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Distribution and epidemiological features of cutaneous leishmaniasis in Asir province, Saudi Arabia, from 2011 to 2020

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A B S T R A C T

Background: Cutaneous leishmaniasis (CL) places a major burden on the health authorities in Saudi Arabia. Information about the geographical reach and seasonality of CL in Asir province remains limited. Therefore, this study aimed to investigate the epidemiological features of CL in southwest Saudi Arabia.
Methods: Retrospective data from CL patients was collected from the regional vector control unit in Asir province over 9 years. Information analysis was performed using R statistic language (version 4.0.5) and the spatial distribution of cases was mapped using QGIS (version 3.20.0).
Results: A total of 1565 CL cases were recorded from 2011 to 2020. Saudi male citizens were at the highest risk of CL infection. However, children under the age of 13 years were most at risk of contracting CL. CL lesions were primarily located on the face and most cases were reported in the winter and autumn seasons. Spatially, the governates of Abha, Sarat-Abidah and Khamis-Mushait had the highest CL infection prevalence. Moreover, a geographical expansion of CL from Abha to Khamis-Mushait governate was noted during past ten years.
Conclusions: This is the first large scale study to investigate the seasonality, spatial distribution and demographics of CL in Asir province. It describes how the geographical change of CL incidence differs in Asir province and reveals those people most at of CL infections. This study highlights the importance of incorporating improved living conditions, school education and public awareness in the development of CL control policies.

1. Introduction

Leishmaniasis is an insect-borne disease that is caused by *Leishmania* parasites that are transmitted by the bite of female phlebotomine sand flies. It is known as one of the neglected tropical diseases (NTD) commonly found in parts of the tropics, subtropics, and southern Europe. Leishmaniasis exists in different clinical forms in patients. Visceral leishmaniasis (VL) is the most severe clinical form that can result in organ failure of bone marrow, spleen and liver. Cutaneous leishmaniasis is the most common clinical form and has less severity than VL. Based on *Leishmania* species, geographical location and host immunity, CL causes skin lesion sores that can either self-heal or develop into other complicated clinical forms such as mucocutaneous leishmaniasis or diffused cutaneous leishmaniasis [1].

The endemicity of leishmaniasis was reported in 92 and 83 countries or territories in 2018 [2]. More than 100,000 new cases of CL worldwide are reported to WHO every year [3]. Several factors are linked to leishmaniasis endemicity such as deforestation, building of dams, irrigation schemes and urbanization [2,3]. The Kingdom of Saudi Arabia (KSA) was announced as one of the top ten countries for CL endemicity in 1996 [4]. However, due to the efforts of the Leishmaniasis Control Program (LCP) managed by Saudi Ministry of Health (MoH), CL cases have sharply decreased. In 2012, this reduced disease incidence now places KSA in fourth position for the most CL endemic area in western Asia [5]. However, the national health authorities still count CL as one of the major health problems that affects several communities [6]. Despite the national control interventions, CL cases are still reported in some regions of KSA. According to the Saudi Ministry of Health (MoH) records, the majority of CL cases are reported in six regions of KSA, namely; Al-Qaseem, Riyadh, Al-Hassa, Asir, Ha'il, and Al-Madinah. Two types of *Leishmania* strains are responsible for CL in KSA: *Leishmania major* (*L. major*) that is transmitted by *Phlebotomus papatasi* and *Leishmania*

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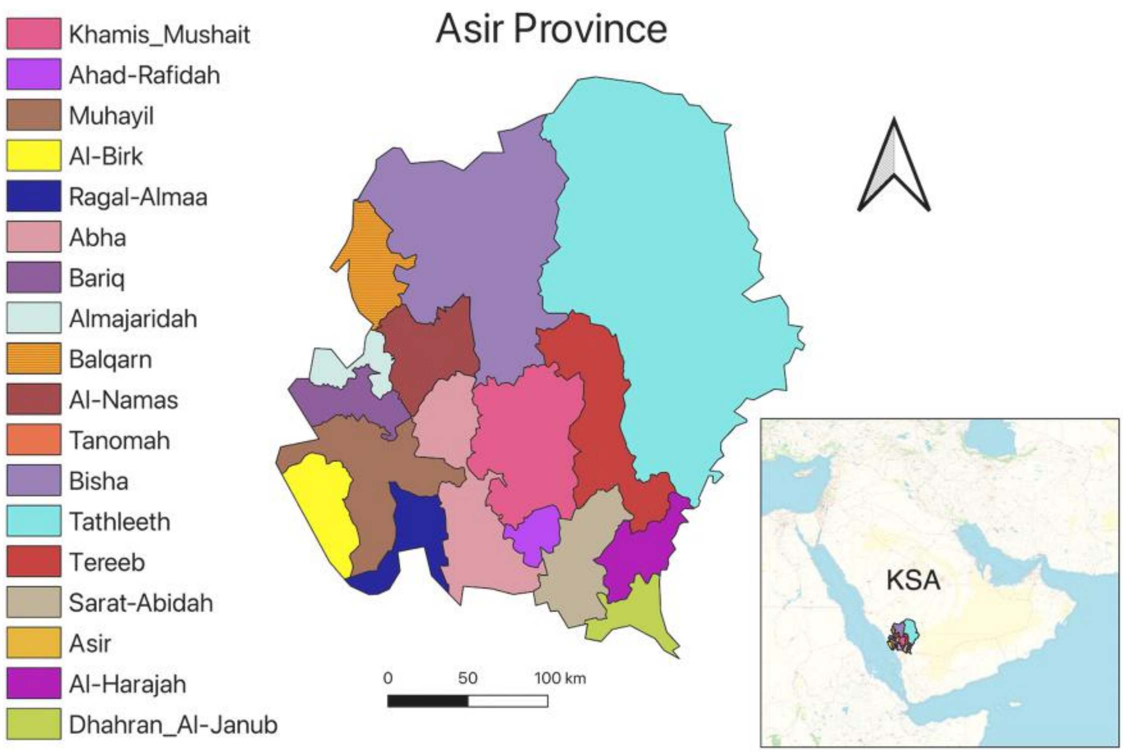

Fig. 1. Map of Asir province of the Kingdom of Saudi Arabia showing locations of Asir governates.

tropica (*L. tropica*) are transmitted by *Phlebotomus sergent* [6] sand-flies.

Asir province is located in the southwest of KSA between latitude (17.266788–21.002471) and longitude (41.242676–44.330078), and is bordered on the north by Makkah Al Mukarramah and Al-Baha province and from the south by Jizan and Najran province. It is divided into sectors that represent different terrains, which range from 300 m to 2800 m above sea level. Most recent studies on CL in Asir province have demonstrated that *L. tropica* is the predominant parasite species and it is mainly transmitted by *Ph. sergenti* [7,8]. However, the stereotyped picture of leishmaniasis in Asir province is not completely clear, although there are some studies that have studied the epidemiology of the disease, but not exclusively in the Asir province [6,7,9].

Because of several building developments and tourist projects underway in Asir province, it is important to fully understand the clinical and epidemiological features of CL in Asir province to mitigate risks. However, because a comprehensive analysis of CL in this district is lacking, this study will fill this gap and evaluate the epidemiological features and spatial distribution of CL in Asir province using retrospective data from 2011 to 2020 using data collected from the Vector Control Unit (VCU) of MoH at Asir province.

2. Methods

2.1. Study area

This study was applied in Asir province that is located in the Southwest of Saudi Arabia. The Asir province is divided into 17 governates namely: Khamis-Mushait, Sarat-Abidah, Tathleeth, Muhayil, Ahad-Rafidah, Abha, Al-Birk, Regal-Almaa, Bariq, Almajaridah, Balqarn, Al-Namas, Tanomah, Bisha, Tereeb, Al-Harajah and Dhahran Al-Janub (Fig. 1).

2.2. Data collection

This retrospective study was applied on data of CL patients reported between 2011 and 2020 and was collected at the Vector Control Unit (VCU) in MoH at Asir province. Each governate regularly reports the incidence of CL to the VCU at Saudi MoH.

2.3. CL diagnosis

According to the National Policy For Management of Cutaneous Leishmaniasis Cases that published by MoH, CL diagnosis is made through combination of clinical history, epidemiological data, and laboratory confirmation. All suspected CL patients with typical appearance of the lesion are referred by the dermatologist to visit the VCU for laboratory diagnosis. Skin lesion scrapping for each patient is made on the slide and stained by giemsa stain and then microscopically examined for the presence of the *Leishmania* parasite. All the CL cases were reported after patients were confirmed microscopically for the presence of *Leishmania* parasite within lesion smear. Additional demographic information about patients such as age, gender, residence, lesion location, number of lesions and date were also recorded by VCU staff.

2.4. Data analysis

All the recorded data was translated into English and digitized in Excel for statistical analysis using R statistic Language v. 4.0.5. [10]. Open-source software QGIS (Quantum GIS version 3.20.0) was used to map the spatial distribution of CL cases in Asir province for the period between 2011 and 2020.

3. Results

A total of 1565 CL cases, 1029 males and 536 females, were reported between 2011 and 2020 to the VCU in Asir province. Overall, from 2011 to 2020, CL prevalence was higher in males than in

Table 1
Number of CL cases based on demographic information in Asir province from 2011 to 2020.

Characteristic	Female, N = 536[a]	Male, N = 1029[a]	p-value[b]	Characteristic	Female, N = 536[a]	Male, N = 1029[a]	p-value[b]
Lesion Number			0.5	Age Group			< 0.001
Multiple	151 (28%)	308 (30%)		0–5	117 (22%)	166 (16%)	
Single	385 (72%)	721 (70%)		6–12	148 (28%)	209 (20%)	
Lesion Location			**0.2**	13–15	35 (6.5%)	76 (7.4%)	
Abdomen	4 (0.7%)	15 (1.5%)		16–18	26 (4.9%)	54 (5.2%)	
Feet-Leg	43 (8.0%)	103 (10%)		19–24	51 (9.5%)	83 (8.1%)	
Hand-Arm	156(29%)	314 (31%)		25–29	28 (5.2%)	102 (9.9%)	
Face	333 (62%)	597 (58%)		30–39	43 (8.0%)	137 (13%)	
Nationality			**< 0.001**	40–49	26 (4.9%)	87 (8.5%)	
Non-Saudi	40(7.5%)	209 (20%)		50–59	28 (5.2%)	56 (5.4%)	
Saudi	496 (93%)	820 (80%)		60–69	22 (4.1%)	27 (2.6%)	
Year			**0.2**	70 +	12 (2.2%)	32 (3.1%)	
2011	38 (7.1%)	102 (9.9%)					
2012	31 (5.8%)	68 (6.6%)					
2013	25 (4.7%)	57 (5.5%)					
2014	32 (6.0%)	74 (7.2%)					
2015	44 (8.2%)	72 (7.0%)					
2016	85 (16%)	131 (13%)					
2017	53 (9.9%)	102 (9.9%)					
2018	60 (11%)	128 (12%)					
2019	96 (18%)	194 (19%)					
2020	72 (13%)	101 (9.8%)					

[a] n (%)
[b] Pearson's Chi-squared test

females in Asir district. Moreover, when considering both sexes, Saudi citizens were more likely to suffer from CL than non-Saudi expatriates (Table 1). Among CL patients, most of the cases reported a single lesion for both males (70%) and females (72%). According to the anatomical location of the lesions, most of lesions were located on the face (62% for females; 58% for males) and hands (29% for females and 31% for males). Both sexes showed a similar age trend in relation to exposure or susceptibility to CL infection. The majority of the notified CL cases were reported in males (26%) and females (50%) younger than 13 years age (Table 1).

Among all age groups, non-school age (0–5) and primary school age (6–12) children consistently had the highest number of CL cases annually during the ten years. In contrast, elderly patients (above 60 years of age) had the lowest cases of CL infection (Fig. 2).

According to the site of lesions, facial lesions were more common among those younger than 13 years old, while lesions on upper and lower limbs were frequently seen in patients aged between 19 and 60 years old (Fig. 3).

CL cases were reported in all months from 2011 to 2020 but with different intensities (Fig. 4A). In general, the highest percentage of CL cases was reported in December (13%) followed by February (9.7%), April (9.1%), January (8.9%) and October (8.9%). The lowest number of cases were reported in August (6.2%) and June (6.4%). Overall, CL seasonality was clear higher number of cases were recorded in spring (avg. 41 cases) and winter (avg. 49 cases) seasons. CL cases in the autumn season in 2016 and from 2018 to 2020 (Fig. 4B) were also elevated.

The endemicity of CL varies in Asir governates. Between 2011 and 2020, most of the CL cases were reported in Abha and Khamis-Mushait governates with 687 and 570 cases respectively. Whereas, CL cases were reported in lesser frequency: Bariq. (7 cases), Tathleeth (4 cases) and Tereeb (11 cases) (Suppl Table 1).

Regarding the incidence rate of CL, through the last ten years, Abha, Sarat-Abidah and Khamis-Mushait governates recorded the highest rate of CL with 188, 152 and 113 cases per 100,000 people. Tathleeth and Muhayil consistently reported the lowest governates incidence of CL with 8 and 11 incidence rates (Suppl Table 1). Through this period, slight fluctuations in incidence rate have been noticed in all governates, except for Khamis-Mushait, Sarat-Abidah and Ahad-Rafidah, where there was a positive trend of CL incidence rate starts nearly from 2016 to 2020, (Fig. 5).

A heatmap of CL endemicity in Asir province from 2011 to 2020 was generated based on longitude and latitude of CL patient locations. In general, CL cases were reported in most of the Asir governates with high incidence level in Abha and Khamis-Mushait. However, the endemicity of CL between Abha and Khamis-Mushait governates has changed over this period; the endemicity of CL was concentrated in Abha governate from 2011 to 2015, which then slightly moved to become highly endemic in Khamis-Mushait governate from 2016 to 2020.

(Fig. 6).

4. Discussion

Epidemiological studies on cutaneous leishmaniasis (CL) have become more important in terms of determining disease patterns and predicting high risk zones of CL in endemic regions. Knowing this information will help improve CL control measures [11,12]. In Saudi Arabia, CL is classified by Saudi MoH as a major public health problem after dengue fever, and Asir province is counted as one of the major endemic CL regions [6,8]. This study investigates, for the first time, the demographic information, seasonality, and spatial distribution of CL based on retrospective data across Asir province.

The current study reveals most CL patients of both sexes suffered from a single lesion on the face, which is a common picture for old world CL as described in previous studies [6,8,12–16]. Interestingly, not all age groups were heavily complaining of facial lesions; the majority of these cases observed in children younger than 13 years old. This study also observed over the ten years of data that younger children, pre-school age (0–5) and primary school age (6–12), were at higher risk of CL infection for both sexes. Nevertheless, other studies in different regions of the KSA concerning teenagers and adult age categories as a most affected groups by CL [6,12,17]. This bias for facial lesions in children could be related to behavior, such as higher exposure to the sandfly by playing and sleeping outdoors during the sunset and night-time or playing near to higher risk areas like livestock waste [18,19]. It may also be that children were bitten inside their homes as the sand fly vector (*Ph. sergenti*) is known to rest indoors [20–22]. However, if this strongly contributed to high infection rates in children, then the CL infection rate should also be high among adult females, as they culturally do their activities inside houses, which contradicts with the current study observations.

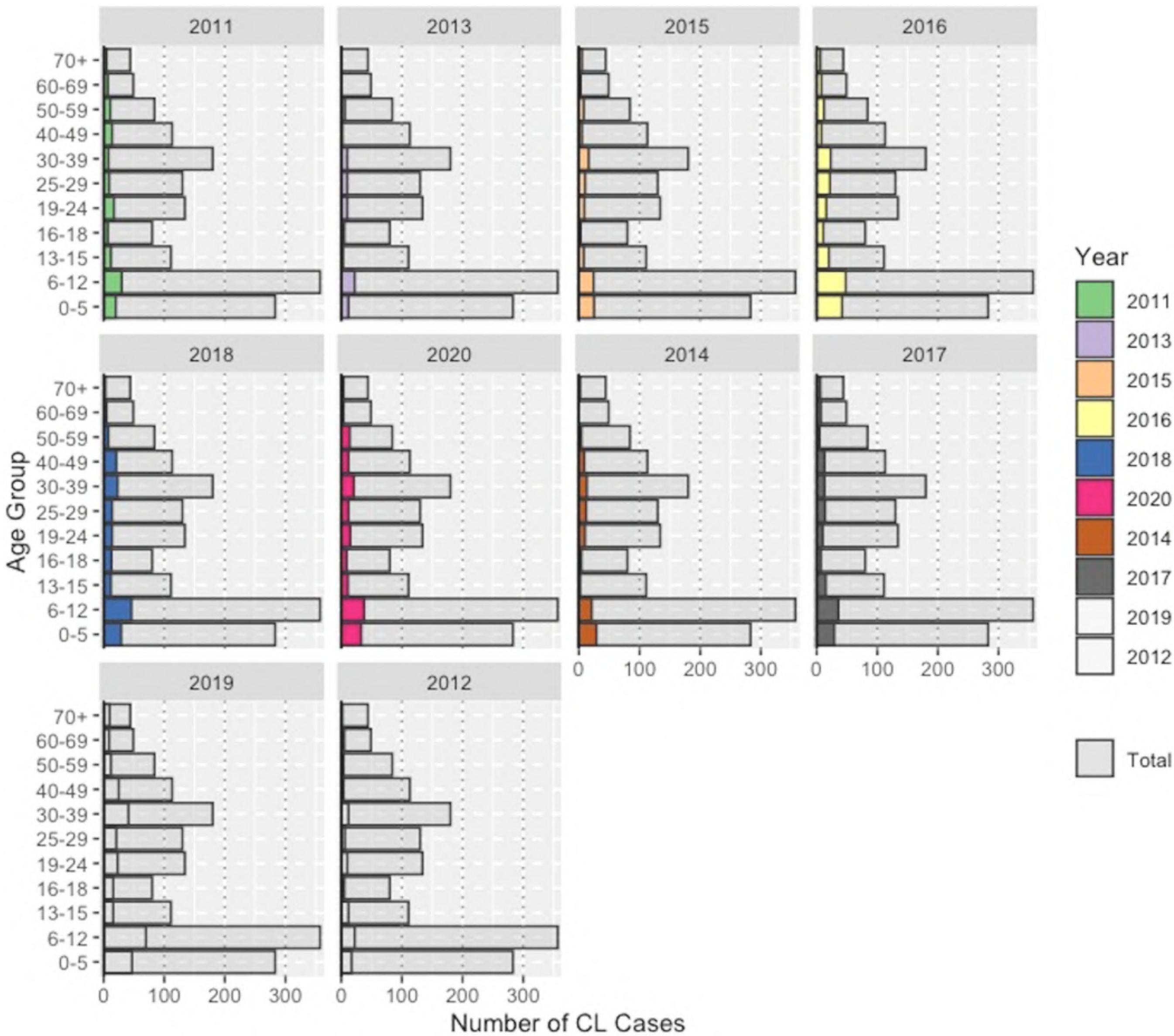

Fig. 2. Age distribution of reported cases of cutaneous leishmaniasis in Asir province from 2011 to 2020.

Another explanation for the higher incidence of CL in children may be related to a young child's immune status against CL, especially as CL infection rates decreased with increased child age [14]. This would indicate a type of acquired exposure immunity where a lack of previous CL infection elevates infection rate for children and decreases prevalence rates among older people [23,24]. This finding highlights the need for a specific intervention by health authorities targeting children, which could be community awareness, children education campaigns and performing a massive diagnostic screen for CL infection on children at schools to reduce CL burden in Asir province. Further investigation on sandfly population and feeding behavior in Asir district are also required for implementing a future framework of indoor vector control strategies. In addition, more investigation on immunological markers can be useful to understand why children are more sensitive to CL infection for vaccine development.

Previous CL studies on populations in Saudi Arabia showed males were highly affected by CL infection more than females (from 2011 to 2020). This difference may be explained by either the use of Islamic catechistic dress for females or the higher risk occupations and behavior of men, which usually involve outdoor activities during night time [6]. When patient nationality was assessed, there was a remarkable difference between Saudis and expatriates in terms of CL infection, in which most of the CL cases were reported on Saudi locals. This outcome comes in agreement with results of previous studies in Al-Madina, Jazan and Al-Taif regions (15,18,19). Usually CL cases are reported in rural and pre-urban areas [19]. In Asir province, rural populations are Saudi locals with their families living close to their domestic animals. This could also help explain why CL is frequently seen in Saudi patients of different age categories in Asir province.

It is thought that temperature is the most important factor associated with the seasonality of cutaneous leishmaniasis in Asir district; with increased temperature during summer, more cases will appear in the following seasons [25]. This concurs with the current study observations where the average number of CL cases is elevated during the autumn and peaks by winter, followed by a general decline to the minimum level by the summer. This observed seasonal trend was similar to other studies in Al-Hassa and Al-Taif regions [12,14,16]. The increased number of CL cases during the winter and

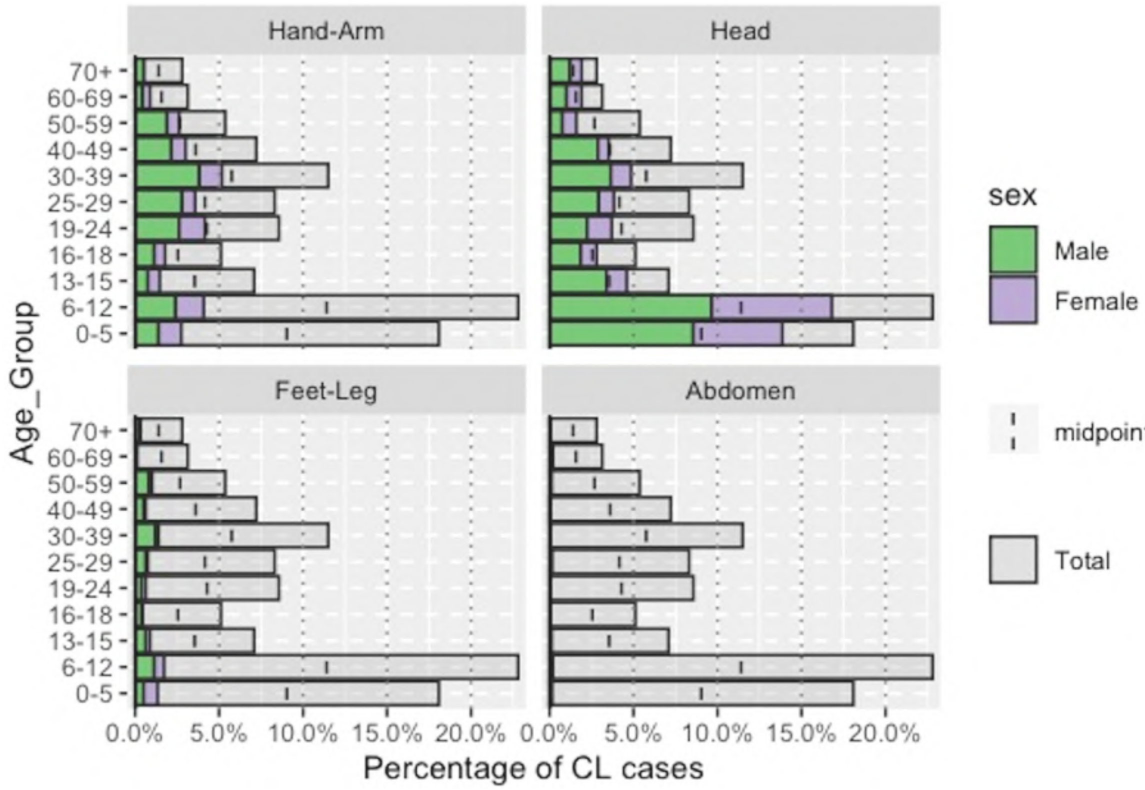

Fig. 3. Distribution of lesions in relation to body sites for gender among notified cases of cutaneous leishmaniasis in Asir province from 2011 to 2020.

autumn seasons, and the persistence of CL infection throughout the year, can be linked to the sand fly activity. According to the vector seasonal activity, *Ph. sergenti* (known vector for *L. tropica* in Asir province) is present throughout the year, but peak densities are during May and June [26]. Usually, CL patients realize the infection 1–2 months after the sandfly bite [27]. Therefore, it is expected to see CL case reports throughout the year with variation in number of cases.

According to these study findings, the current control measurements, such as patient treatment and vector control strategies, are not highly effective at controlling CL as the CL infection rate in Abha governate (the most endemic governate in Asir province) did not fluctuate significantly from 2011 to 2020. Furthermore, the

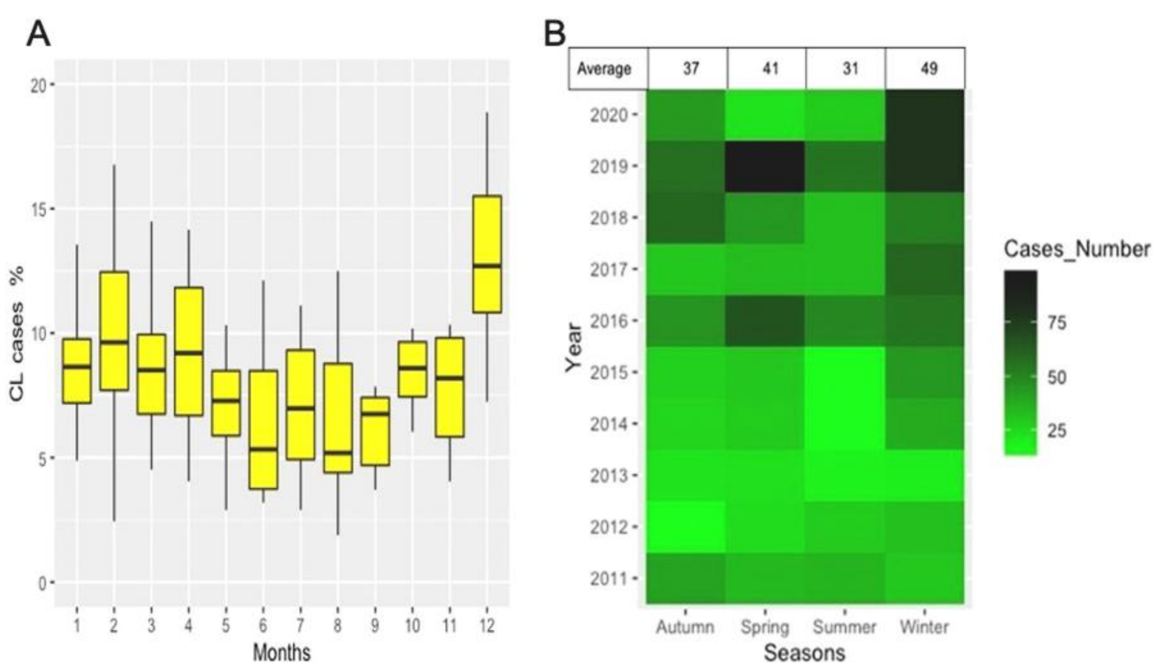

Fig. 4. Frequency distribution of cutaneous leishmaniasis cases according to month and Seasons in Asir province from 2011 to 2020. A) represents boxplot chart of the percentage of CL cases per month for all years. B) represents heatmap of CL cases number per season for each year.

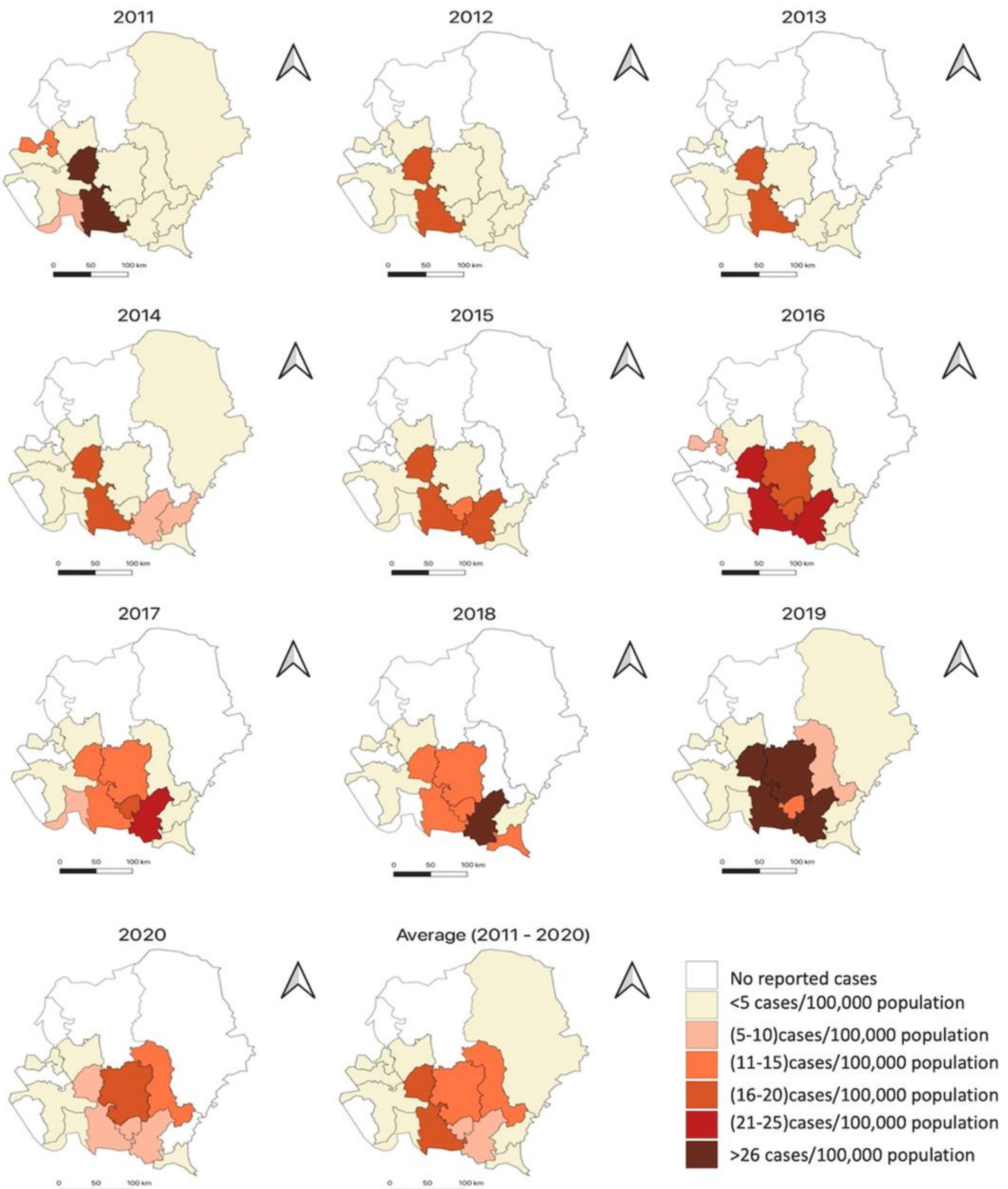

Fig. 5. Categorization of governate endemicity according to the incidence rate of CL cases in Asir province between 2011 and 2020.

endemicity of CL has increased within the previous five years in the Khamis-Mushait, Sarat-Abidah and Ahad-Rafidah governates. Several reasons could underpin the emergence and persistence of CL in this area. Increased urbanization, agricultural and irrigation development, the presence of livestock animals and climate change are all considered to be obstacles to the health control efforts [6,28,29]. Patient treatment also plays an important role in breaking anthroponotic transmission of CL caused by *L. tropica*. Despite that CL treatment is free and easy to access, according to the Al-Salem conclusions in 2019, most of the CL patients infected by *L. tropica*

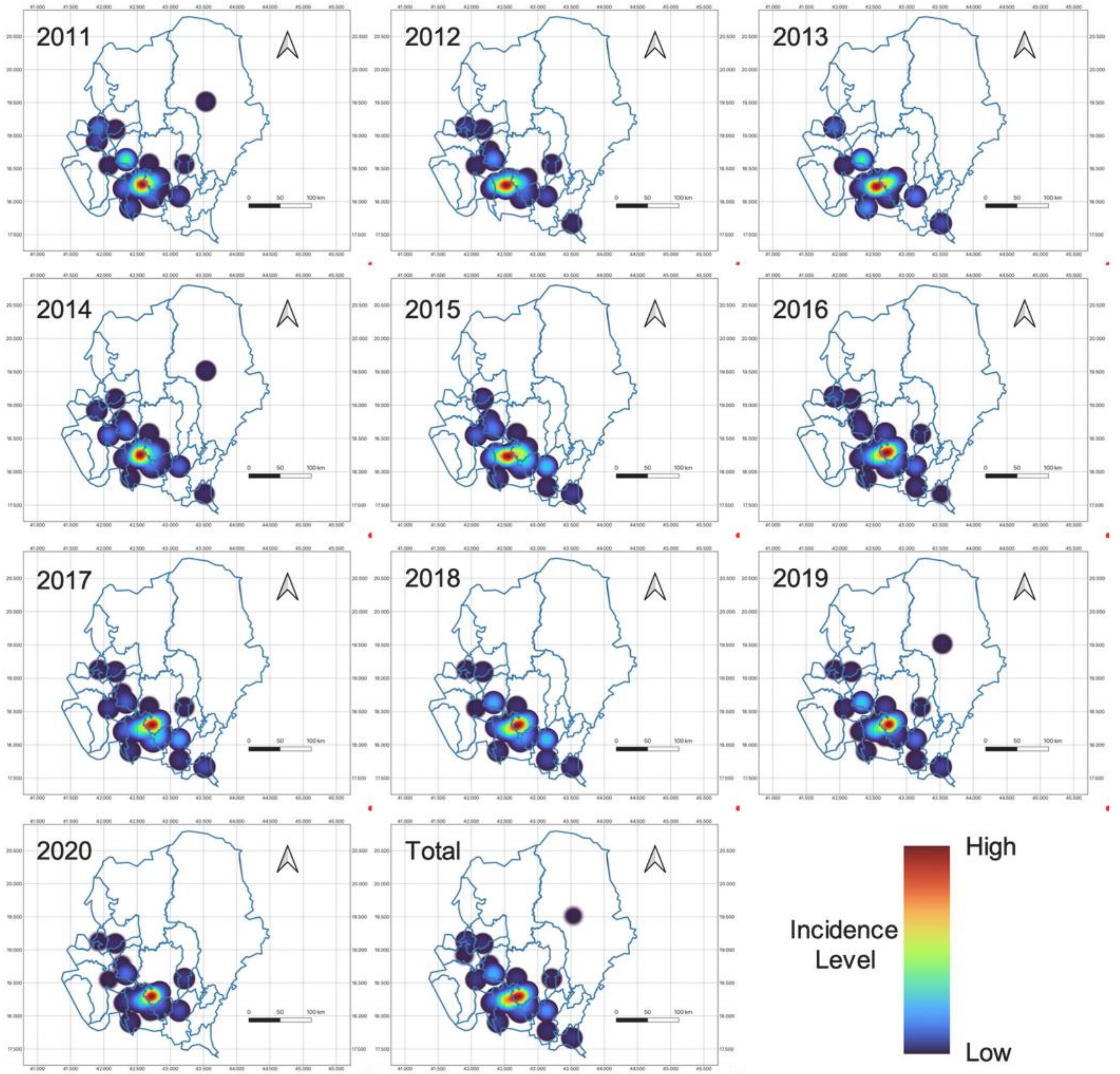

Fig. 6. Heatmap showing the spatial distribution of CL cases in Asir province between 2011 and 2020.

were unresponsive to the current treatment regimen. The treatment is sodium stibogluconate (SSG) and requires a lengthy and painful treatment period where some patients withdraw before the treatment course can be finished [8]. *Leishmania tropica* is dominant in Asir province, and it is known to be more refractory to antimonial drugs like SSG based on previous studies in Iran [30,31]. The antimony action mechanism depends on the ability of SSG to inhibit trypanothione reductase which leads in a lethal imbalance of thiol homeostasis. Therefore, the mode of parasite resistance to antimonials depends mainly on decreasing the intracellular concentration of SSG either by increasing detoxification of SSG through overexpression of gamma- glutamylcysteine synthetase and ornithine decarboxylase genes or decreasing SSG uptake by lowering expression of Aquaglyceroporin gene, or by lowering the activation of SSG by lowering expression of antimonite reductase and thiol dependent reductase [32]. The need to develop effective, less painful, alternative treatment protocols is important and will ultimately help limit CL burdens in Asir province. For this reason, further investigating the treatment efficacy and drug resistance on Asir's CL patients is highly recommended.

In contrast to the Abha governate, other Asir governates had low levels of CL endemicity during the past ten years. Caution should be taken as case underreporting needs to be considered. It is possible that afflicted communities lack of CL awareness and an unsatisfactory treatment protocol could lead to nonreporting or delay in reporting of active CL cases, which will adversely affect the accuracy of CL incidences [6].

This study highlights the possible geographical movement of CL transmission from Abha to Khamis-Mushait governate in Asir province over the past ten years. The geographical move of CL transmission between endemic and non-endemic areas can occur as result of either migration (human or vector) and reservoir host movements caused by the pressure of implemented vector control programs [33]. As a first step towards disease control, it is important to understand the spatial distribution of CL cases to predict higher and potential risk zones for CL transmission. The inclusion of further molecular epidemiology studies will help refine and guide future control strategies in disease hotspots [19]. Moreover, the possible reservoir host of CL has not been determined yet in Asir province. Therefore, to understand the geographical movement of CL, it is advisable to apply a massive epidemiological screen on type of animals present in endemic areas with clinical presentation of typical lesion(s) and molecular typing of leishmania parasite.

A limitation in this analysed dataset spanning a decade of patient data is that the data does not actually represent the true number of CL cases in Asir province. In some governates, namely Bisha and Balqarn, the data was not accessible. In addition, traditional medicine is still favoured and practiced by some of the local populations, which consequently means these CL cases will not be reported to the VCU. A further limitation is based on the ability to diagnose a CL infection. Geimsa-stained slides were used by the VCU of MoH to microscopically confirm CL. The sensitivity of this diagnostic method is not high and so false negative results can occur [23].

5. Conclusion

This study sought to evaluate the spatial distribution and seasonal variation of CL in Asir province using retrospective data from 2011 to 2020 made available by the Asir VCU (Ministry of Health). This study concluded that males and Saudi locals were at higher risk of CL infection. Furthermore, children under 13 years of age were more likely to suffer from CL than other age groups, particularly in the higher seasons (winter and autumn). Finally, this study indicates that there is possible geographical movement of CL transmission from Abha to Khamis-Mushait governate during the past ten years. Based on these findings, this study stresses the importance of improving living conditions, and dedicating resources to enhance school education and public awareness on CL control practices. Further research on vector behavior, host reservoirs, parasite molecular identification and treatment efficacy are highly recommended in Asir province to develop an effective disease control and prevention program.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jiph.2022.05.015.

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Molecular Characterization of *Leishmania* Species among Patients with Cutaneous Leishmaniasis in Asir Province, Saudi Arabia

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Abstract: Anthroponotic cutaneous leishmaniais (ACL) and zoonotic cutaneous leishmaniasis (ZCL) caused by *Leishmania tropica* and *Leishmania major*, respectively, are endemic vector-borne diseases in southern Saudi Arabia. In 2021, an outbreak of cutaneous leishmaniasis occurred in the province of Asir. The main objective of our investigation was to analyze the epidemiological features of CL in southern Saudi Arabia. The ministry of health recorded 194 CL patients between January and December 2021 from the Asir province. Our findings showed that the majority of CL patients (87.1%) originated from the governorates of Khamis-Mushait and Abha. Most of the patients were males (62.3%). While CL affected all age groups, those under 13 years old were the most affected (38.1%). For both genders, CL patients were mostly Saudi citizens (90.7%) compared to non-Saudi expatriates. The majority of CL patients (75.2%) suffered from a single lesion, and the majority of lesions (61.3%) were located on the face. The seasonal prevalence of CL showed two peaks, a small one in July–August and a larger one in March. Of a total of 194 Giemsa slides samples, 188 showed positive amplification of *Leishmania* ITS1 gene. Based on PCR-RFLP and PCR-HMR, 183 patients showed positive amplification of *L. tropica* and five patients showed positive amplification of *L. major*. Phylogenetic analysis revealed a clear distinct separation between *L. major* and *L. tropica* sequences. Our results provided strong evidence of the pre-domination of *L. tropica*, the main etiological agent of ACL in Asir province. We reported for the first time the presence of *L. major*, an etiological agent of ZCL in the study areas. The co-circulation of ACL and ZCL highlighted the complexity of the epidemiology of CL in southern Saudi Arabia, and subsequently, further studies to identify competent vectors and reservoir hosts for the establishment of control strategies are needed.

Keywords: anthroponotic cutaneous leishmaniasis; zoonotic cutaneous leishmaniasis; *Leishmania tropica*; *Leishmania major*; co-circulation; molecular identification

1. Introduction

Leishmaniasis is a neglected vector-borne parasitic disease of public health concern caused by an obligate intracellular parasite belonging to the genus *Leishmania*, which is transmitted to humans through the bite of infected female sandflies during blood feeding [1]. Infections caused by *Leishmania* parasites are major global health problems, with high endemicity in developing countries. This neglected tropical disease affects the health of more than 12 million people worldwide, with two million new cases occurring each year [2,3]. Moreover, the increasing number of coinfections with HIV aggravates the burden of this disease [4]. Depending on both the *Leishmania*-infected vector species and the host immunological responses to the etiological agents, leishmaniasis ranges from asymptomatic to self-healing, advanced muco-cutaneous infection, and eventually fatal visceral leishmaniasis, if left untreated [5]. Recently, leishmaniasis has emerged or re-emerged in many geographical areas generating global health and economic concerns that could affect humans [6], domestic animals [7], and wild animals [8]. Environmental changes, poor sanitation, development of agrarian mega-plans leading to the introduction of new reservoir hosts into communities are considered significant risk factors for leishmaniasis [9–12].

Globally, cutaneous leishmaniasis (CL) is the most common clinical manifestation. Lesions can be single or multiple depending on the number of infected insect bites and *Leishmania* species [13]. Lesions can last for months or even years before healing, leaving permanent scars. Although CL is generally not fatal, the lesions produced may cause substantial disfigurement and severe distress to infected individuals, with lifelong psychological and social consequences [14]. A variety of dermotropic *Leismania* parasite species causes CL. *Leishmania major* and *L. tropica* are the etiological agents of zoonotic cutaneous leishmaniasis (ZCL) and anthroponotic cutaneous leishmaniasis (ACL) in North Africa, the Middle East, and Central Asia [15–17]. In East Africa, CL is mainly caused by *L. aethiopica* [18]. In Central and South America, CL is caused by several *Leishmania* species, including *L. mexicana*, *L. amazonensis*, and *L. venezuelensis* [19]. Cutaneous leishmaniasis caused by viscerotropic parasites, such as *L. infantum* [13] and *L. donovani*, is less prevalent [20]. Cutaneous leishmaniasis is the most common form of the disease in Saudi Arabia. The disease is most prevalent in Al-Hassa oasis, with an outbreak in 1983 reporting 18,000 cases [21]. Since the establishment of the national control program, the incidence has declined [22]. According to the Ministry of Health of Saudi Arabia, the average annual incidence was 2500 cases per year, with Al-Hassa, Al-Madinah, Ha'il, and Al-Qaseem being the most endemic areas [23]. Cutaneous leishmaniasis is spreading to new areas leading to the emergence of new foci [24]. The geographical expansion of CL in Saudi Arabia is mainly related to anthroponotic disturbance of natural ecosystems due to massive urbanization and development of agricultural projects leading to the establishment of a stable cycle of leishmaniasis, including sandfly vectors, rodent reservoirs, and non-immune populations and subsequently increasing risk of transmission [25].

It is well known that Asir Province is a major CL focus, and children are at the highest risk of CL infection [24]. A previous study reported that *L. tropica* is the main parasite circulating in Asir province [26]. However, in the aforementioned study, only a small sample size was included in addition to the use of Giemsa stain as a diagnostic tool limited the outcome of this eco-epidemiological investigation. For a better understanding of the epidemiological situation in Asir province, investigating a large number of CL patients and using molecular approaches to identify circulating *Leishmania* species is highly needed.

2. Materials and Methods

2.1. Study Area and Sample Collection

This study was conducted in Asir province located in the southwest of Saudi Arabia (Figure 1). From January to December 2021, Giemsa stain slides were taken from 194 suspected CL patients' skin lesions. The diagnostic was based on the identification of *Leishmania* parasites within Giemsa-stained slides. Following parasite identification, DNA was extracted from Giemsa-stained slides to identify *Leishmania* species. Demographic information about patients, such as age, gender, residence, lesion location, and number of lesions was also recorded.

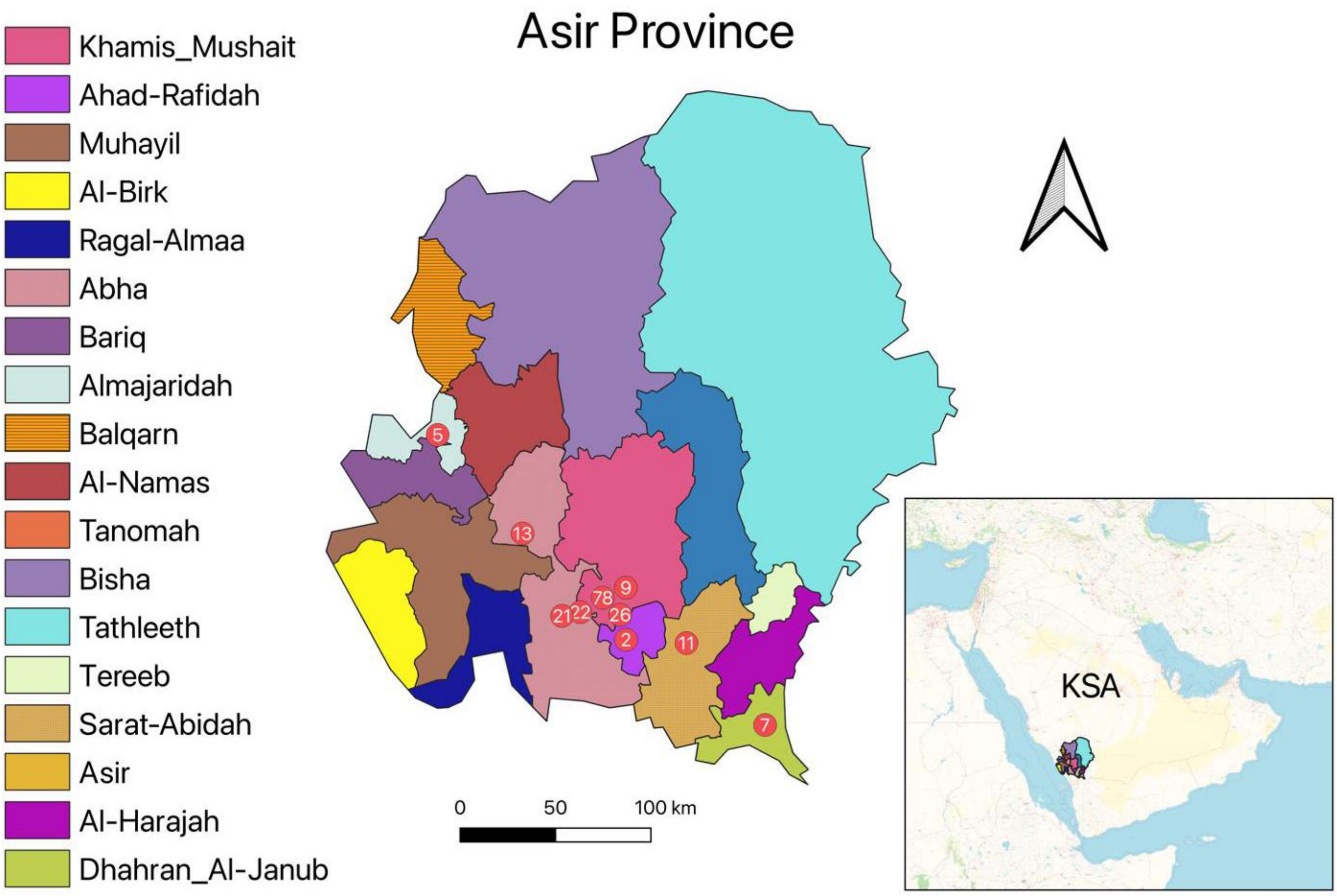

Figure 1. Map of Asir province with the number of CL patients according to their geographical location.

2.2. DNA Extraction

The genomic DNA was extracted from collected Giemsa stain slides using the DNAeasy Blood and Tissue Kit (QIAGEN, Hannover, Germany) according to the manufacturer's instructions. The extracted DNA was kept at −20 °C for further molecular assays.

2.3. *Leishmania* Identification Using PCR-RFLP Assay

Two primers were used for DNA amplification of the *Leishmania* internal transcribed spacer 1 target gene (ITS1) of approximately 320 bp LITSR (5′-CTGGATCATTTTCCGATG-3′) and L5.8S (5′-TGATACCACTTATCGCACTT-3′) [27,28]. The 20 µL reaction mixture contained 1× Dream Taq buffer with 2 mM MgCl2 (Thermo Scientific, Walthman, MA, USA), 0.25 mM dNTPs mix, 500 nM of each primer, and 0.125 U of Dream Taq DNA polymerase (Thermo Scientific, USA). The PCR reactions were performed in a T100 Thermal Cycler (Bio-Rad, Watford, UK). Cycling conditions started with an initial denaturation at 98 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 20 s, annealing at 53 °C for 30, s, and extension at 72 °C for 30 s. This was followed by a final extension at 72 °C for 5 min. After that, the PCR products were digested using the *HaeIII* enzyme. Finally, PCR products were analyzed by gel electrophoresis and stained with SYBR Safe (Invitrogen, Waltham, MA, USA) to improve the DNA visibility under UV light. A positive control with known DNA *Leishmania* species was used to assess PCR efficiency and negative water control to check for any contamination. ITS1-PCR products of the positive samples were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and submitted for sequencing under the forward primer to confirm *Leishmania* species.

2.4. *Leishmania* Identification Using Real-Time PCR-HRM Assay

The high-resolution melt PCR (PCR-HRM) was used to improve the resolution of *Leishmania* species identification. Two primers (F: 5′-CACGTTATGTGAGCCGTTATCC-3′ and R: 5′-GCCTTTCCCACATACACAGC-3′) were used to differentiate between *L. major* and *L. tropica* [28]. PCR reactions were carried out in an HRM capable of performing out CFX Connect Real-Time PCR Detection System (Biorad, UK). The final volume of 20 µL contained 1x Luna Universal qPCR SYBR Green-based master mix (NEB, Cambridge, UK), 500 nM of each primer. Cycling conditions started with 1 min of denaturation at 95 °C, followed by denaturation at 95 °C for 15 s, followed by annealing and extension at 60 °C for 30 s. After 35 cycles, the HRM was carried out by denaturing at 95 °C for 1 min, then reannealing at 50 °C for 30 s, and gradually raising the temperature by 0.1 °C increments every 2 s while recording changes in fluorescence. Samples with a cycle threshold level (Ct) below 33 were treated as positive amplification of *Leishmania*. Samples with melting temperature values of 84. 2 °C and 85.8 °C corresponded to *L. tropica* and *L. major*, respectively.

2.5. Data Analysis

The recorded data were translated into English and digitized in Excel for statistical analysis using R statistical Language v. 4.0.5.

The Open-source software QGIS (Quantum GIS version 3.20.0) was used to map the spatial distribution of CL cases in Asir province in 2021. A Mann–Kendall trend test was used to determine whether or not a trend exists in time series data (monthly CL cases).

2.6. Phylogenetic Analysis

The sequences resulted from our samples were added to other similar sequences obtained from a previous *Leishmania* study performed in Eastern Saudi Arabia [29]. Moreover, more sequences were obtained from GenBank by using blast analysis [30]. Details about the analyzed sequences are shown in Table 1. After the alignment of all sequences by using the MAFFT aligner [31], the maximum likelihood fits of 24 different nucleotide substitution models were done to the alignment, and the Jukes–Cantor model was chosen to be fed as a prior when building the tree since it had the lowest Bayesian information criterion score (BIC) and the maximum likelihood value (lnL). The substitution model estimation was done using the MEGAX software [32].

Table 1. List of all sequences used for phylogenetic analysis with available WHO codes or strain names. Our sequences colored in blue and those of Al-Rashed et al. [29] are colored in green.

Species	Strain Name/WHO Code	Country	GenBank Accession
L. donovani		Eastern Sudan	AJ276259.1
L. infantum		Northern Cyprus	KC998879.1
L. major	MHOM/SU/73/5ASKH	Russia	AJ000310.1
L. major		Turkmenistan	AJ272383.1
L. major	MHOM/SD/90/SUDAN3	Sudan	AJ300481.1
L. major	IPAP/EG/89/SI-177	Spain	DQ295824.1
L. major	Friedlin Chr27:1010784-1011083	Spain	OU755561.1
L. major	MRHO/IR/75/ER	Iran	KU680846.1
L. major	LMJI3	Iraq	KY882277.1
L. major		Jordan	MN604128.1

Table 1. *Cont.*

Species	Strain Name/WHO Code	Country	GenBank Accession
L. major	**SA-Lm1**	**Eastern Saudi Arabia**	OK560721.1
L. major	**SA-Lm101**	**Eastern Saudi Arabia**	OK560817.1
L. major	MHOM/SA/2021/G132	Southern Saudi Arabia	ON872293.1
L. major	MHOM/SA/2021/G110	Southern Saudi Arabia	ON876503.1
L. tropica		Turkey	FJ940894.1
L. tropica		Turkey	FJ940895.1
L. tropica	MHOM/IL/01/LRC-L838	Israel	FN677341.1
L. tropica	MHOM/PS/01/ISL593	Palestine	FN677343.1
L. tropica		Iran	HM004586.1
L. tropica		Southern Iran	JX560467.1
L. tropica		Southern Iran	JX560469.1
L. tropica		Southern Iran	JX560470.1
L. tropica		Southern Iran	JX560482.1
L. tropica	ISER/IL/98/LRC-L747	Israel	LC459350.1
L. tropica	**SA-Lt44**	**Eastern Saudi Arabia**	OK560763.1
L. tropica	**SA-Lt77**	**Eastern Saudi Arabia**	OK560795.1
L. tropica	**SA-Lt78**	**Eastern Saudi Arabia**	OK560796.1
L. tropica		Malaysia	OL413428.1
L. tropica	**MHOM/SA/2021/G02**	**Southern Saudi Arabia**	ON872294.1
L. tropica	**MHOM/SA/2021/G71**	**Southern Saudi Arabia**	ON872333.1
L. tropica	**MHOM/SA/2021/G58**	**Southern Saudi Arabia**	ON872334.1
L. tropica	**MHOM/SA/2021/G51**	**Southern Saudi Arabia**	ON872335.1
L. tropica	**MHOM/SA/2021/G82**	**Southern Saudi Arabia**	ON872336.1
L. tropica	**MHOM/SA/2021/G08**	**Southern Saudi Arabia**	ON872354.1
L. tropica	**MHOM/SA/2021/G54**	**Southern Saudi Arabia**	ON872355.1
L. tropica	**MHOM/SA/2021/G16**	**Southern Saudi Arabia**	ON872356.1
L. tropica	**MHOM/SA/2021/G67**	**Southern Saudi Arabia**	ON872357.1
L. tropica	**MHOM/SA/2021/G32**	**Southern Saudi Arabia**	ON872358.1
L. tropica	**MHOM/SA/2021/G47**	**Southern Saudi Arabia**	ON872359.1

A Bayesian tree was constructed using version v1.10.4 of the BEAST suite [33] with the following prior assumptions: (1) The population size remained constant throughout the time covered by the genealogy, generate a random starting tree under the coalescent process, (2) the Jukes–Cantor substitution model [34], and (3) constant coalescent likelihood with strict clocks (uniform rates across branches) were used as prior and then ran for 10 million iterations. After that, a consensus tree was generated after discarding the first 10% as burn-in using Tree Annotator, which is part of the BEAST suite. The final tree was then visualized and examined using Figtree software [35].

2.7. Ethical Approval

The study was carried out under ethical approval from the Regional Committee for Research Ethics of the ministry of health (Approval Number H-06-B-091).

3. Results

3.1. Socio-Epidemiological Features

Of a total of 194 CL patients (121 males and 73 females) from Asir province reported in 2021, 58.2% (N = 113) originated from the governorate of Khamis–Mushait and 28.8% (N = 56) from the governorate of Abha (Table 2). According to reported data, most of the notified CL cases (38.1%, N = 74) were under 13 years of age (34% males and 46% females). For both genders, Saudi citizens were more likely to suffer from CL than non-Saudi expatriates. The majority of CL patients suffered from a single lesion for both males (80%) and females (67%). The majority of lesions were located on the face (58% for females and 64% for males) and hands (29% for females and 25% for males) (Table 2).

Table 2. Number of cutaneous leishmaniasis cases based on demographic information in Asir during 2021.

Characteristic	Female, N = 73 [1]	Male, N = 121 [1]	*p*-Value [2]	Characteristic	Female, N = 73 [1]	Male, N = 121 [1]	*p*-Value [2]
Nationality			<0.001	Age_group			<0.001
Non-Saudi	0 (0%)	18 (15%)		0–5	15 (21%)	18 (15%)	
Saudi	73 (100%)	103 (85%)		6–12	18 (25%)	23 (19%)	
Lesion Number			0.041	13–15	5 (6.8%)	4 (3.3%)	
Multiple	24 (33%)	24 (20%)		16–18	1 (1.4%)	4 (3.3%)	
Single	49 (67%)	97 (80%)		19–24	9 (12%)	9 (7.4%)	
Lesion Location			0.8	25–29	4 (5.5%)	16 (13%)	
Abdomen	2 (2.7%)	3 (2.5%)		30–39	6 (8.2%)	14 (12%)	
Face	42 (58%)	77 (64%)		40–49	4 (5.5%)	12 (9.9%)	
Feet-Leg	8 (11%)	11 (9.1%)		50–59	5 (6.8%)	10 (8.3%)	
Hand-Arm	21 (29%)	30 (25%)		60+	6 (8.2%)	11 (9.1%)	
Lesion Appearance			>0.9	Lesion size			0.8
Dry	71 (97%)	118 (98%)		<1 cm	6 (8.2%)	10 (8.3%)	
Wet	2 (2.7%)	3 (2.5%)		1–3 cm	56 (77%)	97 (80%)	
				4–5 cm	11 (15%)	14 (12%)	

[1] n (%). [2] Pearson's Chi-squared test; Fisher's exact test.

3.2. Clinical Characteristics of Cutaneous Leishmaniasis

According to the site of lesions, facial lesions were more common among patients under 13 years old, while lesions on the upper and lower limbs were frequently observed in patients aged 19 to 60 years old (Figure 2).

The monthly prevalence of CL is variable, with the highest observed in March (15.4%) and the lowest in July–August (1.5%) (Figure 3). However, no significant trend in the monthly variation of CL cases was observed (*p*-value = 0.2415)

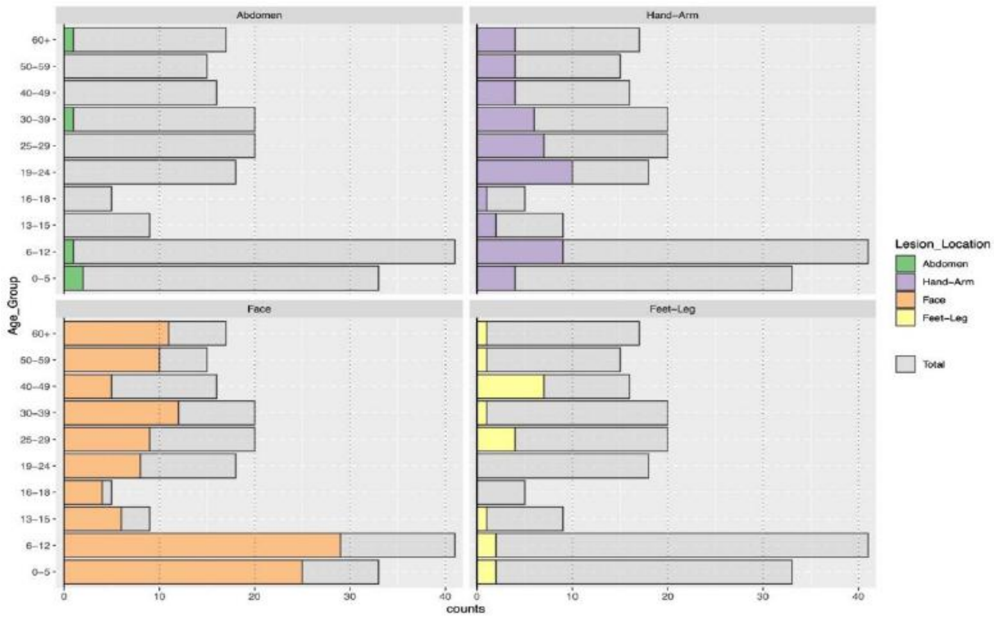

Figure 2. Distribution of lesions in relation to body sites according to age groups.

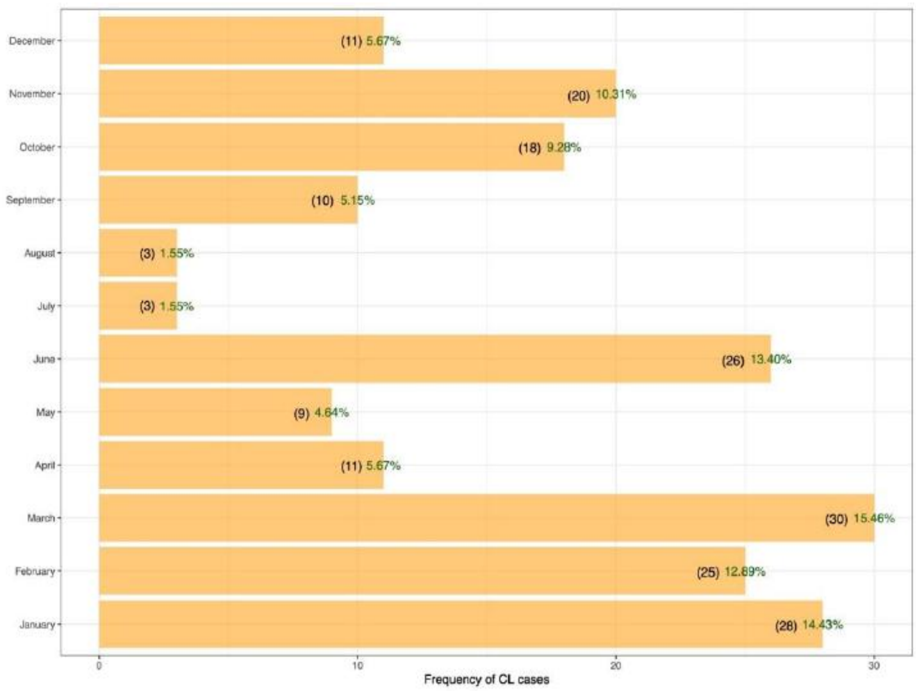

Figure 3. Monthly distribution of cutaneous leishmaniasis cases reported in Asir province during 2021.

3.3. Molecular Characterization of Cutaneous Leishmaniasis in Asir Region

Out of 194 Giemsa slides samples, 179 showed positive amplification of *Leishmania* ITS1 gene (Figure 4). Based on PCR-HMR, 183 patients showed positive amplification of *L. tropica*, and five patients showed positive amplification of *L. major*, yielding a total of 188 positive samples (Figure 5). It is important to note that of a total of 188 positive samples using conventional PCR, 26 showed very weak amplification and, therefore, very weak bands on the gel after RFLP step. As a result, it was difficult to determine the species of *Leishmania* presented in the sample, which then was confirmed by PCR-HMR. Moreover, 9 of 188 samples showed negative results with PCR-RFLP (no bands). These nine samples tested positive by PCR-HMR (cycle Ct = around 29 cycles).

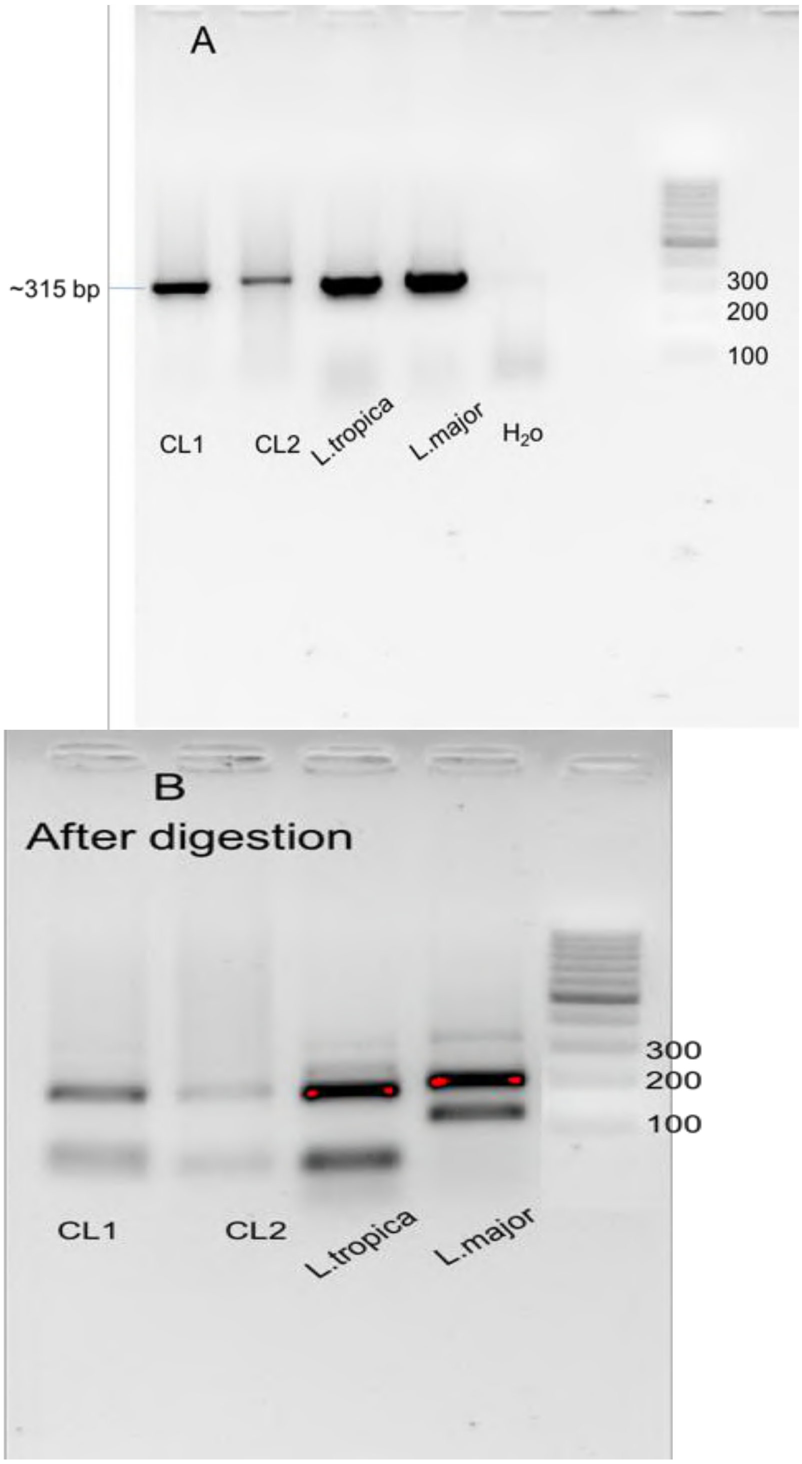

Figure 4. SYBR safe–stained agarose gel showing ITS1 identification. Bands were separated on a 2% agarose gel for 30 min to document differences in RFLP patterns: (**A**) Positive amplification of ITS1 in CL smears 1 and 2. Positive controls have shown positive bands, (**B**) digestion of amplified ITS1 regions of *Leishmania* species with *HaeIII* enzyme. CL1 and CL2 showed positive results corresponding to *L. tropica*.

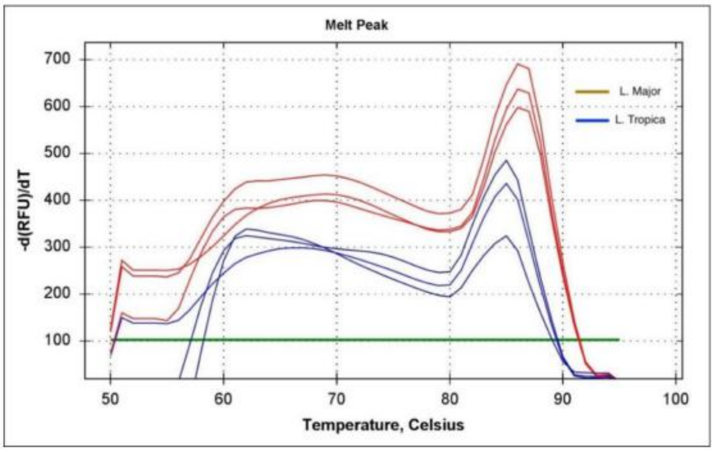

Figure 5. Real-time PCR-HRM analysis of *Leishmania* infection in cutaneous leishmaniasis human samples. The green line is the threshold line that was set to ignore noising peaks below 100.

3.4. Phylogenetic Analysis

For the phylogenetic analysis, we selected samples with strong bands based on geographical location. Out of a total of 188 positive samples, only 13 sequences were analyzed. Phylogenetic analysis revealed a clear distinct separation between *L. major* and *L. tropica* sequences (Figure 6). All sequences of *L. tropica* were clustered together except for one sample (G08), which was taken from a non-Saudi patient, a 36-year-old Sudanese man, and appeared to be grouped with sequences from outside of Saudi Arabia. The Eastern Saudi Arabia sequences were also more clustered with sequences from outside Saudi Arabia. Similarly, *L. major* sequences were relatively clustered together and more grouped with sequences from Jordan. However, Eastern Saudi Arabia sequences were more clustered with more diverse sequences. Moreover, the outgroup sequences from *L. major* were *L. infantum* and *L. donovani*.

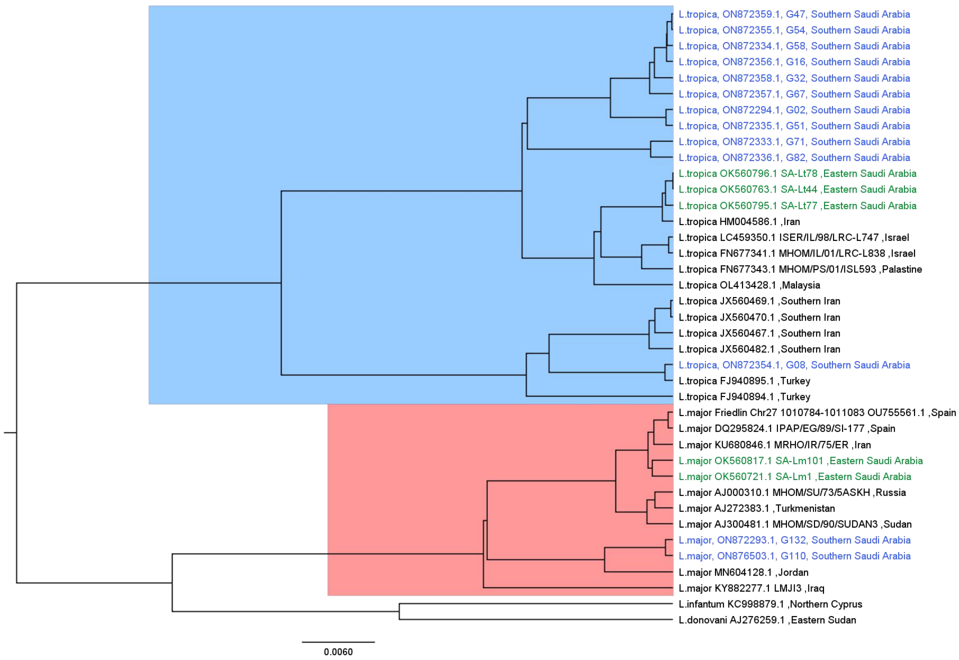

Figure 6. Bayesian phylogenetic tree generated with 39 ITS1 sequences. Our sequences are colored in blue, whereas sequences from eastern Saudi Arabia are colored in green, and the remained sequences from other countries are colored in black.

4. Discussion

A total of 1565 CL cases were reported from the Asir province during 2011–2020 [24], and subsequently, this province is considered endemic for CL. It is of major epidemiological importance to point out that due to under-reporting, the actual number of CL patients in this region is much higher, and subsequently, the percentage of patients evaluated in this study is low. While several cases are annually reported from the Asir province, the epidemiology of CL has not been well documented. A relatively recent study showed the predominance of *L. tropica* in Asir region [26]. However, the aforementioned study is focused on different regions of Saudi Arabia, with a limited sample size in each one. The objective of the present work was to identify circulating *Leishmania* species and to perform a comprehensive study on the epidemiological features of CL in Asir region.

The majority of patients were male (62.37%, N = 194) compared to female (37.62%). The high prevalence of CL among males could be attributed to nightly outdoor activities compared to females. Furthermore, females conventionally cover the outermost portions of their bodies, thus protecting them from infected bites by sandflies. The findings of previous studies performed in Saudi Arabia and in other countries from the Middle East, North Africa, and Central Asia showed a predominance of CL cases among males [36–40], and subsequently, they are in agreement with our results. Only one study reported similar CL prevalence between males and females in Saudi Arabia [21].

The geographical distribution of CL clusters in space with the highest prevalence was observed in the governorates and Abha and Khamis-Mushait. The predominance of cutaneous leishmaniasis caused by *L. tropica* in the south west of Saudi Arabia is related to the distribution of *Ph. sergenti*, the main vector of *L. tropica* [41]. However, infection with *L. major* is more prevalent mainly in the northwest, the center, and the east of Saudi Arabia, where *Ph. papatasi* is the main vector [26,37]. Similar findings were reported in Tunisia, where infections with *L. major* and *L. tropica* are prevalent in the center and in the southwest, respectively [15,42].

While *L. tropica* infection is mostly located on the face with a single lesion, infection with *L. major* is located on the limbs with multiple lesions [13]. In the present study, we showed a predominance of *L. tropica* compared to *L. major*, and therefore, common cases presented with a single lesion, with the face being the most commonly affected site, followed by the hands. Previous studies performed in Saudi Arabia and in North Africa reported similar trends with the majority of cases presenting with a distinct lesion on the face [15,21]. Conversely, the study conducted in Al-Madinah district indicated that the majority of patients have more than one lesion and the majority of them were observed in the lower extremities. Lesions' location and their number on the body sites are dependent on the *Leishmania* parasite species and its vector [13].

The highest prevalence of CL was observed among children under 13 years old. This finding could be explained by the fact that this age group has no previous exposition to sandfly bite, and subsequently, it is the most naïve population in the community. Another study performed in Al-Madinah reported that CL is prevalent in all age groups [38]. However, Amin et al. [22] reported that CL cases from Central Saudi Arabia were mainly reported in the age group of 15–45 years. Similar results were reported in the northwest of Saudi Arabia and in Central Tunisia [36,43].

Taking into account that the number of expatriates involved in the present study is low compared to Saudi Citizens, it is expected that the majority of the cases were among Saudi residents. Similar results were reported by Al-Tawfiq and AbuKhamsin [21], showing a predominance of CL among Saudi citizens (98.3%). Another study reported similar CL prevalence between Saudi citizens and expatriates. [22,36,37]. However, previous studies performed in Arar have shown that CL prevalence was higher among expatriates compared to Saudi citizens [44]. Hence, CL prevalence is not related to nationalities but rather to the exposition site, the level of awareness, and subsequently, the protection against the vector. In addition, CL prevalence is limited by the under-reporting of the disease among communities.

The monthly variation of CL prevalence is the highest in January through March and the lowest in July–August. Cases of CL patients in the northeastern Saudi Arabia were reported in all months of the year with a minimum in June–July [36]. In CL endemic areas located in the East of Saudi Arabia, the number of cases showed a steep increase starting from November, reached a peak during January and February, and then declined by March and April [22]. In Tunisia, lesions that emerged during June–January were caused mostly by *L. major* (64.7%), and lesions that emerged during February–May were caused mainly by *L. tropica* [15].

An entomological investigation performed in the Al Baha region located between Makkah and Asir regions in southwestern Saudi Arabia showed that *Phlebotomus bergeroti* is the most abundant sandfly species, followed by *Ph. sergenti*, and *Ph. papatasi* [45]. The infection prevalence of field-collected *Ph. papatasi* and *Ph. sergenti* from the northwestern Saudi Arabia with *L. major* and *L. tropica* were 23.7% and 31%, respectively [46]. The fat sand rats *Psammomys obesus* and the hyrax, reservoirs of *L. major* and *L. tropica*, respectively [47,48], are present in Saudi Arabia [49]. The aforementioned studies provide strong evidence that the co-circulation of *L. tropica* and *L. major* in CL patients from the Asir province is caused by a zoonotic transmission of both *Leishmania* species involving sandfly vectors (*Ph. tropica* and *Ph. papatasi*) and rodent reservoirs (hyrax and fat sand rats). Hence, investigations on sandfly vectors and potential rodent reservoirs of *Leishmania* species in Asir province are highly needed.

The monthly variation of CL prevalence is related to the seasonal sandfly activity, the seasonal sandfly infection with *Leishmania* parasite, and the incubation period of the disease [50]. *Phlebotomus sergenti* is present in all months of the year, with one major peak in May–June [51]. The seasonal activity of *Ph. papatasi* is bimodal, with a large peak in May–June and a small one in August [45]. Further studies are needed to investigate the monthly variation of CL prevalence caused by *L. tropica* and *L. major* in Asir province.

The predominance of cutaneous leishmaniasis caused by *L. tropica* in southwestern Saudi Arabia is related to the distribution of *Ph. sergenti*, the main vector of *L. tropica* [41]. However, infection with *L. major* is more prevalent mainly in the northwest and in the center of Saudi Arabia, where *Ph. papatasi* is the main vector [37]. Mixed foci were reported in northwestern and central Saudi Arabia [26,52].

While a larger sample size might reflect the phylogenetic relatedness better than a small collection, sequencing all positive samples from 17 governorates in Asir province was not feasible due to financial constraints. The phylogenetic analysis of the selected positive samples showed that *L. tropica* and *L. major* clustered in separate clades, distinct from the *L. donovani* complex (*L. infantum* and *L. donovani*). Our results provided strong evidence that *L. tropica* is the predominant *Leishmania* species circulating in the investigated areas.

Taking into account that *L. tropica* was the only *Leishmania* species so far isolated from field-collected *Ph. sergenti* in Abha, located in Asir province [41], the dominance of *L. tropica*-infection in CL patients was expected. The anthroponotic form of CL (ACL) caused by *L. tropica* and transmitted mainly by *Ph. sergenti* is endemic in southwestern Saudi Arabia [41]. However, the presence of hyrax in southern Saudi Arabia [50] also suggests a zoonotic transmission of *L. tropica*. Thus, more studies to assess the transmission of *L. tropica* among sandfly vectors, potential rodent reservoir hosts, and humans in Saudi Arabia are highly needed. The occurrence of a few cases of CL caused by *L. major*, an etiological agent of ZCL in small micro-foci might be related to the low abundance of *P. papatasi* in southwestern Saudi Arabia [51]. We report for the first time a mixed focus in southwestern Saudi Arabia. Similar findings were reported from the southwest and the center of Tunisia [15,53]. The epidemiology of CL in southwestern Saudi Arabia is highly complex by the high diversity of sandfly vectors and their associated *Leishmania* species, leading to mixed forms of CL caused by different pathogens. Therefore, a better understanding of the ecology of sandfly vectors is highly needed for efficient control to reduce the indoor abundance of sandfly vectors and subsequently reduce the incidence of CL.

Although Giemsa staining is a primary diagnostic tool, the lack of confirmation of amastigotes in the lesion's indirect smears and tissue specimens can easily lead to misdiagnosis [54]. PCR analysis of *Leishmania* species is an accurate and effective tool that has been used in leishmaniasis research. This approach has also been used for the taxonomic differentiation of *Leishmania* species because of its high sensitivity and specificity [55]. However, real-time PCR-based amplification of the *Leishmania* ITS1, followed by HRM, was found to be more sensitive in identifying *Leishmania* infections in CL lesions over the ITS1-PCR [28]. Furthermore, this technique was shown to be highly specific in discriminating between *L. major* and *L. tropica* infections based on their corresponding melting temperatures. Thus, combining PCR-RFLP and PCR-HMR for the epidemiological studies of *Leishmania* in CL focus is useful for accurate detection and characterization of the infecting parasites compared to microscopic examination.

5. Conclusions

Our results provided strong evidence of the pre-domination of *L. tropica*, the main etiological agent of CL in Asir province, Saudi Arabia. It displays a wide clinical polymorphism and, subsequently, should be considered in strategic planning and future diagnosis, treatment, and control programs. We reported for the first time the presence of *L. major*, an etiological agent of ZCL in the study areas. Moreover, this study highlights a valuable tool of the PCR-HRM assay in selecting optimal therapy and treatment regimens, especially in complex localities where more than one *Leishmania* species is present. Further studies to identify competent vectors and reservoir hosts are needed to clarify the epidemiological situation of CL in Asir province.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data in this study are available on request from Y.A.

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