Leishmaniasis is a complex disease with cutaneous, mucocutaneous, or visceral manifestations depending on the parasite species and host immunity. Despite continued elimination efforts, leishmaniasis continues to afflict known and newer endemic regions, where 0.5–0.9 million new cases of visceral leishmaniasis (VL) and 0.6–1.0 million new cases of cutaneous leishmaniasis (CL) occur every year (1). An increase in VL and CL cases from newer foci and atypical disease manifestation pose a challenge to leishmaniasis control programs (2–7). Unlike the known species-specific disease phenotype, parasite variants can cause atypical disease, so that Leishmania species generally associated with VL can cause CL and vice versa.

In India, VL caused by L. donovani parasites in the northeastern region and CL caused by L. tropica in the western Thar Desert represent the prevalent forms of the disease (2). Himachal Pradesh is a more recently leishmaniasis-endemic state in northwest where VL and CL coexist; CL incidence is higher than VL incidence and most cases are attributable to L. donovani instead of L. tropica infection (8,9). Sharma et al. conducted limited molecular analysis of a few CL cases and reported preliminary findings (8). For an in-depth study on the involvement of L. donovani parasites in CL cases, we conducted a comprehensive molecular analysis of CL cases in Himachal Pradesh.

**The Study**

During 2014–2018, an increase in CL cases occurred in Himachal Pradesh; case reports came from different tehsils (i.e., townships) in Kinnaur, Shimla, and Kullu and the previously nonendemic districts of Mandi and Solan (Appendix Table 1, Figure 1, https://wwwnc.cdc.gov/EID/article/26/8/19-1761-App1.pdf). We confirmed 60 CL cases indigenous to the state with detailed patient information, demonstration of the presence of Leishman-Donovan bodies and CL-specific histopathologic changes in skin lesional specimens, and PCR detection of parasitic infection (Appendix).

We conducted PCR and restriction fragment-length polymorphism (RFLP) analysis of parasite species–specific internal transcribed spacer 1 (ITS1) sequences by using appropriate standard controls. We detected the expected ≈320-bp product with a HaeIII RFLP pattern specific to L. donovani in all patient biopsy specimens, indicating L. donovani, L. infantum, or both as the causative agent of infection (Appendix Figure 4) (10).

BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of 44 ITS1 test sequences showed all the samples to be closest to L. donovani, having maximum identity to L. donovani isolates from Bhutan (GenBank accession nos. JQ730001–2) and possibly L. infantum. None of the CL cases were consistent with L. tropica infection, unlike in a previous report (8). To distinguish whether HP isolates were L. donovani, L. infantum, or both and to infer genetic and geographic relatedness between
these isolates and standard reference strains, we performed ITS1 microsatellite repeat analysis and phylogenetic classification (11–13). The 4 ITS1 polymorphic microsatellite repeat analysis indicate HP isolates different from *L. infantum* and closest to the *L. donovani* isolates from Bhutan (Table 1; Figure 1, panel A). We detected a polymorphism in the third poly (TA) microsatellite locus with 5 repeats and

### Table 1. Standard *Leishmania* strains used in ITS1-based microsatellite polymorphism and phylogenetic analysis of cutaneous leishmaniasis isolates, Himachal Pradesh, India, 2014–2018*

| Standard *Leishmania* strains (place of origin) | WHO code | Genbank accession no. | Zymodeme | Disease form | Strain type† | ITS1 polymorphic microsatellite stretches (nucleotide position, bp) | Poly C (24–39) | Poly A (24–39) | Poly TA (61–76) | Poly A (124–134) |
|-----------------------------------------------|----------|----------------------|----------|--------------|--------------|-------------------------------------------------|----------------|----------------|-----------------|-----------------|
| **VL- and CL-causing *L. infantum* and *L. donovani* parasite strains** | | | | | | | | | | | |
| *L. infantum* (Tunisia) | MHOM/TN/80/IPT1 | AJ000289 | MON-1 | VL | A | 3 6 4 8 | | | | |
| *L. donovani* (India) | MHOM/IN/00/DEVI | AJ634376 | MON-2 | VL | H | 2 8 5 7 | | | | |
| *L. donovani* (Sri Lanka) | MHOM/LK/2002/L60c | AM901447 | MON-37 | CL | ND | 2 8 5 7 | | | | |
| *L. donovani* (Bangladesh) | | KT921417 | ND | VL | ND | 2 8 5 7 | | | | |
| *L. donovani* (Kenya) | MHOM/KE/85/NLB323 | AJ000297 | MON-37 | VL | G | 2 8 5 7 | | | | |
| *L. donovani* (Sudan) | MHOM/SD/75/LV139 | AJ000291 | ND | CL | E | 2 8 6 8 | | | | |
| *L. donovani* (Ethiopia) | MHOM/SD/93/9S | AJ634372 | MON-18 | VL | F | 2 9 5 7 | | | | |
| *L. donovani* (China) | MHOM/ET/67/HU3 | AJ634373 | MON-18 | VL | F | 2 9 5 7 | | | | |
| *L. donovani* (HP, India) | MHOM/CN/00/Wangje1 | AJ000294 | MON-35 | VL | C | 3 6 4 7 | | | | |
| *L. donovani* (Bhutan) | MHOM/IN/83/CHANDIGARH Trashigang1 | AM901449 | MON-37 | VL | ND | 2 8 2, TAA, 3 | | | | |
| Samtse1 | JQ730001 | ND | VL | ND | 2 8 2, TAA, 3 | | | | |

| **CL-causing *L. donovani* isolates from Himachal Pradesh‡** | | | | | | | | | | |
| HPCL22 | – | MG982955 | ND | CL | ND | Heterogeneous 2, TAA, 3 | | | | |
| HPCL27 | – | MG982958 | ND | CL | ND | Heterogeneous 2, TAA, 3 | | | | |
| HPCL28 | – | MG982959 | ND | CL | ND | Heterogeneous 2, TAA, 3 | | | | |
| HPCL32 | – | MG982963 | ND | CL | ND | Heterogeneous 2, TAA, 3 | | | | |
| HPCL42 | – | MG982972 | ND | CL | ND | Heterogeneous 2, TAA, 3 | | | | |
| HPCL45 | – | MG982975 | ND | CL | ND | Heterogeneous 2, TAA, 3 | | | | |
| HPCL47 | – | MG982977 | ND | CL | ND | Heterogeneous 2, TAA, 3 | | | | |
| HPCL49 | – | MG982978 | ND | CL | ND | Heterogeneous 2, TAA, 3 | | | | |
| HPCL52 | – | MG982981 | ND | CL | ND | Heterogeneous 2, TAA, 3 | | | | |
| HPCL55 | – | MG982983 | ND | CL | ND | Heterogeneous 2, TAA, 3 | | | | |

| **CL-causing standard WHO *Leishmania* species** | | | | | | | | | | |
| *L. major* | MHOM/SU/73/5ASKH | AJ000310 | MON-4 | CL | ND | 4 6 6 6 | | | | |
| *L. tropica* | MHOM/SU/60/OD | EU326226 | LON-7 | CL | ND | 4 9 1, TTA, 2 | 3, C, 4A | | | |
| *L. mexicana* | MHOM/MX/85/SOLIS | AJ000313 | MON-152 | CL | ND | 2 8 1 | 3, C, 7A | | | |
| *L. braziliensis* | MHOM/BR/00/LTB300 | FN398338 | MON-166 | CL | ND | 2 6 1 | 5 | | | |
| *L. amazonensis* | MHOM/BR/73/M2269 | HG512964 | MON-132 | CL | ND | 2 7 1 | 3, C, 6A | | | |

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*CL, cutaneous leishmaniasis; HP, Himachal Pradesh; ITS1, internal transcribed spacer 1; ND, not determined; VL, visceral leishmaniasis; WHO, World Health Organization.
†ITS sequences strain type according to Kuhls et al. (13).
‡These species represent 10/44 samples used in polymorphic microsatellite analysis.
an atypical insert of TAA and the fourth poly (A) microsatellite tract with 8 repeats; these polymorphisms were identical to the VL-causing \textit{L. donovani} isolates from Bhutan. An \textit{L. donovani} Chandigarh isolate originally from HP is reported to be closest to the Bhutan isolates and matched with HP isolates at

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure1.png}
\caption{ITS1-based molecular analysis of clinical isolates from cutaneous leishmaniasis (CL) patients, Himachal Pradesh, India, 2014–2018. A) Multiple sequence alignment of ITS1 microsatellite repeat sequences of representative parasite isolates from CL patients with those of \textit{L. donovani} complex reference strains from different geographic regions. Sequences were aligned by using BioEdit sequence alignment program (https://bioedit.software.informer.com/7.2). B) Phylogenetic tree of ITS1 sequences from CL test isolates (designated as HPCl, numbered in order of their collection) and standard Leishmania strains. Tree constructed by using maximum-likelihood method with 5,000 bootstraps in the dnaml program of PHYLIP package (http://evolution.genetics.washington.edu/phylip/doc/main.html). GenBank accession numbers are indicated. Scale bar indicates the nucleotide substitution per site. ITS1, internal transcribed spacer 1; RFLP, restriction fragment length polymorphism.}
\end{figure}
isolates from India and elsewhere and the CL-causing L. donovani isolates from Himachal Pradesh into a discrete cluster different from the VL-causing L. donovani from India and elsewhere and the CL-causing L. donovani isolates from Sri Lanka. The Himachal Pradesh CL isolates within the cluster exhibited considerable heterogeneity (Table 1; Figure 1, panel B; Appendix Table 4).

Sequences of the 6-phosphogluconate dehydrogenase gene (6PGDH) exhibit a high degree of polymorphism and have been used to identify Leishmania species and differentiate region-specific zymodemes (14). We performed multiple sequence alignment of the representative partial 6PGDH amino acid sequences from Himachal Pradesh isolates by using the homologous 6PGDH protein sequences of the reference Leishmania isolates to determine their genetic and geographic relatedness (Table 2; Figure 2, panel A; Appendix Table 4, Figure 5). Himachal Pradesh isolates exhibited a 6PGDH sequence specific to Mon-37 and different from Mon-2 (having aspartic acid in place of asparagine) at position 326 (Figure 2, panel A). Thus, CL-causing L. donovani from Himachal Pradesh were distinct from the most common VL-causing India Mon-2 L. donovani and the Bangladesh L. donovani isolate, whereas they were similar to the CL-causing L. donovani isolate from Kerala and CL- and VL-causing Mon-37 isolates from Sri Lanka and the isolates from Kenya, Brazil, and China.

Phylogenetic analysis of 6PGDH amino acid sequences of CL isolates grouped them into a heterogeneous cluster; variants were closer to a viszertropic L. donovani isolate from Sri Lanka and distinct from the VL-causing L. donovani isolates from India and Bangladesh and CL-causing isolates from Kerala and Sri Lanka (Figure 2, panel B). However, the HPClL55 isolate (GenBank accession no. MH1208450) grouped differently. The HPClL49 isolate (GenBank accession no. MH208446) showed relatedness to the standard L. infantum strain, although ITS1 analysis using BLAST and microsatellite repeat sequences showed regions of similarity with L. donovani. ITS1 and 6PGDH sequence analysis suggest that Himachal Pradesh isolates from CL patients consist of heterogeneous L. donovani variants and possibly represent hybrid genotypes.

None of the CL patients had VL-specific symptoms or VL history. Ten of 43 patient blood samples tested positive for rK39 antibody, and 37 of 51 samples were positive for the circulating parasite DNA with L. donovani-specific ITS1 (Appendix Figure 6, panel A, B). The result suggests asymptomatic systemic L. donovani infection in a fraction of CL patients.

**Conclusions**

The presence of leishmaniasis in Himachal Pradesh is not yet well known in India and globally (15). Our epidemiologic study shows newer CL pockets during 2014–2018; thus, the state needs to be recognized as leishmaniasis-endemic by public health authorities (Appendix Figure 1). We conclude that CL cases in Himachal Pradesh are caused by L. donovani variants

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**Table 2. Standard Leishmania strains used in partial 6PGDH amino acid–based phylogenetic analysis of cutaneous leishmaniasis isolates, Himachal Pradesh, India, 2014–2018**

| Species (place of origin) | WHO code | Zymodeme | GenBank accession no. | Pathology |
|---------------------------|----------|----------|-----------------------|-----------|
| L. donovani (India)       | MHOM/IN/0000/DEVI | MON-2 | AM157147 | VL |
| L. major (Turkmenistan)   | MHOM/TM/1973/5ASKH | ND | AY706107 | CL |
| L. infantum               | ND       | ND       | XM_001469106 | ND |
| L. mexicana               | MHOM/28/82/BEL21 | ND | AY386372 | CL |
| L. tropica                | ND       | ND       | AYO45763 | CL |
| L. amazonensis            | ND       | ND       | AY168562 | CL |

*6PGDH, 6-phosphogluconate dehydrogenase gene; CL, cutaneous leishmaniasis; ND, not determined; VL, visceral leishmaniasis; WHO, World Health Organization.
Figure 2. 6PGDH-based molecular analysis of clinical isolates from cutaneous leishmaniasis (CL) patients, Himachal Pradesh, India, 2014–2018. A) Sequence alignment of partial 6PGDH amino acid of CL isolates exhibit replacement of asparagine (N) with aspartic acid (D) at position 326 analogous to visceral leishmaniasis–causing and CL-causing isolates from Sri Lanka. B) Phylogenetic tree for 6PGDH sequences from CL test isolates (designated as HPCL, numbered in order of their collection) and standard Leishmania strains. Tree constructed by using maximum-likelihood method with 5,000 bootstraps in the dnaml program of PHYLIP package (http://evolution.genetics.washington.edu/phylip/doc/main.html). GenBank accession numbers are indicated. Scale bar indicates the amino acid substitution per site. 6PGDH, 6-phosphogluconate dehydrogenase gene; HP, Himachal Pradesh.
distinct from the viscerotropic L. donovani strain from northeast India. The CL isolates in Himachal Pradesh exhibit considerable heterogeneity and indicate the possible existence of genetic hybrids. The scenario appears somewhat similar to Sri Lanka and Kerala, where L. donovani parasites cause cutaneous disease, albeit with differences in the region-specific L. donovani variants. In lieu of the coexistence of VL and CL in Himachal Pradesh, parasite isolates from VL patients also need to be characterized. To understand the biology of atypical L. donovani variants with cutaneous manifestations and to genetically differentiate the dermotropic versus viscerotropic potential of L. donovani variants, comparison of CL- and VL-causing isolates in Himachal Pradesh using whole-genome sequence analysis is required.

L. donovani parasites in the blood of some CL patients represent human reservoirs similar to asymptomatic VL carriers, and the parasite variants have the potential to cause full-blown VL manifestations. An elaborate surveillance program dedicated to the Himachal Pradesh region is urgently required for better diagnosis, treatment, prediction of parasite variants in different afflicted pockets, and prevention of transmission of the disease to other regions.

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References
1. World Health Organization. Leishmaniasis. 2019 [cited 2019 Sep 1]. https://www.who.int/en/news-room/fact-sheets/detail/leishmaniasis
2. Thakur L, Singh KK, Shanker V, Negi A, Jain A, Matlashewski G, et al. Atypical leishmaniasis: a global perspective with emphasis on the Indian subcontinent. PLoS Negl Trop Dis. 2018;12:e0006659. https://doi.org/10.1371/journal.pntd.0006659
3. Sandhya R, Rakesh P, Dev S. Emergence of visceral leishmaniasis in Kollam District, Kerala, southern India. Int J Community Med Public Health. 2019;6:1350–2. https://doi.org/10.18203/2394-6040.ijcmph20190639
4. Kumar NP, Srinivasan R, Anish TS, Nandakumar G, Jambulingam P. Cutaneous leishmaniasis caused by Leishmania donovani in the tribal population of the Agasthyamala Biosphere Reserve Forest, Western Ghats, Kerala, India. J Med Microbiol. 2015;64:157–63. https://doi.org/10.1099/jmm.0.076695-0
5. Siriwardana Y, Zhou G, Deepachandi B, Akarawita J, Wickremaratne C, Warnasuriya W, et al. Trends in recently emerged Leishmania donovani induced cutaneous leishmaniasis, Sri Lanka, for the first 13 Years. BioMed Res Int. 2019;2019:4093603. https://doi.org/10.1155/2019/4093603
6. Sharma NL, Mahajan VK, Negi AK. Epidemiology of a new focus of localized cutaneous leishmaniasis in Himachal Pradesh. J Commun Dis. 2005;37:275–9. PubMed
7. Kumari S, Garg A. Lip leishmaniasis: a new emerging clinical form of cutaneous leishmaniasis from sub-Himalayan Region. Journal of Medical Science and Clinical Research. 2018;06:62–9.
8. Sharma NL, Mahajan VK, Kanga A, Sood A, Katoch VM, Mauricio I, 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. 2005;72:819–24. https://doi.org/10.4269/ajtmh.2005.72.819
9. Sharma NL, Sood A, Arora S, Kanga A, Mahajan V, Negi A, et al. Characteristics of Leishmania spp. isolated from a mixed focus of cutaneous and visceral leishmaniasis in Himachal Pradesh (India). Internet J Third World Med. 2009;7(8).
10. el Tai NO, Osman OF, el Fari M, Presber W, Schönnagl G. Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of Leishmania donovani spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. Trans R Soc Trop Med Hyg. 2000;94:575–9. https://doi.org/10.1016/S0035-9203(00)90093-2
11. Dávila AM, Momen H. Internal-transcribed-spacer (ITS) sequences used to explore phylogenetic relationships within Leishmania. Ann Trop Med Parasitol. 2000;94:651–4. https://doi.org/10.1080/00034983.2000.11813588
12. Yangzom T, Cruz I, Bern C, Argaw D, den Boer M, Velez ID, et al. Endemic transmission of visceral leishmaniasis in Bhutan. Am J Trop Med Hyg. 2012;87:1028–37. https://doi.org/10.4269/ajtmh.2012.12-0211
13. Kuhls K, Mauricio IL, Pratlong F, Presber W, Schönnagl G. Analysis of ribosomal DNA internal transcribed spacer sequences of the Leishmania donovani complex. Microbes Infect. 2005;7:1224–34. https://doi.org/10.1016/j.micinf.2005.04.009
14. Ranasinghe S, Zhang W-W, Wickremasinghe R, Abeygunasekara P, Chandrasekharan V, Athauda S, et al. Leishmania donovani zymodeme MON-37 isolated from an autochthonous visceral leishmaniasis patient in Sri Lanka. Pathog Glob Health. 2012;106:421–4. https://doi.org/10.1179/2047773212Y.0000000054
15. World Health Organization. Leishmaniasis country profile—2015, India. 2017 [cited 2019 Sep 1]. https://www.who.int/leishmaniasis/burden/India_2015-hl.pdf?ua=1

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Leishmania donovani Infection with Atypical Cutaneous Manifestations, Himachal Pradesh, India, 2014–2018

Appendix

Material and Methods

Study Design and Ethics

Lesional biopsies and blood samples were collected from the CL patients reporting at Department of Dermatology, Indira Gandhi Medical College (IGMC), Shimla and Mahatma Gandhi Medical Servies Complex (MGMSC) Khaneri in Rampur, Himachal Pradesh, India. Informed consent was obtained from all the patients in the study. The study was conducted on 60 CL patients, in the age group of 4 to 70 years at the time of diagnosis over the period from 2014 to 2018. Baseline characteristics of the patients with clinical details were compiled from the standard case report forms (Appendix Table 2). Patient history regarding the visit to endemic areas, residence, place where the disease was acquired, age with lesion duration, location and distribution was recorded on the date of sample collection. Lesional 4 mm punch biopsies were taken from the active edge of the lesions from 57 patients and processed for parasite detection, histopathological and molecular studies. Blood samples were taken to access seroprevalence of rK39 antibody. The protocol of the present study was approved by the Institutional Ethics Committee IGMC, Shimla, H.P., Approval no. HFW (MS) G-5 (Ethics)/2014–10886 and Central University of Punjab, Punjab, Approval no. CUPB/IEC/2016/034.

Clinical Diagnostics

Lesional biopsy samples were processed for parasite detection using Giemsa stained touch smears, Hematoxylin and Eosin (H & E) stained paraffin-embedded tissue sections as per standard protocol (1,2). Laboratory grown reference strains, L. donovani and L. major to be used as controls in different experiments, were grown under promastigote supporting growth conditions in RPMI with 5% FBS, Penicillin (100 units/ml) and Streptomycin (100ug/ml).
A part of the lesional biopsy from the CL cases was processed for examining CL specific histopathological changes. The samples were processed in 10% NBF, embedded in paraffin and processed to 4 to 5 µm thick tissue sections. Tissue sections were stained with H&E and examined for epidermal and dermal histopathological changes specific to cutaneous lesions (2).

**Serum Isolation and rK39 Strip Assay**

Blood samples collected from 50/60 patients were used to isolate sera. The rK39 immunochromatographic rapid diagnostic test was performed to determine the seroprevalence of rK39 antibody as per manufacturer instructions (InBios International, Inc. Seattle, WA 98104). Briefly, 20 µl of serum was loaded on to the strip followed with addition of chase buffer solution. Samples were read after 10 minutes and considered positive for the presence of anti-K39 IgG with two distinct red lines corresponding to the, test region and the control region.

**Molecular Analysis**

Skin lesion specimens from 57/60 patients and laboratory-grown *L. donovani* and *L. major* cultures (used as controls) were used to isolate Genomic DNA (gDNA) as described previously (3). gDNA from patients’ blood samples was also isolated to analyze presence of circulating parasite. gDNA from the test and control samples was used to perform species-specific ribosomal internal transcribed spacer 1 region (ITS1) PCR-RFLP assay and 6-phosphogluconate dehydrogenase (6PGDH) gene amplification (Appendix Figure 4, 5). Both ITS1 and 6PGDH amplification products were sequenced for species identification and to decipher genetic relatedness of the HP isolates with other region-specific VL and CL causing *Leishmania* isolates (4–8).

**ITS1 PCR-RFLP**

Parasite specific ITS1PCR amplification was done for all the samples with primers LITSR (5’-CTGGATCATTTTCCGATG-3’) and L5.8S (5’-TGATACCACTTATCGCACCTT-3’) as described previously (3,4). Briefly 50–100 ng of gDNA was used as template and amplified with10 pmol of each primer using Go Taq Green Master mix, 1X (Promega, Cat # M7122) in a reaction volume of 25 µl. Reaction conditions comprised an initial denaturation at 95°C for 2 min, 34 cycles of denaturation at 95°C for 20 sec, annealing at 53°C for 30 sec and extension at 72°C for 1 min with the final extension at 72°C for 6 min. The PCR product of ~ 320 bp size was
subjected to \textit{Hae}III RFLP with overnight \textit{Hae}III digestion at 37°C and run on 2.5% agarose gel to identify the \textit{Leishmania} species.

\textbf{6PGDH PCR}

6PGDH amplification was done with primers 6PGDH-F: AATCGAGCAGCTCAAGGAAG and 6PGDH-R: GAGCTTGGCGAGAATCTGAC as described previously \cite{7,8}. 50–100 ng of gDNA was amplified with 10 pmol of each primer using Go Taq Green Master mix, 1X (Promega, Cat # M7122) in a reaction volume of 25 µl. The reaction conditions comprised denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 30 secs and extension at 72°C for 1 min with the final extension at 72°C for 10 min. The PCR product was run on 1.8% agarose gel.

\textbf{Sequencing and Phylogenetic Analysis}

DNA sequencing of ITS1 and 6PGDH PCR products was done for identification of \textit{Leishmania} species/species variants in relation to CL and VL causing standard \textit{Leishmania} isolates from different regions (Table 1, https://wwwnc.cdc.gov/EID/article/26/8/19-1761-T1.htm; Table 2, https://wwwnc.cdc.gov/EID/article/26/8/19-1761-T2.htm). ITS1 PCR products from 44 samples and 6PGDH PCR products from 28 samples were outsourced for Sanger sequencing. ITS1 sequences from 44 specimens with Accession numbers MG982941 to MG982984 and 6PGDH sequences from 28 specimens with Accession numbers MH208423 to MH208450 were deposited in Genbank (Appendix Table 4).

The homologous gene sequences for ITS1 and 6PGDH of standard WHO \textit{Leishmania} species specific isolates and region specific \textit{L. donovani} isolates were retrieved from the NCBI-GenBank database. ITS1 nt query sequences from 44 specimens were analyzed using BLAST and multiple alignment software, MUSCLE using default parameters \cite{9}. The final alignment was made using BioEdit sequence alignment editor (version 7.0.5.3). The maximum likelihood tree from the aligned sequences was obtained with 5000 bootstraps with default parameters using the \textit{dnaml} program of the phylip package \cite{10}. The final tree was plotted using FigTree software (version 1.4.3). To analyze 6PGDH protein sequences, gene sequences from 28 CL samples were translated into the corresponding homologous protein sequences using translate tool at ExPASy. The representative protein sequences of the seven clusters obtained from the 28 test 6PGDH protein sequences were analyzed in relation to the homologous 6PGDH protein sequences of the
reference *Leishmania* strains using the methods explained previously. The partial 6PGDH sequence alignment was made using Jalview multiple alignment editor version 2.10.4b1 (11). The maximum likelihood tree was obtained with 5000 bootstraps using the *proml* program of the phylip package with default parameters (10).

**Results Section**

**Disease Epidemiology**

During the period from 2014–2018, an increase in CL cases in the routine skin OPD was recorded in IGMC, Shimla and MGMSC, Khaneri, Rampur. A detailed record of patients suspected with CL was taken at the time of diagnosis (Appendix Table 2). In our study on 60 CL patients, there was an almost equivalent frequency of females (51.6%) and males (48.3%) ranging in age from 4 years to 70 years. The majority of the patients belonged to the indigenous students and the farming community. All the patients had localized cutaneous skin lesions predominantly on exposed body parts with involvement of the face in majority of the cases. The time gap between the appearance of lesions and disease diagnostics on the day of sample collection, ranged from 10 days to 2.6 years with most cases reporting within 3–4 months of disease occurrence, indicating a lack of awareness among local population. Most of the patients had one or two raised and itchy lesions presented as plaques, nodules and/or papules, often ulcerated unlike those present in post kala azar dermal leishmaniasis. Around 26% of cases had mucocutaneous like lesions extending to the inner nose consistent with previous reports (12, 13; Appendix Table 2).

**Clinical Diagnostics**

Clinical confirmation of the CL cases was performed by microscopic examination of Giemsa stained lesion touch smears, H & E stained biopsy sections for the presence of LD bodies and by parasite-specific ITS1 and 6PGDH PCR analysis (Appendix Figure 2, panels A–C; Appendix Table 2). Giemsa stained tissue smears were LD positive for 50% (23/46) of the samples processed with non-availability of the smears for 14 patients. 38% (19/50) H&E stained biopsy sections were positive for LD bodies. Only 11 patients demonstrated LD positivity in both the tissue smears and the histologic sections. Patients negative for LD bodies were, however, positive for the infection based on the PCR based parasite detection. Also, the CL
lesion-specific histopathological analysis of biopsy sections performed for 50 patients exhibited characteristic dermal and epidermal changes to variable extents (Appendix Figure 3, panels A–F; Appendix Table 3). The characteristic CL lesion-specific epidermal changes with acanthosis and papillomatosis along with varying degree of keratosis accompanied granulomatous inflammation as accessed by a trained pathologist. These findings confirmed that all the 60 CL cases were positive for Leishmania infection and displayed varying degrees of CL specific lesonal pathologies.

References

1. Bain BJ, Bates I, Laffan MA. Dacie and Lewis practical haematology e-book. London: Elsevier Health Sciences; 2016 [cited 2020 Apr 3]. https://www.elsevier.com/books/dacie-and-lewis-practical-haematology/bain/978-0-7020-6696-2

2. Lever WF, Elder DE, Elenitsas R, Johnson BL, Murphy GF. Lever’s histopathology of the skin. Philadelphia: Wolters Kluwer Health; 2009.

3. Salotra P, Sreenivas G, Pogue GP, Lee N, Nakhasi HL, Ramesh V, et al. Development of a species-specific PCR assay for detection of Leishmania donovani in clinical samples from patients with kala-azar and post-kala-azar dermal leishmaniasis. J Clin Microbiol. 2001;39:849–54. PubMed https://doi.org/10.1128/JCM.39.3.849-854.2001

4. el Tai NO, Osman OF, el Fari M, Presber W, Schönian G. Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of Leishmania donovani spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. Trans R Soc Trop Med Hyg. 2000;94:575–9. PubMed https://doi.org/10.1016/S0035-9203(00)90093-2

5. Dávila AM, Momen H. Internal-transcribed-spacer (ITS) sequences used to explore phylogenetic relationships within Leishmania. Ann Trop Med Parasitol. 2000;94:651–4. PubMed https://doi.org/10.1080/00034983.2000.11813588

6. Yangzom T, Cruz I, Bern C, Argaw D, den Boer M, Vélez ID, et al. Endemic transmission of visceral leishmaniasis in Bhutan. Am J Trop Med Hyg. 2012;87:1028–37. PubMed https://doi.org/10.4269/ajtme.2012.12-0211

7. Siriwardana HV, Noyes HA, Beeching NJ, Chance ML, Karunaweera ND, Bates PA. Leishmania donovani and cutaneous leishmaniasis, Sri Lanka. Emerg Infect Dis. 2007;13:476–8. PubMed https://doi.org/10.3201/eid1303.060242
8. Ranasinghe S, Zhang W-W, Wickremasinghe R, Abeygunasekera P, Chandrasekharan V, Athauda S, et al. *Leishmania donovani* zymodeme MON-37 isolated from an autochthonous visceral leishmaniasis patient in Sri Lanka. Pathog Glob Health. 2012;106:421–4. PubMed https://doi.org/10.1179/2047773212Y.0000000054

9. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004;32:1792–7. PubMed https://doi.org/10.1093/nar/gkh340

10. Felsenstein J. PHYLIP (phylogeny inference package) version 3.6. 2005 [cited 2019 Dec 11]. http://evolution.genetics.washington.edu/phylip.html

11. Clamp M, Cuff J, Searle SM, Barton GJ. The Jalview Java alignment editor. Bioinformatics. 2004;20:426–7. PubMed https://doi.org/10.1093/bioinformatics/btg430

12. Thakur L, Singh KK, Shanker V, Negi A, Jain A, Matlashewski G, et al. Atypical leishmaniasis: a global perspective with emphasis on the Indian subcontinent. PLoS Negl Trop Dis. 2018;12:e0006659. PubMed https://doi.org/10.1371/journal.pntd.0006659

13. Kumari S, Garg A. Lip leishmaniasis: a new emerging clinical form of cutaneous leishmaniasis from sub-Himalayan Region. Journal of Medical Science and Clinical Research. 2018;06:62–9.

14. Sharma NL, Mahajan VK, Negi AK. Epidemiology of a new focus of localized cutaneous leishmaniasis in Himachal Pradesh. J Commun Dis. 2005;37:275–9.

### Appendix Table 1. Expansion of older and newer endemic pockets of atypical CL in Himachal Pradesh

| Blocks/Tehsils | Newer blocks/tehsils | Earlier reported blocks/tehsils | References |
|----------------|---------------------|---------------------------------|------------|
| Kinnaur        | Kalpa, Sangla       | Nichar, Pooh                    | (14)       |
| Shimla         | Shimla, Nankhari, Kotkhai, Mashobra, Theog, Chaupal, Basantpur, Rohru | Rampur, Kumarsain | (13)       |
| Kullu          | Banjar              | Aani, Nirmand                   |            |
| Newer districts | Newer blocks/tehsils |                                 |            |
| Solan          | Kunihar             |                                 |            |
| Mandi          | Karsog, Thunag, Nehri, Sarkaghat | | |

*For older districts, reports cover all reported through 2017.
### Appendix Table 2. Baseline characteristics and clinical findings: cutaneous leishmaniasis patients from Himachal Pradesh

| Total no. of CL cases, N = 60 | 0–20 | 21-40 | >40 | No. (%) by age group, y | Persons with occupations most affected (students and farmers) | Duration till clinical diagnosis | Patients with *MCL*-like lesions | Parasite detection, No. (%) |
|---|---|---|---|---|---|---|---|---|
| Male, 29/60 | 10/29 (34.5) | 14/29 (48.3) | 5/29 (17.2) | 12/20 (60) | 25 d to 2.6 y | 6/29 (20.7) | | Giemsa touch smears LD +ve= 23/46 (50) |
| | | | | | | | | +ve LD+ve= 19/50 (38) |
| | | | | | | | | +ve = 47/57 (~82) |
| | | | | | | | | +ve = 33/55 (60) |
| | +ve LD+ve= 10/21 (47.6) | 10/23 (43.47) | 23/57 (40.3) | 16/55 (29) |
| | -ve LD+ve= 8/21 (38.1) | 13/23 (56.52) | 6/57 (10.5) | 11/55 (20) |
| | Doubtful LD+ve= 2/21 (9.5) | | | |
| | Not Done LD+ve= 8/23 | 6/29 | | |
| | +ve LD+ve= 13/25 (52) | 9/27 (33.3) | 24/57 (42.1) | 17/55 (31) |
| | -ve LD+ve= 10/25 (40) | 18/27 (66.6) | 4/57 (7) | 11/55 (20) |
| | Doubtful LD+ve= 3/25 (12) | | | |
| | Not done LD+ve= 6/31 | 4/31 | | |

| Female, 31/60 | 8/31 (25.8) | 12/31 (38.7) | 11/31 (35.5) | 13/20 (65) | 10 d to 1.6 y | 10/31 (32.3) | | Giemsa touch smears |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | H&E biopsy sections |
| | | | | | | | | +ve = 47/57 |
| | | | | | | | | +ve = 33/55 (60) |
| | +ve LD+ve= 13/25 (52) | 9/27 (33.3) | 24/57 (42.1) | 17/55 (31) |
| | -ve LD+ve= 10/25 (40) | 18/27 (66.6) | 4/57 (7) | 11/55 (20) |
| | Doubtful LD+ve= 3/25 (12) | | | |
| | Not done LD+ve= 6/31 | 4/31 | | |
### Appendix Table 3. Histopathological features of CL specific lesions in ACL patients from Himachal Pradesh

| Histopathological feature | No. (%) ACL cases, N = 50 |
|---------------------------|---------------------------|
| **Epidermal changes**     |                           |
| Acanthosis                | 28 (56)                   |
| Papilomatosis             | 11 (22)                   |
| Hyperkeratosis            | 19 (38)                   |
| Parakeratosis             | 15 (30)                   |
| Spongiosis                | 3 (6)                     |
| Subcorneal blister        | 7 (14)                    |
| **Dermal changes**        |                           |
| Granuloma                 | 22 (44)                   |
| Histiocytes               | 19 (38)                   |
| Epitheloid cells          | 39 (78)                   |
| Plasma cells              | 37 (74)                   |
| Giant cell                | 3 (6)                     |
| Lymphocytes               | 23 (46)                   |
| Neutrophils               | 30 (60)                   |
| Microabscess formation    | 3 (6)                     |
| Occasional eosinophils    | 11 (22)                   |
| Civatte bodies            | 1 (2)                     |
| Fibrosis                  | 3 (6)                     |
| Spongiosis                | 3 (6)                     |
| LD bodies                 | 19 (38)                   |

*ACL, atypical cutaneous leishmaniasis

### Appendix Table 4. Accession numbers of ITS1 and 6PGDH sequences of L. donovani isolates from dermal lesions of cutaneous leishmaniasis patients from Himachal Pradesh

| Sample no. | ITS1 accession no. | 6PGDH accession no. |
|------------|--------------------|----------------------|
| HPCL1      | MG982941           | MH208423             |
| HPCL4      | MG982942           |                      |
| HPCL6      | MG982943           | MH208424             |
| HPCL7      | MG982944           | MH208425             |
| HPCL8      | MG982945           |                      |
| HPCL10     | MG982946           |                      |
| HPCL11     | MG982947           | MH208426             |
| HPCL12     | MG982948           | MH208427             |
| HPCL13     | MG982949           | MH208428             |
| HPCL15     | MG982950           | MH208429             |
| HPCL17     | MG982951           |                      |
| HPCL18     | MG982952           |                      |
| HPCL19     | MG982953           | MH208430             |
| HPCL20     | MG982954           | MH208431             |
| HPCL22     | MG982955           |                      |
| HPCL24     | MG982956           |                      |
| HPCL26     | MG982957           | MH208432             |
| HPCL27     | MG982958           | MH208433             |
| HPCL28     | MG982959           | MH208434             |
| HPCL29     | MG982960           | MH208435             |
| HPCL30     | MG982961           |                      |
| HPCL31     | MG982962           |                      |
| HPCL32     | MG982963           |                      |
| HPCL33     | MG982964           |                      |
| HPCL34     | MG982965           | MH208436             |
| HPCL35     | MG982966           | MH208437             |
| HPCL36     | MG982967           |                      |
| HPCL37     | MG982968           |                      |
| HPCL38     | MG982969           | MH208438             |
| HPCL39     | MG982970           | MH208439             |
| HPCL41     | MG982971           | MH208440             |
| HPCL42     | MG982972           | MH208441             |
| HPCL43     | MG982973           |                      |
| HPCL44     | MG982974           | MH208442             |
| HPCL45     | MG982975           | MH208443             |
| HPCL46     | MG982976           | MH208444             |
| HPCL47     | MG982977           | MH208445             |
| HPCL49     | MG982978           | MH208446             |
Appendix Figure 1. District map of Himachal Pradesh showing geographic distribution of atypical cutaneous leishmaniasis (ACL) cases in the newly endemic state. ACL cases from the skin OPD of reference hospitals (Indira Gandhi Medical College and Mahatma Gandhi Medical Services Complex, Shimla) and our data on patients enrolled in the study form the basis of data for disease distribution. Disease endemicity in state districts is indicated by different colors: yucca yellow shading for previously reported endemic districts and tzavorite green shading for newly emerging endemic districts. Regions with blue and red dots indicate previously reported and newly emerging endemic Blocks/Tehsils in the endemic districts of the state, respectively. The map was created using ArcGIS 10.3 software. The map showing regional distribution of disease was geo-referenced with UTM projection taking WGS84 datum. The unit of measurement for the scale bar is Kilometers.
Appendix Figure 2. A) CL patients with characteristic lesions on exposed parts of the body. B) Giemsa stained tissue touch smears from patients with cutaneous leishmaniasis showing intracellular and extracellular LD bodies (arrowed). Original magnification x100. C) Hematoxylin & Eosin stained CL lesional biopsy sections showing intracellular and extracellular LD bodies (arrowed). Original magnification x100.
Appendix Figure 3. Histopathological characteristics of CL lesional tissue biopsies. Representative histopathological observations on Hematoxylin & Eosin stained tissue biopsy sections from CL patients. A) Mild acanthosis. Original magnification x4. B) Papilomatosis. Original magnification x10. C) Epitheloid cell granuloma (circled). Original magnification x40. D) Langhans giant cell (arrowed). Original magnification x40. E) Diffuse inflammatory cell infiltrate showing plasma cells and epitheloid cells. Original magnification x40. F) Microabscess. Original magnification x40.
Appendix Figure 4: *Leishmania* species-specific ITS1 PCR on DNA isolated from lesional biopsy samples from cutaneous leishmaniasis (CL) patients and *Hae*III PCR–RFLP analysis of ITS1 region in test samples and standard *Leishmania* cultures used as positive controls. ITS1 PCR on cutaneous leishmaniasis patient samples. Lanes: 1, *L. major*; 2, *L. donovani*; 3, 100 bp DNA marker; 4–15, CL test samples; 16, water control. *Hae*III RFLP of ITS1 PCR amplicon for *Leishmania* species identification. Lanes: 1, *L. major*; 2, *L. donovani*; 3, 100 bp DNA ladder; 4–15, CL test samples; 16, water control. ITS1, internal transcribed spacer 1; RFLP, restriction fragment length polymorphism.
Appendix Figure 5: 6PGDH PCR on cutaneous leishmaniasis (CL) patient lesional biopsy test samples. Lanes: 1, 100 bp DNA ladder; 2, *L. donovani*; 3–10, CL patient samples from Himachal Pradesh. 6PGDH, 6-phosphogluconate dehydrogenase gene.

Appendix Figure 6. Systemic circulation of *L. donovani* in CL patients. A) Rapid Immunochromatographic rK39 dipstick test using serum samples from cutaneous leishmaniasis patients and visceral leishmaniasis patients used as positive controls for detection of circulatory *L. donovani* specific anti-rK39 antibody. The single and double band reflects negative and positive results respectively. B)
ITS1 Leishmania species-specific PCR on DNA from blood samples of specific ACL patients. ITS1-PCR product of ~320 bp in test samples and standard L. donovani positive control samples. Lanes: 1, L. donovani (positive control); 2, 50 bp DNA ladder; 3 to 8, CL test samples. HaeIII PCR-RFLP analysis of ITS1. Lanes: 1, L. donovani (positive control); 2, 50 bp DNA ladder; 3 to 8, CL test samples.