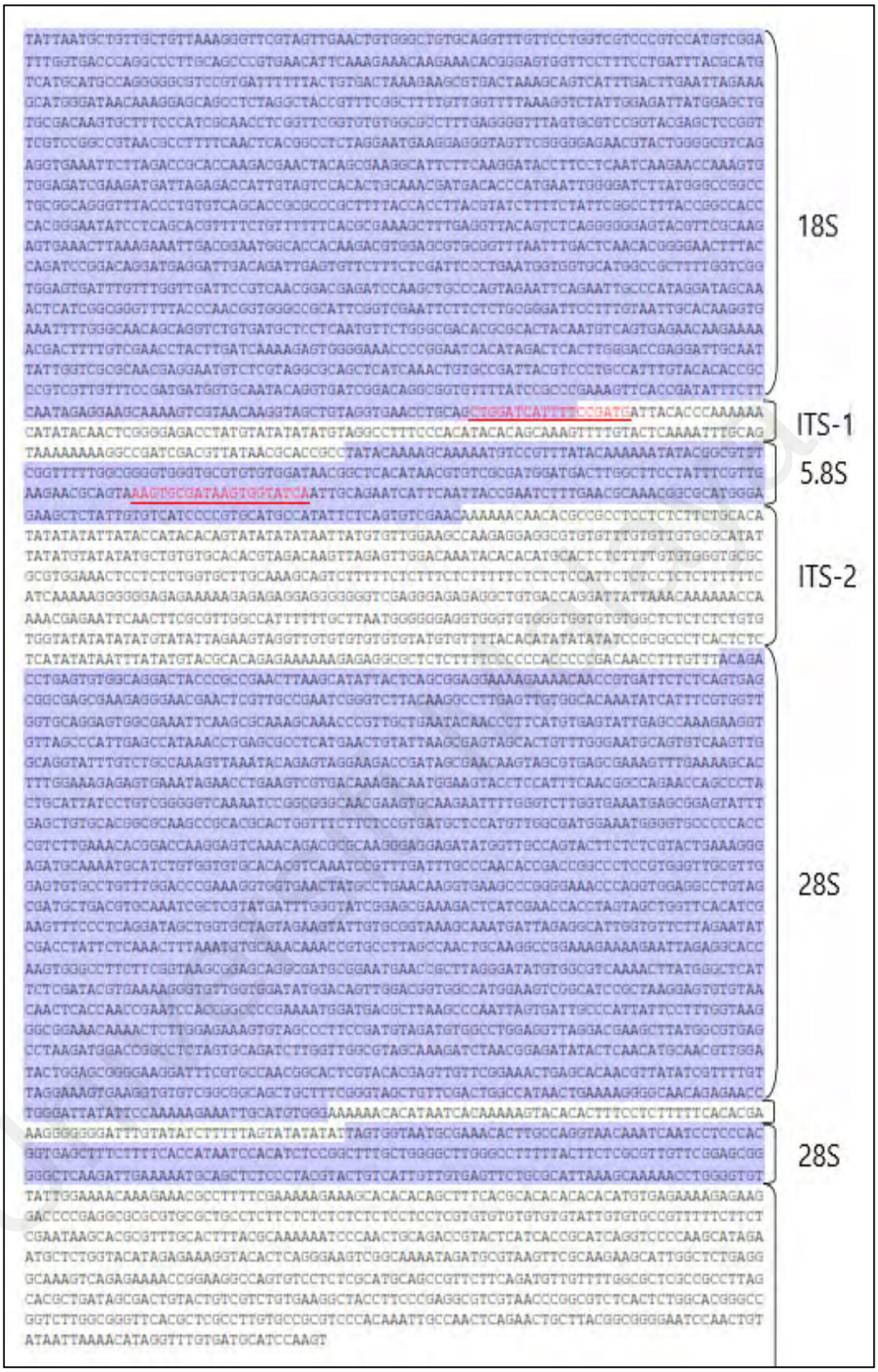

Figure 3.5: SSU rRNA gene of *Leishmania* species with the *ITS-1* gene of interest for the detection of *Leishmania* spp. isolates.

The red font represents oligonucleotide primers used in PCR amplification.

3.10.4 *ITS-1* gene sequencing

3.10.4.1 Gel purification of PCR products

Purification of PCR products were performed using QIAquick® Gel Extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The DNA fragment with expected size was excised from the gel and transferred into a 1.5 mL microcentrifuge tube. Three times of Buffer QG (solubilisation) was added to one volume of the gel (100 mg ~ 100 mL). The mixture was incubated at 50℃ for 10 minutes with occasional vortexing to dissolve the gel. One gel volume of isopropanol was added to the dissolved sample and mixed well. The entire volume of the mixture was transferred to a QIAquick® spin column in a 2 mL collection tube and centrifuged at 13,000 rpm for 1 minute. Flow-through was discarded. Five hundred µL of Buffer QG was added to the QIAquick column and centrifuged at 13,000 rpm for 1 minute. Flow-through was discarded. The column was then washed with 750 µL of Buffer PE and allowed to stand for 2-5 minutes before centrifugation at 13,000 rpm for 1 minute. Flow-through was discarded. The column was then centrifuged again for 1 minute at 13,000 rpm to remove any residual wash buffer.

The dried QIAquick column was transferred into a clean 1.5 mL microcentrifuge tube, then 20-30 µL Buffer EB (10 mM Tris-HCl, pH 8.5) was directly added to the centre of the spin column and incubated for 2-5 minutes before centrifuged at 13,000 rpm for 1 minute. Flow-through was re-eluted to the centre of the spin column before centrifugation at 13,000 rpm for 1 minute.

3.10.4.2 DNA cloning into pGEM®-T vector

Purified PCR product was cloned into pGEM®-T vector through TA cloning method using pGEM®-T vector system (Promega Corp., USA) (Appendix K). All the ligation mixture components were added into a PCR tube as in Table 3.4. The ligation mixture was mixed well and incubated at 4℃ overnight.

Table 3.4: Ligation reaction volumes of pGEM®-T vector

Reagents	Volume (µL)
PCR product	3.0
pGEM®-T vector (50 ng)	1.0
2X rapid ligation buffer	5.0
T4 DNA ligase (3 Weiss units/µL)	1.0
Total volume	10.0

3.10.4.3 Transformation pGEM®-T vector into competent cells

Following the 4℃ overnight incubation, 10 µL of the ligation mixture was added into 100 µL of a pre-thawed competent cell. The cells were incubated on ice for 30 minutes, followed by immediate heat-shock incubation in the water bath at 42 ℃ for 45 seconds, and were returned on ice for another 5 minutes. The cells were diluted (1 mL) with LB broth before incubating in a shaking incubator at 37℃ for 1 hour. The cells were spun down until the pellet appeared. The supernatant was discarded until left 100 µL. The pellet was suspended by tapping the tube until the solution turned cloudy. One hundred µL of the cell suspension was plated onto LB agar containing 100 µg/mL ampicillin and spread carefully with a sterile spreader. All plates were incubated overnight at 37 ℃.

3.10.4.4 Colony PCR of the transformants

Screening of the positive pGEM®-T clones with insert were performed by picking 5-12 colonies from each ampicillin-incorporated LB agar plate incubated overnight. PCR amplification was carried out with M13 forward and reverse primer set as described in Table 3.5.

Table 3.5: Colony PCR reaction volumes.

Reagents	Volume (µL)
5X Green GoTaq® Flexi buffer	2.0
MgCl (25 mM)	1.0
dNTP mix (10 mM)	0.25
GoTaq® Flexi DNA polymerase	0.1
M13-F (10 µM)	0.25
M13-R (10 µM)	0.25
Nuclease free water	6.15
Total volume	10.0

Picked colonies were streaked onto LB agar and also added to the PCR tube which consists of the PCR reaction mixture. Sterile ddH_2O was used as a negative control. PCR amplification was initiated at 95 ℃ for 4 minutes, followed by 35 cycles of denaturation at 95℃ for 30 seconds, annealing at 50℃ for 45 seconds and extension at 72 ℃ for 1 minute. The cycles were eventually completed with a final extension at 72 ℃ for 10 minutes and a holding temperature at 16 ℃.

3.10.4.5 Determination of positive clones

PCR products were analyzed and visualized using agarose gel electrophoresis (see section 3.11.3). Selected positive clones with the expected size were inoculated into 5 mL of LB broth containing ampicillin (100 µg/ mL) and incubated overnight at 37℃ in shaking incubator.

3.10.4.6 Plasmid extraction of positive recombinant clones

DNA plasmids of selected positive clones were isolated according to the manufacturer protocol of the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). Briefly, the overnight culture was centrifuged at 5,000 rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 250 µL Buffer P1. The cell suspension was transferred into a sterile 1.5 mL microcentrifuge tube. This was followed by the addition of 250 µL Buffer P2 then 350 µL Buffer N3 with gentle inversion between each addition. The mixture was spun at 13,000 rpm for 10 minutes. The clear supernatant was transferred to the QIaprep spin column and was centrifuged at 13,000 rpm for 1 minute. Flow-through was discarded and 500 µL Buffer PB was added to the column and was centrifuged again for 1 minute with the same speed.the spin colum was eventually washed with 750 µL Buffer PE before subjected to two rounds of centrifugation at 13,000 rpm for 1 minute. Flow-through was discarded after each centrifugiation. The spin column was placed in a clean 1.5 mL microcentrifuge tube followed by the addition of 50 µL of Buffer EB directly into the membrane and was incubated for 2-3 minutes at room temperature. The column was spun at 13,000 rpm for 1 minute to elute the bound DNA plasmid.

3.10.4.7 Storage and maintenance of positive recombinant clones

Glycerol stock of the verified positive clones were prepared by mixing 1 volume of sterile 50% glycerol with 1 volume of overnight culture (1:1) and were stored at -80 °C. The clones were restreaked onto fresh antibiotic-incorporated plates, and new glycerol stocks were prepared every six months.

3.10.4.8 DNA sequencing

The purified plasmid was sequenced by a commercial laboratory (Apical Scientific Sdn. Bhd., Malaysia). M13 forward (-20) and reverse (-24) universal sequencing primers

were used to perform Sanger dideoxy sequencing, while LITSR-F and L5.8S-R oligonucleotide primers were sent along with the purified gels for direct DNA fragment sequencing by the same company.

3.10.4.9 Confirmation of non-*Leishmania*, *Trypanosoma* samples

Samples that had yielded *Trypanosoma* identity and coverage in BLAST with the *ITS-1* gene were analyzed with another set of oligonucleotide primers that is specifically targeting small subunit ribosomal RNA (*SSU rRNA*) gene of trypanosomes. The TRY927F (5'-GAAACAAGAAACACGGGAG-3‟) forward and TRY927R (5'-CTACTGGGCAGCTTGA-3‟) reverse primers were used for primary PCR reaction while SSU561F (5‟-TGGGATAACAAAGGAGCA-3‟) forward and SSU561R reverse primer (5‟-CTGAGACTGTAACCTCAAAGC-3‟) were used for the nested one (Smith et al., 2008). The PCR reagents concentration and thermal cycler conditions of primary and secondary PCR reactions are described in Table 3.6 and Table 3.7.

Table 3.6: PCR master-mix reagents composition using for the amplification of trypanosomes *SSU rRNA* gene

Reagents	Stock conc.	Volume (µL)
ddH_2O	-	13.6
GoTaq® Flexi Buffer	5X	5.0
$MgCl_2$	25 mM	2.0
dNTPs	10 mM	1.0
Forward (N1 = TRY927-F, N2 = SSU561F)	10 µM	0.5
Reverse (N1 = TRY927-R, N2 = SSU561-R)	10 µM	0.5
GoTaq® DNA polymerase	(5 U/µL)	0.4
DNA template/ddH_2O*	-	2.0
Total reaction		25.0

* ddH_2O for negative control only, N1 = primary reaction, N2 = secondary reaction.

Table 3.7: Thermal cycling conditions of the primary PCR of *Trypanosoma* identification using *small subunit ribosomal RNA* (*SSU rRNA*) gene

Cycle/Step	Temp.	Time	Cycle
Initial Denaturation	94°C	3 min	1
Denaturation	94°C	30 sec	45*
Annealing	53°C	30 sec	
Extension	72°C	1 min	
Final Extension	72°C	6 min	1
Hold	16°C	∞	1

* 35 cycles for secondary PCR.

3.11 Phylogenetic analysis and genetic diversity of *leishmania* isolates

The chromatograms of the forward and reverse sequences of each sample were checked using BioEdit Sequence Alignment Editor Software (BioEdit version 7.2.5), and a sequence consensus was then created for each of the isolates. *Leishmania ITS-1* sequences (n = 54) obtained in the present work from both human and animal samples were subjected to multiple alignments and analysed through the construction of a phylogenetic tree to look at the genetic similarity or diversity among the isolates in the study area using Molecular Evolutionary Genetics Analysis software (Mega version 7.0.26). Nucleotide diversity (π) of the Yemeni *Leishmania tropica* isolates was analysed via DNA Sequence Polymorphism software (DnaSP version 6.12.03). The difference between haplotypes from the different sites of the study area as well as the difference between human and animal haplotypes was also analysed using DnaSP.

Furthermore, a total of 313 *ITS-1* sequences of *L. tropica* obtained by previous studies in different countries were retrieved from the GenBank. Majority of these sequences were from Iran (n = 96) followed by Syria (n = 58), Morocco (n = 41) and China (n = 27). Moreover, three additional sequences previously published for Yemen were retrieved from the GenBank (accession numbers GU561643.1, GU561644.1 and HG512919.1), making the total number of sequences from Yemen 57. Overall, the final dataset included 367 sequences of *L. tropica* of different hosts (Appendix L). Haplotype network was constructed based on the median joining method using PopART v. 1.7 (Leigh & Bryant, 2015). DnaSP v.6.12.03 was used to identify haplotypes and estimate the genetic diversity indices including nucleotide (N_d) and haplotype (H_d) diversities, with their corresponding standard deviations, and the average number of variable sites (nucleotide differences) (Rozas et al., 2017). In addition, other genetic diversity indices including average number of pairwise nucleotide differences (*Kxy*), nucleotide

substitution per site (*Dxy*), number of net nucleotide substitutions per site (*Da*), and genetic differentiation index based on the frequency of haplotypes ($\boldsymbol{G_{ST}}$) between populations were also calculated by DnaSP v.6.12.03.

Moreover, Tajima's *D* (Tajima, 1989) and Fu's *Fs* (Fu, 1997) were used to test neutrality using the DnaSP version 6.12.03. These indices (i.e. Tajima‟s *D* and Fu‟s *Fs*) were used to determine whether there was any deviation from the assumption of neutrality mutation or selective neutrality of the tested populations. Tajima‟s *D* and Fu‟s *Fs* values are sensitive to demographic expansion or purifying selection, which usually leads to large negative values, whereas positive values indicate no deviation from neutrality, which is representative of stationary populations. Tajima‟s *D* represents the difference between the mean pairwise nucleotide differences (π) and the number of variable sites (*S*) relative to their standard error. Moreover, Fu‟s *Fs* represents the number of derived nucleotide variants observed only once in a sample with the mean pairwise difference between sequences. *P*-values < 0.1 and < 0.02 were considered statistically significant for Tajima‟s *D* and Fu‟s *Fs*, respectively (Kariyawasam et al., 2017).

Genetic differentiation was determined by calculating the inter-population pairwise genetic differentiation index (also called pairwise fixation index) (F_{ST}: Wright‟s *F*-statistics) using DnaSP v.6.12.03. F_{ST} values indicate the level of genetic differentiation, and the values range between 0 (means complete sharing of genetic material) and 1 (means the two populations do not share any genetic diversity, or the populations are fixed) (Barbosa et al., 2019). A standard scale of fixation index is F_{ST} < 0.05 represent little genetic difference, F_{ST} = 0.05–0.15 represents moderate genetic difference, F_{ST} = 0.15 and 0.25 represents high genetic difference, and F_{ST} > 0.25 represents strong genetic difference (Barbosa et al., 2019; Kariyawasam et al., 2017).

Moreover, this scale was simplified as $F_{ST} < 0.05$ represents non-significant genetic differentiation and $F_{ST} > 0.15$ represents significant genetic differentiation (Frankham et al., 2002). Moreover, number of migration (*Nm*) was also estimated using formula ($Nm = (1\text{-}F_{ST})/4F_{ST}$) to assess the gene flow level among *L. tropica* populations in the studied areas (Hudson et al., 1992). The *Nm* value was categorised into three levels: high (≥ 1.0), medium (0.250–0.99) and low (< 0.249) (Govindaraju, 1988).

3.12 Statistical analysis

The data were reviewed and double-checked before and after data entry. Statistical analysis of epidemiological data was performed using *IBM SPSS Statistics* (version 20). For descriptive data, frequencies and percentages were used to express the prevalence of infection and to present qualitative variables, while the median (IQR) was used to express quantitative data. The quantitative data were examined for normality by the Shapiro-Wilk test.

3.12.1 Analysis of factors associated with CL

The chi-square test (χ^2) or Fisher"s exact test was used where applicable to examine the association between the dependent variable (CL infection) and explanatory variables, such as age, sex, education level, occupation and housing characteristics. Crude odds ratios (CORs) and corresponding 95% confidence intervals (CIs) were computed.

A conceptual framework that considered the hierarchical relationships between risk factors and CL was constructed (Figure 3.6). The potential risk factors were categorised into five blocks or levels in the analysis: socioeconomic characteristics (Block 1), household and indoor conditions (Block 2), environmental and outdoor conditions and animal characteristics (Block 3), knowledge about CL and sandfly vector factors (Block 4), and behavioural and health characteristics (Block 5). Following a hierarchical approach (Coura-Vital et al., 2011; Victora et al., 1997), the blocks of

variables were added to the multivariate analysis in sequence, as shown in the proposed conceptual framework. Variables that showed *P* values of ≤ 0.25 in the univariate analysis were included in the logistic regression models (Bendel & Afifi, 1977). Age and sex variables were considered confounders; thus, they were included in all analyses. Then, variables in each block „block model" that attained *P* values of < 0.10 were included in the next-level model (Victora et al., 1997). Adjusted odds ratios (AORs) and corresponding 95% CIs were computed.

The backward elimination method was used to develop the stepwise unconditional logistic regression models, and likelihood ratio statistics were used to adjust the models. Variables with significance levels of $P < 0.05$ in the final model were considered significant predictors of CL. Dummy variables were used to code categorical explanatory variables in the logistic regression models since this provided a better data fit.

3.12.2 Analysis of KAP data and associated factors

With regards to KAP data, scores were developed for the participants" responses on questionnaire items related to knowledge, attitude and practices following the methods described previously (Alharazi et al., 2021; Devipriya et al., 2021). In brief, KAP-based scores were developed for each participant, with each correct response was assigned a score of 1 while each incorrect or "don"t know" response was assigned a score of 0. The participants" knowledge about CL was assessed based on the responses given to five items. A total score was calculated for each participant based on a scale of 0 to 5, with 0 being the lowest and 5 being the highest (if the responses to all the questions were correct). A CL-related knowledge score between 0 and 3 was considered as "poor knowledge" while scores from 4 to 5 were considered as "good knowledge". Similarly, participants" knowledge about the sandfly vector was evaluated using five questions and

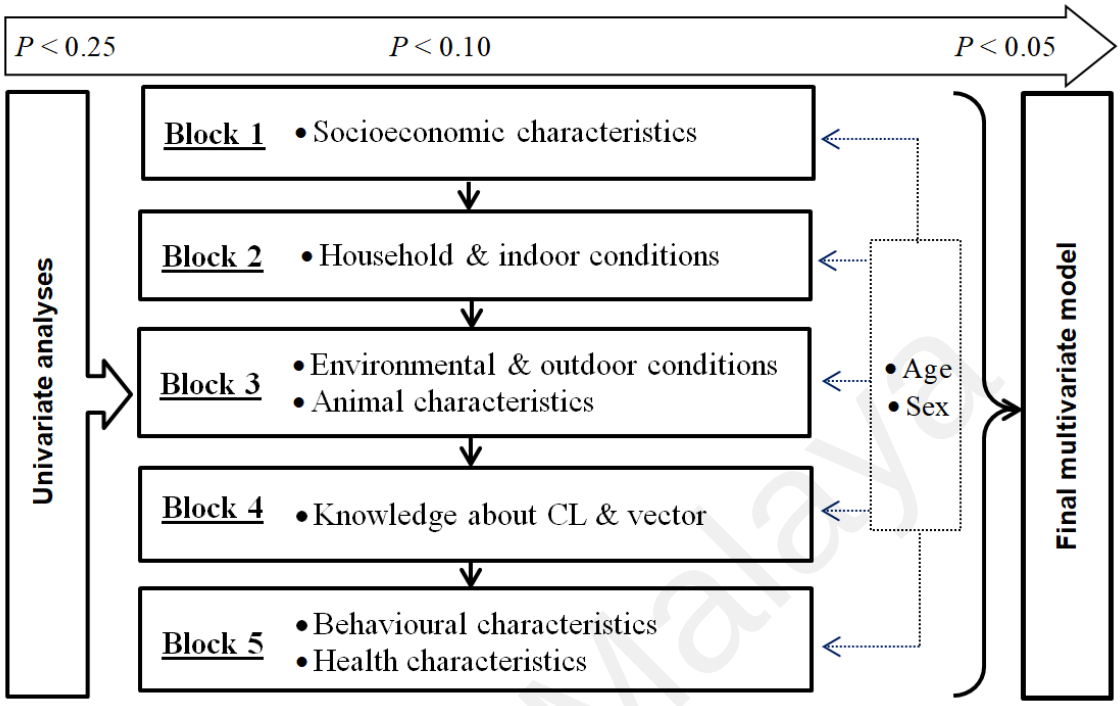

Figure 3.6: Hierarchical framework for assessing risk factors associated with CL infection in the Utmah district, western Yemen.

graded on a scale of 0 to 5. Participants‟ scores of 0 to 3 were considered as “poor sandfly-related knowledge” while scores of 4 to 5 indicating “good knowledge”.

Moreover, the participants‟ attitude towards CL was assessed using four questions with a score of 0 to 3 indicating a “negative attitude” while a score of 4 to 5 was considered as a “positive attitude”. In addition, participants‟ practices related to CL were evaluated using 10 items and graded on a scale of 0 to 10. Participants with a total score of 0 to 5 were considered to have “poor CL-related practices” while participants with a score of 6 to 10 were deemed to have “good CL-related practices”.

Associations between participants‟ KAP variables and socio-demographic factors including age, sex, residence, education level, occupation, household monthly income and the presence of confirmed CL cases in the household were examined by the Chi-square test (χ^2) or Fisher‟s exact test. Moreover, multivariate backward logistic regression analyses were performed to identify the significant predictors of good CL- and sandfly-related knowledge, attitude towards CL and good CL-related practices. All variables with a *P* value of ≤ 0.25 in the univariate analyses were included in the logistic regression analyses (Bendel & Afifi, 1977). Adjusted odds ratios (AORs) with corresponding 95% CIs were computed and the P value of less than 0.05 was considered statistically significant.

CHAPTER 4

RESULTS

4.1 General characteristics of the study participants

Two hundred eighty-nine households were involved in this study. The majority of the households are considered having poor housing conditions, with 81.7% of the houses were built of mud with cracked walls and wooden roofs while only 18.3% of the houses were built of brick, concrete or stone, with no cracks. Almost all (93.8%) of the households had unimproved sources for drinking water, such as rain, wells and springs whereas only 6.2% of the houses had improved sources for drinking water. Overall, there was no proper disposal of waste and garbage. Moreover, only 80 households (27.7%) had a total monthly household income of YER50,000 (equivalent to US$75) and above, while 209 households (72.3%) had a total monthly household income of less than YER50,000. With regards to family size, 43.3%, 40.1% and 16.6% of the households had more than 10, 6–01 and ≤ 5 family members, respectively.

A total of 612 individuals resided in the involved 289 households from the escarpment areas of the Utmah district were involved in this study. They aged between five months old and 88 years old, with a median age of 25 (IQR 10–54 years old). The majority of the participants (79.9%) were males, and only 20.1% were females; the study included 210 (34.3%) children below 18 years of age and 402 (65.7%) adults. A total of 226 (36.9%) were from the Bani Bahr sub-district, while 180 (29.4%) were from the Alsomal sub-district. The majority of the participants resided in houses built of stone and used unimproved water supplies for drinking water, such as rain, wells and springs, and there was no proper disposal of waste and garbage (Appendix G). Distribution of participants according to age, sex and location are shown in Table 4.1.

4.2 Prevalence and distribution of cl

Based on the physical examinations, 33% (202/612) of the participants had active skin lesions and/or scars that met the clinical criteria for a case of CL. Overall, 14.7% (90/612) of the participants had active skin lesions and were considered suspected CL cases. However, *Leishmania* parasites were detected in 65.4% (53/81) of the suspected cases; nine cases refused to undertake skin scraping sampling. That said, the overall prevalence of confirmed CL among the studied participants was 8.7% (53/612); 37 (6.0%) cases were confirmed by microscopy, while 53 (8.7%) by molecular examination, which included the 37 microscopy-positive cases. The density of amastigotes in about two-thirds (67.6%) of the microscopy-positive samples was of 1+ while densities of 2+, 3+ and 4+ were recorded in 8.1%, 10.8% and 13.5% of the positive samples, respectively.

Table 4.1 shows the distribution of CL infection according to age, sex and location. The distribution of confirmed cases according to age group was statistically significant (χ^2 = 41.284; $P < 0.001$), with the highest prevalence (23.9%) reported among children aged ≤ 10, while the lowest prevalence (3.8%) was among those aged 31–50. The youngest patient was a seven-month-old female from Razeh sub-district, whereas the oldest patient was an 80-year-old male from Alsomal. Furthermore, the prevalence of confirmed CL among females was significantly higher than among males. On the other hand, the prevalence of CL was comparable among the four sub-districts, with the highest prevalence of CL found in the Hemyar Alwasat (10.7%) and Alsomal (10.0%) sub-districts.

Table 4.1: Prevalence and distribution of cutaneous leishmaniasis status among the participants according to age, sex, and location (n=612)

Variable	N	Lesion only	Scar only	Lesion and scar	Total infection	*P*	Confirmed CL infection	*P*
Overall (%)	612	56 (9.2)	112 (18.3)	34 (5.6)	202 (33.0)		53 (8.7)	
Age (years)								
≤10	113	25 (22.1)	9 (8.0)	11 (9.7)	45 (39.8)	0.099	27 (23.9)	<0.001
11–17	97	7 (7.2)	19 (19.6)	5 (5.2)	31 (32.0)	0.664	6 (6.2)	0.915
18–30	140	11 (7.9)	26 (18.6)	9 (6.4)	46 (32.9)	0.535	8 (5.7)	0.971
31–50	159	7 (4.4)	39 (24.5)	4 (2.5)	50 (31.4)	0.690	6 (3.8)	0.548*
>50	103	6 (5.8)	19 (18.4)	5 (4.9)	30 (29.1)	1	6 (5.8)	1
Sex								
Female	123	20 (16.3)	15 (12.2)	14 (11.4)	49 (39.8)	0.071	23 (18.7)	<0.001
Male	489	36 (7.4)	97 (19.8)	20 (4.1)	153 (31.3)	1	30 (6.1)	1
Location								
Alsomal	180	19 (10.6)	33 (18.3)	3 (1.7)	55 (30.6)	0.331	18 (10.0)	0.877
Bani Bahr	226	13 (5.8)	49 (21.7)	14 (6.2)	76 (33.6)	0.585	16 (7.1)	0.364
Razeh	150	20 (13.3)	19 (12.7)	11 (7.3)	50 (33.3)	0.576	13 (8.7)	0.651
Hemyar Alwasat	56	4 (7.1)	11 (19.6)	6 (10.7)	21 (37.5)	1	6 (10.7)	1

* The difference was examined using Fisher‟s exact test (otherwise, Chi Square test was used).

4.3 Clinical presentation of cutaneous leishmaniasis

Clinically, 202 participants out of 612 had skin manifestations in the forms of active lesion/s and/or scar/s reflecting current or past CL infection (Table 4.2). Of these, 90 participants had active skin lesion/s, altogether harbouring at least 134 skin lesions (Appendix I). The majority of the patients (63.3%) had a single lesion, while 36.7% had multiple lesions. Most (*81.3%)* of the lesions were of the dry type, while *18.7*% were wet lesions (Figure 4.1). Over half the patients had nodulo-ulcerative lesions (53.0%), whereas 16.4% and 12.7% had plaque and nodular lesions, respectively. Most (75.4%) of the lesions were on the face, with 33.6%, 22.4% and 12.7% on the cheeks, nose and lips, respectively (Figure 4.1). Ears, arms, legs, and neck were also involved. On the other hand, 146 participants had scars reflecting potentially healed CL lesions. The majority of the scars (67.8%) were on the face, with 30.8% and 24.0% on the cheeks and nose, respectively.

Table 4.2: Clinical presentation of cutaneous leishmaniasis active lesions according to number, site, size, and type

Characteristics of lesions	Total (n = 81)	Positive for CL (n = 53)	Negative for CL (n = 28)
Number			
1	50 (61.7)	29 (54.7)	21 (75.0)
2	24 (29.6)	19 (35.8)	5 (17.9)
3	4 (4.9)	3 (5.7)	1 (3.6)
4	3 (3.7)	2 (3.8)	1 (3.6)
Type			
Papule	4 (4.9)	2 (3.8)	2 (7.1)
Nodule	21 (25.9)	14 (26.4)	7 (25.0)
Ulcerating nodule	32 (39.5)	21 (39.6)	11 (39.3)
Plaque	17 (21.0)	13 (24.5)	4 (14.3)
Ulcerating plaque	5 (6.2)	2 (3.8)	3 (10.7)
Others	2 (2.5)	1 (1.9)	1 (3.6)
Size (mm)			
< 10	21 (25.9)	13 (24.5)	8 (28.6)
10–20	40 (49.4)	27 (50.9)	13 (46.4)
21–30	14 (17.3)	10 (18.9)	4 (14.3)
> 30	6 (7.4)	3 (5.7)	3 (10.7)
Site			
Cheek	32 (39.5)	24 (45.3)	8 (28.6)
Nose	23 (28.4)	17 (32.1)	6 (21.4)
Lips	10 (12.3)	6 (11.3)	4 (14.3)
Chin	2 (2.5)	1 (1.9)	1 (3.6)
Ear	2 (2.5)	1 (1.9)	1 (3.6)
Upper limbs	10 (12.3)	4 (7.5)	6 (21.4)
Lower limbs	2 (2.5)	0 (0.0)	2 (7.1)
Appearance			
Dry	44 (54.3)	30 (56.6)	14 (50.0)
Wet	21 (25.9)	11 (20.8)	10 (35.7)
Mixed	16 (19.8)	12 (22.6)	4 (14.3)

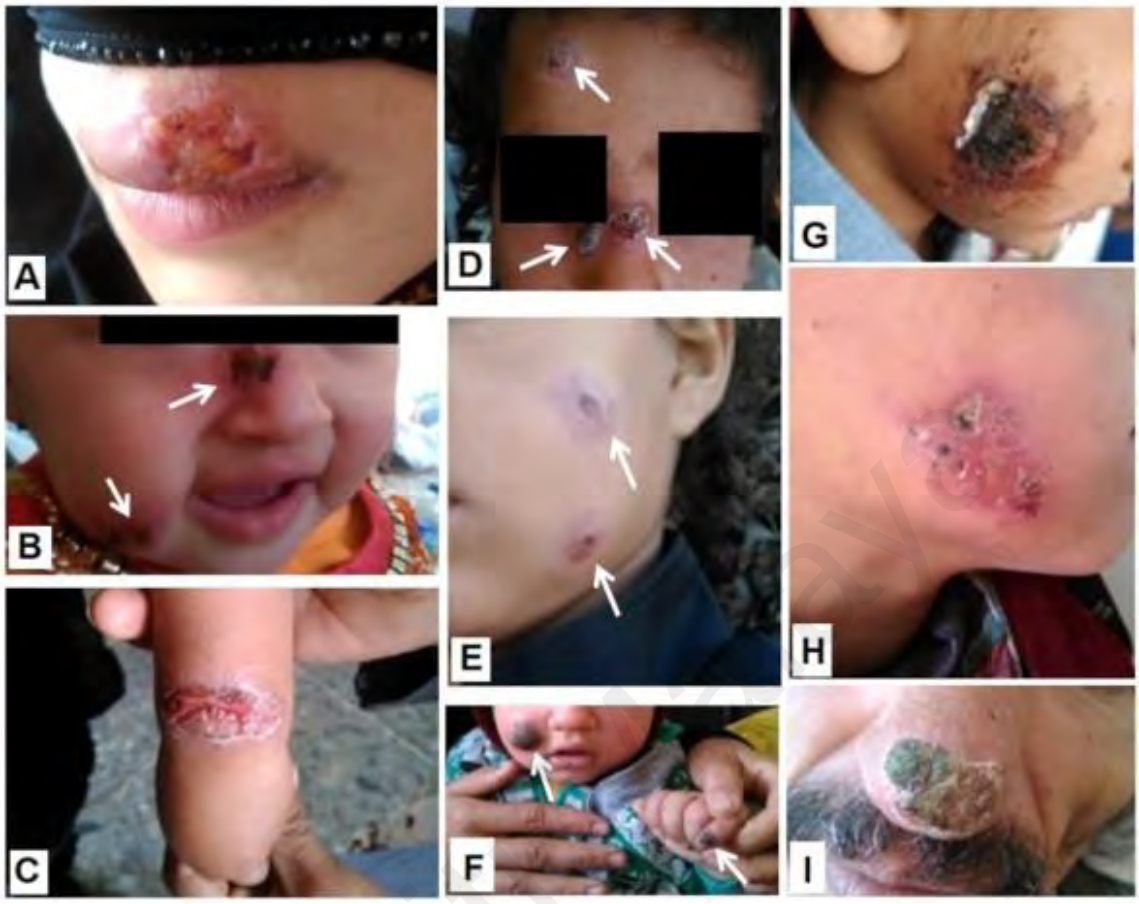

Figure 4.1: Clinical features of cutaneous leishmaniasis in Utmah district, western Yemen.

(A) mucocutaneous involvement of the upper lip of a young woman; (B) multiple nodulo-ulcerative lesions on the nose and cheek in an infant; (C) ulcerative scaly lesion on the forearm of a toddler; (D) multiple nodulo-ulcerative lesions on the nose and forehead; (E) multiple nodulo-ulcerative lesions on the left cheek; (F) nodulo-ulcerative lesions on the cheek and little finger of an infant; (G) plaque with the effect of traditional therapy; (H) leishmaniasis recidivans; and (I) chronic lesion on the nose.

4.4 Factors associated with cutaneous leishmaniasis

Different case-control comparisons were performed to assess the associations between CL and demographic, socioeconomic and behavioural factors among the participants. The general demographic and socioeconomic characteristics of the study participants involved in this analysis are shown in Table 4.3. A total of 223 participants were involved in this analysis (53 confirmed cases of CL and 170 controls). The median age for cases was 13 years old (IQR 7–28), and it was 30 years old (IQR 15–45) for the control group, with the majority (50.9%) of cases and 31.8% of controls within the age group of 0–10 and 31–50 years old, respectively. More than half (56.6%) of the cases and the majority (88.2%) of the controls were males. Among the cases, 73.4% were non-educated, while 52.4% of the controls were non-educated. Poverty prevails in the areas studied, with about 69.8% of the cases and 70% of the controls having an overall family monthly income of less than YER50,000 (US$75). The majority of the cases (90.6%) and controls (77.6%) lived in poor houses built of stone and with cracked walls, with 94.3% and 93.5% of the participants, respectively, using unimproved water supplies, such as underground wells, rain, uncovered cisterns/troughs and springs for drinking and for domestic use.

Table 4.3: General demographic and socioeconomic characteristics of CL cases and controls, Utmah district, western Yemen (n = 223)

Variables	Cases (n=53) n (%)	Controls (n=170) n (%)
Age (years)		
≤10	27 (50.9)	21 (12.4)
11–17	6 (11.3)	30 (17.6)
18–30	8 (15.1)	38 (22.4)
31–50	6 (11.3)	54 (31.8)
>50	6 (11.3)	27 (15.9)
Sex		
Female	23 (43.4)	20 (11.8)
Male	30 (56.6)	150 (88.2)
Level of education		
Non-educated	39 (73.6)	89 (52.4)
Primary	10 (18.9)	47 (27.6)
Secondary and above	4 (7.5)	34 (20.0)
Occupation		
Unemployed adults	3 (5.7)	19 (11.2)
Schoolchildren (or younger)	32 (60.4)	45 (26.5)
Farmers	14 (26.4)	46 (27.1)
Self-employed daily workers	3 (5.7)	44 (25.9)
Government employees	1 (1.9)	16 (9.4)
No. of household members		
≤5	7 (13.2)	31 (18.2)
6–01	22 (42.5)	75 (44.1)
>01	24 (45.3)	64 (37.6)
Household monthly income		
<50,000 YER	37 (69.8)	119 (70.0)
≥50,000 YER	16 (30.2)	51 (30.0)
Housing conditions		
Good (brick, concrete or stone, no cracks in walls)	5 (9.4)	38 (22.4)
Poor (mud wall, cracks in walls)	48 (90.6)	132 (77.6)
Sources of drinking water		
Improved (piped)	3 (5.7)	11 (6.5)
Unimproved (rain, well, springs)	50 (94.3)	159 (93.5)

Values are n (%); YER, Yemeni Riyal; (US$1 = YER650)

4.4.1 Univariate analysis of factors associated with cutaneous leishmaniasis

Tables 4.4–4.7 summarise the univariate analyses of the associations between the main potential risk factors in their grouping blocks and CL among the participants. Table 4.4 demonstrates significantly higher prevalence of CL in female participants and children aged 10 or younger compared with males and those aged 10 or older. Similarly, non-educated participants and schoolchildren and younger children had significantly higher prevalence of CL compared with participants with a secondary or above level of education and unemployed adult participants, respectively. Internal displacement into the area, family size and household monthly income were not associated with the likelihood of having CL.

Among the household and indoor factors, Table 4.5 demonstrates that among these participants, living in poor housing conditions (houses with mud and/or cracked walls and wooden roofs) (OR = 2.76; 95%CI = 1.03, 7.43), having no access to improved toilet facilities (OR = 1.95; 95%CI = 1.03, 3.69) and living with family members with a typical ulcerating disease (OR = 4.68; 95%CI = 1.82, 12.1) were associated higher prevalence of CL. In contrast, participants who kept livestock animals on the ground floor of their houses (OR = 0.33; OR = 0.14, 0.75) had a significantly lower prevalence of CL compared with those who kept their animals in shelters outside the house. Of note, the source of drinking water, the number of household windows, having bed nets and not using screens for house windows were not associated with the odds of CL; nonetheless, the latter was only included in the multivariate model (P = 0.149).

Table 4.4: Univariate analyses of demographic and socioeconomic factors (Block 1) associated with cutaneous leishmaniasis among rural participants in Utmah district, Dhamar, Yemen

Variables	Confirmed cutaneous leishmaniasis			
	Case (n=53)	Control (n=170)	COR (95% CI)	*P*
Age group (years)				
≤10	27 (50.9)	21 (12.4)	5.79 (2.02, 16.6)	0.001*
11–17	6 (11.3)	30 (17.6)	0.90 (0.26, 3.13)	0.868
18–30	8 (15.1)	38 (22.4)	0.95 (0.30, 3.05)	0.928
31–50	6 (11.3)	54 (31.8)	0.50 (0.15, 1.70)	0.260
> 50	6 (18.2)	27 (56.2)	0	
Sex				
Female	23 (43.4)	20 (11.8)	5.75 (2.81, 11.7)	< 0.001*
Male	30 (56.6)	150 (88.2)	1	
Family size				
>01 (large)	24 (45.3)	64 (37.6)	1.66 (0.65, 4.27)	0.290
6–01	22 (41.5)	75 (44.1)	1.30 (0.50, 3.35)	0.588
≤5	7 (13.2)	31 (18.2)	1	
Educational level				
Non educated	39 (73.6)	89 (52.4)	3.73 (1.24, 11.2)	0.014*
Primary education	10 (18.9)	47 (27.6)	1.81 (0.52, 6.25)	0.345
Secondary or higher	4 (7.5)	34 (20.0)	1	
Occupation				
Others	4 (7.5)	60 (35.3)	0.42 (0.09, 2.06)	0.274**
Farmers	14 (26.4)	46 (27.1)	1.93 (0.50, 7.49)	0.337
Schoolchildren (or younger)	32 (60.4)	45 (26.5)	4.50 (1.23, 16.5)	0.016†
Unemployed adults	3 (5.7)	19 (11.2)	1	
Household monthly income				
<50,000 YER	37 (69.8)	119 (70.0)	0.99 (0.51, 1.94)	0.979
≥50,000 YER	16 (30.2)	51 (30.0)	1	
Internal displacement status‡				
Yes	7 (16.7)	16 (11.2)	1.53 (0.58, 4.01)	0.383
No	36 (85.7)	126 (88.1)	1	

YER, Yemeni Riyal; (US$1=YER650); COR, Crude odds ratio; CI, Confidence interval
* Variable included in the block multivariable analysis ($P \leq 0.25$), model A
** The difference was examined using Fisher's exact test (otherwise, Chi-Square test was used)
† Variable not included in the block multivariable analysis due to high correlation with age
‡ There were 38 participants with missing data in this variable

Table 4.5: Univariate analyses of household and indoor factors (Block 2) associated with cutaneous leishmaniasis among rural participants in Utmah district, Dhamar, Yemen

Variables	Confirmed cutaneous leishmaniasis			
	Case (n=53)	Control (n=170)	COR (95% CI)	*P*
Housing conditions				
Poor	48 (90.6)	132 (77.6)	2.76 (1.03, 7.43)	0.037*
Good	5 (9.4)	38 (22.4)	1	
Source of drinking water				
Unimproved source (spring, well, rain)	50 (94.3)	159 (93.5)	1.15 (0.31, 4.30)	0.832
Improved source (piped water)	3 (5.7)	11 (6.5)	1	
Presence of improved toilet in the house				
No	23 (43.4)	48 (28.2)	1.95 (1.03, 3.69)	0.039*
Yes	30 (56.6)	122 (71.8)	1	
Number of household windows				
>10	6 (11.3)	17 (10.0)	1.30 (0.46, 3.69)	0.622
6–10	25 (47.2)	72 (42.4)	1.28 (0.66, 2.46)	0.462
1–5	22 (41.5)	81 (47.6)	1	
Screened household windows				
No	49 (92.5)	144 (84.7)	2.21 (0.74, 6.65)	0.149*
Yes	4 (7.5)	26 (15.3)	1	
Having bed nets				
No	46 (86.8)	142 (83.5)	1.29 (0.53, 3.16)	0.569
Yes	7 (13.2)	28 (16.5)	1	
Presence of other family members with ulcerating disease				
Yes	11 (20.8)	9 (5.3)	4.68 (1.82, 12.1)	0.001*
No	42 (79.2)	161 (94.7)		
Location of livestock animals barn/shelter				
On the ground floor	12 (22.6)	74 (43.5)	0.33 (0.14, 0.75)	0.007*
Inside the one-storey house	23 (43.4)	60 (35.3)	0.77 (0.37, 1.61)	0.483
Outside the house	18 (34.0)	36 (21.2)	1	

COR, Crude odds ratio; CI, Confidence interval
Poor house type: mud walls, cracks in walls and wooden roof.
Good house type: built of brick or stone with no cracks in walls.
* Variable included in the block multivariable analysis ($P \leq 0.25$), model B

With regards to environmental and outdoor factors (Table 4.6), the presence of open water sources near the house (OR = 1.95; 95%CI = 1.05, 3.64) and owning goats (OR = 2.06; 95%CI = 1.10, 3.90) were significantly associated with a higher prevalence of CL. In contrast, owning dogs was significantly associated with a lower prevalence of CL (OR = 0.51; 95%CI = 0.27, 0.95). Of note, sightings of field rodents in the surrounding area was associated with a higher prevalence of CL, whereas the presence of bats in the vicinity and owning donkeys were associated with a lower prevalence of the disease; however, the associations were not statistically significant. Nonetheless, these factors were included in the multivariate analysis (model C) following the $P \leq 0.25$ inclusion criterion.

Table 4.7 shows that a significant level of risk of acquiring CL was associated with a poor level of knowledge about CL transmission (OR = 2.25; 95%CI = 1.08, 4.68) and also about sandflies (OR = 2.10; 95%CI = 1.06, 4.15). In the last block for behavioural and health factors (Table 4.7), a significant association was found between the prevalence of confirmed CL and sleeping outside (OR = 2.61; 95%CI = 1.31, 5.18). Not using bed nets was included in the block multivariate regression model ($P = 0.088$). However, knowledge about CL prevention, working outside at night, sleeping with the windows open, using insecticide, wearing long sleeve clothes, travelling outside the district and having chronic diseases were not independently associated with the risk of acquiring CL ($P > 0.05$).

Table 4.6: Univariate analysis of environmental and outdoor conditions and animal characteristics (Block 3) associated with cutaneous leishmaniasis among the participants

Variables	Confirmed cutaneous leishmaniasis			
	Case (n=53)	Control (n=170)	COR (95% CI)	*P*
Proximity to open water source				
Near (≤250 meters)	28 (52.8)	62 (36.5)	1.95 (1.05, 3.64)	0.034*
Far (>250 meters)	25 (47.2)	108 (63.5)	1	
Seen wild rabbits in the last 3 months				
Yes	16 (30.2)	46 (27.1)	1.17 (0.59, 2.29)	0.657
No	37 (69.8)	124 (72.9)	1	
Seen field rodents in the last 3 months				
Yes	20 (37.7)	44 (25.9)	1.74 (0.90, 3.33)	0.096*
No	33 (62.3)	126 (74.1)	1	
Presence of bats in the vicinity				
Yes	42 (79.2)	147 (86.5)	0.60 (0.27, 1.32)	0.201*
No	11 (20.8)	21 (13.5)	1	
Owning domestic animals				
Yes	47 (88.7)	154 (90.6)	0.82 (0.30, 2.20)	0.684
No	6 (11.3)	16 (9.4)	1	
Owning dogs				
Yes	23 (43.4)	102 (60.0)	0.51 (0.27, 0.95)	0.033*
No	30 (56.6)	68 (40.0)	1	
Owning cats				
Yes	31 (58.5)	111 (65.3)	0.75 (0.40, 1.41)	0.369
No	22 (41.5)	59 (34.7)	1	
Owning goats				
Yes	34 (64.2)	79 (46.5)	2.06 (1.10, 3.90)	0.025*
No	19 (35.8)	91 (53.5)	1	
Owning sheep				
Yes	16 (30.2)	61 (35.9)	0.89 (0.44, 1.76)	0.447
No	37 (69.8)	109 (64.1)	1	
Owning cows				
Yes	38 (71.7)	126 (74.1)	0.86 (0.44, 1.68)	0.727
No	15 (28.3)	44 (25.9)	1	
Owning donkeys				
Yes	19 (35.8)	80 (47.1)	0.63 (0.33, 1.19)	0.152*
No	34 (64.2)	90 (52.9)	1	
Owning chicken				
Yes	21 (39.6)	66 (38.8)	1.03 (0.55, 1.94)	0.917
No	32 (60.4)	104 (61.2)	1	

COR, Crude odds ratio; CI, Confidence interval
* Variable included in the block multivariable analysis ($P \leq 0.25$), model C

Table 4.7: Univariate analyses of participants' awareness about the disease and vector (Block 4), and behavioural and health factors (Block 5) associated with cutaneous leishmaniasis among the participants

Variables	Confirmed cutaneous leishmaniasis			
	Case (n=53)	Control (n=170)	COR (95% CI)	*P*
AWARENESS FACTORS[†]				
Knowledge about CL transmission				
Poor	42 (79.2)	107 (62.9)	2.25 (1.08, 4.68)	0.028*
Good	11 (20.8)	63 (37.1)	1	
Knowledge about CL prevention				
Poor	34 (64.2)	96 (56.5)	1.38 (0.73, 2.61)	0.322
Good	19 (35.8)	74 (43.5)	1	
Knowledge about sandfly vector				
Poor	39 (73.6)	97 (57.1)	2.10 (1.06, 4.15)	0.031*
Good	14 (26.4)	73 (42.9)	1	
BEHAVIOURAL AND HEALTH FACTORS				
Working outside during night				
Yes	12 (22.6)	29 (17.1)	1.42 (0.67, 3.04)	0.360
No	41 (77.4)	141 (82.9)	1	
Sleeping outside during night				
Yes	19 (35.8)	30 (17.6)	2.61 (1.31, 5.18)	0.005**
No	34 (64.2)	140 (82.4)	1	
Sleeping with windows open				
Yes	45 (84.9)	136 (80.0)	1.41 (0.61, 3.26)	0.425
No	8 (15.1)	34 (20.0)	1	
Using insecticide				
No	50 (94.3)	156 (91.8)	1.50 (0.41, 5.42)	0.537
Yes	3 (5.7)	14 (8.2)	1	
Using bed nets				
No	51 (96.2)	150 (88.2)	3.40 (0.77, 13.5)	0.088**
Yes	2 (3.8)	20 (11.8)	1	
Wearing long sleeve clothes				
No	31 (58.5)	85 (50.0)	1.41 (0.76, 2.63)	0.280
Yes	22 (41.5)	85 (50.0)	1	
Travelled outside the district in the last one year				
Yes	12 (22.6)	52 (30.6)	0.66 (0.32, 1.37)	0.264
No	41 (77.4)	118 (69.4)	1	
Having chronic diseases (comorbidities)				
Yes	9 (17.0)	37 (21.8)	0.74 (0.33, 1.64)	0.452
No	44 (83.0)	133 (78.2)	1	

COR, Crude odds ratio; CI, Confidence interval
Variable included in block multivariable analyses ($P \leq 0.25$): * model D, ** final model
[†] Data based on responses of adult participants and parents/guardians of participating children

4.4.2 Multivariate analysis of factors associated with cutaneous leishmaniasis

Table 4.8 demonstrates the multivariate hierarchical logistic regression analyses. In model A, being aged 10 or younger or of the female sex was identified as a significant risk factor of CL, with likelihood ratios of 4.28 and 3.84, respectively. The occupation variable, specifically the "schoolchildren and younger" category, was closely associated with the age variable, and the inclusion of both factors in the model led to instability. Moreover, in these rural settings, housewives and students are also engaged in farm activities and are, therefore, at risk of infection, and this resulted in an unclear grouping. Consequently, occupation was not included in model A and subsequent analyses. It should be noted that when an "engaged in farming activities" variable was separately assessed, participants engaged in farm work had significantly higher likelihood ratios, but this analysis is not reported here due to a potentially close link between occupation categories and age. In model B, sharing a residence with a family member with ulcerating skin lesions and keeping livestock animals on the ground floor of the house remained significant factors of CL. Moreover, poor housing conditions variable was included in model C *(P* = 0.091). In model C, only the presence of open water sources near the household was retained as a significant CL risk factor. Furthermore, owning goats was included in the final model *(P* = 0.076). In model D, significant associations of both awareness-related factors were not retained; however, having poor level of knowledge about sandfly vector was included in the final model (*P* = 0.098).

In the final model (Table 4.8), six variables were identified as the significant predictors of CL among the studied participants. Hosmer–Lemeshow test, used for the inferential goodness-of-fit test, indicated that the models were sufficient in explaining the data (with final model: χ^2=6.153; *P*=0.630). Age of 10 years or below (AOR = 3.49; 95%CI = 1.44, 8.48), female (AOR = 5.57; 95%CI = 2.31, 15.65), living in poor

housing conditions with mud-plastered and cracked walls (AOR = 3.84; 95%CI = 1.13, 14.77), presence of other family members with ulcerating skin lesions (AOR = 3.89; 95%CI = 1.19, 12.76), and sleeping outside (AOR = 3.58; 95%CI = 1.63, 8.96) were the significant risk factors of CL in the final model. Moreover, keeping livestock animals on the ground floor of the house remained protective (AOR = 0.25; 95% CI = 0.11, 0.62). Interestingly, another regression model (not shown) showed a similar protective association of keeping animals on the ground floor of the house when compared to sharing the one-storey household space with the animals (AOR = 0.35; 95% CI = 0.21, 0.85) and also compared to both groups (i.e. outside and inside one-storey household) merged in one reference category (AOR = 0.39; 95% CI = 0.21, 0.77). By contrast, proximity to open water sources, owning goats, knowledge about CL transmission, knowledge about sandflies, and using of bed nets were not retained as significant factors in the final model ($P > 0.05$).

Table 4.8: Multivariable logistic regression models of factors associated with cutaneous leishmaniasis among the participants in Utmah district, Dhamar, Yemen

Variables	Confirmed cutaneous leishmaniasis		
	AOR	95% CI	*p*
MODEL A*			
Age (≤10 years)	4.28	1.94, 9.43	< 0.001
Sex (female)	3.84	1.75, 8.44	0.001
Educational level (Non educated)	1.38	0.54, 3.52	0.503
MODEL B**			
Housing conditions (poor)	2.64	0.86, 8.16	0.091
Presence of improved toilet in the house (no)	1.12	0.48, 2.58	0.794
Screened household windows (no)	2.16	0.61, 7.71	0.237
Presence of other family members with skin ulcerating disease (yes)	6.35	2.24, 18.0	0.001
Location of livestock animals barn/shelter (ground floor)	0.30	0.13, 0.69	0.005
MODEL C***			
Proximity to open water sources (≤250 meters)	2.26	1.03, 4.93	0.041
Seen field rodents in the last 3 months (yes)	1.75	0.76, 4.00	0.187
Presence of bats in the vicinity (yes)	1.03	0.35, 3.00	0.963
Owning dogs (yes)	0.44	0.15, 1.29	0.135
Owning goats (yes)	1.93	0.89, 4.19	0.093
Owning donkeys (yes)	0.71	0.32, 1.57	0.397
MODEL D†			
Knowledge about CL transmission (poor)	1.26	0.54, 3.12	0.625
Knowledge about sandfly vector (poor)	1.95	0.88, 4.56	0.098
FINAL MODEL‡			
Age (≤ 10 years)	3.49	1.44, 8.48	**0.006**
Sex (female)	5.57	2.31, 15.65	**< 0.001**
Housing conditions (poor)	3.84	1.13, 14.77	**0.029**
Presence of other family members with skin ulcerating disease (yes)	3.89	1.19, 12.76	**0.024**
Location of livestock animals barn/shelter (ground floor)	0.25	0.11, 0.62	**0.003**
Proximity to open water sources (≤250 meters)	1.49	0.67, 3.33	0.330
Owning goats (yes)	1.32	0.51, 3.91	0.627
Knowledge about sandfly vector (poor)	1.58	0.69, 3.68	0.279
Sleeping outside during night (yes)	3.58	1.63, 8.96	**0.002**
Using bed nets (yes)	0.41	0.07, 2.52	0.337

AOR, adjusted odds ratio; CI, Confidence interval

* Model A involved covariates from univariate analysis with *P*-value ≤0.25 (Block 1)

** Model B involved covariates from univariate analysis with *P*-value ≤0.25 (Block 2) and adjusted for covariates from model A with *P*-value <0.1, age and sex

*** Model C involved covariates from univariate analysis with *P*-value ≤0.25 (Block 3) and adjusted for covariates from model B with *P*-value <0.1, age and sex

† Model D involved covariates from univariate analysis with *P*-value ≤0.25 (Block 4) and adjusted for covariates from model C with *P*-value <0.1, age and sex

‡ The final model involved covariates from univariate analysis with *P*-value ≤0.25 (Block 5) and adjusted for covariates from model D with *P*-value <0.1, age and sex

4.5 Assessment of respondents' knowledge, attitudes and practices about cutaneous leishmaniasis

A total of 289 household heads (75.1% male, 24.9% female) from the selected areas of the Utmah District participated in this KAP survey. The majority of them were males (75.1%, 217/289) while 72 (24.9%) were females, with their median age was 42 years (IQR 32–54 years). Overall, 152 (52.6%) of the respondents were non-educated, while 47.4% had attained at least six years of formal education (primary education), with 73 (25.3%) had secondary and above education. A total of 130 (45.0%) were mainly involved in farming activities, 88 (30.4%) were self-employed daily workers, 26 (9.0%) were government employees, and 45 (15.6%) were unemployed.

4.5.1 Respondents' knowledge about cutaneous leishmaniasis

Table 4.9 shows the results of the respondents" knowledge about the signs and symptoms, transmission, prevention and treatment of CL. It was found that all the respondents had prior knowledge about the disease in its local name. However, only 69.6% (201/289) had heard of the scientific terms „*Leishmania'* or „leishmaniasis", and only 4.2% (12/289) correctly identified the disease as a fly-induced skin disease. The respondents mentioned some local names to describe the CL lesion, such as *sheqna* (lesion spreads among all family members), *bada'h* or *budda* (popular/nodular lesion at the initial stage), *bulla* (wet lesion) and *akela* (skin-eating, *ulcerative* skin *lesion*). Knowledge about the scientific terms was mostly through medical teams who visited the area (52.2%; 105/201), followed by the clinics where they sought treatment (26.4%; 53/201) and 19.9% (40/201) from family members, friends and neighbours. Over half of the respondents (51.9%; 150/289) did not know the cause of CL, but 9.3% attributed it to sandflies and 17.6% to mosquitoes (not specifically sandflies). Moreover, 8.7% of the respondents believed that CL is transmitted from animals and 6.9% from infected individuals, respectively.

Table 4.9: Knowledge about cutaneous leishmaniasis among the study respondents (n = 289)

Variables	Response	n (%)
Know the disease by the term	Leishmania or leishmaniasis	201 (69.6)
	Local names only	76 (26.2)
	Fly-induced skin disease	12 (4.2)
Source of information about the term leishmaniasis (n=201)	Medical team visits	105 (52.2)
	Clinic	53 (26.4)
	Family, friends & neighbours	40 (19.9)
	School	3 (1.5)
Knowledge about causes and transmission of CL	Bites of mosquitoes/insects	55 (18.6)
	Bites of sandflies	27 (9.3)
	Houseflies	13 (4.5)
	Through animals	25 (8.7)
	Human-to-human	20 (6.9)
	Do not know	150 (51.9)
Knowledge about symptoms of CL	Skin lesion	149 (55.0)
	Skin wound	52 (18.0)
	Skin scar	46 (15.9)
	Itching and redness	32 (11.1)
Knowledge about prevention methods of CL	Treating patients	41 (14.2)
	Using insecticide	33 (11.4)
	Improved personal hygiene	29 (10.0)
	Isolating patients	26 (9.0)
	Using bed nets	21 (7.3)
	Do not know	139 (48.1)
Knowledge about treatment options of CL	Chemotherapy	83 (28.7)
	Herbal medicine	59 (20.4)
	Cauterisation	34 (11.8)
	No treatment	16 (5.5)
	Self-heal	10 (3.5)
	Do not know	87 (30.1)
Overall knowledge about CL	-	-
Total mean score ± SD	-	3.3 ± 1.2
Good knowledge (score 4–5)	-	148 (51.2)
Poor knowledge (score 0–3)	-	141 (48.8)

Regarding symptoms of CL, 55.0% correctly mentioned skin lesions, 18.0% skin wounds and 15.9% skin scars. When respondents were asked about the prevention of CL, 48.1% (139/289) could not cite any preventive measure, while 42.9% (124/289) had correct knowledge: 11.4% mentioned using insecticides, 14.2% mentioned treating infected patients and 7.3% mentioned using bed nets. Regarding the treatment of CL, 30.1% of the respondents could not mention any treatment method, while 60.9% (176/289) mentioned one correct treatment method, with 28.7%, 20.4% and 11.8% of the respondents correctly believing that chemotherapy, herbal medicine and cauterisation, respectively, are methods of treatment of CL. Based on the scoring system, 51.2% of the respondents had a good level of knowledge about CL while 48.8% had a poor level.

4.5.2 Respondents' knowledge about the sandfly vector

Although all the respondents had prior knowledge about the sandfly in its local name, only 26.6% (77/289) of them had heard the term „sandfly" in its Arabic translation (Table 4.10). Moreover, 36.0% (104/289) were able to describe and differentiate sandflies from other common mosquitoes and insects based on some correct feature of the vector. Regarding breeding places of sandflies, a majority (54.7%; 158/289) of the respondents did not know the answer, while 17.0%, 8.3% and 5.5% mentioned domestic animal barns, valleys and caves, respectively. Only 42.9% of the respondents had knowledge about the biting time of sandflies. Moreover, 65.4% (189/289) of the respondents were unable to mention any control measures against sandflies, while 34.6% of them mentioned correct measures, such as spraying insecticides (18.3%), screening of windows (4.8%) and using bed nets (3.8%). Overall, only 33.9% of the respondents had a good or satisfactory level of knowledge about sandflies, while about two-thirds (66.1%) had a poor level.

Table 4.10: Knowledge about the sandfly vector among the study respondents (n = 289)

Variables	Response categories	n (%)
Know the sandfly vector by the term	Sandfly	77 (26.6)
	Only local name	212 (73.4)
Source of information about the term sandfly (n=77)	Medical team visits	20 (26.0)
	Clinic	5 (6.5)
	Family, friends & neighbours	52 (67.5)
Can identify and differentiate sandflies from other mosquitoes and common flies	Yes	104 (36.0)
	No	185 (64.0)
Know breeding places of sandflies	Cattle barns	49 (17.0)
	Valleys	24 (8.3)
	Old houses	22 (7.6)
	Caves	16 (5.5)
	Wells	11 (3.8)
	Deserts	9 (3.1)
	Do not know	158 (54.7)
Know biting time of sandflies	During night time	124 (42.9)
	During day time	21 (7.3)
	At any time	51 (17.6)
	Do not know	93 (32.2)
Know methods to control sandflies	Spraying insecticides	53 (18.3)
	Screening of windows	14 (4.8)
	Cleanliness	13 (4.5)
	Using bed nets	11 (3.8)
	Using mosquito coil smoke	9 (3.1)
	Do not know	189 (65.4)
Overall knowledge about sandflies	-	-
Total mean score ± SD	-	2.6 ± 1.3
Good knowledge (score 4–5)	-	98 (33.9)
Poor knowledge (score 0–3)	-	191 (66.1)

4.5.3 Respondents' attitudes and practices concerning cutaneous leishmaniasis

Table 4.11 shows that a total of 246 respondents (85.1%) considered CL to be a serious disease. The majority (76.5%) believed that CL is a curable disease, while 9.0% believed that the disease cannot be cured. Moreover, only 27.3% of the respondents believed that CL can be prevented. In addition, 45.7% (132/289) believed that CL is a stigmatising disease, with a significantly higher percentage of them being female compared to male (56.9% vs. 41.9%; $\chi^2 = 4.908$; $P = 0.027$). Overall, it was found that only 38.1% (110/289) of the respondents had an overall positive or favourable attitude towards CL.

Table 4.12 shows the results of the respondents" CL-related practices. Widespread poor practices were observed among the respondents, with only 16.3% (47/289) of them having good CL-related practices in general. The results revealed that only 9% (26/289) of the respondents used bed nets and 9.3% (27/289) used insecticides, and 13.8% (40/289) had screened windows with nets. Similarly, only 18.3% (53/289) of the respondents lived in good houses (built of brick or stone with no cracks in the walls). Moreover, 35.6% and 30.4% of the respondents work and sleep outside at night, respectively.

Table 4.11: Attitudes towards CL among the study respondents (n = 289)

Variables	Response categories	n (%)
Is CL a serious disease?	Yes*	246 (85.1)
	No	28 (9.7)
	Do not know	15 (5.2)
Is CL curable?	Yes*	221 (76.5)
	No	26 (9.0)
	Do not know	42 (14.5)
Is CL preventable?	Yes*	79 (27.3)
	No	167 (57.8)
	Do not know	43 (14.9)
I believe that having close contact with individuals infected with CL is safe	Yes*	175 (60.6)
	No	60 (20.8)
	Do not know	54 (18.7)
Is CL a stigmatising disease?	Yes	132 (45.7)
	No*	157 (54.3)
Overall attitude status		
Total mean score ± SD	-	3.1 ± 1.2
Positive attitude (score 4-5)	-	110 (38.1)
Negative attitude (score 0-3)	-	179 (61.9)

* Correct responses were assigned a score of 1 and other responses were assigned 0.

Table 4.12: Practices of respondents with regards to CL in Utmah district, western Yemen

Variables	Response categories	n (%)
Using bed nets	Yes*	26 (9.0)
	No	263 (91.0)
Using insecticide spray in the household	Yes*	27 (9.3)
	No	262 (90.7)
Living in a good house (no cracks in walls)	Yes*	53 (18.3)
	No	236 (81.7)
Living near open water sources (≤ 250 meters)	Yes	179 (61.9)
	No*	110 (38.1)
Working outside during night	Yes	103 (35.6)
	No*	186 (64.4)
Sleeping outside during night	Yes	88 (30.4)
	No*	201 (69.6)
Wearing long sleeve clothes	Yes*	149 (51.6)
	No	140 (48.4)
Keeping windows closed during night	Yes*	87 (30.1)
	No	202 (69.9)
Screening of windows with nets	Yes*	40 (13.8)
	No	249 (86.2)
Keeping livestock animals outside the house	Yes*	68 (23.5)
	No	221 (76.5)
Overall practice status		
Total mean score ± SD	-	3.9 ± 1.7
Good (score 6-10)	-	47 (16.3)
Poor (score 0-5)	-	242 (83.7)

* Correct responses were assigned a score of 1 and other responses were assigned 0.

4.5.4 Treatment-seeking behaviour and practices concerning CL

Table 4.13 shows that 56.7% of the respondents mentioned that they would go to the nearest health centre when they have skin lesions, while 28.4% would primarily/preferably use herbal remedies for the treatment of skin lesions, and 10.4% indicated that they do not treat skin lesions. However, when questioned about how they had previously treated skin lesions (probably due to CL) for themselves or their family members, a substantial number (50%; 79/158) stated they used traditional herbal remedies, followed by seeking treatment from health centres (31.7%). Interestingly, 13.9% (22/158) used traditional methods, such as cauterisation of the lesions with very hot metal objects (spoons, knives or iron skewers), or applying caustic materials or strong acids, such as battery acid. While the majority of those who applied traditional remedies (55.7%; 44/79) used herbal remedies from traditional healers, some medicinal plants were also mentioned, including *Aloe vera, Calotropis procera, Ficus palmate*, *Acalypha fruticosa* and *Prunus dulcis*.

4.5.5 Factors associated with KAP concerning CL and the sandfly vector

Table 4.14 shows that the percentage of respondents with monthly household incomes of ≥ YER50,000 who had a good level of knowledge about CL was significantly higher than that of respondents with monthly household incomes below YER50,000 (61.2% vs. 47.4%; $\chi^2 = 4.462$; $P = 0.035$). Similarly, the percentages of respondents with good level of knowledge about CL were higher among respondents who lived in the same household as confirmed CL cases (62.3%) and those from Hemyar Alwasat sub-district (66.7%) compared with those lived in households without CL cases (48.7%) and those from Alsomal (52.9%); however, the differences were not statistically significant ($P = 0.075$ and $P = 0.232$, respectively). On the other hand, the percentages with good level

Table 4.13: Treatment-seeking behaviour and related practices of respondents in relation to CL in the Utmah district, western Yemen

Variables	n (%)
Treatment-seeking behaviour of skin lesions (as a first line activity)	
Go to hospital/clinic	164 (56.7)
Use traditional herbal remedies	82 (28.4)
Cauterisation	13 (4.5)
Do nothing	30 (10.4)
Treatment of previous CL-suspected lesions (n=158)	
Medication from health centres	50 (31.7)
Traditional medicinal plants	79 (50.0)
Cauterisation	13 (8.2)
Application of acids	9 (5.7)
Did nothing	7 (4.4)
Medicinal plants used by the respondents (n=79)	
Scientific name (local name/part used)	
Aloe vera (Saber/leaf latex & gel)	8 (10.1)
Calotropis procera (Oshar/milky latex)	6 (7.6)
Ficus palmata (Hamat/milky latex)	5 (6.3)
Acalypha fruticosa (Enshit/leaves)	5 (6.3)
Prunus dulcis (Lauz/flowers)	3 (3.8)
Ready herbal remedies from traditional healers	44 (55.7)
Did not remember	8 (10.1)

of knowledge about CL were lower among females (41.7%), respondents from Razeh sub-district (43.5%) and those working as farmers (42.3%) when compared with males (54.4%), those from Alsomal (52.9%), and not working respondents (53.3%); however, the differences were not statistically significant ($P > 0.05$).

Regarding knowledge about the sandfly vector, Table 4.14 demonstrates that the percentage of those possessing a good level of knowledge was significantly higher in respondents over 40 years of age (40.5%) compared to that of their younger counterparts (27.0%) ($\chi^2 = 5.972$; $P = 0.014$). By contrast, the percentage of respondents with a good level of knowledge about sandflies was significantly lower among respondents from the Bani Bahr sub-district (27.9%) compared to those from Alsomal (44.7%) ($\chi^2 = 5.941$; $P = 0.015$). Similarly, the percentage with good level of knowledge was low among respondents from Hemyar Alwasat (29.2%) and Razeh sub-districts when compared to that of respondents from Alsomal (44.7%), and among those who lived in the same household with confirmed CL cases (24.5%) compared to those who lived in households without CL cases (36.0%); however, the differences were not statistically significant ($P > 0.05$).

Table 4.14: Association of respondents' knowledge towards CL and the sandfly vector with their socio-demographic factors

Variables	Knowledge about CL**				Knowledge about sandfly†			
	Poor	**Good**	**COR (95% CI)**	***P***	**Poor**	**Good**	**COR (95% CI)**	***P***
Age (years)								
> 40	77 (52.0)	71 (48.0)	0.77 (0.48, 1.22)	0.259	88 (59.5)	60 (40.5)	1.85 (1.13, 3.04)	0.014*‡
18–40	64 (45.4)	77 (54.6)	1		103 (73.0)	38 (27.0)	1	
Gender								
Female	42 (58.3)	30 (41.7)	0.60 (0.35, 1.03)	0.062‡	44 (61.1)	28 (38.9)	1.34 (0.77, 2.32)	0.303
Male	99 (45.6)	118 (54.4)	1		147 (67.7)	70 (32.3)	1	
Location								
Hemyar Alwasat	8 (33.3)	16 (66.7)	1.78 (0.69, 4.60)	0.232‡	17 (70.8)	7 (29.2)	0.51 (0.19, 1.36)	0.172‡
Bani Bahr	54 (48.6)	57 (51.4)	0.94 (0.53, 1.65)	0.825	80 (72.1)	31 (27.9)	0.48 (0.26, 0.87)	0.015*‡
Razeh	39 (56.5)	30 (43.5)	0.68 (0.36, 1.30)	0.243‡	47 (68.1)	22 (31.9)	0.58 (0.31, 1.12)	0.105‡
Alsomal	40 (47.1)	45 (52.9)	1		47 (55.3)	38 (44.7)	1	
Level of education								
Secondary & above	33 (45.2)	40 (54.8)	1.19 (0.67, 2.07)	0.561	50 (68.5)	23 (31.5)	089 (0.49, 1.61)	0.687
Primary	33 (51.6)	31 (48.4)	0.92 (0.51, 1.64)	0.766	41 (64.1)	23 (35.9)	1.08 (0.59, 2.01)	0.808
Non educated	75 (49.3)	77 (50.7)	1		100 (65.8)	52 (34.2)	1	

Table 4.14: Continued

Variables	Knowledge about CL**				Knowledge about sandfly†			
	Poor	Good	COR (95% CI)	*P*	Poor	Good	COR (95% CI)	*P*
Occupation								
Government employees	9 (34.6)	17 (65.4)	1.65 (0.61, 4.48)	0.322	17 (65.4)	9 (34.6)	1.17 (0.42, 3.27)	0.761
Self-employed workers	36 (40.9)	52 (59.1)	1.26 (0.61, 2.61)	0.526	62 (70.5)	26 (29.5)	0.93 (0.43, 2.03)	0.852
Farmers	75 (57.7)	55 (42.3)	0.64 (0.33, 1.27)	0.200‡	81 (62.3)	49 (37.7)	1.34 (0.65, 2.76)	0.428
Not working	21 (46.7)	24 (53.3)	1		31 (68.9)	14 (31.1)	1	
Monthly household income								
≥ 50,000 YER	31 (38.8)	49 (61.2)	1.76 (1.04, 2.97)	0.035*‡	49 (61.2)	31 (38.8)	1.34 (0.79, 2.29)	0.282
< 50,000 YER	110 (52.6)	99 (47.4)	1		142 (67.9)	67 (32.1)	1	
Presence of confirmed CL cases among family members								
Yes	20 (37.7)	33 (62.3)	1.74 (0.94, 3.20)	0.075‡	40 (75.5)	13 (24.5)	0.58 (0.29, 1.14)	0.110‡
No	121 (51.3)	115 (48.7)	1		151 (64.0)	85 (36.0)	1	

Values are number (%). COR, Crude odds ratio. CI, Confidence interval.
* Significant association ($P < 0.05$), ** Based on scores shown in Table 4.9, † Based on scores shown in Table 4.10. ‡ Included in multivariate analyses ($P \leq 0.25$)

Table 4.15 shows the association of respondents‟ attitude towards CL with the socio-demographic factors. The percentage of respondents with a good or positive attitude towards CL was significantly lower among females (26.4%), respondents from Razeh (23.2%) and those who lived in a household with a confirmed CL case (42.5%) when compared with males (41.9%) (χ^2 = 5.543; P = 0.019), those from Alsomal (41.2%) (χ^2 = 5.564; P = 0.018) and those who lived in households without CL cases (41.1%) (χ^2 = 5.043; P = 0.025). Moreover, the percentage of respondents with good level of attitude was high among those who had good knowledge about CL compared to their peers who had poor level of knowledge (43.2 vs. 32.6%); however, the difference was not statistically significant (P = 0.063).

The results in Table 4.16 show that the percentage of individuals with good practices related to CL was significantly high among respondents who had a secondary and above education level compared to non-educated respondents (24.7 vs. 13.2%; χ^2 = 4.646; P = 0.031). Similarly, a significantly higher percentage of respondents who had good CL-related practices were those with monthly household incomes of ≥ YER50,000 compared to those with low monthly household incomes (23.8% vs. 13.4%; χ^2 = 4.553; P = 0.033). Also, presence of confirmed CL cases among family members was associated with high percentage of good practices; however, the association was not statistically significant (χ^2 = 2.223; P = 0.136).

Table 4.15: Association of respondents' attitudes towards cutaneous leishmaniasis with their socio-demographic factors

Variables	Attitudes towards CL[†]			
	Positive	Negative	COR (95% CI)	*P*
Age (years)				
> 40	87 (58.8)	61 (41.2)	1.32 (0.82, 2.12)	0.258
18–40	92 (65.2)	49 (34.8)	1	
Gender				
Female	53 (73.6)	19 (26.4)	0.50 (0.28, 0.90)	0.019[*‡]
Male	126 (58.1)	91 (41.9)	1	
Location				
Hemyar Alwasat	16 (66.7)	8 (33.3)	0.72 (0.28, 1.85)	0.488
Bani Bahr	60 (54.1)	51 (45.9)	1.21 (0.69, 2.15)	0.505
Razeh	53 (76.8)	16 (23.2)	0.43 (0.21, 0.87)	0.018[*‡]
Alsomal	50 (58.8)	35 (41.2)	1	
Level of education				
Secondary & above	41 (56.2)	32 (43.8)	1.30 (0.74, 2.29)	0.363
Primary	43 (67.2)	21 (32.8)	0.81 (0.44, 1.51)	0.513
Non educated	95 (62.5)	57 (37.5)	1	
Occupation				
Government employees	15 (57.7)	11 (42.3)	1.47 (0.54, 3.97)	0.450
Self-employed workers	60 (68.2)	28 (31.8)	0.93 (0.43, 2.01)	0.860
Farmers	74(56.9)	56 (43.1)	1.51 (0.74, 3.08)	0.251
Not working	30 (66.7)	15 (33.3)	1	
Monthly household income				
≥ 50,000 YER	48 (60.0)	32 (40.0)	1.12 (0.66, 1.90)	0.675
< 50,000 YER	131 (62.7)	78 (37.3)	1	
Presence of confirmed CL cases among family members				
Yes	40 (75.5)	13 (24.5)	0.47 (0.24, 0.92)	0.025[*‡]
No	139 (58.9)	97 (41.1)	1	
Level of knowledge about CL				
Good	84 (56.8)	64 (43.2)	1.57 (0.97, 2.54)	0.063[‡]
Poor	95 (67.4)	46 (32.6)	1	
Level of knowledge about sandflies				
Good	57 (58.2)	41 (41.8)	1.27 (0.77, 2.09)	0.344
Poor	122 (63.9)	69 (36.1)	1	

Values are number (%). COR, Crude odds ratio. CI, Confidence interval
[*] Significant association ($P < 0.05$)
[†] Based on scores shown in Table 4.11
[‡] Included in multivariate analysis ($P \leq 0.25$)

Table 4.16: Association of respondents' practices towards cutaneous leishmaniasis with their socio-demographic factors

Variables	Practices towards CL[†]			
	Poor	Good	COR (95% CI)	*P*
Age (years)				
> 40	123 (83.1)	25 (16.9)	1.10 (0.59, 2.06)	0.767
18–40	119 (84.4)	22 (15.6)	1	
Gender				
Female	58 (80.6)	14 (19.4)	1.35 (0.67, 2.69)	0.399
Male	184 (84.8)	33 (15.2)	1	
Location				
Hemyar Alwasat	19 (79.2)	5 (20.8)	1.23 (0.40, 3.81)	0.911
Bani Bahr	94 (84.7)	17 (15.3)	0.84 (0.39, 1.81)	0.662
Razeh	59 (85.5)	10 (14.5)	0.79 (0.33, 1.89)	0.779
Alsomal	70 (82.4)	15 (17.6)	1	
Level of education				
Secondary & above	55 (75.3)	18 (24.7)	2.16 (1.06, 4.39)	0.031[*‡]
Primary	55 (85.9)	9 (14.1)	1.08 (0.46, 2.52)	0.859
Non educated	132 (86.8)	20 (13.2)	1	
Occupation				
Government employees	19 (73.1)	7 (26.9)	1.89 (0.67, 6.34)	0.251
Self-employed workers	68 (77.3)	20 (22.7)	1.60 (0.62, 4.12)	0.329
Farmers	117 (90.0)	13 (10.0)	0.60 (0.22, 1.62)	0.313
Not working	38 (84.4)	7 (15.6)	1	
Monthly household income				
≥50,000 YER	61 (76.2)	19 (23.8)	2.01 (1.05, 3.86)	0.033[*‡]
<50,000 YER	181 (86.6)	28 (13.4)	1	
Presence of confirmed CL cases among family members				
Yes	48 (90.6)	5 (9.4)	0.48 (0.18, 1.28)	0.136[‡]
No	194 (82.2)	42 (17.8)	1	
Level of knowledge about CL				
Good	121 (81.8)	27 (18.2)	1.35 (0.72, 2.54)	0.350
Poor	121 (85.8)	20 (14.2)	1	
Level of knowledge about sandflies				
Good	79 (80.6)	19 (19.4)	1.40 (0.74, 2.66)	0.302
Poor	163 (85.3)	28 (14.7)	1	
Level of attitude towards CL				
Positive	96 (87.3)	14 (12.7)	0.65 (0.33, 1.27)	0.202[‡]
Negative	146 (81.6)	33 (18.4)	1	

Values are number (%). COR, Crude odds ratio. CI, Confidence interval
[*] Significant association ($P < 0.05$)
[†] Based on scores shown in Table 4.12
[‡] Included in multivariate analysis ($P \leq 0.25$)

4.5.6 Multivariate analysis of factors associated with KAP about cutaneous leishmaniasis and sandfly vector

The multivariate logistic regression analyses (Table 4.17) show that females (AOR = 0.53; 95% CI = 0.32, 0.82) and respondents working as farmers (AOR = 0.51; 95% CI = 0.33, 0.83) were less likely to have good knowledge about CL compared to their male and non-working counterparts. Moreover, respondents who lived in the same household with confirmed CL cases were about two times (AOR = 1.94; 95% CI = 1.03, 3.66) more likely to have good CL-related knowledge compared to those in households without confirmed CL cases. Also, the results demonstrate that the odds of having good knowledge about sandflies increased by about two times among respondents over 40 years of age compared to younger respondents (AOR = 1.95; 95% CI = 1.18, 3.22).

Interestingly, respondents who lived with confirmed CL cases in the same household were 0.46 times (AOR = 0.46; 95% CI = 0.23, 0.93) less likely to have a good attitude towards CL compared to those living in households without CL cases. Likewise, respondents from Razeh were less likely to have a positive attitude towards CL compared to those from Alsomal (AOR = 0.42; 95% CI = 0.23, 0.82). Regarding practices, respondents with monthly household incomes of ≥ YER50,000 were more inclined to follow good CL-related practices (AOR = 2.06; 95% CI = 1.07, 3.96) when compared to their peers who had lower incomes. All of the other socio-demographic factors were not retained in the respective multivariate analyses (Table 4.17).

Table 4.17: Multivariate analysis for predictors of respondents' knowledge, attitudes and practices towards cutaneous leishmaniasis

Variables	AOR	95% CI	P
Knowledge about CL			
Gender (female)	0.53	0.32, 0.82	**0.026***
Location (Hemyar Alwasat)	2.13	0.87, 5.23	0.097
Location (Razeh)	0.75	0.42, 1.33	0.317
Occupation (farmers)	0.51	0.33, 0.83	**0.006***
Monthly household income (≥ 50,000 YER)	1.49	0.91, 2.70	0.110
Presence of confirmed CL cases among family members (yes)	1.94	1.03, 3.66	**0.040***
Knowledge about sandflies			
Age (> 40 years)	1.95	1.18, 3.22	**0.012***
Location (Hemyar Alwasat)	0.50	0.18, 1.36	0.174
Location (Razeh)	0.65	0.34, 1.23	0.183
Location (Bani Bahr)	0.60	0.35, 1.01	0.054
Presence of confirmed CL cases among family members (yes)	0.57	0.29, 1.14	0.112
Attitude towards CL			
Gender (female)	0.50	0.30, 1.03	0.061
Location (Razeh)	0.42	0.23, 0.82	**0.012***
Presence of confirmed CL cases among family members (yes)	0.46	0.23, 0.93	**0.030***
Level of knowledge about CL (good)**	1.55	0.94, 2.56	0.088
Practices related to CL			
Level of education (secondary & above)	1.71	0.83, 3.55	0.147
Monthly household income (≥ 50,000 YER)	2.06	1.07, 3.96	**0.031***
Presence of confirmed CL cases among family members (yes)	0.46	0.17, 1.25	0.128
Level of attitude towards CL (positive)†	0.580	0.29, 1.16	0.122

AOR, Adjusted odds ratio. CI, Confidence interval.
* Significant predictor ($P < 0.05$)
** Based on scores shown in Table 4.9
† Based on scores shown in Table 4.11

4.6 Occurrence of *Leishmania species* in animals

A total of 122 domestic and wild animals were involved in this study (Table 4.18). Blood samples were collected on filter papers and genomic DNA were extracted and subjected to gene amplification through nested PCR followed by direct sequencing as for human samples. *Leishmania tropica* was detected in goats, cows, donkeys, bulls, rabbits, bats, dogs and rats. On the other hand, it was not detected in the examined sheep and cats. The results showed that 16.4% (20/122) of the examined animals were found positive for *L. tropica*. This species (i.e. *L. tropica)* was the only *Leishmania* species reported among the studied animals. The highest number of *L. tropica* cases was found among goats (n = 11) which accounted for 9.0% (11/122) of the overall prevalence among the studied animals. Within the same kind of animal, dogs had the highest prevalence rate of *L. tropica* (33.3%; 2/6) followed by bulls (28.6%; 2/7). The prevalence among rats and bats was 25% (1/4), each.

Interestingly, *Trypanosoma lewisi* was reported in seven (5.7%) animal blood samples (3 sheep, 1 bull, 1 rabbit 1 cat and 1 rat) (Appendix E). Of these, the rat was found to harbour co-infection of L. tropica and T. lewisi. Interestingly, cases of *L. tropica* were detected in animals from all four sub-districts while cases of *T. lewisi* were detected in three areas from three different sub-districts (although bordering each other), namely Halma waBani Ayoub in Alsomal, Bani Asad in Bani Bahr, and Bani Aywah in Razeh (Figure 4.2).

Table 4.18: Occurrence and distribution of *Leishmania tropica* and *Trypanosoma lewisi* infections among animal hosts at the study area (n = 122)

Reservoir host	No. examined	*Leishmania tropica* n (%)	*Trypanosoma lewisi* n (%)
Goats (*Capra hircus*)	47	11 (23.4)	0 (0.0)
Sheep (*Ovis aries*)	23	0 (0.0)	3 (13.0)
Cows (*Bos taurus*)	13	1 (7.7)	0 (0.0)
Bulls (*Bos taurus*)	7	2 (28.6)	1 (14.3)
Donkeys (*Equus asinus*)	9	1 (11.1)	0 (0.0)
Dogs (*Canis lupus* familiaris)	6	2 (33.3)	0 (0.0)
Cats (*Felis catus*)	4	0 (0.0)	1 (25.0)
Rabbits (*Oryctolagus cuniculus*)	4	1 (20.0)	1 (20.0)
Bats (*Rousettus aegyptiacus*)	4	1 (25.0)	0 (0.0)
Rats (*Rattus norvegicus*)	4	1 (25.0)	1 (25.0)
Total	**122**	**20 (16.4)**	**7 (5.7)**

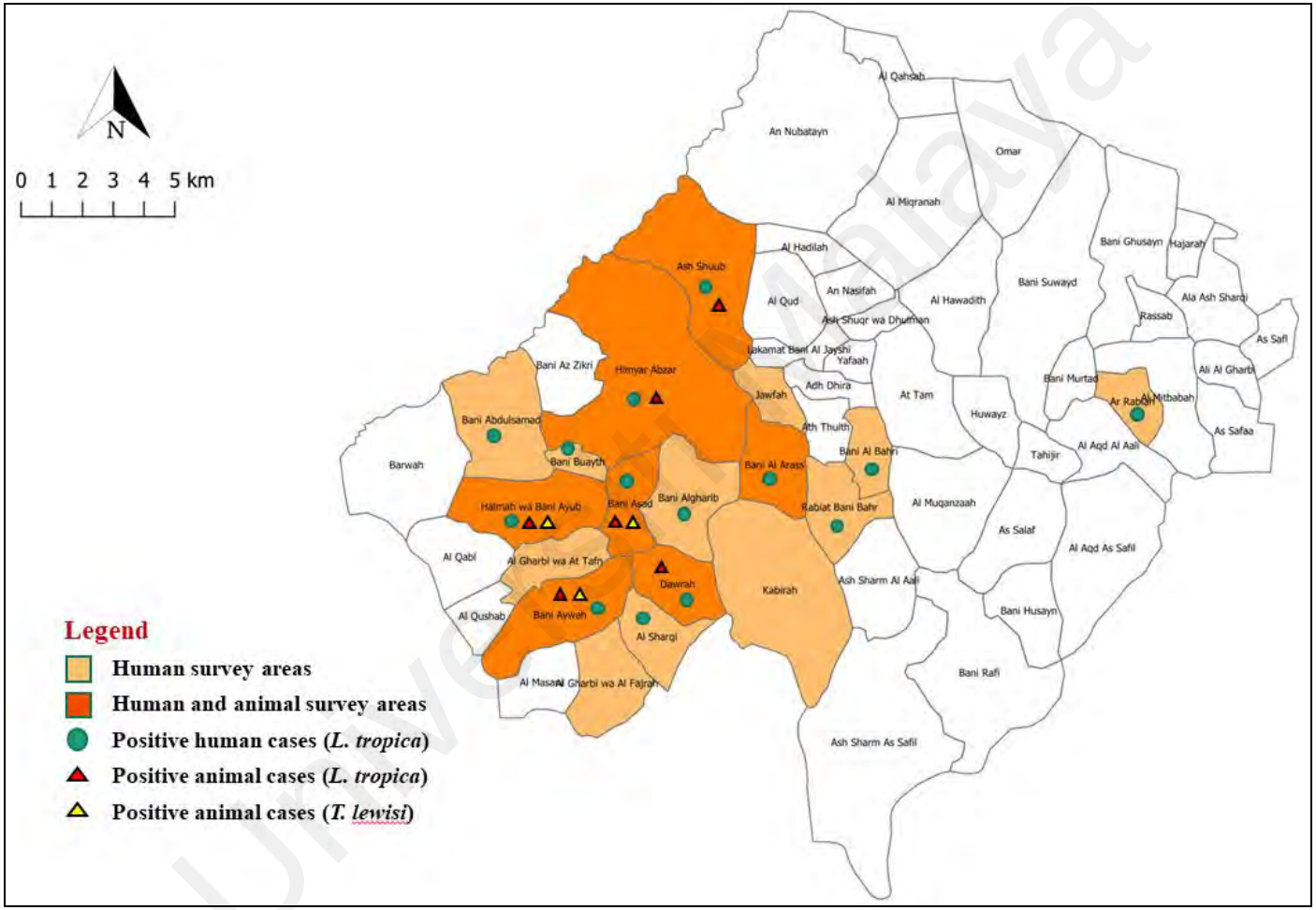

Figure 4.2: A map showing the areas with positive cases of *L. tropica* and *T. lewisi* in Utmah district, Dhamar governorate, western Yemen

4.7 Genetic analysis of human and animal *Leishmania tropica* populations isolated from the study area

4.7.1 Phylogenetic analysis

The DNA samples extracted from the human CL skin scraping/slits and animal skin scraping and blood samples were amplified by nested PCR for ITS-1, and amplicons of ~ 300-350 bp were generated and sequenced. *Leishmania tropica* was the only causative agent identified by this study (Figure 4.3). Fifty-four samples were successfully sequenced and revealed clear sequences for constructing a phylogenetic tree for the four different sub-districts and areas involved in the present study. Out of them, 34 were sequences derived from human samples (accession numbers PP217232–PP217265) while 20 sequences were from animals (accession numbers PP217266–PP217285); the sequences were deposited in the GenBank (Appendix L).

The phylogenetic analysis of human and animal sequences segregated six different *L. tropica* haplotypes: 3 from human and 4 from animal (Figure 4.4). A total of 47 sequences (32 of human isolates and 15 of animal isolates) were classed into the same haplotype (dominant haplotype). Moreover, two sequences of human isolates (isolates no. H3 from Razeh and H205 from Bani Bahr) were classed into two different haplotypes. Likewise, five sequences of animal isolates were assigned into 3 new haplotypes, with one haplotype involved three sequences (isolates no A45 and A118 of goats from Alsomal and A95 of a bull from Razeh). All sequences formed separation from the outgroup *Leishmania aethiopica* while the reference *L. tropica* was assigned into a cluster between the study sequences (Figure 4.4).

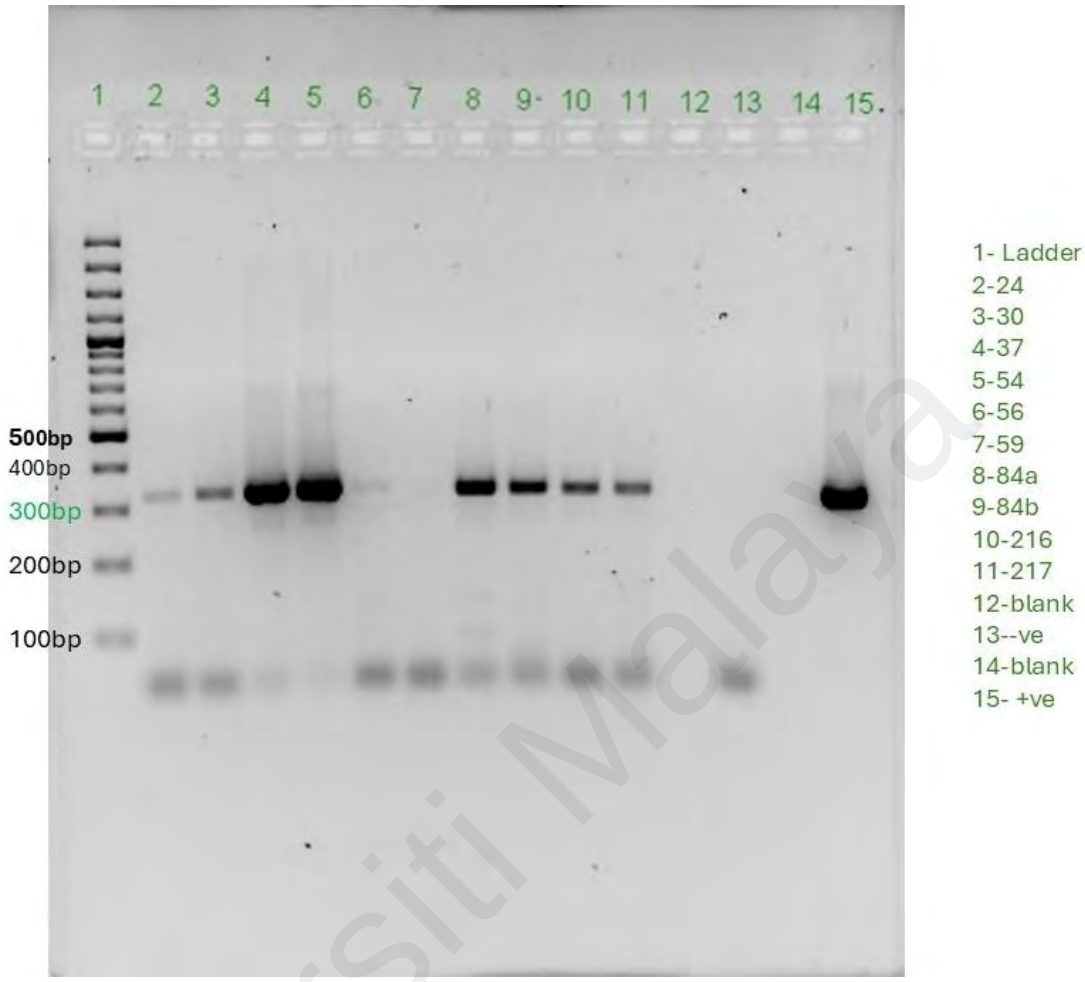

Figure 4.3: Agarose (2.5%) gel electrophoresis of nested polymerase chain reaction products in isolates of patients with cutaneous leishmaniasis.

Lane 1: molecular weight (ladder); lane 2-11: patients' samples; lane 12 & 14: blank; lane 13: negative control; lane 15: *Leishmania tropica* positive control.

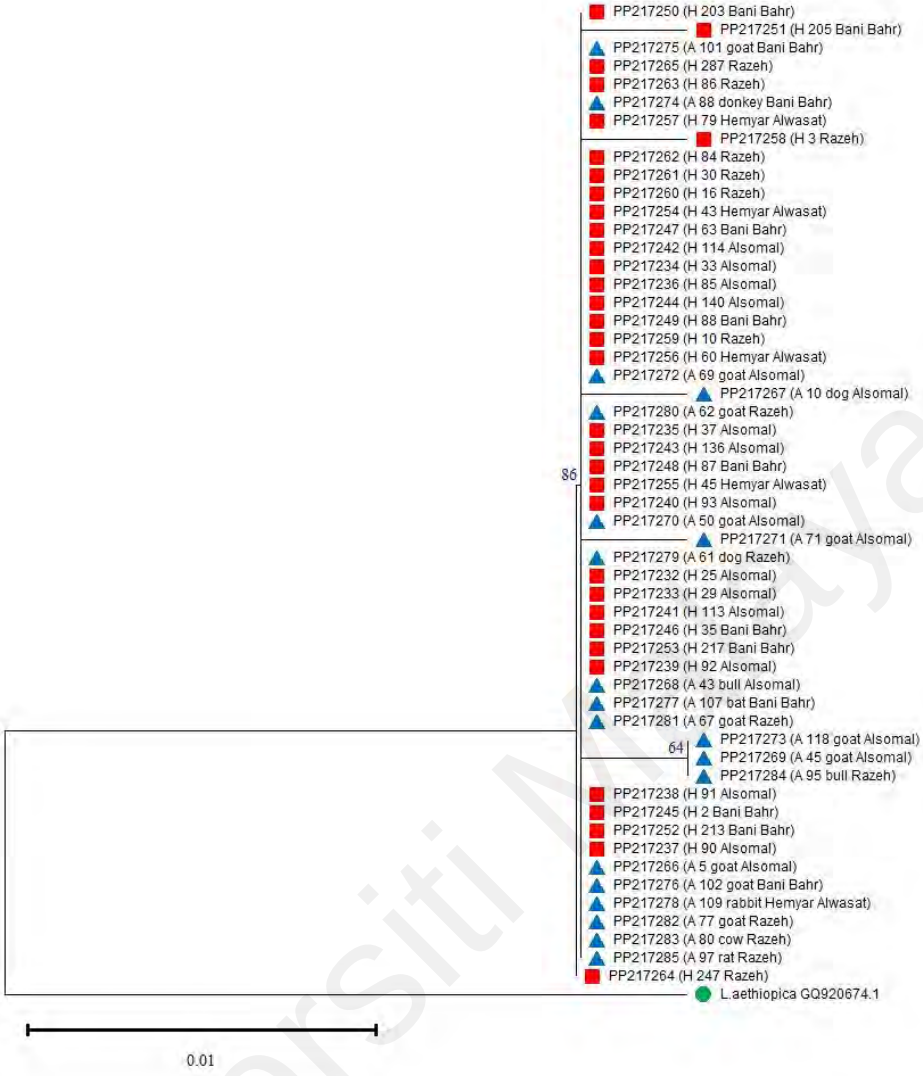

Figure 4.4: Neighbour-Joining phylogenetic tree with 1000 bootstrap replications for the phylogenetic analysis of internal transcribed spacer one (*ITS1*) sequences from animal and human *Leishmania tropica* isolates from Utmah district.

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches in blue numbers. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Red square: *L. tropica* sequences from studied humans; Blue triangle: *L. tropica* sequences from studied animals; *Leishmania aethiopica* 18S ribosomal RNA gene sequence (GQ920674.1) retrieved from GenBank was used as an outgroup. The numbers denote

each sequence are their accession numbers, with samples number and area are given in parentheses.

4.7.2 Genetic diversity analysis

Six polymorphic (segregating) sites (3 from human and 4 from animal) were detected among the sequences of *L. tropica* isolates obtained in this study. Details on genetic diversity of both *L. tropica* populations from human and animal are shown in Table 4.19. It was found that the average number of nucleotide differences (*k*) was 0.255 with total nucleotide diversity (N_d) of 0.00078 ± 0.00026. Likewise, the overall haplotype diversity (H_d) was low (0.242 ± 0.077). It was found that *L. tropica* population from animals had higher H_d and N_d values than those from human; however, these genetic diversity differences were statistically not significant ($P > 0.10$). Table 4.20 shows inter-population differentiation indices between the two *L. tropica* populations. The fixation index (F_{ST}) and its related analog estimate *Gst* demonstrated low values (0.050 and 0.041, respectively), with *Nm* value of 4.65, indicating that *L. tropica* is not genetically differentiated between the studied areas of Utmah district. Likewise, inter-population pairwise nucleotide differences (*Kxy*) between both populations and the average number of nucleotide substitutions per site (*Dxy*) showed very low values (0.309 and 0.001, respectively), indicating no genetic differentiation (Table 4.20).

Table 4.19: Genetic analysis of human and animal *L. tropica* populations

	Population 1 (Animal)	**Population 2 (Human)**	**Overall**
Number of sequences	20	34	54
Number of segregating sites	3	2	5
Number of haplotypes	4	3	6**
Haplotype diversity (H_d)*	0.432 ± 0.126	0.116 ± 0.074	0.242 ± 0.077
Nucleotide diversity (N_d)*	0.00144 ± 0.00047	0.00036 ± 0.00023	0.00078 ± 0.00026
Average no. of nucleotide differences (k)	0.468	0.118	0.255

* Values are mean ± standard deviation.

** One haplotype is shared between both populations.

Table 4.20: Inter-population differentiation indices between the two *L. tropica* populations

Population 1	Population 2	F_{ST}	*Nm*	*Kxy*	*Dxy*	G_{ST}	*Da*
Human	Animal	0.050	4.65	0.309	0.0010	0.041	0.0001

F_{ST}, fixation index; *Nm*, gene flow and population migration among populations; *Kxy*, the average number of pairwise nucleotide differences between populations; *Dxy*, the average number of nucleotide substitutions per site between populations; G_{ST}, genetic differentiation index based on the frequency of haplotypes between populations; *Da*, the number of net nucleotide substitutions per site.

At sub-district level, the phylogenetic tree showed that Alsomal sub-district had the highest number of haplotypes (n = 4) followed by Razeh (n = 3) while Hemyar Alwasat had only a single haplotype (Table 4.21). The difference in genetic diversity between isolates from the different areas was also examined. Out of the different six haplotypes existed in the study area, isolates from Alsomal were found to carry all animal haplotypes (including one haplotype shared with humans), while 3 haplotypes were detected in isolates from Razeh (one human, one animal and one shared). Isolates from Hemyar Alwasat areas were found to carry a single haplotype (shared haplotype). Accordingly, *L. tropica* population from Alsomal sub-district showed the highest haplotype (H_d = 0.348± 0.128) and nucleotide diversities (N_d = 0.0012± 0.0005) when compared with *L. tropica* populations from other sub-districts. The overall analysis showed a difference in the genetic diversity between isolates from different areas (χ^2 = 10.753); however, this difference was not statistically significant (P = 0.769) (Table 4.21).

In the same vein, pairwise fixation index in the *L. tropica* populations varied from F_{ST} = -0.026, with *Nm* value = infinite (between Alsomal and Razeh) to F_{ST} = 0.025, with *Nm* = 9.75 (between Alsomal and Hemyar Alwasat), indicating no genetic differentiation with high gene flow (Table 4.22). These results were supported by the low values of average numbers of nucleotide differences and nucleotide substitutions per site between *L. tropica* populations that ranged from *Kxy* = 0.077 and *Dxy* = 0.00024 (between Bani Bahr and Hemyar Alwasat) to *Kxy* = 0.311 and *Dxy* = 0.0010 (between Alsomal and Razeh) (Table 4.22).

Table 4.21: Genetic analysis of parasite's populations from different areas

	Location (sub-districts)			
	Alsomal	**Razeh**	**Bani Bahr**	**Hemyar Alwasat**
Number of sequences	21	15	13	5
Number of segregating sites	3	2	1	0
Number of haplotypes	4	3	2	1
Haplotype diversity (H_d)*	0.348± 0.128	0.257±0.142	0.154± 0.126	ND
Nucleotide diversity (N_d)*	0.0012± 0.0005	0.0008±0.0005	0.0005± 0.00039	ND
Average no. of nucleotide differences (k)	0.372	0.267	0.154	ND

* Values are mean ± standard deviation, ND, not determined due to lack of segregating polymorphism.

Table 4.22: Inter-population differentiation indices between the *L. tropica* populations from studied areas

Population 1	Population 2	F_{ST}	*Nm*	*Kxy*	*Dxy*	G_{ST}	*Da*
Alsomal	Razeh	-0.026	∞	0.311	0.0010	-0.016	-0.00002
Alsomal	Bani Bahr	0.018	13.64	0.267	0.0008	0.005	0.00001
Alsomal	Hemyar Alwasat	0.025	9.75	0.190	0.0006	0.041	0.00001
Razeh	Bani Bahr	0.000	∞	0.210	0.0006	0.003	0.00000
Razeh	Hemyar Alwasat	0.000	∞	0.133	0.0004	0.025	0.00000
Bani Bahr	Hemyar Alwasat	0.000	∞	0.077	0.0002	0.014	0.00000

F_{ST}, fixation index; *Nm*, gene flow and population migration among populations estimated by a pairwise method according to Hudson et al. (Hudson et al., 1992); *Kxy*, the average number of nucleotide differences between populations; *Dxy*, the average number of nucleotide substitutions per site between populations; G_{ST}, genetic differentiation index based on the frequency of haplotypes between populations; *Da*, the number of net nucleotide substitutions per site.

4.8 Global genetic analysis of *Leishmania tropica* populations

4.8.1 Global genetic differentiation and diversity of *L. tropica*

A total of 367 *ITS-1* sequences belonging to *L. tropica* were included in this analysis (Table 4.23). Of these, 54 sequences were from the present study, representing Yemen, while 313 *ITS-1* sequences were obtained from GenBank based on previous studies (Appendix L). The analysis showed 54 variable (segregating) sites producing a total of 54 haplotypes, suggesting a relatively low level of divergence for the involved sequences.

The haplotype network analysis revealed that the haplotype diversity of all populations included in the analysis was 0.605 ± 0.028 when pooled together. Moreover, Tajima‟s *D* (-2.583; $P < 0.01$) and Fu‟s *Fs* tests (-33.210; $P < 0.02$) resulted in significantly negative values (Table 4.23). These results rejected neutrality for all populations, suggesting a recent population expansion or selective sweep. At a country level, the analysis of the genetic diversity of *L. tropica* in the populations with at least 10 sequences showed that China had the highest haplotype diversity (0.869±0.054), followed by Morocco (0.788±0.053). Meanwhile, the highest nucleotide diversity (N_d) of *L. tropica* isolates was observed in Morocco origins (0.0069) followed by Turkey (0.0063) and China (0.0053). In comparison, both haplotype (0.321±0.080) and nucleotide (0.0031±0.0004) diversities of *L. tropica* in Yemen were significantly lower than in the rest of the *L. tropica* geographic range.

For neutrality tests, negative values were observed for both Tajima‟s *D* test and Fu‟s *Fs* for sequences from all countries except those from Saudi Arabia (0.748 and 0.403, respectively) and Israel (1.443 and 1.137, respectively) that showed non-significant positive values (Table 4.23). Tajima‟s *D* test and Fu‟s *Fs* values were both significantly negative ($P < 0.05$ and $P < 0.02$, respectively) for Iran (-1.873 and -

17.966), Syria (-1.819 and -7.491), Morocco (-1.848 and -8.029) and China (-1.976 and -12.773) indicating recent population expansions. For Yemen, the negative Tajima‟s *D* value was significant (-1.935; $P < 0.05$) while Fu‟s *Fs*, which is more powerful in detecting population expansion, was not significantly negative (-9.498; $P > 0.02$), indicating an excess of rare haplotypes over what would be expected under neutrality.

Table 4.23: Genetic polymorphism in the whole dataset of 367 *ITS-1* sequences belonged to *L. tropica*

Country	*N*	*H*	*S*	*k*	H_d	N_d	Neutrality tests	
							Tajima's *D*	Fu's *Fs* tests
Yemen[**]	57	9	7	0.3797	0.321± 0.080	0.0013± 0.0004	-1.935[*]	-9.498
Iran	96	16	16	0.6272	0.464±0.063	0.0029±0.0005	-2.302[*]	-17.966[*]
Syria	58	11	11	0.8530	0.650±0 0.041	0.0039± 0.0005	-1.819[*]	-7.491[*]
Morocco	41	14	16	1.6988	0.788± 0.053	0.0069±0.0012	-1.848[*]	-8.029[*]
China	27	15	15	1.6581	0.869±0.054	0.0053±0.0009	-1.976[*]	-12.773[*]
Saudi Arabia	14	3	2	0.8022	0.670± 0.082	0.0034±0.0006	0.748	0.403
Turkey	13	5	7	1.3333	0.705±0.122	0.0063± 0.0020	-1.551	-1.043
Israel	11	2	1	0.5455	0.545± 0.072	0.0025±0.0003	1.443	1.137
Afghanistan	10	4	4	1.0667	0.711± 0.117	0.0037± 0.0011	-0.943	-0.742
All countries	367	54	54	0.9327	0.605± 0.028	0.0051± 0.0004	-2.583[*]	-33.210[*]

Results for all countries and countries with ≥ 10 sequences are shown.

N, no. of sequences; H, no. of haplotypes; S, no. of segregating/variable sites; k, average no. of nucleotide differences; H_d, haplotypes diversity; N_d, nucleotide diversity.

[*] Tajima's *D*: significant, $P < 0.05$, Fu's *Fs*: significant, $P < 0.02$.

[**] For Yemen, 54 sequences from this study plus other 3 sequences from the GenBank.

4.8.2 Global haplotype network analysis of *L. tropica*

The median-joining haplotype network constructed for all 367 *L. tropica ITS-1* sequences revealed rather short distances (Figure 4.5). The results revealed the dominance of one haplotype (H_1) that contained 224 sequences from 17 countries, including 86% (49/57) of the sequences from Yemen (48 sequences of the present study plus one previously published sequence). Similarly, H_1 contained the majority of sequences of Iran (73/96), Morocco (19/41) Turkey (7/13), and China (13/27). Interetingly, all sequences from Israel (11/11) were classed into this dominant haplotype.

The second largest node was H_6 that contained 54 sequences from 13 countries, with about half (48.1%, 26/58) of them were from Syria. As depicted in (Figure 4.5), these two haplotypes (i.e. H_1 and H_6) were distributed throughout all continents of the Old World, from the West of Africa to China in East Asia through Greece and Turkey. All other haplotypes were basically originated from H_1.

Moreover, 13 haplotypes were non-unique (H_1, H_5, H_6, H_7, H_10, H_12, H_13, H_15, H_20, H_22, H_45, H_47, and H54) while most of the generated haplotypes (75.9%, 41/54) were unique. Most of the unique haplotypes were from Iran (8), Syria (8), Morocco (7) and China (7). In this global haplotype network, the 54 sequences from Yemen that provided by the present study were represented by five haplotypes, three of which (H_2, H_3 and H_4) were unique and novel, whereas one novel haplotype (H_5) was represented by three sequences from animals (2 from goats and 1 from a bull). Moreover, most of the sequences of the present study (n = 48) were classed into H_1. Besides, two of the previously published sequences from Yemen were classed into H_6. Although there were two different countries in H7 (Saudi Arabia and Malaysia), the isolate from Malaysia (accession number OL413428.1) was an imported

case of CL reported in a 10-year-old Saudi child who had travelled from Saudi Arabia before being diagnosed with the disease in Malaysia (Tan et al., 2022).

Considering the host, haplotypes H_1, H_5, H_6, H_7, H_13, H_20, H_45 and H_47 were non-unique groups that shared sequences of humans and animals. For instance, H_1 involved sequences of human *L. tropica* isolates from different countries as well as lizard from China, goat, dog, rabbit, cow, bull, rat, and bat from Yemen, sandflies from Greece, Morocco and Ghana, and jackal from Israel. H_6 involved sequences from China (lizard), Iran (dog), Ghana (sandfly), Saudi Arabia, Yemen, Pakistan and Russia (human). Likewise, H_13 involved sequences from Iran (human) and China (lizard). H_9, H_20 and H_22 involved sequences from Morocco only (human and sandfly). H_47 involved sequences from Tunisia (human and sand rat) and Libya (sandfly). On the other hand, H_5 involved sequences from Yemen only (goats and bull), whereas H_7, H_10, H_12 and H_15 were groups that involved only human haplotypes; H_7 (from Saudi Arabia plus the Malaysian sequence imported from Saudi Arabia), H_10 (from Iran and Afghanistan), and H_12 (from Iran, Syria and Turkey).

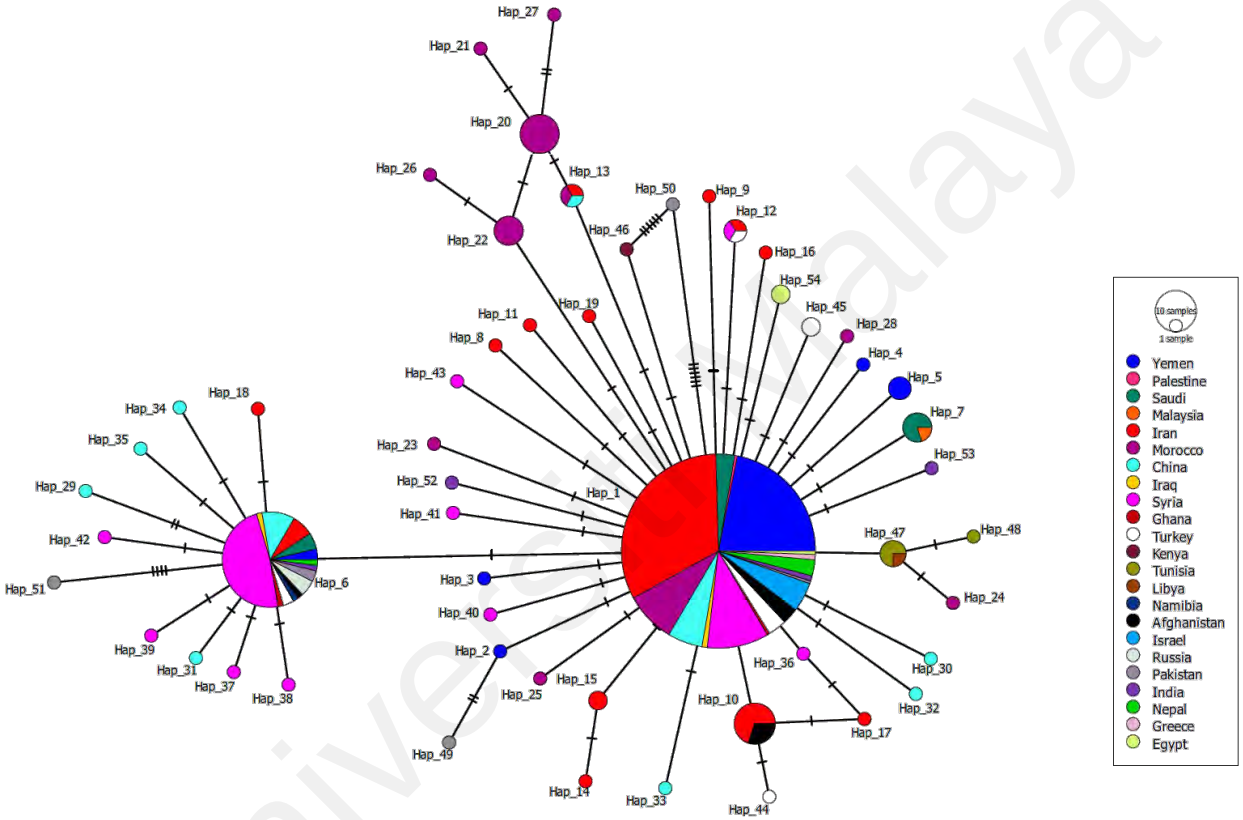

Figure 4.5: Haplotype network analysis of 367 *ITS-1* sequences belonged to *L. tropica*.

The dataset involved Yemeni human and animal isolates obtained by this study (n = 54) together with other sequences obtained from the GenBank representing different geographical locations (n = 313). (H_n) denotes a distinct haplotype number. Circles represent individual haplotypes; their colours reflect the country of origin while the size of each circle reflects the frequency of each haplotype. Branches lengths are proportional to the number of nucleotide substitutions indicated by cross marks. Distance between nodes and the branch lengths have no meaning.

Pairwise comparisons of sequences from Yemen with other countries with ≥ 10 sequences that formed related clusters when analyzed with other *L. tropica* sequences obtained from the GenBank were performed in order to comprehensively assess the genetic differentiation and diversity of the sampled isolates (Table 4.24). The results demonstrated that fixation index and *Gst* estimates demonstrated the highest values (F_{ST} = 0.295 and G_{ST} = 0.170), with *Nm* value of 0.597 (between Yemen and Syria), suggesting a high level of genetic differentiation and medium gene flow between the two *L. tropica* populations. These results are supported by the inter-population pairwise nucleotide differences (*Kxy*) between both populations and the average number of nucleotide substitutions per site (*Dxy*) that showed relatively low values (*Kxy* = 0.777 and *Dxy* = 0.0042, respectively) (Table 4.24). The second highest values were reported with Morocco, with F_{ST}, *Nm*, *Kxy* and *Dxy* were 0.234, 0.818, 1.067, 0.0058, and 0.311, respectively, also indicating level of genetic differentiation and medium gene flow between the two *L. tropica* populations (i.e. Yemen and Morocco). On the other hand, a non-significant fixation index (F_{ST} = 0.020), with a very high *Nm* = 12.250 was reported in *L. tropica* populations from Yemen and Iran, indicating very low genetic differentiation with high gene flow.

Table 4.24: Pairwise population genetics indices between different populations of *L. tropica* obtained from Yemen and other countries

Population 1	Population 2	F_{ST}	*Nm*	*Kxy*	*Dxy*	G_{ST}	*Da*
Yemen	Iran	0.020	12.250	0.408	0.0022	0.008	0.0001
Yemen	Syria	0.295	0.597	0.777	0.0042	0.170	0.0013
Yemen	Saudi Arabia	0.138	1.562	0.625	0.0034	0.090	0.0005
Yemen	Morocco	0.234	0.818	1.067	0.0058	0.099	0.0013
Yemen	Turkey	0.026	9.365	0.668	0.0036	0.050	0.0001
Yemen	China	0.123	1.783	0.855	0.0046	0.080	0.0006
Yemen	Afghanistan	0.117	1.887	0.533	0.0029	0.053	0.0003
Yemen	Israel	0.018	13.639	0.141	0.0008	0.024	0.0000

F_{ST}, fixation index; *Nm*, gene flow and population migration among populations estimated by a pairwise method according to Hudson et al. (Hudson et al., 1992); *Kxy*, the average number of nucleotide differences between populations; *Dxy*, the average number of nucleotide substitutions per site between populations; G_{ST}, genetic differentiation index based on the frequency of haplotypes between populations; *Da*, the number of net nucleotide substitutions per site.

At a regional framework, further analysis of genetic differentiation and diversity for the all 367 *L. tropica ITS-1* sequences was performed in order to have a clearer picture on the distribution of haplotypes across different geographical regions. Table 4.25 shows the regional grouping and the identities of detected haplotypes and infected hosts. Western Asia region is characterized by a variety of hosts including human, sandfly and different kinds of animal. However, human was the only host included from Syria, Saudi Arabia, Palestine and Iraq. By contrast, Eastern Asia region included animal *L. tropica* isolates (only lizard). Most of sandfly-isolated *L. tropica* populations were from Africa plus Israel (Western Asia) and Greece (Europe).

Similarly, another median-joining haplotype network was constructed by gathering countries within their respective regions. Figure 4.26 shows the regional-based median-joining haplotype network constructed for the all 367 *L. tropica ITS-1* sequences following similar haplotype network analysis showed in Figure 4.5. It can be basically postulated that CL-causing *L. tropica* populations from Southern Asia (particularly Iran) and Western Asia (particularly Syria) regions are the ancestral populations that spread eastward to China and westward to Africa. These regions have the largest number of *L. tropica* haplotypes and the largest distribution area. Moreover, *L. tropica* population from China were classed into the two predominant haplotypes (H_1 and H_6) while *L. tropica* population from Africa, specifically from Morocco, had well-separated group of haplotypes H_20–H_28, that have been found only in Morocco and might form a new distinct country-specific group.

Table 4.25: Regional-based distribution of *L. tropica* haplotypes

Region	Country	Haplotype (no. of sequences)	Host (*no. of haplotypes*)*
Western Asia	Yemen	Hap_1 (49), Hap_2 (1), Hap_3 (1), Hap_4 (1), Hap_5 (3), Hap_6 (2)	Human (4), goat (3), dog (1), bull (1), cow (1), rabbit (1), rat (1), bat (1)
	Syria	Hap_1 (23), Hap_6 (26), Hap_12 (1), Hap_36 (1), Hap_37 (1), Hap_38 (1), Hap_39 (1), Hap_40 (1), Hap_41 (1), Hap_42 (1), Hap_43 (1)	Human (11)
	Saudi Arabia**	Hap_1 (7), Hap_6 (3), Hap_7 (4)	Human (3)
	Israel	Hap_1 (11)	Human (1), Jackal (1), fox (1), dog (1), sandfly (1),
	Palestine	Hap_1 (1)	Human (1)
	Iraq	Hap_1 (2), Hap_6 (1)	Human (2)
Southern Asia	Iran	Hap_1 (73), Hap_6 (4), Hap_8 (1), Hap_9 (1), Hap_10 (7), Hap_11 (1), Hap_12 (1), Hap_13 (1), Hap_14 (1), Hap_15 (2), Hap_16 (1), Hap_17 (1), Hap_18 (1), Hap_19 (1)	Human (14), dog (1)
	Afghanistan	Hap_1 (6), Hap_6 (1), Hap_10(3)	Human (3)
	Pakistan	Hap_1 (1), Hap_6 (2), Hap_49 (1), Hap_50 (1), Hap_51 (1)	Human (5)
	India	Hap_1 (2), Hap_6 (1), Hap_52 (1), Hap_53 (1)	Human (4)
	Nepal	Hap_1 (6), Hap_6 (1)	Human (2)
Eastern Asia	China	Hap_1 (13), Hap_6 (6), Hap_13 (1), Hap_29 (1), Hap_30 (1), Hap_31 (1), Hap_32 (1), Hap_33 (1), Hap_34 (1), Hap_35 (1)	Lizard (10)
Africa	Morocco	Hap_1 (19), Hap_13 (1), Hap_20 (9), Hap_21 (1), Hap_22 (5), Hap_23 (1), Hap_24 (1), Hap_25 (1), Hap_26 (1), Hap_27 (1), Hap_28 (1)	House mouse (1), human (6), sandfly (7)
	Egypt	Hap_1 (1), Hap_54 (2)	Human (2)
	Namibia	Hap_6 (1)	Unspecified (1)
	Ghana	Hap_1 (1), Hap_6 (1)	Sandfly (2)
	Kenya	Hap_46 (1)	Unspecified (1)
	Libya	Hap_47 (1)	Sandfly (1)
	Tunisia	Hap_47 (3), Hap_48 (1)	Human (1), sand rat (2)
Europe	Turkey	Hap_1 (7), Hap_6 (2), Hap_12 (1), Hap_44 (1), Hap_45 (2)	Human (4), unspecified (1)
	Greece	Hap_1 (2)	Sandfly (1), Human (1)
	Russia	Hap_6 (3)	Human (1)

* Different hosts can be classed into the same haplotype.

** One sequence for Malaysia was included in Western Asia as it was from an imported case from Saudi Arabia.

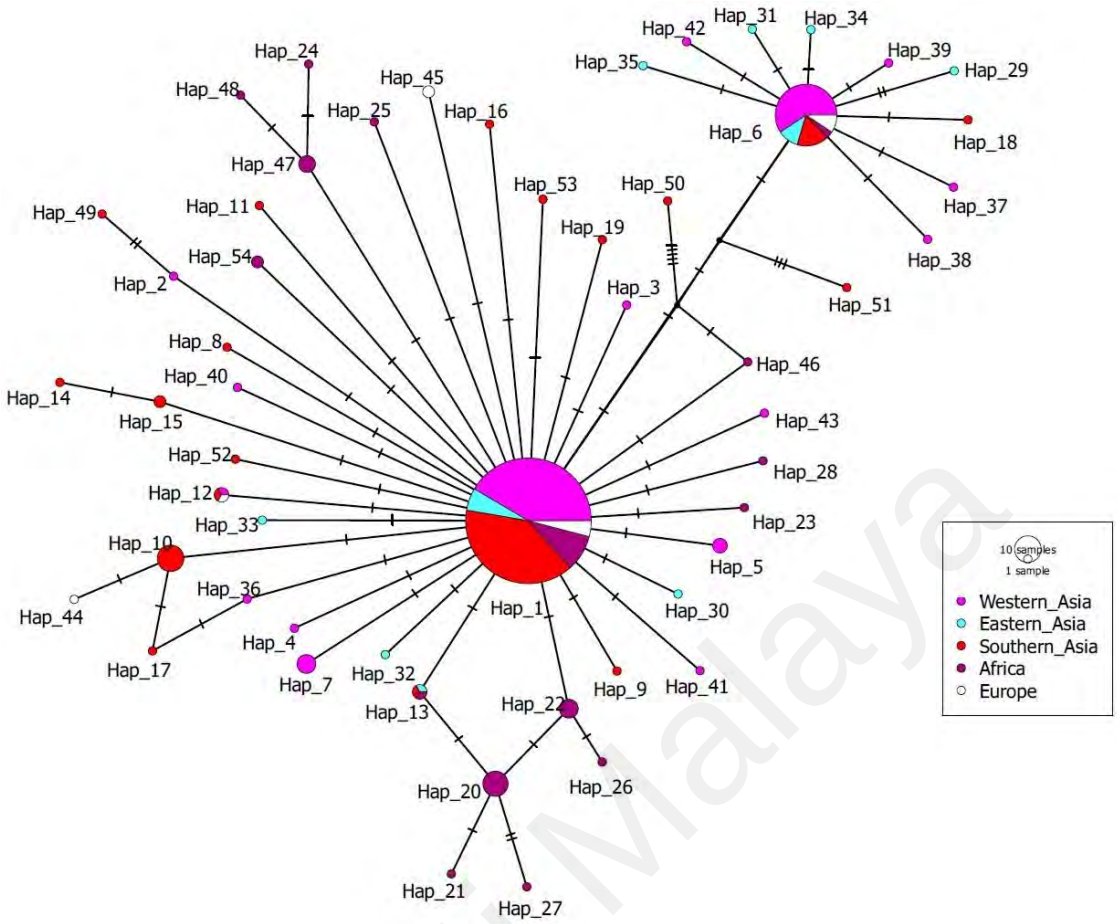

Figure 4.6: Regional-based haplotype network analysis of 367 *ITS-1* sequences belonged to *L. tropica*.

(H_n) denotes a distinct haplotype number. Circles represent individual haplotypes; their colours reflect the region of origin while the size of each circle reflects the frequency of each haplotype. Branches lengths are proportional to the number of nucleotide substitutions indicated by cross marks. Size was minimized to emphasis common non-unique haplotypes.

Table 4.26 shows that Southern Asia had the highest number of segregating sites producing 22 haplotypes followed by Africa with 21 segregating sites producing 19 haplotypes, whereas Europe showed 7 segregating sites with 5 haplotypes only. Moreover, Eastern Asia, represented only by China, had the highest haplotype diversity (similar to results in Table 4.23), followed by Africa (0.829±0.043) while Southern Asia had the lowest *Hd* (0.532±0.054) followed by Western Asia region, including Yemen, (0.559±0.041). Interestingly, Tajima‟s *D* and Fu‟s *Fs* test were negative in all populations, the Tajima‟s *D* and Fu‟s *Fs* values were significantly negative in all except European and Australian populations.

Table 4.27 shows the matrix of inter-geographic genetic differentiation represented by the fixation index (F_{ST}) and the gene flow (*Nm*) of *L. tropica* populations from the included regions. Interestingly, African *L. tropica* sequences showed pairwise moderately significant ($P < 0.05$) genetic differentiation with high gene flow when compared with *L. tropica* populations from other regions, with the values of F_{ST} ranged from 0.124 to 0.157 and *Nm* values ranged 1.342 to 1.766. The F_{ST} values between Europe and Eastern Asia were found to be negative, indicating no genetic differentiation between these *L. tropica* populations.

Table 4.26: Regional genetic polymorphism in the whole dataset of 367 *ITS-1* sequences belonged to *L. tropica*

Region	***N***	***H***	***S***	***k***	H_d	N_d	**Neutrality tests**	
							Tajima's *D*	**Fu's *Fs* tests**
Western Asia**	145	18	18	0.69061	0.559±0.041	0.00340±0.00034	-2.14506*	-19.085*
Southern Asia	124	22	28	0.84041	0.532±0.054	0.00410±0.00072	-2.49649*	-25.422*
Eastern Asia	27	15	15	1.65812	0.869± 0.054	0.00525±0.00087	-1.97610*	-12.773*
Africa	53	19	21	1.93977	0.829±0.043	0.00818±0.00121	-1.98314*	-13.080*
Europe	18	5	7	1.18954	0.693±0.086	0.00561±0.00156	-1.40996	-0.817
Total	367	54	54	0.93265	0.605± 0.028	0.00507±0.00040	-2.58329*	-33.210*

N, no. of sequences; H, no. of haplotypes; S, no. of segregating/variable sites; k, average no. of nucleotide differences; H_d, haplotypes diversity; N_d, nucleotide diversity.

* Tajima"s *D*: significant, $P < 0.05$, Fu"s *Fs*: significant, $P < 0.02$.

** One sequence for Malaysia was included in Western Asia as it was from an imported case from Saudi Arabia.

Table 4.27: Matrix of regional pairwise genetic fixation index (F_{ST}) and gene flow (*Nm*) between different populations of *L. tropica*

		Gene flow (*Nm*)				
		Western Asia	**Southern Asia**	**Eastern Asia**	**Africa**	**Europe**
Fixation index (F_{ST})	**Western Asia**	-	5.306	35.464	1.342	35.464
	Southern Asia	0.045	-	3.128	1.766	8.083
	Eastern Asia	0.007	0.074	-	1.384	∞
	Africa	0.157	0.124	0.153	-	1.486
	Europe	0.007	0.030	-0.005	0.144	-

CHAPTER 5

DISCUSSION

5.1 Overview

Yemen, much like other countries in the region that have been impacted by civil wars and armed conflict, is suffering from increasing trends of infectious diseases. As health system falters, different infectious diseases creep back in Yemen, with the occurrence of several outbreaks and epidemics (Al-Mekhlafi, 2018; Alsabri et al., 2022). The situation has been exacerbated by the famine and internal displacement as well as the disruption of infectious diseases control programmes including the routine immunization programmes (UNHCR, 2017).

Cutaneous leishmaniasis is one of Yemen's public health issues and the most prevalent skin disease. The disease is endemic in all regions across the country with the majority of reported cases come from highland areas such as Dhamar, Hajjah, Al-Bayda and Lahj governorates, with more than 12,000 CL cases reported in 2019 (MOPHP, 2019; WHO, 2022). According to previous reports, the overall CL incident rate (IR) increased from 13 per 100.000 in 2013 (Dahnan & Al-Mahaqri, 2013) to 19.6 per 100.000 in the first half of 2020 (Al Daari et al., 2022). However, it is believed that the disease is largely underreported, and official data may only indicate the peak of an iceberg of underreported cases of CL, particularly among women and children in rural areas (Al-Kamel, 2016b; Ali, 2009; Du et al., 2016).

This present study is the first to provide community-based information on the prevalence and risk factors of CL in Yemen using microscopic and molecular examinations. It is also the first study to provide comprehensive results on the genetic

structure and haplotype diversity of *L. tropica* among human and animal populations from Yemen in relation to regional and global *L. tropica* populations‟ structure and diversity. Moreover, the study evaluated the population‟s knowledge, attitude and practices concerning the disease and its vector as well as investigated the role of non-human mammals in the transmission of leishmaniasis in the study areas.

5.2 Prevalence and distribution of cutaneous leishmaniasis

The present study revealed that CL is highly endemic in the Utmah district in the western highlands of Yemen. Overall, 14.7% (90/612) of the participants from 289 households had active skin lesions and were considered to be suspected CL cases, with 8.7% (53/612) confirmed positive for CL. This high prevalence corresponds to the increasing trend of CL cases being reported in rural Yemen, particularly since the ongoing civil war began in March 2015.

This finding complements previous observations by the only two local community-based studies conducted on CL (Alkulaibi et al., 2019; Asmaa et al., 2017). In Shara‟b district, Taiz, southwestern Yemen, 18.8% of 525 participants aged 1–60 were found to be infected with CL through microscopic examination (Asmaa et al., 2017). Considering microscopic examination only, the prevalence of CL in the present study was 6%, which is much lower than the percentage reported in Taiz governorate. Similarly, another study conducted in the Utmah district between March and July 2015 (i.e. during the first five months of the widespread civil war) reported that 215 out of 1,165 participants (18.5%) were found positive by the leishmanin skin test (LST), with a positivity rate of 37% (175/471) in the escarpment areas, including a detection rate of 5.5% (26/471) for active lesion and a detection rate of 31.7% (149/471) for scars of healed lesions (Alkulaibi et al.,

2019). Indeed, the LST (also known as the Montenegro skin test) is performed through intradermal injection of *Leishmania* antigens to monitor past exposure and immunity to *Leishmania* (Carstens-Kass et al., 2021). A previous community-based serological survey was conducted among 285 children in VL-endemic rural districts in Sana‟a governorate, north Yemen, and demonstrated that 34.7% of the children were seropositive for VL (Al-Shamahy, 1998). Obviously, these findings revealed the high endemicity of leishmaniasis in rural Yemen.

During 2016–2018, residents and officials in the Utmah district called for urgent action to fight CL, which had swept into the district after the start of the civil war (Anonymous, 2016). The situation was similar in the bordering districts, Kosmah, Mazhar and Aljubain of Raymah governorate, where over 2,000 new cases of CL, mostly in children, were recorded between July 2018 and April 2019 (Al-Dhubaibi, 2019; Anonymous, 2019). These outbreaks reflect the presence of epidemiological factors that contribute to the transmission of CL in these communities. Therefore, in the absence of effective prevention and control interventions, it is more likely that the incidence of CL will increase and CL will continue to have devastating consequences in these communities.

In the present study, 14.7% (90/612) of the participants from 289 different households had active skin lesions and were considered to be suspected CL cases; therefore, the reported prevalence represented active infections. This shocking prevalence corresponds to the increasing trend of CL cases being reported in rural Yemen, particularly since the ongoing civil war began in March 2015. Indeed, during the period of this study, the Yemeni health authorities declared some outbreaks and new foci of CL transmission in all governorates except Socotra Island, with over 12,000 cases reported in 2019 (MOPHP, 2019). The highest annual number of cases was in the Amran governorate, followed by

Sana‟a, Almahweet and Dhamar (Alkulaibi et al., 2019; Anonymous, 2016; MOPHP, 2019). This situation has been exacerbated by a severe shortage of leishmaniasis treatment since 2017 and inadequate response or resistance to treatment (Al-Kamel, 2016a; Khatri & Haider, 1999b).

Previous cross-sectional community-based studies conducted in other Middle Eastern countries reported a high prevalence of CL. For instance, all 500 residents of a small village in the Khushab District in the province of Punjab, Pakistan, were screened for CL, and 109 (21.8%) cases were identified and treated (Anwar et al., 2007). Moreover, among 1,000 participants from three villages in Shiraz, southwestern Iran, where CL was endemic, a prevalence of 23.2% was reported, which included 7% active lesions and 16.2% scars (Razmjou et al., 2009). Similarly, in a new focus on CL in a rural district in Khorassan province, northeast Iran, 15.3% of 541 schoolchildren and 13.4% of 807 participants from four villages had active lesions and/or scars suspected to be due to CL (Yaghoobi-Ershadi et al., 2003). A large-scale community-based survey conducted among 10,596 individuals in Kabul, Afghanistan, reported that 224 (2.1%) and 1,421 (13.4%) had active CL lesions or scars, respectively (Reithinger et al., 2010). In the same vein, large-scale community-based studies in Tigray, northern Ethiopia, demonstrated that 2.3–6.7% and 7.3–20.9% of the studied population had active CL lesions and scars, respectively (Bsrat et al., 2015; Yohannes et al., 2019). Moreover, the prevalence of active CL reported by a house-to-house survey in Kibet town, central Ethiopia, was 10.4% (59/566) (Negera et al., 2008). Among 523 primary schoolchildren in a CL hotspot in Ochollo town, southwestern Ethiopia, 5.5% had CL active lesions, and 59.9% had scars, making a total prevalence of 65.8% (Bugssa et al., 2014).

In 2013, the estimated number of CL cases in Yemen exceeded 180,000, with more than 20,000 new cases occurring per year (Al-Kamel, 2016a; Al-Kamel, 2017). The impact of armed conflicts on the spread of NTDs, including leishmaniasis in Yemen, has not yet been assessed. A recent study in Sana‟a City found a significant association between internal displacement and the prevalence of VL (Abu-Hurub et al., 2022); however, our study did not find such association with CL, possibly due to small number of displaced participants in this study.

In a Yemeni-like war-torn situation, Syria has been engulfed in a bloody civil war since March 2011, forcing about 6.9 and 5.6 million people to be internally and externally displaced, respectively, and destroying over 50% of healthcare facilities (WHO, 2023). The annual incidence of CL in Syria between 2004 and 2008 was estimated to be 23,000 cases per year. However, the annual incidence increased dramatically, with over 58,000 cases of CL reported in 2011, over 86,000 cases in 2015 and 82,000 cases in 2018 (Muhjazi et al., 2019). Reports from many Syrian conflict-affected towns revealed a massive upsurge in CL cases, with prevalence rates exceeding 25%, particularly among schoolchildren (Alasaad, 2013; SOHR, 2021). This increasing trend of CL cases has also been widespread in neighbouring countries, including Lebanon, Jordan and Turkey, due to the Syrian refugee influx (Bizri et al., 2021). Therefore, it is possible to hypothesise a dramatic increase in the total number of new CL cases in Yemen after 2015, primarily as a result of the ongoing civil war that has led to the world‟s greatest humanitarian crisis and a paralysed healthcare system as well as an internal displacement of about 4.5 million people, particularly from urban to rural areas (Al-Mekhlafi, 2018; Alsabri et al., 2022; WB, 2021). The Task Force on Population Movement (TFPM) has identified large pockets of internal displacement within seven consolidated areas; one of them is the Utmah district (TFPM, 2017).

However, the impact of ongoing armed conflicts on the incidence and distribution of vector-borne infectious diseases, including CL in Yemen needs further investigations.

With regard to disease control in Yemen, case detection is passive, and there are no national control programmes for leishmaniasis and its sandfly vector and potential reservoirs. Recently, CL was included in the electronic Diseases Early Warning System (eDEWS), a health facility-based disease surveillance system established in 2013 that covers 31 communicable diseases. However, eDEWS is used by only one-third of all health facilities in Yemen, and this represents an essential challenge to its functionality (Dureab et al., 2020). On the other hand, the Regional Leishmaniasis Control Centre (RLCC), a charitable non-governmental organisation founded in early 2013, has made invaluable efforts through treatment and awareness campaigns targeting leishmaniasis in certain areas in Yemen where CL is endemic (Al-Kamel, 2016c). On average, the RLCC has been seeing about 67–100 patients per month for free diagnosis and treatment (Pentostam® injections) (ISID, 2016). Unfortunately, these efforts have been affected by a scarcity of resources, the ongoing civil war and instability (Al-Kamel, 2016a).

Previous hospital-based studies among clinically suspected cases in different governorates in Yemen demonstrated that CL is prevalent across the country, with the highest number of cases reported in Hajjah (northwestern), Al-Bayda (central) and Taiz (southwestern) governorates (Al-Kamel, 2016b; Alharazi et al., 2016; Khatri et al., 2016; Khatri et al., 2009; Muthanna et al., 2022). Studies among patients with suspected cases who were attending healthcare centres and were examined by microscopy and/or PCR showed high detection rates in countries in Middle Eastern and East African regions, such as Saudi Arabia, Pakistan, Iran and Ethiopia (Hawash et al., 2018; Iqbal et al., 2022; Khosrotaj et al., 2022). In rural Yemen, patients usually utilise herbal and traditional

remedies for different diseases (such as cauterisation by applying acids or heating on skin lesions caused by CL), and they seek treatment from health centres only if the symptoms get worse. Thus, the passively detected rates reported by the hospital-based studies can be considered an underestimation of the real burden.

5.3 Clinical presentation of cutaneous leishmaniasis

In general, CL manifests as skin lesions, mainly ulcers, which can persist for months, sometimes years, with the severity of infection determined largely by the interactions between the *Leishmania* parasite species and/or strain and the host‟s immune response (Cecilio et al., 2022; Uzun et al., 2018). The present study showed that the dry type, single, localised, facial and centrally ulcerated nodule was the most common clinical phenotype of CL among the studied subjects. This finding is consistent with most previous studies in Yemen (Al-Kamel, 2016b; Asmaa et al., 2017; Khatri et al., 2016; Khatri et al., 2009; Mogalli et al., 2016; Muthanna et al., 2022). Several previous studies showed that the dry type of CL is generally caused by *L. tropica* and *L. infantum,* whereas the wet type of CL is caused by *L. major* (Khatri et al., 2016; Uzun et al., 2018). However, other studies found no correlation between causal *Leishmania* parasite species and clinical presentation (wet or dry or mixed appearance of lesions) (Khan et al., 2016)

Interestingly, mucocutaneous involvement of the nose and lips was observed in five patients. Some reports from central and north Yemen have documented the presence of MCL and occasionally described it as CL with involvement of mucous membrane (Al-Kamel, 2016b; Khatri et al., 2016). MCL is a destructive disease that mainly affects the mucous membranes of the mouth and nose and does not heal spontaneously. It is mainly caused by *L. braziliensis* infection; however, MCL cases by other species of *Leishmania* including *L. tropica*, *L. infantum* and *L. major* have been occasionally reported (Shirian et

al., 2013). The maximum number of multiple lesions among the studied subjects was four lesions; however, Mogalli et al. (Mogalli et al., 2016) found up to 16 lesions in one patient, while Khatri et al. (Khatri et al., 2016) found four patients with 13, 17, 19, and 71 lesions each in northwestern Yemen. These findings revealed high exposure to infected sandfly bites in rural Yemen.

Interestingly, a significantly higher percentage of lesions on the face was reported among children (90.1%) compared with adults (69.2%), and this agrees with (Asmaa et al., 2017), which reported that the highest percentage of lesions among adults were on the upper and lower limbs, while the face was the most affected among children. Similar findings were reported by studies in other countries (Hawash et al., 2018; Iqbal et al., 2022). Unlike adults, children might be less aware of the need to protect their faces from flies, whereas adults, specifically males, wear traditional attire that leaves their arms and legs exposed. By contrast, the face was also the most affected among the studied adult females, which was due to them covering their bodies except the face because of *customs* and the traditional clothing they tend to wear.

5.4 Risk factors associated with cutaneous leishmaniasis

The present study showed that age was a strong factor influencing the occurrence of CL among the participants, with the most affected group being children 10 years of age or younger (23.9%), and this agrees with previous studies in Yemen (Al-Kamel, 2016b; Alharazi et al., 2016; Alkulaibi et al., 2019; Asmaa et al., 2017; Nassar et al., 2021) and in other countries, including Saudi Arabia (Alraey, 2022; Hawash et al., 2018), Pakistan (Iqbal et al., 2022; Kayani et al., 2021), Afghanistan (Ghatee et al., 2020) and Iran (Karimi et al., 2021; Khosrotaj et al., 2022). These studies showed that the prevalence of

CL increases with age through early childhood and levels off by 15 years of age. The higher prevalence among young children could be due to their increased activity and potentially higher exposure to sandfly bites by playing outdoors near sandfly breeding areas, like livestock sheds and coops during dusk and night-time or in the early morning (Maroli et al., 2013).

By contrast, previous studies conducted abroad showed a higher prevalence of CL among the 15–40 age group (Akhlagh et al., 2019; Amin et al., 2013; Zeleke et al., 2021). On the other hand, previous LST-based studies conducted in Yemen and elsewhere demonstrated that the prevalence of CL increased with age, with individuals aged over 45 having the highest positivity rate (Akuffo et al., 2021; Alkulaibi et al., 2019; Bettaieb et al., 2014). Nonetheless, this result can be expected in CL-endemic areas, as LST detects active and past exposure that also increases with age.

Contrary to expectations, the present study reported a significantly higher prevalence among females compared with males (18.7% vs 6.1%). Inconsistent with this finding, several previous studies in Yemen reported significantly higher infection rates in males compared with females (Alharazi et al., 2016; Khatri et al., 2016; Mogalli et al., 2016). Findings on male preponderance for CL have also been reported in other countries, including Saudi Arabia (Alraey, 2022; Amin et al., 2013; Hawash et al., 2018), Libya (Abdellatif et al., 2013), Ethiopia (Custodio et al., 2012; Zeleke et al., 2021), Ghana (Akuffo et al., 2021), and Sri Lanka (Siriwardana et al., 2010). Consistent with the findings of the present study, few previous reports in Yemen and elsewhere showed that females were more prone to CL than males (Al-Kamel, 2016b; Khosrotaj et al., 2022; Nassar et al., 2021; Reithinger et al., 2003). In rural areas of Yemen, women complete all household

activities and are also exclusively responsible for the livestock, including stall feeding, milking, cleaning barns/sheds and dealing with manure and waste.

Overall, the present study showed that young children aged ≤10 and adult females accounted for 66% (35/53) of the confirmed CL cases. This finding suggests that the majority of infected individuals (i.e. young children and adult females) were bitten in the household vicinity that may contain livestock sheds and coops and also livestock waste (e.g. dried dung), providing optimum breeding and rest places for sandflies. Many previous studies in anthroponotic CL foci in different countries demonstrated that *Phlebotomus sergenti* (*L. tropica* main vector) showed endophilic and exophilic behaviours and were found primarily indoors or both indoors and outdoors (Al-Zahrani et al., 1997; Maroli et al., 2013; Orshan et al., 2010). Thus, the transmission cycle of anthroponotic *L. tropica* probably occurs in the indoor or immediate outdoor environments.

In the rural areas of Yemen, women frequently gather in front of their households at dusk for a few hours, when most CL transmission probably occurs, and young children play or sleep near their mothers; thus, they all are more exposed to sandfly bites. Moreover, it is largely believed that stigma, in addition to poverty, prevents CL-infected females from seeking treatment and medical care at health centres (Al-Kamel, 2017; Kassi et al., 2008), and this may explain the higher proportion of CL cases among males in most of the previous hospital-based studies. Nonetheless, it should be taken into account that the identified association could be due to the low number of female participants involved in this study.

In addition to age and sex, the present study revealed that sharing a residence with family members with ulcerating skin diseases, living in poor housing conditions with cracked walls, sleeping outside and keeping livestock animals on the ground floor of the

house were significant predictors of CL among the study population. A clustering of CL cases, particularly caused by *L. tropica* among households and neighbourhoods when residents have presented with typical skin lesions, has been reported in different settings (Brooker et al., 2004; Custodio et al., 2012; Kariyawasam et al., 2015; Kayani et al., 2021; Ngere et al., 2020; Reithinger et al., 2010; Shita et al., 2022). Occasionally, CL may be considered a family infection due to the exposure of more than one individual to the biting of infected sandflies in the same environment (Uzun et al., 2018). Hence, the family members of infected individuals and close neighbours should be screened for the disease and should also be included in the risk group for CL as well as VL, which also has significant endemicity in the Utmah district and other areas in Yemen (Abu-Hurub et al., 2022; Al-Kamel, 2016b; Al-Selwi et al., 2018; Al-Shamahy, 1998).

The present study revealed an increased risk of CL in poor housing conditions with cracked walls, implying an almost three-fold increase in the likelihood of CL infection among participants who lived in houses with mud-plastered stone walls or un-plastered brick walls. This is consistent with many previous studies conducted elsewhere (Dires et al., 2022; Eshetu & Mamo, 2020; Kariyawasam et al., 2015; Ngere et al., 2020; Siriwardana et al., 2010; Younis et al., 2020). Wall cracks and crevices represent suitable daytime resting and hiding environments for sandflies after feeding and imply a higher risk of indoor exposure to vector bites and CL infection. This could be propagated by poor household sanitary conditions, as reported elsewhere (Kayani et al., 2021; Younis et al., 2020). The present study showed a significant association between the unavailability of improved toilet facilities in a house and CL; however, this association was not retained in the multivariate analysis.

Moreover, outdoor sleeping, especially during summer nights, is a common habit in rural and semi-urban areas in Yemen, where the majority of villagers guard or harvest their crops. This period coincides with the breeding season of sandflies (El Sawaf et al., 2016), although sandflies in northwestern and southwestern Yemen are found throughout the year, with more than one peak between April and September. Thus, sleeping outside in these CL-endemic areas will increase individuals‟ exposure to sandfly bites, and the findings of the current study showed that the likelihood of being diagnosed with CL was 3.58 times higher in cases who slept outside, and this agrees with findings reported elsewhere (Custodio et al., 2012; Kayani et al., 2021; Khan et al., 2021; Shita et al., 2022). Previous studies in neighbouring countries demonstrated that most sandfly species are either crepuscular, with peak biting activity either soon after sunset and before dawn, or nocturnal, with the highest activity reported around midnight (Aklilu et al., 2017). However, a sandfly‟s habitat and biting activity can vary within its geographical range depending on different variables such as temperature, humidity, habitat availability and the presence and abundance of vertebrate hosts (Aklilu et al., 2017; Doha & Samy, 2010). Therefore, further studies on the feeding behaviour and activity of the sandfly vectors in CL endemic areas in Yemen are highly warranted.

The current study showed that the location of livestock shelters/barns was a significant predictor of CL, with the likelihood of CL infection 25% lower among cases who kept animals on the ground floor of their houses compared with those who kept animals in separate shelters near the households as well as those who shared a one-storey house with livestock. A similar protective association was reported by previous studies elsewhere (Bern et al., 2005; Bern et al., 2000; Schenkel et al., 2006). On the other hand, previous studies in Yemen and other countries found that keeping animals in houses or in

peridomestic areas increases the likelihood of contracting CL (Araujo et al., 2016; Kayani et al., 2021; Kolaczinski et al., 2008; Nassar et al., 2021). Indeed, the role of domestic animals in *Leishmania* parasite infection is still controversial. Some studies suggested that domestic animals can be potential reservoir hosts for leishmaniasis caused by *L. infantum*, *L. major* and *L. donovani* (Kushwaha et al., 2022; Rezaei et al., 2022), yet there are no similar reports on *L. tropica*. Moreover, adult sandflies often inhabit animal shelters, as they may provide optimum conditions for their breeding, thereby increasing indoor vector density and risk exposure to humans (Bern et al., 2010; Votypka et al., 2012). On the other hand, animals may serve as an alternative blood meal source for sandflies and therefore mitigate CL transmission risk among their owners (Zooprophylaxis) (Bern et al., 2005; Bern et al., 2000; Kolaczinski et al., 2008). In rural Yemen, livestock animals are traditionally housed on the ground floor of a double/triple-storey house or share the one-storey house with their owners, although some keep their animals in a shed attached to the house. Thus, these people are in very close proximity to animals. Moreover, local veterinarian services are lacking; thus, animals are not routinely screened for infectious diseases. Nevertheless, feeding behaviour of the sandfly and the role of livestock in CL transmission in these areas needs further investigations.

In addition, the present study found significant associations between CL infection and a low educational level, a lack of improved toilets in the house, goats ownership, the presence of open water sources near the house and the knowledge about CL transmission. However, these associations were not retained in the multivariate hierarchical logistic regression analyses. These findings are not in line with the results of previous studies conducted elsewhere and identified low levels of education, living near irrigation or open water sources, the presence of goats in the house, and poor sanitation conditions in the

household as significant risk factors of CL (Eshetu & Mamo, 2020; Kayani et al., 2021; Ngere et al., 2020; Shahryari et al., 2022; Younis et al., 2020).

Moreover, the present findings showed a significantly lower prevalence of CL among dog owners, and this is contrary to previous findings that dog ownership was a significant risk factor for a higher prevalence of CL (Eshetu & Mamo, 2020; Votypka et al., 2012). Although *L. tropica* is mainly considered to be anthroponotic, canine infections by *L. tropica* have been reported in different Middle Eastern countries (Alanazi et al., 2019a; Baneth et al., 2022). Other studies have indicated the possibility of dogs being the reservoirs for CL infections caused by *L. donovani* (Jambulingam et al., 2017; Nawaratna et al., 2009). In areas where *L. infantum* has a zoonotic cycle, canine infections are common (Bourdeau et al., 2020; Dantas-Torres et al., 2012). Interestingly, a previous study conducted in VL-endemic rural areas in Sana‟a governorate, Yemen, documented natural infection of *Leishmania* species in 50% of studied feral dogs (Al-Shamahy, 1998).

5.5 Knowledge, attitude and practices about cutaneous leishmaniasis and sandfly vector

Although all the participants had prior knowledge about the disease by its local name and 69.6% (201/289) of them knew it by the scientific term „*Leishmania'*, the overall knowledge about the disease and its sandfly vector was unsatisfactory. Only 9.3% (27/289) of the participants had correct knowledge about the role of sandflies in CL transmission, while about one third of them (36.0%) were able to identify and differentiate sandflies from other mosquitoes and house flies. These findings are consistent with those reported by the only previous KAP local survey conducted in Taiz Governorate, southwestern Yemen (Alharazi et al., 2021). However, only 22.3% of the participants from Taiz had a good level

of knowledge about CL, which is much lower than that reported by the present study (51.2%). The higher awareness about CL‟s name and its clinical presentation can be attributed to the endemicity of the disease in the study area.

As the present study reported, 33% (202/612) of the participants had active skin lesions and/or scars that met the clinical criteria for a case of CL, and at least one confirmed case of CL was reported in 18.3% (53/289) of the studied households. Moreover, the majority of the participants (86.9%) had seen CL cases among household members or other individuals within the community. On the other hand, the poor level of knowledge about the disease‟s transmission among the study participants could be due to the lack of a CL control programme in the study area. Although a majority of the respondents heard about the term „leishmaniasis‟ through medical teams who visited the area and its clinics, CL-related health education was not provided to these communities. The mobile medical outreach teams visited the villages mainly to improve vaccination rates and provide basic healthcare and treatment for a wide range of diseases.

In comparison with KAP surveys conducted in CL-endemic areas in other countries, a previous study in rural communities of southern Ethiopia found that all heads of households studied did not know the mode of CL transmission and had never heard of sandflies, with only 19% (80/422) having a good level of CL-related knowledge (Alemayehu et al., 2023). Similarly, very poor levels of knowledge about the disease and its vector were reported in endemic areas of Kerala, India (Nandha et al., 2014) and Central Morocco (Mounia et al., 2022). On the other hand, a previous study in central Iran demonstrated that almost all (97.9%) participants were aware that CL is transmitted by sandflies; however, only 28.6% were able to identify a sandfly (Saberi et al., 2012).

In addition, the present study showed that about half and two thirds of the participants could not cite any preventive measures against CL and sandflies, respectively. These findings agree with those reported previously in southern Yemen (61.2% and 58.2%, respectively), although both populations did not receive CL-related health education (Alharazi et al., 2021). Despite the high endemicity of CL in the remote districts of Khyber Pakhtunkhwa, Pakistan, a very low level of awareness was reported, with only 2% of 844 CL-suspected individuals having knowledge about basic preventive measures of CL (Ahmad et al., 2022). These differences can be due to CL endemicity, the education levels of participants and the implementation of CL-targeted health education programmes. People living in CL endemic areas should be aware of sandfly characteristics, feeding behaviour, breeding sites and control measures. They should also perceive sandflies as the vectors of CL so that they minimise exposure and adopt effective control measures (Irum et al., 2021; Pardo et al., 2006).

The present study demonstrated that about one third of the participants had a positive attitude towards CL, with only 27.3% believing that CL is a preventable disease. Previous studies from Yemen and Saudi Arabia found that 36.6% and 19.3% of the participants, respectively, assumed that CL can be prevented (Alharazi et al., 2021; Moussa et al., 2019). On the contrary, the majority of participants in northern Ethiopia (82%) and southern Iran (69%) had positive attitudes about the prevention of CL (Sarkari et al., 2014; Tesfay et al., 2021). The endemicity of CL for many decades, coupled with the lack of health education interventions, may result in negative attitudes regarding the possibility of CL prevention. Interestingly, the present study revealed that 45.7% of the participants, particularly females, believed that CL is a stigmatising disease. This concurs with a previous study conducted among CL-infected females in Sana‟a and Radaa governorates

(Al-Kamel, 2017). In the present study, most CL lesions were on the face, particularly on the cheeks, nose and lips, and the infected female participants have expressed their strong sense of shame and embarrassment of being seen in public. CL-associated stigmatisation might prevent infected individuals, particularly females, from seeking treatment at health centres. This may also exacerbate their health outcomes and influence their educational attainment (Al-Kamel, 2017; Grifferty et al., 2021).

In regards to treatment-seeking behaviour, over half of the studied participants declared that they would go to the nearest health centre as a first line activity to seek treatment for skin lesions; however, in reality, 50% (79/158) used traditional remedies to treat previous CL-suspected lesions while 31.7% used chemotherapy. Some medicinal plants were cited by the participants, including *Aloe vera* and *Calotropis procera*. These findings are consistent with previous studies in Yemen (Alharazi et al., 2021) and southern Ethiopia (Alemayehu et al., 2023). Moreover, other studies from northern Ethiopia (Tesfay et al., 2021) and India (Nandha et al., 2014) found that 90% and 100% of participants with CL, respectively, were treated solely by traditional herbal remedies. In addition, about 14% (22/158) of the studied participants used cauterisation by placing very hot metal objects or applying battery acid on the lesions, practices that may lead to permanent deformities and severe complications (Weigel et al., 1994). Awareness of CL treatments should be improved, and adequate chemotherapy should be made available and accessible in endemic areas. Due to desperate shortages of drugs created by the ongoing civil war, few people travelled to main cities, especially Sanaʻa, which caused additional burdens.

Poverty prevails in the studied communities. Thus, poor housing and environmental conditions that favour CL transmission represent critical challenges that may hinder the control and elimination of the disease in Utmah. The findings showed that the majority of

the population did not adopt any control measure, with a very small proportion of the households owning bed nets and/or using insecticides. In the CL-endemic rural communities of Tigray, Ethiopia, bed nets and insecticides were not used at all by any households, despite a high awareness of CL (Tesfay et al., 2021). Likewise, previous studies from Ethiopia and Pakistan reported that negligible proportions of the participants used bed nets and insecticides (Ahmad et al., 2022; Alemayehu et al., 2023). By contrast, higher percentages (up to 50%) of households that used bed nets and insecticidal sprays to control sandflies were reported in Pakistan (Irum et al., 2021) and India (Garapati et al., 2018). Moreover, a significant proportion of the studied participants reported working and sleeping outside during the night. Outdoor activities during the night-time are common in rural communities in Yemen and other countries where considerable proportions of villagers work in their farms or guard their crops at night; thus, they are exposed to sandfly bites (Tesfay et al., 2021). Therefore, health education intervention about the vector of CL is crucial for the endemic areas to adopt necessary preventive and control measures (Pardo et al., 2006). In addition, the distribution of bed nets and insecticides to prevent malaria is expected to also help against sandflies (Coulibaly et al., 2018). However, the activities of the Yemeni National Malaria Control Programme have been largely affected by the unstable political situation and civil war since 2010 (Abdul-Ghani et al., 2021).

In the present study, the knowledge about CL was found to be associated with sex, occupation and the presence of CL-confirmed cases in the household. The female participants and those working as farmers were less likely to have good CL-related knowledge compared to their counterparts. This can be due to their lower educational levels. Females also had lower exposure to CL-related knowledge at health centres or from health workers visiting the villages, as cultural customs prevent females from talking to

men and foreign individuals. Similar findings were reported in Yemen and elsewhere (Alemayehu et al., 2023; Alharazi et al., 2021). The presence of CL-confirmed cases in the household is associated with a good level of CL-related knowledge that may be gathered during efforts to manage and treat skin lesions.

Moreover, the findings revealed that age is a significant determinant of knowledge about sandflies, with participants aged over 40 years old having almost twice the odds of having better knowledge compared to younger participants. This finding concurs with that reported previously from Yemen (Alharazi et al., 2021). In CL-endemic areas, it is expected that elder people gain knowledge about the disease and sandflies over time. In addition, this study found that participants who lived with confirmed CL cases in the same household and those from the Razeh sub-district had significantly lower odds of having a positive attitude towards CL. Although the prevalence of CL-confirmed cases across the studied sub-districts was comparable, the observed district-wise difference in the attitude towards CL could be due to participants‟ experience with the disease or their educational level. The Razeh sub-district had a significantly higher proportion of non-educated participants when compared to other sub-districts. Furthermore, only monthly household income was retained as a significant predictor of participants‟ CL-related practices. Indeed, CL is considered to be a poverty-related NTD (Alvar et al., 2006). Poverty, represented by low income and limited financial resources, has adverse effects on the prevention-related practices of vector-borne diseases by limiting the opportunities for families to improve housing conditions or to adopt protective measures, such as bed nets and insecticides or repellents (Moya-Salazar et al., 2021).

5.6 Occurrence of leishmaniasis in animals

The present study is the first to provide preliminary evidence on the existence of potential non-human mammal reservoir hosts for CL in Yemen. Ten different types of domestic and wild animals (goats, sheep, cows, donkeys, bulls, rabbits, bats, dogs, cats, and rats) were examined for the presence of *Leishmania* parasites. The results showed that 16.4% (20/122) of the examined animals were positive for the *Leishmania* parasite, with the highest prevalence was found among goats (23.4%, 11/47). Interestingly, *L. tropica* being the sole causative agent, and it was detected in all types of the studied animals except for cats and sheep.

In Yemen, canine leishmaniasis was reported in human VL endemic foci in Taiz and Sana‟a governorates (Al-Shamahy, 1998; Rioux et al., 1989). Several studies to investigate zoonotic leishmaniasis were conducted in other countries but only included one or two types of animals, with dogs and rodents being the most studied animals (Alanazi et al., 2021; Alanazi et al., 2019a; Alanazi et al., 2019b; Alsaad & Hameed, 2021; Baneth et al., 2022; Caldart et al., 2017; Cassan et al., 2018; Kassahun et al., 2015b; Lemma et al., 2017; Luis et al., 2013; Sosa-Bibiano et al., 2022). A previous survey of wild and domestic animals for VL in Jiashi County, China, identified *Leishmania infantum* in domestic animals such as sheep, goats, cattle and donkeys (Gao et al., 2015).

Similarly, *L. tropica* has been reported in dogs (Alanazi et al., 2019a; Baneth et al., 2022), golden jackals and red foxes (Talmi-Frank et al., 2010b), several types of rodents such as mice, rats, and gerbils (Kassahun et al., 2015b), black rats and North African gundi (Bousslimi et al., 2012), and rock hyrax (Svobodova et al., 2006; Talmi-Frank et al., 2010a), cats (Aksulu et al., 2021; Baneth et al., 2022), lizards (Zhang et al., 2016) and bats (Kassahun et al., 2015a). To our best knowledge, the current study is the first that

documented *L. tropica* parasites in donkeys and bulls from the old world. However, a recent study from Pakistan reported *L. tropica* in 11 cows (*Bos taurus*) and 6 goats (*Capra hircus*) for the first time (Rasheed et al., 2023).

The present study reported *L. tropica* in a bat from Bani Bahr sub-district. A previous study from Ethiopia reported that rodents and bats could have adequate features to be naturally infected by *Leishmania* and could subsequently play a role in its transmission cycle (Kassahun et al., 2015a). The longevity and the ability of bats to disperse could facilitate the maintenance and dispersal of *Leishmania* and could subsequently play a role in its transmission and epidemiology (Kassahun et al., 2015a). Similarly, rodents have a worldwide geographical distribution, with a great ability to move and reproduce, making them capable of transmitting and spreading a variety of infectious diseases including leishmaniasis (Jamil et al., 2021).

A previous study from Ethiopia demonstrated that none of the *L. tropica*-positive bats or rodents showed any visible clinical signs that could be attributed to CL. Nonetheless, regardless of the presence of cutaneous lesions, previous studies have shown that PCR can detect *Leishmania* DNA in blood and different tissues of animals (Manna et al., 2004). Moreover, CL species that infect humans are known for their ability to invade other animal hosts' visceral organs (Laskay et al., 1995). In addition, several studies have confirmed the presence of CL species in different organs of animals, such as the spleen, liver, and ear tissue in addition to the blood and skin biopsies (Alanazi et al., 2021; Baneth et al., 2022; Cassan et al., 2018; Castro et al., 2020; Kassahun et al., 2015b; Pasa et al., 2015; Shapiro et al., 2013).

Since the first discovery of canine leishmaniasis in Tunisia in 1908 (Nicolle & Comte, 1908), dogs have been considered the main animal reservoir for zoonotic VL. Moreover, several studies from different countries reported *L. tropica* and *L. major* CL in stray and domestic dogs (Alanazi et al., 2021; Alanazi et al., 2019a; Alanazi et al., 2019b; Alsaad & Hameed, 2021; Baneth et al., 2022). In a recent study from Iraq that aimed at examining skin biopsies and blood samples of dogs, 96% of the skin biopsies and 20.7% of the blood samples were positive for *L. major*; however, *L. tropica* was detected in 4.2% and 7% of symptomatic and asymptomatic dogs, respectively (Alsaad & Hameed, 2021). Similarly, a study conducted in Saudi Arabia between 2016 and 2018 to investigate the presence of *Leishmania* parasites in 526 dogs reported 4.0% for *L. major* and 1.9% for *L. tropica* (Alanazi et al., 2019a).

With regards to the new world, CL was detected in several reservoir hosts including bats (Castro et al., 2020), opossums (Cardoso et al., 2015), equines (Silva et al., 2021), canines (Paniz Mondolfi et al., 2019), rodents (Andrade et al., 2015; Sosa-Bibiano et al., 2022), sloths (Gonzalez et al., 2015), and cats (Mendoza et al., 2022; Paniz Mondolfi et al., 2019). However, up until this point, only wild rodents have been recognised as having the capacity to act as potential reservoirs (Andrade et al., 2015). Therefore, further investigations on bigger sample sizes and more kinds of animals are warranted to enable better understanding about the animal types that have the capacity to act as potential reservoirs of leishmaniasis.

Unanticipatedly, the present study detected *Trypanosoma lewisi* in seven blood samples (3 sheep, 1 bull, 1 rabbit 1 cat and 1 rat), with the rat (in Bani Bahr sub-district) was found co-infected with *L. tropica* and *T. lewisi.* To the best of found knowledge, this is the first report on this parasite from Yemen. A previous study on 431 invasive and native

rodents in southern Senegal found that about 40 rats were infected with *L. major* and 67 with *T. lewisi*, with 12 of them were co-infected with the two detected parasites (Cassan et al., 2018). *Trypanosoma lewisi* is a globally distributed non-pathogenic parasite that is commonly transmitted by many species of rat fleas to different rodent species mainly *Rattus* species, mice and also kangaroo rats in America (Desquesnes et al., 2016). It also can infect other mammals sporadically, with unknown zoonotic risk to humans. However, *T. lewisi* has recently emerged as potentially pathogenic for humans. Several cases of *T. lewisi* were documented among human, especially young babies, in Africa and Southeast Asia including one fatal case (Truc et al., 2013). Accordingly, potential zoonotic epidemic cannot be ignored; and thus, further evaluation and field investigations on this infection in endemic areas are warranted.

5.7 Population genetic structure and haplotype diversity of human and animal *Leishmania tropica* populations

All confirmed CL cases reported by the present study were found due to *L. tropica*, and this is consistent with several findings reported earlier in Yemen (Mahdy et al., 2010; Mogalli et al., 2016). Among 143 CL-patients from Hajah, North Yemen, *L. tropica*, was the only *Leishmania* parasite species detected using ITS1-PCR and RE analysis (Mogalli et al., 2016). Similarly, out of 53 smears collected from patients with suspected CL in Sana‟a, 22 (41.5%) were microscopy-positive and only 16 of them were successfully sequenced; *L. tropica* was the sole causative agent detected (Mahdy et al., 2010). Other previous studies in different regions of Yemen have also demonstrated the predominance of *L. tropica*, with very small proportions of CL cases by *L. infantum* and *L. donovani* (Khatri et al., 2016; Khatri et al., 2009; Pratlong et al., 1995b). In most countries of the MENA region, ZCL

caused by *L. major* is the main form of the disease, whereas CL in Yemen is thought to be caused exclusively by an anthroponotic cycle (Du et al., 2016; Karami et al., 2023). The only documented CL case by *L. major* in Yemen was reported in Taiz, southwestern Yemen (Rioux et al., 1986). Interestingly, *L. tropica* has been implicated in many human cases with life-threatening VL in different countries, such as Saudi Arabia, Iran, Kenya and India; thus, early diagnosis and treatment and the implementation of sustained control strategies are crucial (Magill et al., 1993; Thakur et al., 2018).

In comparison with findings from neighbouring countries, *L. major* has been reported as the predominant species in different regions of Saudi Arabia; however, *L. tropica* is considered the predominant species in Asir and Jazan, the southwestern regions of Saudi Arabia bordering Yemen (Al-Rashed et al., 2022; Al-Salem et al., 2019). This variation in the geographical distribution of *Leishmania* species could be due to the impact of climate and altitude conditions on the sandflies. In the southwestern parts of the Arabian Peninsula (including the Sarawat range from Asir and Jazan in southwestern Saudi Arabia to Dhamar and Taiz in southwestern Yemen), *P. sergenti* (*L. tropica* main vector) was only found in the highlands (≥ 1000m above sea level), while *P. Papatasi* (*L. major* main vector) was rare and restricted to the arid lowland areas (≤ 600m above sea level) (Al-Salem et al., 2019; El Sawaf et al., 2016).

Although *L. tropica* is the predominant causative agent of CL in Yemen, the epidemiology of the disease is not fully studies. The transmission cycle of *L. tropica* is primarily thought to be anthroponotic; however, zoonotic transmission may be possible in some areas. *L. tropica* is characterised by great enzymatic diversity, and its wide geographic distribution makes it the most challenging species of *Leishmania* to control. However, its epidemics are less severe than those caused by *L. major*. On the other hand, a

previous study claimed that *L. tropica* and *L. major* are not likely to be found in the same climate and altitude unless patients travelled from *L. tropica* endemic area to another endemic area with *L. major* and vice versa (Al-Salem et al., 2019). The authors‟ explanation was that the vector of *L. tropica* is not found at low altitudes (600 m or less above sea level) because this altitude's climate conditions and ecology do not allow proper transmission of *L. tropica* by its main vector, *Phlebotomus sergenti*.

In the present study, phylogenetic analysis of *ITS-1* gene sequences from 54 isolates showed that human and animal isolates were classified into six groups (haplotypes). All human sequences except two (samples no. H3 & H205) plus 15 of the animal sequences were found to be similar and classified in the same group. The CL caused by *L. tropica* is considered mainly anthroponotic; however, sporadic zoonotic outbreaks have been reported in different countries such as Saudi Arabia, Greece, Jordan, Israel, and Kenya (Jacobson et al., 2003). Thus, sequenced isolates of the current study from animal and human samples on the same cluster of the phylogenetic tree may support the hypothesis that *L. tropica* can be zoonotic. In Tunisia, sand rats, *Potamonautes obesus* and *P. vexillaris,* are known as the main reservoir hosts of zoonotic CL due to *L. major*, and could be the hosts to *L. tropica* and *L. infantum* simultaneously (Ben Othman et al., 2018). Moreover, these results were supported by a recent study that analysed a vast group of *ITS-1* sequences of *L. tropica* obtained from the GenBank across many endemic countries and found some haplotypes contain both human and non-human isolates (Charyyeva et al., 2021). However, further investigations including ecological niche modeling of main reservoir hosts and sandflies vectors of *L. tropica* in endemic regions are highly required.

The present findings showed that five animal and two human isolates were assigned into five new clusters; one included three animal isolates, while four others included one

isolate each (Figure 4.4). Of these five animal isolates, four (3 from goats and one from a dog) were from Halmah waBani Ayoub (Alsomal sub-district). The fifth isolate was collected from a bull in the Dawrah area of Razeh sub-district. The population movement could be one explanation for these results. A study conducted in two Indian CL foci located near each other found that *L. tropica* multilocus microsatellite typing (MLMT) sequences were genetically separated into two distinct groups, clustered from both foci on two clades (Krayter et al., 2014). The lack of association between *L. tropica* haplotypes and their geographical origins may be due to the proximity of the foci, which allows the movement of infected individuals in both directions, facilitating the circulation of *L. tropica* MLMT strains between the foci. Moreover, these may explain the existence of unique haplotypes of *L. tropica* in areas close to each other.

In addition, the present study showed that the phylogenetic analysis of human and animal sequences segregated six different *L. tropica* haplotypes: 3 from human and 4 from animal (Figure 4.4). The reported number is higher than that reported from Yemen by previous studies that detected only two haplotypes (Charyyeva et al., 2021; Mahdy et al., 2010). However, such comparison might be unsafe due to the very low number of *L. tropica* sequences analysed by previous studies (only 17 sequences). In comparison with other countries, number of haplotypes in the current study is lower than that reported from other countries such as Iran (Ghatee et al., 2018) and Morocco (Charyyeva et al., 2021; Daoui et al., 2022; El Hamouchi et al., 2019; El Kacem et al., 2021). A recent study analysed the genetic diversity of 58 sequences of *L. tropica* in three endemic foci in Morocco and found 29 polymorphic sites that produced 13 haplotypes in the studied population (Daoui et al., 2022). The study also found that the distribution of haplotypes was not uniform, with some haplotypes being more common in certain areas than others.

Similar findings were reported by another Moroccan study that conducted in two endemic foci and identified 29 polymorphic sites that led to 14 haplotypes (El Kacem et al., 2021). Likewise, a previous study analysed 93 *L. tropica* sequences from one province (Azilal) in Morocco and identified 27 polymorphic sites that produced 13 haplotypes (El Hamouchi et al., 2019). A 4-years earlier study from the same province (Azilal) analysed 31 *L. tropica* sequences isolated from *Phlebotomus sergenti* sandflies and segregated them into 16 haplotypes (Ajaoud et al., 2015).

These series of findings from Morocco revealed different patterns of population structure and haplotype diversity, suggesting that the number of haplotypes of *L. tropica* can vary based on the geographic region and the type of population. This might explain the low haplotype diversity (0.242 ± 0.077) and non-significant genetic differentioation (F_{ST} = 0.050) found for the Yemeni *L. tropica* sequences sampled from the study area. Moreover, the sampled *L. tropica* population had significantly negative value for Tajima‟s *D* (-1.935; $P < 0.05$) while Fu‟s *Fs* tests, which is more powerful in detecting population expansion (Ramos-Onsins & Rozas, 2002), did not depart significantly from neutrality ($P > 0.02$), indicating an excess of rare haplotypes over what would be expected under neutrality.

Although the studied sub-districts belonging to Utmah district might be considered as a one area due to the small total area of the district, genetic diversity indices from Alsomal sub-district were higher than those from the other sub-districts; however, the difference was not statistically significant. A strong association between the level of genetic diversity in the *Leishmania* parasite and the effective population size has been suggested, with the genetic diversity is higher in small populations (Banuls et al., 2007). Other biological, demographic and environmental factors such as existence of other *Leishmania* species, global climate change, travelling, and internal and cross-borders migration due to

armed conflicts can also impacted the population genetic structure and genetic diversity (Banuls et al., 2007; Spotin et al., 2023). For instance, Iranian *L. tropica* population showed the second lowest haplotype diversity, after Yemen, compared to other populations, and this could be due to the fact that in Iran *Leishmania major* is the main causative species of CL competing the non-native species, *L. tropica*. Another example is H_7 which contained five sequences (4 from Saudi Arabia and 1 from Malaysia) as the Malaysian isolate was from a Saudi child who had come from Saudi Arabia a few weeks before his infection had been diagnosed in Malaysia (Tan et al., 2022).

The global genetic diversity analysis for the 367 *L. tropica* sequences showed high haplotype diversity (0.605 ± 0.028) but low nucleotide diversity (0.0051 ± 0.0004). Tajima‟s *D* (-2.583) and Fu‟s *Fs* tests (-33.210) values were significantly negative, indicating selective sweep and an excess allele numbers probably due to recent population expansion. The median-joining haplotype network for the 367 sequences showed a relatively low level of divergence. Fifty-four polymorphic sites produced 54 haplotypes; of these 13 were non-unique (i.e. with >1 instance in the dataset). Moreover, the results demonstrated the dominance of one haplotype (H_1) which contained 224 sequences from 17 countries, extending from Morocco to China. This haplotype (i.e. H_1) involved the majority of the sequences of many countries including Yemen, Iran, Morocco, Syria, Turkey, and China. Moreover, all sequences from Israel were classed into this dominant haplotype. Another large group was H_6 that contained 54 sequences from 13 countries, including the majority of Syrian sequences (26/58). Significantly negative neutrality test values (Tajima‟s *D* and Fu‟s *Fs* tests) were found for populations from China, Iran, Syria, and Morocco, indicating population expansion events. On the other hand, positive neutrality test values were observed in both Israel and Saudi Arabia sequences, suggesting a

deficiency of alleles, as would be expected from a recent population bottleneck (positive Fu‟s *Fs* tests), and a balancing selection (positive Tajima‟s *D*) (Kariyawasam et al., 2017).

The regional-based haplotype network analysis showed that Southern Asia (particularly Iran) and Western Asia (particularly Syria) regions showed the largest number of *L. tropica* haplotypes and the largest distribution area; therefore, it can be basically postulated that CL-causing *L. tropica* populations from these regions are the ancestral populations that spread eastward to China and westward to Africa. Moreover, the Western Asia region is characterized by variety of hosts including human, sandfly and different kinds of animal. However, human was the only host included from Syria, Saudi Arabia, Palestine and Iraq. By contrast, Eastern Asia region contained sequences of animal *L. tropica* isolates (only lizard). Most of sandfly-isolated *L. tropica* were from Africa plus Israel from Western Asia and Greece from Europe. Nevertheless, more data are required from endemic countries to enable better understanding of *L. tropica* populations‟ genetic structure and to explore potential regional or host specificity.

These findings are in agreement with a recent study that also found two dominant haplotypes (Charyyeva et al., 2021). A study from Morocco showed a similar pattern with about 80% (74/93) of the analysed *L. tropica* sequences classed in a dominant haplotype while the 19 remaining sequences formed the other 12 haplotypes (El Hamouchi et al., 2019). Similarly, a recent study identified 11 haplotypes when analysed 29 sequences from different countries, with a dominant haplotype that piled 18 sequences from six different countries (Al-Jawabreh et al., 2023). At a country level, the dominance of one haplotype could be considered as a sign of its fitness in the sandfly vector and human hosts within the same area or country (El Hamouchi et al., 2019). Therefore, studies that involved both the host and sandfly vector, taking into consideration the reservoir hosts, from different

endemic countries are highly warranted to enable better understanding of population genetic structure and haplotype diversity of *L. tropica*.

On the other hand, when sequences from Yemen compared with sequences of other countries, strong (F_{ST} > 0.25) and high (F_{ST} = 0.15–0.25) genetic differentiation levels were reported between Yemen and Syria and between Yemen and Morooco, respectively. These results are in line with the pattern of haplotype distribution that showed the clear obvious separation of Syrian haplotypes, dominating H_6, and also the tendency of Moroccan sequences to form a new dominant, but might be restricted, haplotype. These findings are consistent with findings reported by recent studies (Al-Jawabreh et al., 2023; Charyyeva et al., 2021; El Hamouchi et al., 2019).

Utmah district has almost homogenous epidemiological profiles, including population and environmental characteristics; thus, the current findings can be generalised to other rural populations of the district where CL is hyperendemic. On the other hand, the findings may not necessarily be generalisable to the other rural areas of Yemen because of differences in environments, population dynamics, and infection endemicity. Nonetheless, further large-scale community-based studies are warranted to validate the present findings.

CHAPTER 6

CONCLUSION

6.1 Conclusion

Cutaneous leishmaniasis (CL) is one of Yemen's major public health issues and one of the most important neglected tropical diseases in the country (Tabbabi et al., 2019; Hotez et al., 2012). The disease is endemic in all regions across the country with the majority of reported cases come from highland areas such as Dhamar, Hajjah, Al-Bayda and Lahj governorates. The present study is the first to provide community-based information on the prevalence and risk factors of CL in Yemen using microscopic and molecular examinations. It is also the first study to provide comprehensive results on the genetic structure and haplotype diversity of *Leishmania tropica* among human and animal populations from Yemen in relation to regional and global *L. tropica* populations‟ structure and diversity. Moreover, the study evaluated the population‟s knowledge, attitude and practices concerning the disease and its vector as well as investigated the role of non-human mammals in the transmission of leishmaniasis in Utmah district of Dhamar governorate, western Yemen.

The present study revealed an alarmingly high prevalence of confirmed CL cases (8.7%) among the rural population in the Utmah district, western Yemen. Being ≤ 10 years old, being female, living in poor housing conditions with cracked walls, living in the presence of other family members with typical ulcerating skin diseases, sleeping outside and keeping animals on the ground floor were the key factors significantly associated with CL among the studied population.

Moreover, this study demonstrated insufficient knowledge about transmission, prevention and the sandfly vector of CL among the rural population of the Utmah district, western Yemen. The results also showed that about one third of the participants had a positive attitude towards CL, while only 16.3% had good CL-related practices. Although CL was perceived as a serious health problem in the study areas, the findings of the present study revealed insufficient knowledge about CL and its sandfly vector, poor attitudes towards prevention and poor CL-related practices. Age, sex, presence of CL-confirmed cases in the same household, occupation and household monthly income were the significant predictors associated with KAP towards CL among the participants. The reported poor levels of KAP towards CL revealed that these could be a direct consequence of the lack of a national control programme against leishmaniasis. They could also be due to the fact that the disease is being neglected by health authorities and public health policymakers.

Furthermore, the study revealed that *L. tropica* was the only causative agent of CL reported among the study population. Moreover, 16.4% of different types of domestic and wild animals from the study area were found positive for *L. tropica*. The phylogenetic analysis of human and animal sequences segregated six different *L. tropica* haplotypes (3 from human and 4 from animal). Fixation index (F_{ST}) value for the studied sequences from Utmah district was 0.050, with gene flow (*Nm*) value of 4.65, indicating no genetic differentiation and high gene flow between the human and animal *L. tropica* populations. The global genetic differentiation and diversity analysis of 367 *ITS-1 L. tropica* sequences showed 54 segregating sites producing a total of 54 haplotypes, suggesting a relatively low level of divergence for the involved sequences. Moreover, CL-causing *L. tropica* populations from Southern Asia (particularly Iran) and Western Asia (particularly Syria)

regions are the ancestral populations that spread eastward to China and westward to Africa. These regions have the largest number of *L. tropica* haplotypes and the largest distribution area. Therefore, studies that involved both the host and sandfly vector, taking into consideration the reservoir hosts, from different endemic countries, especially undersampled countries, are highly warranted to enable better understanding of population genetic structure and haplotype diversity of *L. tropica*.

6.2 Recommendations

1. An effective leishmaniasis prevention and control programme is urgently needed to combat this devastating disease in the Utmah district and other areas endemic to CL in rural Yemen. The programme should include active surveillance and provide protective materials such as insecticide-treated nets, insecticide spraying (indoor residual spraying and spraying of vector resting sites), and health education about disease ecology and prevention.
2. The control programme can be integrated with that for other vector-borne diseases endemic in rural Yemen, such as malaria and dengue fever.
3. There is a need for health education and community mobilisation campaigns for behavioural change and to improve community awareness about the disease and its vector in endemic areas.
4. Scaling up and promoting public programmes and NGOs-coordinated initiatives such as the Regional Leishmaniasis Control Centre (RLCC) at the national level and involving women and youths in those initiatives should also be considered.
5. Imperatively, training female health workers to perform skin scraping sampling for the diagnosis of CL as well as to provide health education about CL prevention will

encourage women to seek treatment for their skin lesions and to be aware of reducing exposure to sandflies in households.

6. Providing treatment to infected individuals may encourage the participation of females in these communities; however, this was not possible in the present study. Moreover, using non-invasive diagnostic sampling techniques may be essential to improving participation in surveys targeting CL.
7. Moreover, in such fragile and conflict-affected settings, the provision of mobile health clinics targeting CL and other infectious diseases can be the proper approach to maximising vulnerable population coverage, particularly females and children.
8. Despite the high prevalence of leishmaniasis in rural Yemen, information on sandfly fauna is very limited, whereas data on the possible reservoir hosts are lacking. Thus, further studies on the sandfly populations‟ ecology and dynamics and their possible reservoir hosts in rural Yemen are urgently needed to enable a better understanding of disease transmission and to design effective prevention and control strategies against this devastating disease in Yemen.

6.3 Strengths and limitations of the study

The present study has a number of strengths:

1. It provides the first community-based data on one of the most neglected of NTDs in Yemen after the civil war began.
2. The study design allowed the determination of the prevalence of CL in hard-to-reach target areas through a community-based cross-sectional survey and a case-control comparison that allowed for the identification of risk factors.

3. The use of the molecular assay to screen skin-scraping samples increased the prevalence from 6% (by microscopy) to 8.7%.
4. The study used a conceptual hierarchical framework approach to assessing the determinants of CL with a large number of variables included in this approach. This approach took into account the hierarchical relationships between the potential risk factors and, therefore, enabled a better evaluation and interpretation of the web of causation.

On the other hand, some limitations must be considered when interpreting the current findings.

1. The small number of females and internally displaced participants compared with males and those who permanently reside in the district, respectively, may influence the statistical analysis of group differences. Due to custom and tradition in this region and in many other rural areas in Yemen, direct interviews of females by foreigners are prohibited. Due to the small number of cases and certain observations of interest, the confidence intervals for some results are quite large, which necessitates caution in interpreting the findings.
2. Although sample size for the case-control study has been done with care, the numbers obtained has been considered for the total survey and not only for patients, which may have limited the outcome and interpretations.
3. The unmatched case-control comparisons employed did not allow controlling for potential confounding factors such as sex and age differences and socioeconomic status among participants at the study design stage. Although the effect of major potential confounders was adjusted for using the multivariable logistic regression, taking account

of hierarchal relationships between demographic, socioeconomic, environmental and behavioural risk factors, there could potentially be residual confounding.

4. Out of 81 clinically suspected individuals, only 53 were confirmed as positive CL cases using both microscopy and molecular methods; representing 65.4% positivity rate. This could be attributed to several factors. For instance, some patients may have undergone treatment -whether chemical or herbal- that could diminish positive test results. Additionally, infections might have been older and resolved spontaneously, yet the lesions remained active due to secondary bacterial infections. Furthermore, the quality of some samples was suboptimal, which could impact the effectiveness of the employed methods. The direct *ITS-1*-PCR showed a sensitivity ranging from 63.5% to 100%, and the *ITS-1* nested PCR approach can substantially increase the sensitivity and overcame the false-negative results. Nonetheless, false negative results of *ITS-1* nested PCR were observed by some studies. Previous studies reported significant associations between the *ITS-1*-PCR results and some factors such as the age and immune status of infected individuals, location and duration of lesions, type and quality of specimen, parasite strain type, parasite loads in specimen, and presence of inhibiting factors in the extracted DNA (Mahdy et al., 2010; Goto et al., 2010; Ramirez et al., 2000).
5. The selection of the study areas was based on accessibility and security; thus, sampling from the Samah sub-district and many other areas within the four studied sub-districts was not possible. This also limited the ability to investigate some important factors, such as the seasonality of infections and the displacement of people into the area.
5. Although 122 animals were involved in this study, only a small number of positive cases were observed among the animals (n = 20). Nevertheless, animal part of the study

is considered as a pilot study to provide preliminary information on the presence of potential animal reservoir hosts of CL in the area.

6. The sampling was conducted in the time of ongoing civil war, the situation is very risky, and some areas were not safely accessible.
7. Due to Covid-19 nationwide MCO in Malaysia, a delay in performing lab work was unavoidable.

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