Genotypic and phylogenic analyses of cutaneous leishmaniasis in Al Ahsa, Eastern Saudi Arabia during the coronavirus disease 2019 pandemic: First cases of *Leishmania tropica* with the predominance of *Leishmania major*

Abdullatif S. Al-Rashed¹, Reem Al Jindan¹, Salma Al Jaroodi¹, Ahmed Al Mohanna², Ahmed Abdelhady³ & Ayman A. El-Badry*(¹²³)

During the coronavirus disease 2019 lockdown period, a surge in sandflies and cutaneous leishmaniasis (CL) cases was observed in Al-Ahsa, Saudi Arabia. Skin punch biopsies were obtained from 100 patients clinically diagnosed with CL in Al-Ahsa who had no travel history in the last 6 months. Impression smears were used following a three-step polymerase chain reaction (PCR) protocol using genus-specific primers targeting kDNA and ITS1. *Leishmania* speciation was determined by ITS1 PCR/nested PCR-restriction fragment length polymorphism and sequencing. A phylogenetic tree was constructed. The associated patient characteristics were analyzed. Using internal transcribed spacer one (ITS1)-PCR/nested PCR, 98 cases were considered true-positive CL. *Leishmania major* was the predominant species, and *Leishmania tropica* was identified in three cases. Microscopy had poor sensitivity and perfect specificity. Direct ITS1-PCR missed nine cases. Sex, residence, and treatment outcome were significantly associated with the occurrence of *Leishmania*; distribution of skin lesion(s) and treatment outcome were significantly associated with *Leishmania* genotype. This is the first time that *L. tropica* was identified as a cause of CL in human in Al-Ahsa, in addition to the predominant zoonotic species, *L. major*. We recommend using ITS1-nested PCR for negative cases by ITS1-PCR. Further exploration of *Leishmania* transmission dynamics in vectors and reservoir animals is essential for designing effective preventive measures.

Leishmaniasis, a vector-borne disease, is a complex disorder caused by a heterogeneous group of flagellated protozoans of one genus, *Leishmania*, and belongs to the Trypanosomatidae family that has a worldwide distribution. Female sandfly vectors are the principal *Leishmania* transmitter.

Leishmaniasis is categorized into a spectrum of diseases according to its form. It is primarily classified into cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis, and visceral leishmaniasis. CL is considered the predominant form of leishmaniasis and is presented with one or multiple ulcerated lesions in exposed areas of the human skin.²³

¹Department of Microbiology, College of Medicine, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia. ²Vector Borne and Diseases Prevention Center, Al Ahsa, Saudi Arabia. ³Ministry of Health, Riyadh, Saudi Arabia. *email: aelbadry@kasralainy.edu.eg
There are more than 20 species in the genus *Leishmania*. Species can be classified according to their geographical distribution into Old and New World leishmaniasis2.

Old World CL cases are caused predominantly by the zoonotic *Leishmania (L.) major* and the *L. tropica* in Pakistan, India, Iran, Palestine, Jordan, Yemen, and Saudi Arabia4-10, *L. major*, *L. infantum*, and *L. aethiopica* in Saharan Africa and Ethiopia11-13, *L. infantum*, *L. major*, and *L. tropica* in Turkey and Cyprus14,15; and *L. infantum* in Spain and Portugal16. *Leishmania (Viannia)* species is the predominant source of New World CL in Central and South America17,18.

According to the World Health Organization (WHO), approximately 600,000–1 million new cases of leishmaniasis are diagnosed worldwide annually19. Of these new cases, 70% of them are diagnosed in the Middle East region19. Saudi Arabia is considered the fourth country most endemic with CL in the Middle East region20. Various Saudi provinces are endemic to CL, including the Al-Ahsa oasis, Al Qassim, and Al-Madinah Al-Munawwara21. In a previously published retrospective study, the incidence of CL in Al-Ahsa declined from 1956 confirmed cases in 2000 to 457 cases in 201022.

Presumptive CL diagnosis can be established based on clinical presentation; however, establishing the definitive diagnosis of CL is relatively challenging, especially in endemic areas23. To date, microscopic identification of amastigotes in stained smears and promastigotes in tissue cultures are considered the gold standard diagnostic method23. Despite the high specificity of these techniques, they have limited sensitivity, are time-consuming, and cannot differentiate between *Leishmania* species24. Molecular techniques, such as polymerase chain reaction (PCR)-restriction fragment length polymorphism (PCR–RFLP), real-time PCR, and sequencing, are increasingly utilized for *Leishmania* speciation25-30. Multiple distinct genomic regions of *Leishmania* species have been evaluated using many different primer's targets by PCR, such as kinetoplast DNA (kDNA), internal transcribed spacer one (ITS1) and ITS2, mini-exon, small subunit rRNA (ssu rRNA), and heat shock protein 70 (hsp70)26-30.

Documenting the molecular profile of *Leishmania* species in Saudi Arabia can aid in providing effective treatment regimens for patients. Furthermore, exploring *Leishmania* transmission dynamics in vectors will help in designing high-quality preventative measures. Despite the prevalence and endemicity of the disease in Saudi Arabia, the genetic diversity of *Leishmania* species remains poorly studied21,31-34.

The current study aimed to identify *Leishmania* and speciate *Leishmania* molecularly from skin punch biopsy of clinically diagnosed CL cases from Al-Ahsa, Eastern Saudi Arabia.

**Methods**

**Study area and study type.** This was a cross-sectional diagnostic study conducted in Al-Ahsa City, Eastern Region, Saudi Arabia (coordinates, 25°23′00″N 49°36′00″E) in collaboration with the Vector-Borne Diseases Prevention Center, Al-Ahsa.

**Ethical considerations.** The study protocol was approved by the Institutional Review Board (IRB) Committee of the Deanship of Scientific Research at Imam Abdulrahman Bin Faisal University, and all methods were performed according to the relevant guidelines and regulations of the IRB Committee of Deanship of Scientific Research at Imam Abdulrahman Bin Faisal University (reference number: IRB-PGS-2020-01-427/ IRB-PSG-2020-01-186) and Ministry of Health, Saudi Arabia IRB Committee (KFHH RCA #08–25–2020). The purpose of the present study was comprehensively explained to all of the participants and the children's parents/guardians, and the collection of specimens was completed after obtaining their informed consent.

**Sample collection.** Skin biopsies were collected by punch biopsy (Kai Industries Co., Ltd., Japan) from patients clinically diagnosed with CL who were referred to the referral district leishmaniasis clinic at Al-Yahya Primary Health Care Center, Al-Ahsa, for the treatment of leishmaniasis.

Clinically suspected CL skin lesions were primarily diagnosed by the presence of ulcerated lesions or infiltrative erythematous nodules on exposed areas of the body of patients living in Al-Ahsa who had a history of exposure to sandfly bites. All patients diagnosed with CL who were referred to the leishmaniasis clinic during the study period were included in this study.

One punch biopsy was obtained from the borders of the lesion under sterile conditions. A total of 100 skin biopsies were performed between September 2020 and March 2021.

The related patients' demographic and clinical data were collected using a designed questionnaire, including date of diagnosis, sex, age, nationality, place of residence, occupation, level of education, number of lesions, size, site, distribution and duration, family history of CL, treatment regimen, and response to treatment.

**Sample processing and microscopy.** An impression smear was prepared by pressing the biopsy on the slide multiple times and then was stained with Giemsa stain. The slides were examined for amastigotes by 100× oil-immersion light microscopy following practical guide for the diagnosis of leishmaniasis.35,36 Subsequently, the sample was stored at ~20 °C for molecular analysis.

**Molecular assays.** Commercial genomic DNA extraction kits (QIAamp Fast DNA Tissue Kit, Qiagen, Germany) were used to extract the genomic DNA from skin specimens following the manufacturer's guidelines. Extracted DNA was stored at 4 °C until processed. DNA extracted from samples was amplified for *Leishmania* DNA using three PCRs, a genus-specific kDNA PCR29 and two genus- and species-specific PCRs, the ITS1 PCR29 and ITS1 nested PCR (ITS1 nPCR)29. ITS1 nPCR was performed using two sets of two pairs of primers, the primers R221 and R332 targeting ssu rRNA and the nested primers LITS and L5.8S targeting ITS1. All samples were processed using a modified protocol of genus-specific primers targeting kDNA29 as a first-step screen-
ing tool, followed by the protocol published by Schönian et al. which uses two genus- and species-specific PCRs (Fig. 1).

A total of 25 µL was used for all PCR amplifications. Each reaction had 12.5 µL of Master mix, one µL of each primer, 7.5 µL RNA-free water, and three µL of sample’s DNA which was around 25 ng. *Leishmania major* MHOM/TM/82/Lev and *Leishmania tropica* MHOM/SU/80/K28 strains were used as positive control and RNA-free water were used as negative controls, respectively. PCR inhabitation control reactions were carried out to exclude false-negative results. All PCR reagents were purchased from Promega (USA). Primers were purchased from Macrogen (Seoul, South Korea). Table 1 shows the primers used and reaction cycling conditions applied for each PCR assay used in this study.

All PCR amplified products were analyzed by electrophoresis on 1% agarose at 120 V in 1 × Tris–acetate–ethylenediaminetetraacetic acid buffer and were visualized under ultraviolet light.

**Genotyping of Leishmania species.** *Leishmania* species were determined using ITS1 PCR/nPCR-RFLP and sequencing. All positive PCR products from direct ITS1 PCR and ITS1 nPCR were analyzed using restriction enzyme analysis for species identification.

Each PCR-amplified product (10 µL) was digested with 1 U *HaeIII* enzyme (MOLEQULE-ON, New Zealand) at 37 °C for 15 min, following the kit guidelines. The RFLP product was examined on MetaPhor (4%) agarose gel (Lonza, Switzerland) and visualized under ultraviolet light.

The ITS1 PCR/nPCR products were processed by the Macrogen Company (South Korea) for sequencing. Sequencing was conducted in both directions.

All obtained sequences were submitted to the GenBank database (accession numbers OK560721-OK560817). All sequences were compared to the reference ITS1 sequences published in GenBank using BLAST (http://www.ncbi.nlm.nih.gov). DNA sequences were aligned and trimmed using the BioEdit software (version 7.2.5.0; http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Neighbor-joining analysis was used to generate the phylogenetic tree using MEGAX software. Bootstrap analyses with 1000 replicates were performed to assess the robustness of the constructed phylogenetic tree.

### Table 1. PCR targets, primers, amplicon size and thermal cyclic conditions of the study PCR assays.

| PCR target | Primer | Amplicon size | Thermal conditions | References |
|------------|--------|---------------|--------------------|------------|
| kDNA | 13A: 5′-GTGCGGAGGGCGTCT 3′ 13B: 5′-ATTTTACCAACCCCGATT-3′ | 120 BP | 94 C (4 min), [94 C (30 s), 60 C (40 s), 72 C (1 min) for 35 cycles], 72 C (7 min) | 29 |
| ITS1 | LITSR: 5′-CGGATCATTCGCCGATG-3′ L5.8S: 5′-TGTACCACTACGCCACTT-3′ | 320 BP | 95 C (2 min), [95 C (30 s), 53 C (40 s), 72 C (1 min) for 34 cycles], 72 C (7 min) | 37 |
| ssu rRNA | R221: 5′-GGTTCTTTTCCCTGATTACG-3′ R332: 5′-GGCGGTAAAGGCCGATAT-3′ | 603 BP | 94 C (3 min), [94 C (1 min), 56 C (1 min s), 72 C (2 min) for 37 cycles], 72 C (6 min) | 38 |
Statistical analyses. Based upon previous published studies, ITS1 nPCR for negative cases by ITS1 PCR was used for accurate molecular diagnosis and was considered the reference standard diagnostic test. The sample was considered positive if it was positive by direct ITS1 PCR or ITS1 nPCR. Data were analyzed using the Statistical Package for the Social Sciences (SPSS) software version 26 (SPSS Inc., Chicago, IL, USA). Descriptive statistics for categorical variables are presented as percentages and frequencies. Statistical significance was determined using the chi-squared tests, where the $P$ value was less than 0.05. Specificity, positive predictive value (PPV), sensitivity, negative predictive value (NPV), accuracy, and kappa agreement were determined to evaluate the performance of each diagnostic method. Data are presented as tables, when appropriate.

Ethical approval. The study protocol was approved by the IRB Committee of the Deanship of Scientific Research at Imam Abdulrahman Bin Faisal University (reference number: IRB-PGS-2020–01-427/IRB-PGS-2020–01-186) and Ministry of Health, Saudi Arabia IRB Committee (KFHH RCA #: 08–25–2020).

Informed consent. The purpose of the present study was comprehensively explained to all of the participants and the children's parents/guardians, and the collection of specimens was completed after obtaining their informed consent.

Results

Microscopic examination. Out of the 100 stained impression smears from skin punch biopsies, 33% (33/100) were positive for Leishmania amastigotes (Fig. 2), whereas for 67% (67/100) of the smears, Leishmania amastigotes could not be observed.

Compared to the reference standard method, smear microscopy had perfect specificity (100%) and PPV (100%), but had poor sensitivity (31.6%) and NPV (2.9%). The results showed poor agreement with the reference standard (kappa = 0.18) (Tables 2, 3).

Polymerase chain reaction assays. Using the reference standard, 98 of the 100 cases were considered true-positive CL cases, whereas two of the 100 cases were considered true-negative cases. Based on direct ITS1 PCR 89 of the 98 (90.8%, $P < 0.001$) cases were detected, it had a sensitivity of 90.8%, poor NPV (18.2%), and

Table 2. Results of direct ITS1 PCR, kDNA PCR and microscopy of stained smear in detection of CL among all study individuals. *NA: No statistics are computed because kDNA is a constant.
perfect specificity (100%) and PPV (100%). The level of kappa agreement between the direct ITS1 PCR and reference standard was fair (kappa = 0.28) (Tables 2, 3). kDNA PCR was positive in all sample reactions with two false-positive cases (Tables 2, 3).

Identification of *Leishmania* species using restriction fragment length polymorphism analysis. Identification of *Leishmania* species was performed by digesting PCR products for the 98 true-positive samples using *HaeIII* restriction enzyme to distinguish each sample's RFLP pattern (Fig. 3). The predominant species was *L. major*; it was identified in 95 (96.9%) of the total patients’ sample. For the first time in Al-Ahsa, *L. tropica* was identified in three patients (3.1%) (Table 4).

Sequencing analysis. Sequencing analysis confirmed RFLP results with 100% similarity of the study DNA sequences with the sequences previously submitted to the GenBank, as shown in the constructed phylogenetic tree (Fig. 4).

Sociodemographic and clinical data of study individuals and their association with the occurrence of cutaneous leishmaniasis and *Leishmania* species. Most patients were men (90%) and lived in rural areas (98%). More than half (61%) of the patients with CL were aged 15–44 years. There was no travel history for the participants in the last 6 months prior to the presentation.

Skin lesions in the patients with CL were distributed in different body areas, and 50% of the lesions were distributed in the legs. Most of the patients (76.5%) had skin lesions at one body site. The patient presented with

| Leishmania species | Number | Percentage |
|--------------------|--------|------------|
| *L. major*         | 95     | 96.9%      |
| *L. tropica*       | 3      | 3.1%       |

Table 4. Results of genotyping using RFLP analysis.

![Image of Agarose gel](image-url)  
**Figure 3.** MetaPhor (4%) Agarose gel for *Leishmania* speciation isolated from clinical specimen of CL patients by analysis of restriction pattern of ITS1 PCR/nPCR products using restriction enzyme (*HaeIII*). Lane 1: 50 bp DNA ladder; lane 2: *L. major* MHOM/TM/82/Lev positive control (203 and 132 bp); lane 3: *L. tropica* MHOM/SU/80/K28 positive control (200 and 57 bp); lanes 4–6 & 8–12: *L. major* samples; lane 7: *L. tropica* sample.

![Table 3](image-url)  
**Table 3.** Diagnostic performance and Kappa agreement of the study diagnostic tests. *NA*: No statistics are computed because kDNA is a constant. **Key for Kappa:** ˂0 = Poor agreement, 0.01-0.20 = Slight agreement, 0.21-0.40 = Fair agreement, 0.41-0.60 = Moderate agreement, 0.61-0.8 = Substantial agreement, 0.81-1.00 = Almost perfect agreement.
various cutaneous lesions (Fig. 5). Ulcerative plaque lesions were the most common presentation (28%), followed by ulcerative lesion (19%), then nodular (17%), and plaque (13%) lesions.

The patients were treated according to the Saudi Ministry of Health CL treatment guidelines. The choice of therapy was decided according to the clinical presentation, stage, number, and sites of the CL skin lesions. Most of the patients (94/98, 96%) were treated with sodium stibogluconate either intramuscularly or intra-lesionally. Only four (4%) patients were treated with oral fluconazole. Complete resolution of lesions after the entire course

Figure 4. Dendrogram shows the Neighbor-joining phylogenetic tree of \textit{L. major} (SA-L.m 1–9 and 39–98 in one cluster, 10–38 in one cluster, 99, 100 and 101) and \textit{L. tropica} (SA-L.t 44, 77 and 78) study isolates relied on ITS1-5.8S rDNA gene sequences in comparison to reference strains (their accession number is located before their names). Similar sequences were clustered in the tree. Bootstrap analysis was relied on 1000 replicates. The distance scale at the bottom of the tree represents the number of differences between the sequences.
of treatment was achieved in 95 (96.9%) patients (Fig. 6). One patient required a second course of oral fluconazole for 4 weeks after the first course to achieve complete resolution. One patient had recurrence of the lesions after a full course of therapy (lesions appeared after 4 months of complete resolution).

The sociodemographic and clinical data of the patients were analyzed for their association with the molecular diagnosis of *Leishmania* and *Leishmania* species using ITS1 PCR/nPCR-RFLP (Tables 5 and 6). Among the studied patient characteristics, only sex, residence, and treatment outcome were associated with the occurrence of *Leishmania* with statistical significance (*P* < 0.05). Distribution of skin lesion(s) and treatment outcome were statistically significantly associated with the genotype of *Leishmania* (*P* < 0.05).

**Discussion**

Molecular assays, such as PCR, are highly specific and sensitive for the diagnosis of infectious diseases, including CL. The molecular identification of *Leishmania* species was performed by following a modified protocol for genus-specific primers targeting kDNA as the first screening step, followed by the use of two genus- and species-specific PCRs, ITS1 PCR and ITS1 nPCR, using the first ssu rRNA PCR (Fig. 1). This modification aimed to study the use of kDNA primer as a first-step screening tool to be implemented in routine diagnostic laboratories and thus overcome the possible false-negative results by Schönian et al. In our study, 98 of the 100 samples were considered true positive using our reference standard molecular method. Treatment was initiated by the clinical team if the sample was true positive.

The PCR of kDNA was positive in all samples, including two false-positive results. Clinical confirmation of false positivity was performed in the case without initiation of the CL treatment protocol. Both patients showed spontaneous resolution of the lesions after two weeks. kDNA PCR was able to detect as low as 10–4 parasites in patient samples. Compared to other DNA targets, the kDNA target has abundant copy numbers (almost 10,000 copies) that are 50–250-fold higher than those of other PCR targets. Based on our findings, the use of kDNA as a screening tool is discouraged as it may lead to false-positive results and is an extra step, whereas using ITS1 nPCR for negative cases by ITS1 PCR allows for accurate diagnosis and speciation of *Leishmania*. Other studies have suggested the use of kDNA as a screening tool with excellent performance. Further comprehensive studies using larger sample sizes are required to assess kDNA PCR as a first-step screening tool. Based on direct ITS1-PCR, 89 of the 98 (90.8%, *P* < 0.001) cases were detected with 100% specificity and 90.8% sensitivity. Most published studies have reported similar results, with a sensitivity ranging from 63.5% to 100% and a specificity ranging from 93 to 100%. The different methods used for sample collection in published studies could explain the wide range of sensitivities. Studies using punch biopsy or aspirate showed higher performance. Direct ITS1-PCR PPV was 100%, whereas the NPV was poor (18.2%). The poor NPV value compared to other
published studies\textsuperscript{23,32,48,49} can be explained by our inclusion criteria, which only recruited patients referred to the regional CL clinic with a presumptive diagnosis of CL. Implementing ITS1 nPCR for negative samples using ITS1 PCR as a reference standard enhanced the sensitivity and overcame the false-negative results.

Sequencing analysis confirmed the ITS1 PCR/nPCR-RFLP results for \textit{Leishmania} species identification. In the Al-Ahsa region, \textit{L. major} was the predominant zoonotic species. Nevertheless, \textit{L. tropica} was identified in three of the study participants. There was no travel history for any of the study individuals in the last six months prior to presentation. To the best of our knowledge, this is the first study to identify \textit{L. tropica} in human CL cases in the Al-Ahsa region.

Previously, Al-Salem et al.\textsuperscript{33} identified only \textit{L. major} in clinical samples from the Al-Ahsa region. A similar species distribution was identified in other regions of Saudi Arabia. El-Beshbishy et al.\textsuperscript{31,32} found that \textit{L. major} and \textit{L. tropica} were the CL species present both in field-caught sandflies\textsuperscript{31} and in clinical samples\textsuperscript{32} in Al-Madinah Al-Munawwarah, western province of Saudi Arabia. The predominant \textit{Leishmania} species was \textit{L. major}. Rasheed et al.\textsuperscript{50} identified \textit{L. major}, \textit{L. tropica}, and a few cases of \textit{L. infantum} \textit{Leishmania donovani} in Qassim, central region of Saudi Arabia. In Asir and Jazan in southwestern Saudi Arabia, \textit{L. tropica} is considered the predominant species\textsuperscript{33}. Despite findings from all previous studies in Saudi Arabia, which exclusively reported the presence of the anthroponotic type of \textit{L. tropica}, the zoonotic form of \textit{L. tropica} was reported in Al-Ahsa in seven of the 175 samples collected from stray dogs by Alenezi et al.\textsuperscript{51}. They only used two rounds of the less discriminative \textit{kDNA} PCRs for genus and species identification to identify \textit{L. tropica} in dogs, and they did not specify whether the samples had been collected from transported or local dogs.

In our study, the identification for the first time of \textit{L. tropica} in human samples in the Al-Ahsa region can be explained by the migration of sandflies from other regions of Saudi Arabia during the COVID-19 pandemic lockdown that led to the cessation of vector-borne disease prevention programs and affected sandfly surveillance. The WHO and many experts have concluded that vector densities and health threats from vector-borne diseases can increase and even double as a result of restrictions in human mobility due to the COVID-19 lockdown, which may adversely affect the epidemiology of vector-borne diseases\textsuperscript{52–55}.

More generally, the pattern of \textit{Leishmania} species distribution in certain localities can easily change over time due to changes in the environment with rapid urbanization, vector movement, vector potency, vector control measures, animal transportation, and climate changes\textsuperscript{56}. 

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Resolution of the CL lesions. A-C: Post inflammatory hyperpigmentation and scarring in a different exposed body sites after a full course of treatment. (Once weekly 0.5 mL per lesion (50 mg) intralesional sodium stibogluconate for 6 weeks course).}
\end{figure}
Despite the poor performance of microscopic examination as a diagnostic tool, it is still considered the gold standard for diagnosing CL in clinically diagnosed patients in many laboratories worldwide. Microscopy was limited by poor sensitivity, with missed diagnosis in 65 (66.3%) patients. Other studies have reported similar low sensitivity (37–62.6%) of microscopy to detect Leishmania amastigotes in stained skin smears. The poor performance of this tool is explained by the need for a high number of viable parasites in the sample to be visualized, the extreme demand for an expert microscopist to read the smears, and the flawed methods of sample collection. Therefore, a reliable and cost-effective diagnostic tool for the diagnosis of CL is essential.

Multiple studies have addressed the relationship between the clinical and epidemiological characteristics of CL cases and the occurrence of Leishmania and Leishmania species. In the current study, the area of residence of the study participants and the clinical outcome of treatment were significantly associated with PCR results, whereas family history, distribution of cutaneous lesions, and factors related to indoor dwelling environment were significantly associated with ITS1-PCR RFLP results. Further studies are needed to confirm our results.

## Conclusion
To the best of our knowledge, this comprehensive molecular study fills a gap in the knowledge regarding the identification of the prevalent Leishmania species causing CL in the Al-Ahsa region. For the first time, our study detected human cases of anthroponotic L. tropica in Al-Ahsa. For accurate molecular diagnosis and speciation of Leishmania, we recommend using ITS1 nPCR for negative cases by ITS1 PCR. Further studies on vectors and animal reservoirs are essential to identify Leishmania transmission dynamics in the Al-Ahsa region. This crucial information can help guide the future planning of control methods to prevent the spread of CL in this region.

### Table 5. Socio-demographic data of study individuals in association with Leishmania [ITS1 PCR/nPCR results] and Leishmania species [ITS1 PCR/nPCR-RFLP results]. Presented as number (percentage). P value is significant if > 0.05. *Preschool: Participants still did not reach school age.

| Leishmania and Leishmania species [ITS1 PCR/nPCR results] (n = 100) | Positive (RFLP results) | Total | Negative | Total | P value |
|-------------------------------------------------------------|------------------------|-------|----------|-------|---------|
| | L.major | L.tropica | | | | |
| **Sex** | | | | | |
| Male | 86 (96.6) | 3 (3.4%) | 0.576 | 89 (98.9) | 1 (1.1) | 90 | 0.057 |
| Female | 9 (100) | 0 (0%) | | 9 (90) | 1 (10) | 10 | |
| **Nationality** | | | | | |
| Saudi | 38 (97.4) | 1 (2.6%) | 0.816 | 39 (97.5) | 1 (2.5) | 40 | 0.773 |
| Non-Saudi | 57 (96.6) | 2 (3.4%) | | 59 (98.3) | 1 (1.7) | 60 | |
| **Residence** | | | | | |
| Rural | 94 (96.9) | 3 (3.1%) | 0.858 | 97 (99) | 1 (1) | 98 | 0.001 |
| Urban | 1 (100) | 0 (0%) | | 1 (50) | 1 (50) | 2 | |
| **Employment** | | | | | |
| Employee | 76 (97.4) | 2 (2.6%) | 0.573 | 78 (98.7) | 1 (1.3) | 79 | 0.309 |
| Unemployed | 19 (95) | 1 (5%) | | 20 (95) | 1 (5) | 21 | |
| **Level of education** | | | | | |
| Illiterate | 17 (94.4) | 1 (5.6%) | 0.445 | 18 (100) | 0 (0) | 18 | 0.827 |
| Pre-school | 7 (87.5) | 1 (12.5%) | | 8 (100) | 0 (0) | 8 | |
| Primary School | 18 (94.7) | 1 (5.3%) | | 19 (95) | 1 (5) | 20 | |
| Intermediate School | | | | | |
| Secondary School | 15 (100) | 0 (0%) | | 15 (100) | 0 (0) | 15 | |
| Bachelor | 30 (100) | 0 (0%) | | 30 (96.8) | 1 (3.2) | 31 | |
| **Age group** | | | | | |
| 1–4 | 2 (100) | 0 (0%) | 0.108 | 2 (100) | 0 (0) | 2 | 0.061 |
| 5–9 | 3 (75) | 1 (25%) | | 4 (80) | 1 (20) | 5 | |
| 10–14 | 7 (100) | 0 (0%) | | 7 (100) | 0 (0) | 7 | |
| 15–44 | 58 (96.7) | 2 (3.3%) | | 60 (98.4) | 1 (1.6) | 61 | |
| > 45 | 25 (100) | 0 (0%) | | 25 (100) | 0 (0) | 25 | |
| **Total** | 95 (96.9) | 3 (3.1%) | | 98 (98) | 2 (2) | 100(100) | |
Data availability
The datasets generated and/or analyzed in the current study are available in the GenBank database (accession numbers: OK560721–OK560817).

Received: 17 February 2022; Accepted: 10 June 2022
Published online: 24 June 2022

References
1. Aronson, N. et al. Diagnosis and treatment of leishmaniasis: clinical practice guidelines by the Infectious Diseases Society of America (IDSA) and the American Society of Tropical Medicine and Hygiene (ASTMH). Am. J. Trop. Med. Hyg. 96, 24–45 (2017).
2. Garcia, L. S. & Procop, G. W. Diagnostic medical parasitology. Man. Commer. Methods Clin. Microbiol. Int. Ed. 284–308 (2016).
3. Spec, A., Escota, G. V., Chrisler, C. & Davies, B. Comprehensive Review of Infectious Diseases. (2019).
4. Al-Hucheimi, S. N., Sultan, B. A. & Al-Dhalimi, M. A. A comparative study of the diagnosis of Old World cutaneous leishmaniasis in Iraq by polymerase chain reaction and microbiologic and histopathologic methods. Int. J. Dermatol. 48, 404–408 (2009).
5. Bhutto, A. M. et al. Cutaneous leishmaniasis caused by Leishmania (L.) major infection in Sindh province. Pakistan. Acta Trop. 111, 295–298 (2009).
6. Katakura, K. Molecular epidemiology of leishmaniasis in Asia (focus on cutaneous infections). Curr. Opin. Infect. Dis. 22, 126–130 (2009).
7. Azmi, K. et al. Genetic, serological and biochemical characterization of Leishmania tropica from foci in northern Palestine and discovery of zymodeme MON-307. Parasit. Vectors 5, 1–14 (2012).
8. Mahdy, M. A. K. et al. Molecular characterization of Leishmania species isolated from cutaneous leishmaniasis in Yemen. PLoS ONE 5, e12879 (2010).
9. Mošek, I. M., Schónian, G., Kanani, K. & Shafian, B. Leishmania major cutaneous leishmaniasis outbreak in the Jordanian side of the Northern Jordan Valley. Pathog. Glob. Health 112, 22–28 (2018).
10. Abuzaid, A. A. et al. Cutaneous Leishmaniasis in Saudi Arabia: A comprehensive overview. Vector Borne Zoonotic Dis. 17, 673–684 (2017).
11. Gadisa, E. et al. Leishmania (Kinetoplastida): species typing with isoenzyme and PCR–RFLP from cutaneous leishmaniasis patients in Ethiopia. Exp. Parasitol. 115, 339–343 (2007).
12. Rjaajoui, M. et al. New clindicepidemiologic profile of cutaneous leishmaniasis, Morocco. Emerg. Infect. Dis. 13, 1358 (2007).
13. Bennoush-Milka, R. et al. Primary Leishmania infantum MON-80 endonasal leishmaniasis in Tunisia. Ann. Dermatol. Venereol. 135, 389–392 (2008).
14. Antoniou, M. et al. Leishmania donovani leishmaniasis in Cyprus. Lancet. Infect. Dis. 8, 6–7 (2008).

Table 6. Clinical data of the study individuals in association with Leishmania [ITS1 PCR/nPCR results] and Leishmania species [ITS1 PCR/nPCR-RFLP results]. *Other: Sites include abdomen, chest, shoulder, and buttock. Significant values are in bold.
56. Mendoza-Roldan, J. et al. Leishmania infantum and Dirofilaria immitis infections in Italy, 2009–2019: changing distribution patterns. Parasit. Vectors 13, 1–8 (2020).
57. Kumar, R., Bumb, R. A., Ansari, N. A., Mehta, R. D. & Salotra, P. Cutaneous leishmaniasis caused by Leishmania tropica in Bikaner, India: parasite identification and characterization using molecular and immunologic tools. Am. J. Trop. Med. Hyg. 76, 896–901 (2007).
58. Sharma, N. L. et al. Localized cutaneous leishmaniasis due to Leishmania donovani and Leishmania tropica: preliminary findings of the study of 161 new cases from a new endemic focus in Himachal Pradesh, India. Am. J. Trop. Med. Hyg. 72, 819–824 (2005).
59. Bari, A. & Rahman, S. Correlation of clinical, histopathological, and microbiological findings in 60 cases of cutaneous leishmaniasis. Indian J. Dermatology Venereol. Leprol. 72, 28 (2006).
60. Qureshi, N. A., Ali, A., Rashid, U. & Ali, N. Prevalence of Leishmania tropica in school boys of Khyber agency, FATA near Pak-Afghan border. Acta Trop. 164, 90–94 (2016).
61. El-Badry, A. A., El-Dwibe, H., Basyoni, M. M. A., Al-Antably, A. S. A. & Al-Bashier, W. A. Molecular prevalence and estimated risk of cutaneous leishmaniasis in Libya. J. Microbiol. Immunol. Infect. 50, 805–810 (2017).
62. Manomat, J. et al. Prevalence and risk factors associated with Leishmania infection in Trang Province, southern Thailand. PLoS Negl. Trop. Dis. 11, e0006095 (2017).
63. Bisetegn, H. et al. Clinical, parasitological and molecular profiles of Cutaneous Leishmaniasis and its associated factors among clinically suspected patients attending Borumeda Hospital, North-East Ethiopia. PLoS Negl. Trop. Dis. 14, e0008507 (2020).
64. Bamorovat, M. et al. Risk factors for anthroponotic cutaneous leishmaniasis in unresponsive and responsive patients in a major focus, southeast of Iran. PLoS ONE 13, e0192236 (2018).
65. Ngere, I. et al. Burden and risk factors of cutaneous leishmaniasis in a peri-urban settlement in Kenya, 2016. PLoS ONE 15, e0227697 (2020).

Author contributions
This study was designed and the first manuscript was written by A.R.A., A.J., and E.B. and was reviewed by A.J. and E.B. Clinical samples and related data were collected by A.A. and A.R., sandflies were collected, and impression smears were stained and microscopically examined by A.M. and reviewed by A.R. and E.B. Molecular assays were performed by A.J., A.R., and E.B. Data interpretation and reporting results were obtained by A.R., A.J., A.J., and E.B. All the authors have read and approved the final version of the manuscript.

Funding
This project was funded by the Deanship of Scientific Research at the Imam Abdulrahman Bin Faisal University (project number 2020–197-Med).

Competing interests
The authors declare no competing interests.

Additional information
Correspondence and requests for materials should be addressed to A.A.-E.-B.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022