# Source Tracing of *Leishmania donovani* in Emerging Foci of Visceral Leishmaniasis, Western Nepal

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We sequenced *Leishmania donovani* genomes in blood samples collected in emerging foci of visceral leishmaniasis in western Nepal. We detected lineages very different from the preelimination main parasite population, including a new lineage and a rare one previously reported in eastern Nepal. Our findings underscore the need for genomic surveillance.

*Leishmania* spp. are parasitic protozoans that cause human leishmaniasis in multiple forms, including visceral leishmaniasis (VL), which affects the internal organs. For decades, the Indian subcontinent (ISC)—a geographic region that includes Bangladesh, Bhutan, India, Maldives, Nepal, Pakistan, and Sri Lanka—was the most endemic region for VL in the world. In 2005, a regional elimination program was launched in India, Nepal, and Bangladesh, aiming to reduce VL annual incidence to <1 case/10,000 population at subdistrict and district levels (*1*). Before the start of the program, VL in Nepal was confined mainly to 12 VL endemic districts (out of 77), located in the eastern lowlands. Recently, VL cases in Nepal have spread westward, as well as from lowlands to hilly and even mountainous areas, resulting in a current total of 23 official VL endemic districts, with many more districts reporting likely indigenous cases (*1*). Cutaneous leishmaniasis is also becoming more common (*2*), and combined cases of VL and cutaneous leishmaniasis have been reported, without any information to date on the parasite species and genotype involved. There is clearly a need for a postelimination surveillance system adapted to this new epidemiologic profile.

Molecular surveillance of infectious diseases may provide the most relevant information for control programs, such as following the evolution of epidemics in time and space, characterizing of new transmission cycles, conducting outbreak studies and source identification, and detecting new variants with new clinical features (*3*). Currently, no molecular surveillance is being implemented for leishmaniasis in the world, despite the existence of suitable technologies. We previously showed the feasibility and added value of direct whole genome sequencing (SureSelect sequencing [SuSL-seq]; Agilent Technologies, https://www.agilent.com) of *L. donovani* in host tissues, without the need for parasite isolation and cultivation (*4*).

Here, we demonstrate the proof-of-principle of SuSL-seq for genome surveillance of leishmaniasis, in the context of the reported expansion of VL to the western regions of Nepal. We collected blood samples in 2019 and stored them on DNA/RNA Shield (Appendix). We performed sequencing on 3 samples with the highest amounts of DNA, positive for *Leishmania*, and originating from 3 different districts in Nepal (Dolpa, Darchula, and Bardiya) (Appendix Table 1, Figure 1) and compared them with our database of *L. donovani* genome sequences from the ISC. All samples showed a high genome coverage (Appendix Table 2). The database comparison samples originated from 204 cultivated isolates (2002–2011) from Nepal, India, and Bangladesh (*5*); 52 clinical samples (2000–2015) from Nepal (*4*); and 3 isolates (2002, 2010) from Sri Lanka (*6*,*7*). Altogether, these earlier studies reported 4 main genotypes: a large core group (CG), genomically very homogeneous, in the lowlands of India, Nepal, and Bangladesh; a small ISC1 population, genomically very different from CG, in hilly districts of Eastern Nepal; a single divergent isolate from Nepal, BPK512; and a Sri Lanka (SL) cluster. New phylogenomic analyses (Figure) revealed that the samples from the 3 new foci from western Nepal were clearly distinct from CG and SL: one ISC1-related lineage (024) had not been reported previously, and the 2 other lineages (022 and 023) clustered together with BPK512.

It is premature to conclude that ISC1-related (024) and BPK512-like (022, 023) parasites are expanding, spreading, and replacing CG in a postelimination phase. However, a study based on single-locus genotyping showed a much higher proportion of ISC1 and unclassified genotypes (and a strong decrease of CG) during 2012–2014 compared with 2002–2011 (*9*). Considering the genomic differences between these lineages and CG and their transmissibility by *Phlebotomus argentipes* (*10*), we recommend particular attention to the further evolution of parasites in regions of the ISC. Our previous work evidenced several important functional differences between isolates from

ISC1 and CG (Appendix), and we found in this investigation allele differences in 8 of 10 genes previously shown to be involved in *L. donovani* drug resistance (Appendix Figure 2). Of particular interest, those genetic variants are common in the ISC1 group and in the BPK512 but never found in CG parasites. Without experimental confirmation, it is difficult to speculate about the exact impact of this polymorphism on the resistance to antileishmanial drugs, but it is clear that these parasites are genetically (and, likely, functionally) very diverse from the CG parasites, which were the main target of the recent elimination efforts.

Molecular surveillance requires a method applicable on routine samples collected in any type of field settings. We demonstrate that small amounts of blood from routine examination of patients with VL could be successfully used for direct, sensitive, and untargeted whole-genome analysis of *Leishmania*. Our optimized SuSL-seq protocol enables highly discriminatory genotyping and targeted analysis of the genetic variation within selected loci as well as untargeted searching for new markers related to a clinical or epidemiologic question. Our research supports the need for genomic surveillance of VL—in particular in

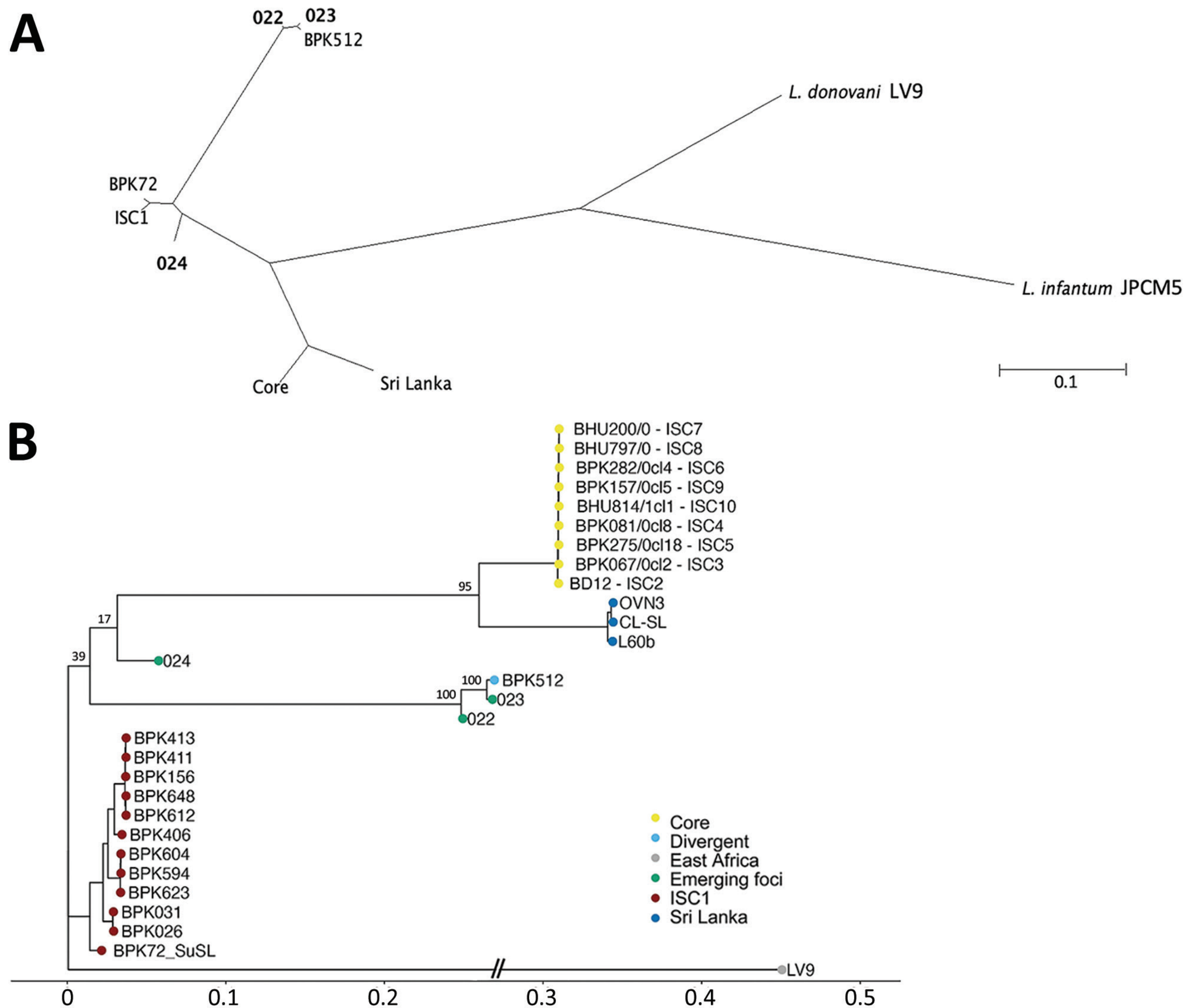


**Figure.** Phylogenetic analyses of *Leishmania donovani* from the ISC, including Nepal, and reference sequences. Trees were based on genomewide single-nucleotide polymorphisms using RAxML (*8*). A) Unrooted phylogenetic network of the *L. donovani* complex, showing samples representing the emerging foci (bold text). B) Rooted phylogenetic tree of reference strains of *L. donovani* from the ISC, showing the branching of 3 samples (022, 023, and 024) originating from emerging foci. Important bootstrap values are indicated on the branches. The West-African LV9 strain is included as an outgroup. BPK72_SuSL represents an ISC1 sample analyzed using SureSelect sequencing (Agilent Technologies; https://www.agilent.com), confirming that the branching of the emerging foci is not a result of a technical artifact. Scale bars indicate number of single-nucleotide polymorphism differences. ISC, Indian subcontinent.

the context of the current elimination program in the ISC—and demonstrate the applicability of SuSL-seq to molecular surveillance of blood. Continued collaborations will be required to translate these new approaches for VL surveillance to the specific needs of the region.

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Genomic sequence reads of the parasites from the 3 new foci are available on the European Nucleotide Archive (https://www.ebi.ac.uk/ena) under accession no. PRJNA991731.

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## References

1. Pandey K, Dumre SP, Shah Y, Acharya BK, Khanal L, Pyakurel UR, et al. Forty years (1980–2019) of visceral leishmaniasis in Nepal: trends and elimination challenges. Trans R Soc Trop Med Hyg. 2023;117:460–9. https://doi.org/10.1093/trstmh/trad001
2. Pandey K, Bastola A, Haiyan G, Pyakurel UR, Pandey BD, Dumre SP. Emergence of cutaneous leishmaniasis in Nepal. Trop Med Health. 2021;49:72. https://doi.org/10.1186/s41182-021-00359-3
3. Domagalska MA, Dujardin JC. Next-generation molecular surveillance of TriTryp diseases. Trends Parasitol. 2020;36:356–67. https://doi.org/10.1016/j.pt.2020.01.008
4. Domagalska MA, Imamura H, Sanders M, Van den Broeck F, Bhattarai NR, Vanaerschot M, et al. Genomes of *Leishmania* parasites directly sequenced from patients with visceral leishmaniasis in the Indian subcontinent. Rogers MB, editor. PLoS Negl Trop Dis. 2019;13:e0007900. https://doi.org/10.1371/journal.pntd.0007900
5. Imamura H, Downing T, Van den Broeck F, Sanders MJ, Rijal S, Sundar S, et al. Evolutionary genomics of epidemic visceral leishmaniasis in the Indian subcontinent. eLife. 2016;5:e12613. https://doi.org/10.7554/eLife.12613
6. Franssen SU, Durrant C, Stark O, Moser B, Downing T, Imamura H, et al. Global genome diversity of the *Leishmania donovani* complex. eLife. 2020;9:e51243. https://doi.org/10.7554/eLife.51243
7. Zhang WW, Ramasamy G, McCall LI, Haydock A, Ranasinghe S, Abeygunasekara P, et al. Genetic analysis of *Leishmania donovani* tropism using a naturally attenuated cutaneous strain. PLoS Pathog. 2014;10:e1004244. https://doi.org/10.1371/journal.ppat.1004244
8. Kozlov AM, Darriba D, Flouri T, Morel B, Stamatakis A. RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. Bioinformatics. 2019;35:4453–5. https://doi.org/10.1093/bioinformatics/btz305
9. Rai K, Bhattarai NR, Vanaerschot M, Imamura H, Gebru G, Khanal B, et al. Single locus genotyping to track *Leishmania donovani* in the Indian subcontinent: Application in Nepal. PLoS Negl Trop Dis. 2017;11:e0005420. https://doi.org/10.1371/journal.pntd.0005420
10. Seblova V, Dujardin JC, Rijal S, Domagalska MA, Volf P. ISC1, a new *Leishmania donovani* population emerging in the Indian sub-continent: Vector competence of *Phlebotomus argentipes.* Infect Genet Evol. 2019;76:104073. https://doi.org/10.1016/j.meegid.2019.104073

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# *Enterocytozoon bieneusi* Infection after Hematopoietic Stem Cell Transplant in Child, Argentina

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## Appendix

### Ethical clearance, clinical and data procedures

Ethical approval was obtained from the Institutional Review Committee of B.P. Koirala Institute of Health Sciences, Dharan, Nepal, as well as from the Nepal Health Research Council, Kathmandu, Nepal. In addition, ethical approval was obtained by the Institutional Review Board of the Institute of Tropical Medicine, Antwerp, and the Ethics Committee of the University Hospital of Antwerp, Belgium. Collaborating VL treatment centers were asked to collect a 2ml venous blood sample from all consenting newly diagnosed VL patients before the start of any treatment in January and February 2019. VL was diagnosed in line with the clinical algorithm recommended in the National Guidelines, i.e., fever >2 weeks in combination with splenomegaly and a positive rK39 Rapid Diagnostic Test (RDT). In addition, information was collected on basic demographic factors as well as the village of residence and travel history in the 2 years before the start of symptoms (Appendix Table 1, Appendix Figure 1). Samples were collected in DNA/RNA shield and stored at room temperature until transportation to the laboratory facilities of the Central Department of Microbiology at Tribhuvan University, Kathmandu, Nepal, where they were stored at −20°C until DNA extraction. DNA extracts were shipped to the laboratory facilities of the Institute of Tropical Medicine for further sequencing.

## Laboratory procedures

### DNA extraction and sequencing

200µl of blood mixed with 200µl DNA/RNA Shield was used to extract DNA with QIAamp DNA Mini Kit (Qiagen) according to manufacturer's instructions with the following modifications: 30µl Proteinase K and 300µl ethanol were used instead of 20µl and 200µl, respectively. DNA concentration was verified using the Qubit broad-range DNA quantification kit (Thermo Fisher Scientific), and the % of *Leishmania* DNA in the samples was estimated using qPCR as described previously (*1*). In our previous study (*1*), we demonstrated that a percentage of *Leishmania* DNA of 0.006% was found to be the lowest limit for suitable analysis of genome diversity. In present study, the *Leishmania* % in the selected samples was 0.13, 0.16 and 0.03 in samples 022, 023, and 024, respectively. SureSelect (Agilent Technologies) was used to capture *Leishmania* genomic DNA following standard SureSelect XTHS Target Enrichment system protocol for Illumina Multiplexed Sequencing platforms. Prior the genome capture, DNA was concentrated using AMPure XP beads (Beckman, Coulter) to obtain ≈10ng of total genomic DNA in 7 µl that was subjected to enzymatic fragmentation using SureSelect XT HS Fragmentation kit (Agilent Technologies). Custom designed oligonucleotide baits were used at 1:10 stock dilution.

Sequencing was conducted on the Illumina NovaSeq platform using 2x150 bp paired reads at GenomeScan (Netherlands), for which 51.49, 51.59 and 50.35 million raw reads were obtained for sample 022, 023 and 024 respectively (Appendix Table 2).

### Bioinformatic procedures

In addition to the newly sequenced data, additional sequencing data were obtained from previous publications: i) genomes describing the population structure of *L. donovani* in Nepal, India and Bangladesh (*2*), ii) sequencing data obtained from samples from Nepal using the SureSelect technology (*1*), similar to the approach used for the three outbreak samples in this report, iii) three genomes originating from Sri Lanka (*3*,*4*), and iv) the *L. infantum* sequencing data submitted under the accession number ERR1913337 (*5*). All publicly available sequencing data were downloaded using the SRAtoolkit software.

The reads were mapped to the reference genome *L. donovani* available at NCBI (accession number GCF_000227135.1) using BWA (version 0.7.17 (*6*)) with a seed length set to

50 17 (*6*). Only properly paired reads with a mapping quality higher than 30 were selected using SAMtools (*7*). Duplicate reads were removed using the RemoveDuplicates command in the Picard software (version 2.22.4, http://broadinstitute.github.io/picard/). SNP calling was performed using the Genome Analysis ToolKit (GATK) (*8*) pipeline (version 4.1.4.1) following the GATK best practices approach: 1) GATK HaplotypeCaller enabling the production of GVCF formatted files, 2) GVCF files of all samples were combined using the GATK CombineGVCF command, 3) genotyping was performed via the GATK GenotypeGVCF command, and 4) filtering of the SNPs and indels was carried out following the "best practices" approach as suggested on the GATK support site using the SelectVariants and VariantFiltration commands. Regions in the vcf-file corresponding to known drug resistance markers were selected using BCFtools, and visualized using the pheatmap function in R.

Phylogenetic trees were constructed using RAxML (*9*). First, the VCF files containing biallelic SNPs were selected using BCFtools (*7*) and were converted to Phylip format using the vcf2phylip.py script (https://github.com/edgardomortiz/vcf2phylip). RAxML was then executed with the GTR+G substitution model, using 1000 bootstrap replicates. The *L. infantum* JPCM5 or the *L. donovani* LV9 genome was employed as an outgroup. The resulting phylogenetic trees were visualized using ggtree (*10*) for rooted phylogenetic trees and SplitsTree (*11*) for unrooted phylogenetic networks.

## Results from the analyses of specific genes reported to be involved in drug resistance

We selected 10 loci that were previously shown to be involved in *L. donovani* resistance to known antileishmanial drugs:

- Antimony: Aquaglyceroporin 1, AQP1 LDBPK_310030 (*12*); ABC transporter MRPA, MRPA LDBPK_230290 (*13*)
- Amphotericin B: sterol C5-desaturase, C5D LDBPK_231560 (*14*); sterol C24-methyltransferase, SMT LDBPK_362520 (*14*)
- Miltefosine: *Leishmania donovani* miltefosine transporter, LdMT LDBPK_131590 (*15*); Beta-subunit of LdMT, LdRos3 LdBPK_320540 (*15*) and genes part of miltefosine sensitivity locus (*16*): 3,2-trans-enoyl-CoA isomerase 1 and 2, TECI1

LdBPK_312320 and TECI2 LdBPK_312400; helicase-like protein, HELI LdBPK_312390; 3′-nucleotidase/nuclease, NUC LdBPK_312380.

For each of the three new *L. donovani* genomes, the sequence of the 10 loci was studied in detail. The 10 loci were well-covered and in 8 out of the 10 genes we found at least one homozygous single nucleotide polymorphism (SNP), which results in a missense mutation or a frameshift in at least 2 out of 3 samples from new emerging loci (Appendix Figure 2). No significant changes were observed for LdRos3 and SMT.

**Functional differences previously reported between ISC1 and CG isolates**

Noteworthy, all results here compiled concern the analysis of isolated and cultivated parasites.

In a first study, we demonstrated that CG parasites were intrinsically more tolerant to trivalent antimonials than ISC1 ones. This phenotype was driven by the amplification of a locus containing MRPA, a gene involved in $Sb^{III}$ sequestration (*13*)

In a second study, we made an integrated genomic and metabolomic profiling of ISC1 vs CG isolates (*17*). We found several genomic differences including SNPs, CNV and small indels in genes coding for known virulence factors, immunogens and surface proteins. With respect to the metabolome, we found differences in several functional groups and pathways, essentially:

(i) **Lipid metabolism**, with 19 glycerophospholipids (GPLs) showing significantly different levels between both groups: GPLs are involved in a wide array of cellular functions including host cell infection

(ii) **Urea cycle**. In ISC1 versus CG we detected a higher concentration of citrulline and a lower concentration of argininosuccinate: Mutants for argininosuccinate synthase genes have shown a lower virulence than WT parasites.

(iii) **Nucleotide salvage pathway**. This pathway is essential, since *Leishmania* cannot synthesize the purine ring de novo and is therefore dependent on salvaging these from host purines. Our previous results suggested that ISC1 parasites might be better at salvaging nucleotides from their environment.

In a third study, we experimentally demonstrated that ISC1 and CG strains are developing similarly in natural ISC vector *Phlebotomus argentipes*, suggesting that *P. argentipes* is a fully competent vector for ISC1 parasites (*18*).

Altogether, these experimental studies demonstrate differences between ISC1 and CG in antimonial susceptibility and predict major functional differences, including virulence. Taking into account that ISC1 can easily be transmitted by *P. argentipes*, particular attention is required to monitor the fate of ISC1-related population in the region, especially in a post-VL elimination context. Genomic surveillance can clearly be done with the approach here used, but this should be complemented by phenotyping of the new detected *L. donovani* variants.

**References**

1. Domagalska MA, Imamura H, Sanders M, Van den Broeck F, Bhattarai NR, Vanaerschot M, et al. Genomes of *Leishmania* parasites directly sequenced from patients with visceral leishmaniasis in the Indian subcontinent. Rogers MB, editor. PLoS Negl Trop Dis. 2019 Dec 12; 13(12):e0007900. Available from: https://dx.plos.org/10.1371/journal.pntd.0007900

2. Imamura H, Downing T, Van den Broeck F, Sanders MJ, Rijal S, Sundar S, et al. Evolutionary genomics of epidemic visceral leishmaniasis in the Indian subcontinent. eLife. 2016;5:e12613. PubMed https://doi.org/10.7554/eLife.12613

3. Franssen SU, Durrant C, Stark O, Moser B, Downing T, Imamura H, et al. Global genome diversity of the *Leishmania donovani* complex. eLife. 2020;9:e51243. PubMed https://doi.org/10.7554/eLife.51243

4. Zhang WW, Ramasamy G, McCall LI, Haydock A, Ranasinghe S, Abeygunasekara P, et al. Genetic analysis of *Leishmania donovani* tropism using a naturally attenuated cutaneous strain. PLoS Pathog. 2014;10:e1004244. PubMed https://doi.org/10.1371/journal.ppat.1004244

5. Camacho E, González-de la Fuente S, Rastrojo A, Peiró-Pastor R, Solana JC, Tabera L, et al. Complete assembly of the *Leishmania donovani* (HU3 strain) genome and transcriptome annotation. Sci Rep. 2019;9:6127. PubMed https://doi.org/10.1038/s41598-019-42511-4

6. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25:1754–60. PubMed https://doi.org/10.1093/bioinformatics/btp324

7. Danecek P, Bonfield JK, Liddle J, Marshall J, Ohan V, Pollard MO, et al. Twelve years of SAMtools and BCFtools. Gigascience. 2021 Feb 1; 10(2):giab008. Available from: https://pubmed.ncbi.nlm.nih.gov/33590861/

8. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 2010;20:1297–303. PubMed https://doi.org/10.1101/gr.107524.110

9. Kozlov AM, Darriba D, Flouri T, Morel B, Stamatakis A. RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. Bioinformatics. 2019;35:4453–5. PubMed https://doi.org/10.1093/bioinformatics/btz305

10. Yu G. Using ggtree to Visualize Data on Tree-Like Structures. Curr Protoc Bioinformatics. 2020;69:e96. PubMed https://doi.org/10.1002/cpbi.96

11. Huson DH, Bryant D. Application of phylogenetic networks in evolutionary studies. Mol Biol Evol. 2006;23:254–67. PubMed https://doi.org/10.1093/molbev/msj030

12. Potvin JE, Leprohon P, Queffeulou M, Sundar S, Ouellette M. Mutations in an Aquaglyceroporin as a Proven Marker of Antimony Clinical Resistance in the Parasite *Leishmania donovani.* Clin Infect Dis. 2021;72:e526–32. PubMed https://doi.org/10.1093/cid/ciaa1236

13. Dumetz F, Cuypers B, Imamura H, Zander D, D'Haenens E, Maes I, et al. Molecular Preadaptation to Antimony Resistance in *Leishmania donovani* on the Indian Subcontinent. MSphere. 2018;3:e00548–17. PubMed https://doi.org/10.1128/mSphere.00548-17

14. Pountain AW, Weidt SK, Regnault C, Bates PA, Donachie AM, Dickens NJ, et al. Genomic instability at the locus of sterol C24-methyltransferase promotes amphotericin B resistance in *Leishmania* parasites. PLoS Negl Trop Dis. 2019;13:e0007052. PubMed https://doi.org/10.1371/journal.pntd.0007052

15. Pérez-Victoria FJ, Sánchez-Cañete MP, Castanys S, Gamarro F. Phospholipid translocation and miltefosine potency require both *L. donovani* miltefosine transporter and the new protein LdRos3 in *Leishmania* parasites. J Biol Chem. 2006;281:23766–75 . PubMed https://doi.org/10.1074/jbc.M605214200

16. Carnielli JBT, Dave A, Romano A, Forrester S, de Faria PR, Monti-Rocha R, et al. 3'Nucleotidase/nuclease is required for Leishmania infantum clinical isolate susceptibility to miltefosine. EBioMedicine. 2022;86:104378. PubMed https://doi.org/10.1016/j.ebiom.2022.104378

17. Cuypers B, Berg M, Imamura H, Dumetz F, De Muylder G, Domagalska MA, et al. Integrated genomic and metabolomic profiling of ISC1, an emerging *Leishmania donovani* population in the Indian subcontinent. Infect Genet Evol. 2018;62:170–8. PubMed https://doi.org/10.1016/j.meegid.2018.04.021

18. Seblova V, Dujardin JC, Rijal S, Domagalska MA, Volf P. ISC1, a new *Leishmania donovani* population emerging in the Indian sub-continent: Vector competence of *Phlebotomus argentipes.* Infect Genet Evol. 2019;76:104073. PubMed https://doi.org/10.1016/j.meegid.2019.104073

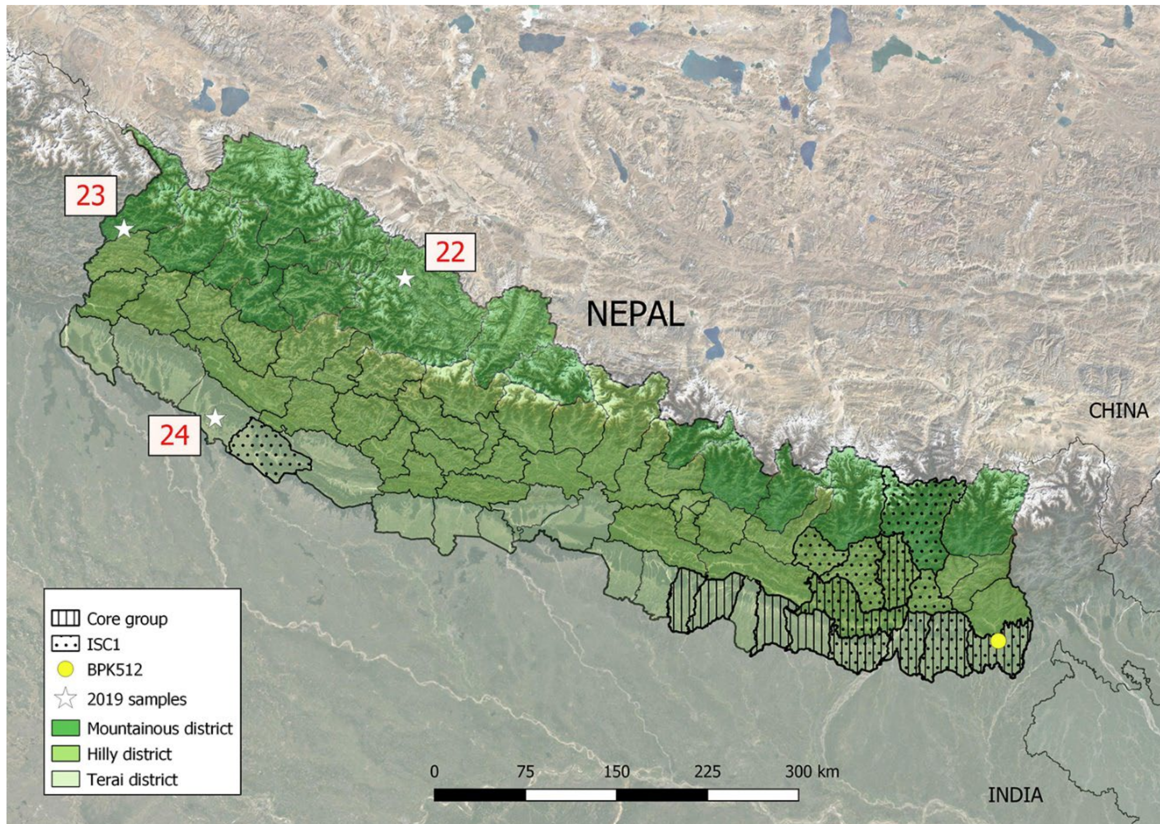
**Appendix Table 1.** Clinical and epidemiologic data of patients*

| **Sample code** | **022** | **023** | **024** |
|---|---|---|---|
| **Sequencing code** | 105328–001–022 | 105328–001–023 | 105328–001–024 |
| **date_sample_collection** | 21/01/2019 | 24/01/2019 | 07/02/2019 |
| **District** | Dolpa | Darchula | Bardiya |
| **Village** | Se-Phoxundo rural Municipality | Juga Rural Municipality | Madhuwan Municipality |
| **type_disease** | VL | VL | VL |
| **Past history of VL or PKDL** | No | Past history of VL x 6 Month | No |
| **past_drug_used** | NA | L-AmB | NA |
| **current_drug_used** | L-AmB | L-AmB + PMM | L-AmB |
| **date_treatment_start** | 21/01/2019 | 24/01/2019 | 07/02/2019 |
| **initial_outcome** | improved | improved | improved |
| **final_outcome** | no data | cured | cured |
| **travel_history_VL endemic areas** | traveled history to Surkhet districts | no travel history to VL endemic areas in Nepal & India | History of travel to Uttrakhand, India |

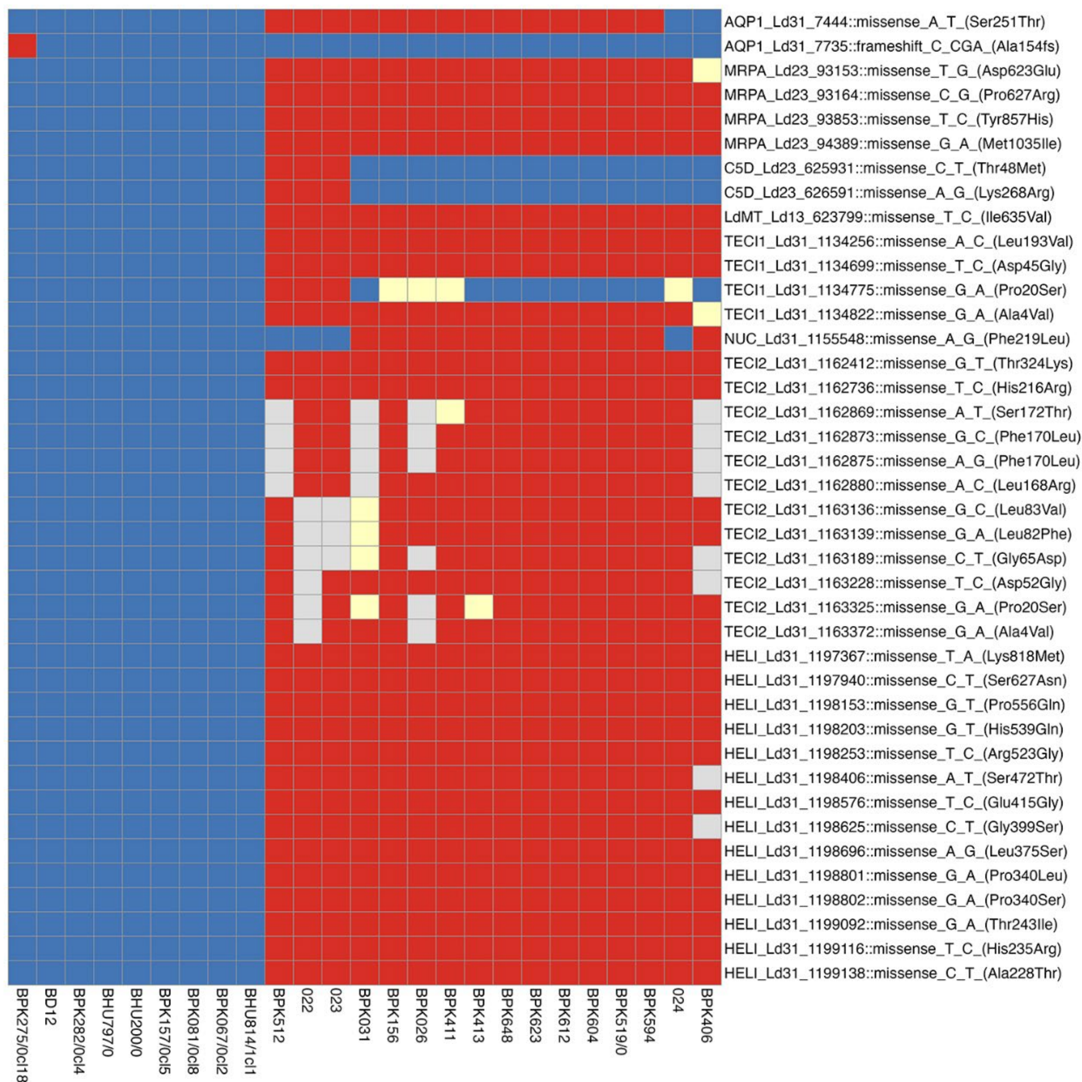
*VL, Visceral Leishmaniasis; PKDL, Post Kala Azar Dermal Leishmaniasis; NA, not applicable; L-AmB, Liposomal Amphotericin B; PMM, Paromomycin

**Appendix Table 2**. Alignment statistics of the three SureSelect processed samples. The estimated percentage of *L. donovani* DNA is the percentage obtained via qPCR analysis as described elsewhere (*1*). The enrichment factor is calculated as the ratio of the percentage of *L. donovani* reads versus the estimated percentage of *L. donovani* DNA obtained via qPCR. The percentage of *L. donovani* with 5x coverage is the percentage of the genome covered with at least 5 reads, and for which SNP detection can be performed.

| Sample | Total no. reads | No. reads mapping to *Leishmania donovani* | *L. donovani* DNA, estimated % | *L. donovani* reads, % | Enrichment factor | *L. donovani* with 5× coverage, % |
|---|---|---|---|---|---|---|
| 022 | 51,494,551 | 5,450,630 | 0.13 | 10.58 | 81 | 85.06 |
| 023 | 51,591,488 | 11,615,012 | 0.16 | 22.51 | 141 | 90.39 |
| 024 | 50,354,911 | 4,423,258 | 0.03 | 8.78 | 293 | 85.83 |




**Appendix Figure 1.** Geographic origin of the three 2019 samples. The map shows the 77 districts of Nepal and those in which parasites of the core group (vertical hatched) and ISC1 (dotted) were detected from 2000 to 2015. Map was done with qGIS version 3.28.4.




**Appendix Figure 2.** Heatmap showing the distribution of single nucleotide polymorphisms (SNPs) in genes reported to be associated with drug-resistant phenotypes. The color scheme represents different SNP categories: blue indicates the absence of SNPs, yellow indicates heterozygous SNPs, red indicates homozygous SNPs and gray indicates that not sufficient reads where present to do reliable SNP prediction. The naming convention for SNPs follows the format of the gene of interest, position in the genome, type of mutation, and its effect on the corresponding protein. Samples: BPK512, CG and ISC1 isolates used for the phylogenomic analysis of Figure 1 together with the 3 blood samples of 2019 (022, 023, 024).