# ANÁLISIS EPIDEMIOLÓGICO, GENÓMICO Y PROTEÓMICO DE *Leishmania infantum* EN COLOMBIA

**ADRIANA CATHERINE CASTILLO CASTAÑEDA**

**Documento de tesis presentado como requisito para optar al título de Doctora en Ciencias Biomédicas y Biológicas y Doctora en Salud y Desarrollo en los Trópicos**

**DOCTORADO EN CIENCIAS BIOMÉDICAS Y BIOLÓGICAS**
**UNIVERSIDAD DEL ROSARIO**
**BOGOTÁ D.C., COLOMBIA**
**DOCTORADO EN SALUD Y DESARROLLO EN LOS TRÓPICOS**
**UNIVERSIDAD DE SALAMANCA**
**SALAMANCA, ESPAÑA**

**2024**

# ANÁLISIS EPIDEMIOLÓGICO, GENÓMICO Y PROTEÓMICO DE *Leishmania infantum* EN COLOMBIA

Estudiante:
Adriana Catherine Castillo Castañeda

Director:
Juan David Ramírez González, Ph.D.
Profesor Titular
Facultad de Ciencias Naturales Universidad del Rosario

Directora:
Luz Helena Patiño Blanco, Ph.D.
Directora Técnica
Centro de Investigaciones en Microbiología y Biotecnología-UR (CIMBIUR)
Facultad de Ciencias Naturales Universidad del Rosario

Director:
Raúl Manzano Román, Ph.D.
Profesor Permanente Laboral
Centro Investigación Enfermedades Tropicales Universidad de Salamanca (CIETUS)
Facultad de Farmacia Universidad de Salamanca

Tutor:
Julio López Abán Ph.D.
Centro Investigación Enfermedades Tropicales Universidad de Salamanca (CIETUS)
Facultad de Farmacia Universidad de Salamanca

Documento de tesis presentado como requisito para optar al título de Doctora en Ciencias Biomédicas y Biológicas y Doctora en Salud y Desarrollo en los Trópicos

DOCTORADO EN CIENCIAS BIOMÉDICAS Y BIOLÓGICAS
UNIVERSIDAD DEL ROSARIO
BOGOTÁ D.C., COLOMBIA
DOCTORADO EN SALUD Y DESARROLLO EN LOS TRÓPICOS
UNIVERSIDAD DE SALAMANCA
SALAMANCA, ESPAÑA
2024

PROGRAMA DE DOCTORADO EN SALUD Y DESARROLLO EN LOS TRÓPICOS
ESCUELA DE DOCTORADO "STUDII SALAMANTINI"
UNIVERSIDAD DE SALAMANCA

**Dr. Juan David Ramírez González,** Profesor Títular de la Facultad de Ciencias Naturales de la Universidad del Rosario; **Dra. Luz Helena Patiño Blanco,** Directora Técnica del Centro de Investigaciones en Microbiología y Biotecnología-UR (CIMBIUR) de la Facultad de Ciencias Naturales de la Universidad del Rosario; y **Dr. Raúl Manzano Román,** Profesor Permanente Laboral de la Universidad de Salamanca.

INFORMAN:

Que la Tesis Doctoral realizada bajo nuestra dirección por D. Adriana Catherine Castillo Castañeda, con el título: ***"Análisis epidemiológico, genómico y proteómico de Leishmania infantum en Colombia"***, reúne los requisitos de calidad, originalidad y presentación exigibles a una investigación científica, y está en condiciones de ser sometida a la valoración del Tribunal encargado de juzgarla en la modalidad de Tesis por compendio de publicaciones para optar al GRADO DE DOCTORA por la Universidad de Salamanca.

Para que así conste a los efectos oportunos, firman la presente en Salamanca, a 25de abril de 2024.

Dr. Juan D. Ramírez González | Dra. Luz Helena Patiño Blanco | Dr. Raúl Manzano Román

V$^{a}$B$^{o}$

Julio López

Tutor Dr. Julio López Abán

La presente Tesis Doctoral titulada *“**Análisis epidemiológico, genómico y proteómico de Leishmania infantum en Colombia**”*, realizada por D. Adriana Catherine Castillo Castañeda, bajo la dirección del Dr. Juan David Ramírez, y codirección del Dr. Raúl Manzano y de la Dra. Luz Helena Patiño, corresponde a un compendio de artículos originales publicados en revistas científicas de prestigio internacional e indexadas en el Science Citation Reports, cuyos datos bibliométricos se detallan en el Anexo I. A continuación, se describen todos los artículos producidos como resultados de la presente tesis, su título, afiliaciones y autores, junto a la referencia completa de la revista científica donde fueron publicados.

**Artículo primero:**

**Título:** Spatial and Temporal Variability of Visceral Leishmaniasis in Colombia, 2007 to 2018
**Autores:** Adriana Castillo-Castañeda[1], Giovanny Herrera[1], Martha S. Ayala[2], Patricia Fuya[3], and Juan David Ramírez[1]
**Afiliación:**
[1] Centro de Investigaciones en Microbiología y Biotecnología-UR (CIMBIUR), Facultad de Ciencias Naturales, Universidad del Rosario, Bogotá, Colombia.
[2] Grupo de Parasitología, Instituto Nacional de Salud, Bogotá, Colombia.
[3] Grupo de Entomología, Instituto Nacional de Salud, Bogotá, Colombia.
**Revista:** The American Journal of Tropical Medicine and Hygiene
**Referencia completa:** Castillo-Castañeda A, Herrera G, Ayala MS, Fuya P, Ramírez JD. Spatial and Temporal Variability of Visceral Leishmaniasis in Colombia, 2007 to 2018. Am J Trop Med Hyg. 2021 Jul 7;105(1):144-155. doi: 10.4269/ajtmh.21-0103. PMID: 34232908; PMCID: PMC8274786

**Artículo segundo:**

**Título:** Development of an Amplicon-Based Next-Generation Sequencing Protocol to Identify *Leishmania* Species and Other Trypanosomatids in Leishmaniasis Endemic Areas.
**Autores:** Luz H. Patiño[a], Adriana C. Castillo-Castañeda[a], Marina Muñoz[a], Jesus E. Jaimes[a], Nicolas Luna-Niño[a], Carolina Hernández[a], Martha S. Ayala[b] , Patricia Fuya[b], Claudia Mendez[c], Carlos E. Hernández-Pereira[d], Lourdes Delgado[d], Claudia M. Sandoval-Ramírez[e], Plutarco Urbano[f], Alberto Paniz-Mondolfi[d,g] , Juan David Ramírez[a]
**Afiliación:**
[a] Centro de Investigaciones en Microbiología y Biotecnología-UR (CIMBIUR), Facultad de Ciencias Naturales, Universidad del Rosario, Bogotá, Colombia.
[b] Grupo de Parasitología, Instituto Nacional de Salud, Bogotá, Colombia.
[c] Grupo de Investigación en Enfermedades Tropicales del Ejército (GINETEJ), Laboratorio de Referencia e Investigación, Dirección de Sanidad Ejército, Bogotá, Colombia.

[d] Instituto de Investigaciones Biomédicas IDB/Incubadora Venezolana de la Ciencia, Barquisimeto, Venezuela

[e] Grupo de Investigaciones en Ciencias Básicas y Aplicadas para la Sostenibilidad (CIBAS), Facultad de Ciencias Exactas, Naturales y Agropecuarias, Universidad de Santander, Bucaramanga, Colombia

[f] Grupo de Investigaciones Biológicas de la Orinoquia, Universidad Internacional del Trópico Americano (Unitropico), Yopal, Colombia

[g] Icahn School of Medicine at Mount Sinai, New York, New York, USA

**Revista:** Microbiology Spectrum

**Referencia completa:** Patiño LH, Castillo-Castañeda AC, Muñoz M, Jaimes JE, Luna-Niño N, Hernández C, Ayala MS, Fuya P, Mendez C, Hernández-Pereira CE, Delgado L, Sandoval-Ramírez CM, Urbano P, Paniz-Mondolfi A, Ramírez JD. Development of an Amplicon-Based Next-Generation Sequencing Protocol to Identify *Leishmania* Species and Other Trypanosomatids in Leishmaniasis Endemic Areas. Microbiol Spectr. 2021 Oct 31;9(2):e0065221. doi: 10.1128/Spectrum.00652-21. Epub 2021 Oct 13. PMID: 34643453; PMCID: PMC8515931.

**Artículo tercero:**

**Título:** Amplicon-based next-generation sequencing reveals the co-existence of multiple *Leishmania* species in patients with visceral leishmaniasis

**Autores:** Adriana Castillo-Castañeda[a], Luz H. Patiño[a], Marina Muñoz[a], Martha S. Ayala[b], Maryi Segura[b], Jessica Bautista[b], Maryia V. Shaban[c], Alberto Paniz-Mondolfi[d], Juan David Ramírez[a].

**Afiliación:**

[a] Centro de Investigaciones en Microbiología y Biotecnología-UR, Facultad de Ciencias Naturales, Universidad del Rosario, Bogotá, Colombia.

[b] Grupo de Parasitología, Instituto Nacional de Salud, Bogotá, Colombia.

[c] Incubadora Venezolana de la Ciencia/Centro de Investigaciones Biomédicas IDB, Barquisimeto, Venezuela.

[d] Department of Pathology, Molecular and Cell-Based Medicine, Division of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY, USA.

**Revista:** International Journal of Infectious Diseases

**Referencia complete:** Castillo-Castañeda A, Patiño LH, Muñoz M, Ayala MS, Segura M, Bautista J, Shaban MV, Paniz-Mondolfi A, Ramírez JD. Amplicon-based next-generation sequencing reveals the co-existence of multiple *Leishmania* species in patients with visceral leishmaniasis. Int J Infect Dis. 2022 Feb;115:35-38. doi: 10.1016/j.ijid.2021.11.029. Epub 2021 Dec 1. PMID: 34863923.

**Artículo cuarto:**

**Título:** An overview of the trypanosomatid (Kinetoplastida: Trypanosomatidae) parasites infecting several mammal species in Colombia.

**Autores:** Adriana C. Castillo-Castañeda[1], Luz H. Patiño[1], Maria Fernanda Zuñiga[1], Omar Cantillo-Barraza[1,2], Martha S. Ayala[3], Maryi Segura[3], Jessica Bautista[3], Plutarco Urbano[4], Jeiczon Jaimes-Dueñez[5] and Juan David Ramírez[1,6]

**Afiliación:**

[1] Centro de Investigaciones en Microbiología y Biotecnología-UR (CIMBIUR), Facultad de Ciencias Naturales, Universidad del Rosario, Bogotá, Colombia.

[2] Grupo de Biología y Control de Enfermedades Infecciosas (BCEI), Universidad de Antioquia, Medellín, Colombia.

[3] Grupo de Parasitología, Instituto Nacional de Salud, Bogotá, Colombia.

[4] Grupo de Investigaciones Biológicas de La Orinoquía, Universidad Internacional del Trópico Americano (Unitropico), Yopal, Colombia.

[5] Grupo de Investigación en Ciencias Animales GRICA, Facultad de Medicina Veterinaria y Zootecnia, Universidad Cooperativa de Colombia UCC, Bucaramanga, Colombia.

[6] Department of Pathology, Molecular and Cell-Based Medicine, Molecular Microbiology Laboratory, Icahn School of Medicine at Mount Sinai, New York, NY, USA.

**Revista:** Parasites & Vectors

**Referencia completa:** Castillo-Castañeda AC, Patiño LH, Zuñiga MF, Cantillo-Barraza O, Ayala MS, Segura M, Bautista J, Urbano P, Jaimes-Dueñez J, Ramírez JD. An overview of the trypanosomatid (Kinetoplastida: Trypanosomatidae) parasites infecting several mammal species in Colombia. Parasit Vectors. 2022 Dec 16;15(1):471. doi: 10.1186/s13071-022-05595-y. PMID: 36522757; PMCID: PMC9756507.

**Artículo quinto:**

**Título:** Validation of Oxford nanopore sequencing for improved New World *Leishmania* species identification via analysis of 70-kDA heat shock protein

**Autores:** Luz Helena Patiño[1], Nathalia Ballesteros[1], Marina Muñoz[1], Jesús Jaimes[1], Adriana C. Castillo-Castañeda[1], Roy Madigan[2], Alberto Paniz-Mondolfi[3] & Juan David Ramírezand Juan David Ramirez[1,3]

**Afiliación:**

[1]Centro de Investigaciones en Microbiología y Biotecnología-UR (CIMBIUR), Facultad de Ciencias Naturales, Universidad del Rosario, Bogotá, Colombia

2Animal Hospital of Smithson Valley, 286 Singing Oaks, Ste 113, Spring Branch, TX, 78070, USA

[3]Department of Pathology, Molecular and Cell-Based Medicine, Molecular Microbiology Laboratory, Icahn School of Medicine at Mount Sinai, New York, NY, USA.

**Revista:** Parasites & Vectors

**Referencia completa:** Patiño LH, Ballesteros N, Muñoz M, Jaimes J, Castillo-Castañeda AC, Madigan R, Paniz-Mondolfi A, Ramírez JD. Validation of Oxford nanopore sequencing for improved New World *Leishmania* species identification via analysis of 70-kDA heat shock protein. Parasit Vectors.

2023 Dec 18;16(1):458. doi: 10.1186/s13071-023-06073-9. PMID: 38111024; PMCID: PMC10726620.

**Artículo sexto:**

**Título:** Revisiting the heterogeneous global genomic population structure of *Leishmania infantum*

**Autores:** Luz H. Patino[1], Adriana Castillo-Castaneda[1], Marina Munoz[1], Carlos Muskus[2], Matilde Rivero-Rodriguez[3,4], Alveiro Perez-Doria[3], Eduar E. Bejarano[3] and Juan David Ramirez[1]

**Afiliación:**

[1] Centro de Investigaciones en Microbiologia y Biotecnologia-UR (CIMBIUR), Facultad de Ciencias Naturales, Universidad del Rosario, Bogotá, Colombia.

[2] Programa de Estudios y Control de Enfermedades Tropicales (PECET), Facultad de Medicina, Universidad de Antioquia, Medellín, Colombia.

[3] Grupo de Investigaciones Biomédicas, Universidad de Sucre, Sincelejo, Colombia.

[4] Candidata a doctor en Medicina Tropical, Universidad de Cartagena-SUE Caribe, Colombia.

**Revista:** Microbial Genomics

**Referencia completa:** Patino LH, Castillo-Castañeda A, Muñoz M, Muskus C, Rivero-Rodríguez M, Pérez-Doria A, Bejarano EE, Ramírez JD. Revisiting the heterogeneous global genomic population structure of *Leishmania infantum*. Microb Genom. 2021 Sep;7(9):000640. doi: 10.1099/mgen.0.000640. PMID: 34491157; PMCID: PMC8715437.

**Artículo séptimo:**

**Título:** Characterizing *Leishmania infantum*-induced resistance to trivalent stibogluconate (SbIII) through deep proteomics.

**Autores:** Castillo-Castañeda, Adriana[1,2]; Patiño, Luz H[1]; Manzano, Raúl[2]; López-Abán Julio[2]; Muro Antonio[2]; Ramírez, Juan David[1,3]

**Afiliación**:

[1] Centro de Investigaciones en Microbiología y Biotecnología-UR (CIMBIUR), Facultad de Ciencias Naturales, Universidad del Rosario, Bogotá, Colombia.

[2] Infectious and Tropical Diseases Research Group (e-INTRO), Biomedical Research Institute of Salamanca-Research Centre for Tropical Diseases at the University of Salamanca (IBSAL-CIETUS), Faculty of Pharmacy, University of Salamanca, 37008 Salamanca, Spain

[3] Department of Pathology, Molecular and Cell-Based Medicine, Molecular Microbiology Laboratory, Icahn School of Medicine at Mount Sinai, New York, NY, USA.

**Referencia completa:** Castillo-Castañeda, A.C., Patiño, L. H., Manzano R., López-Abán, J., Muro A., and Ramírez, J. D. Characterizing *Leishmania infantum*-induced resistance to trivalent stibogluconate (SbIII) through deep proteomics. (Sometido)

“Somos nuestra memoria, somos ese quimérico museo de
formas inconstantes, un montón de espejos rotos”

Jorge Luis Borges.

A mi abuela materna que ha sido el
amor más puro que ha podido existir.

# AGRADECIMIENTOS

Tengo tanto por agradecer y tantas personas a quienes dirigir mi gratitud que resulta difícil expresarlo aquí de manera completa. Confieso que esto lo dejé de último y quizá no recuerde a todos, lo intentaré.

En primer lugar, agradezco a Dios y al universo por permitirme estar aquí y ahora, por la oportunidad de vivir y sentirme viva, y por la fortuna de dedicarme a lo que me apasiona: las Enfermedades Transmitidas por Vectores, en especial mi querida leishmaniasis visceral. Contribuir, en mayor o menor medida, a combatir una enfermedad que afecta a un grupo reducido de personas llena mi corazón de satisfacción. Trabajar por aquellos que forman parte de esos pocos casos, pero que representan el 100% de una enfermedad olvidada, es mi mayor motivación.

A mi madre, Carmenza, le agradezco por su constante aliento y sacrificio para brindarme educación. Gracias por cuidar de mí en momentos de dificultad, por tus consejos, amor incondicional y bendiciones. A mi hermana Yuli, mi confidente y amiga, gracias por ser parte de mis motivos para ser feliz. A mi tía Eliza, mi segunda madre, gracias por enseñarme valentía y generosidad.

A mi compañero de vida, Mauricio, le doy gracias a Dios por tenerte a mi lado. Has sido testigo de mi proceso, mis altibajos, mis logros y mis pérdidas, y a pesar de todo, sigues ahí, apoyándome y amándome. Bendito ese tiempo de pandemia que nos reencontró.

A la Dra. Marthica Ayala, quien despertó mi amor por los parásitos y me inspiró a ayudar a aquellos que están a cientos de kilómetros de distancia, pero cercanos en mi corazón: los pacientes. Al profesor Juan, por su apoyo en mis investigaciones y su ánimo constante, por enseñarme a investigar y aterrizar, a ser realista. A LuzH, por ser un ejemplo de fortaleza y determinación. A los profesores Julio y Raúl, y a mis maestras proteómicas Nieves y Rosa, gracias por su invaluable apoyo y enseñanzas.

A mis amigos Carito, Jessi, Maryi, Cata, Leidy, Vivi, Chiqui y Natha, por estar presentes en los momentos difíciles y en los momentos de celebración. Agradezco a los amigos del CIMBIUR: Amparito, Caro, Liss, Gio, Tati, Lau, David y Jesús, a los que la pandemia trajo y se quedaron. A Claudia, Pilar y Lina, por su amor y cuidado durante mi recuperación. Por los abrazos y las largas conversaciones. Dios los bendiga infinito a todos.

Finalmente, a la universidad, por la beca académica como asistente graduado que me permitió llevar a cabo mis estudios doctorales, por la beca de la pasantía y la experiencia docente que adquirí estos años. De la Universidad del Rosario he aprendido lo que debo y no debo ser en mi futuro, el bien y el mal lo aprendí a partir de la experiencia.

Una vez más, gracias a todos por su apoyo y amor incondicional.

P.D.: A mí misma, que he superado las dificultades y vencido las limitaciones autoimpuestas, gracias por perdonarme y quererme. Agradezco a mí misma por creer en mí y por nunca rendirme. ¡Brindo por mi fuerza y resiliencia!

# ABREVIATURAS

AMB: anfotericina B
CNV: Variación de número de copía (Copy Number Variation)
INS: Instituto Nacional de Salud
LNR: Laboratorio Nacional de referencia
LC: Leishmaniasis cutánea
LM: Leishmaniasis mucosa
LV: Leishmaniasis visceral
MIL: Miltefosina
NGS: secuenciación de siguiente generación (Next-Generation Sequencing)
OMS: Organización Mundial de la Salud
PP: Paramomicina
Sb: Estibogluconato de sodio
SbV: Estibogluconato de sodio pentavalente
SbIII: Estibogluconato de sodio trivalente
SNP: Polimorfismo de nucleótido simple (Single Nucleotide Polymorphism)
SIVIGILA: Sistema Nacional de Vigilancia Epidemiológica

# TABLA DE CONTENIDO

# LISTA DE FIGURAS Y TABLAS

## LISTA DE FIGURAS

## LISTA DE TABLAS

## RESUMEN

La leishmaniasis es una enfermedad transmitida por vectores (ETV) que engloba diferentes formas clínicas, como la cutánea (LC), mucosa (LM) y visceral (LV). En conjunto, estas parasitosis se consideran enfermedades tropicales desatendidas, asociadas a la pobreza y el olvido. A nivel mundial, las especies del complejo *Leishmania donovani* son los principales agentes causales de la LV, seguidas en menor medida por las especies del complejo *L. enrietti.* En regiones como Centro y Sur América, la cuenca del Mediterráneo y el este de África, es la especie *L. infantum* la más prevalente. A nivel de Sur América, aunque Colombia es uno de los países endémicos para la LV; en el país, poco se estudia sobre la epidemiología de la enfermedad, la biología de *L. infantum* y los patrones evolutivos de este parásito.

Actualmente, la LV presenta diversas problemáticas, entre las que destacan: *i)* la aparición de casos en nuevas zonas de transmisión*, ii)* la coinfección con el virus de la inmunodeficiencia humana (VIH) y otros agentes infeccioso, *iii)* la falta de desarrollo de herramientas para identificar posibles eventos de coinfección, y iv) el incremento en la falla terapéutica debido principalmente a la circulación de cepas resistentes a los tratamientos de primera línea como el Estibogluconato de sodio (Sb), la Anfotericina B (AMB) y la Miltefosina (MIL). Ante la gravedad de la enfermedad, la vulnerabilidad de las poblaciones expuestas, la expansión de los focos de transmisión, las lagunas en el conocimiento de la resistencia y la necesidad apremiante de identificar nuevos blancos terapéuticos; ha surgido el interés de estudiar la especie *L. infantum* a nivel local, generando datos relevantes que puedan ser útiles y extrapolables a nivel regional y mundial.

Considerando el contexto anterior, nuestra propuesta de investigación se enfocó en abordar la LV desde cuatro perspectivas clave, utilizando herramientas bioestadísticas, epidemiológicas, moleculares y bioinformáticas: 1) Se realizó un análisis de los datos oficiales y de las publicaciones científicas disponibles para describir las características demográficas de los pacientes, estudiar los cambios en los patrones espaciales y temporales de la distribución de casos de LV en Colombia durante el periodo de 2007 y 2018, especialmente en términos de incidencia; así mismo, se exploró la relación entre la distribución de especies del vector *Lutzomyia* spp. y la incidencia de casos a nivel nacional. 2) A partir de la innovadora propuesta de utilizar secuenciación basada en amplicones (amplicon-based sequencing) para un fragmento de el gen HSP70, se logró detectar la presencia de eventos de coinfección por diversas especies de *Leishmania* e incluso *Trypanosoma* spp. en muestras de vectores, mamíferos silvestres y domésticos, y pacientes con LC, LV; además, se identificó la circulación de especies de *Leishmania* que no habían sido descritas previamente en algunos departamentos de Colombia. 3) Al comparar el genoma de 4 aislamientos de *L. infantum* de Colombia contra genomas de esta especie provenientes de diferentes partes del mundo, se consiguió caracterizar la diversidad genética de la especie tanto dentro como entre los grupos (clasificados por ubicación geográfica: América, Europa, África, Asia y Colombia), identificando cuatro poblaciones definidas de la especie y sus distribuciones geográficas; además, se observó una relación filogenética más estrecha entre los genomas de Colombia con los de Europa y el norte de África que con respecto a los genomas de la región; así mismo, se encontraron interesantes diferencias entre las secuencias de nucleótidos de los principales antígenos del parásito usados en el diagnóstico de la LV. Finalmente, 4) Mediante el empleo de proteómica de alta profundidad, se

caracterizaron cualitativa y cuantitativamente los cambios proteicos en *L. infantum* con resistencia inducida al SbIII, los promastigotes de esta línea resistente tuvieron cambios en cinética de su crecimiento, pues aunque los dos grupos (silvestre y resistente) alcanzaron la fase logarítmica, en el último tomo más tiempo; adicionalmente, la línea resistente tuvo una importante producción de proteínas relacionadas con la transcripción y traducción, el metabolismo de lípidos, el metabolismo de carbohidratos, la compensación energética y la biogénesis de peroxisomas; mientras que, se observó un panorama de reducción de las proteínas asociadas al metabolismo del hierro y de metales.

La presente tesis destaca la importancia de abordar la leishmaniasis visceral desde diversas perspectivas, empleando una amplia gama de métodos y herramientas centrados en la investigación no solo del parásito, sino también de sus vectores y reservorios, permitiendo una mejor comprensión de la enfermedad a nivel local y global. Por un lado, se ha analizado el impacto de diversos factores sobre la LV a lo largo del tiempo y el espacio en Colombia. Por otro lado, el desarrollo de una estrategia eficaz para la identificación simultánea de diferentes especies de *Leishmania* y *Trypanosoma* en un mismo individuo ha expuesto la ocurrencia de eventos de coinfección, relacionando su influencia en la gravedad y el curso de la enfermedad en países endémicos para ambas patologías. Asimismo, el estudio de la diversidad genética de los parásitos autóctonos ha contribuido a comprender mejor el flujo genético y las relaciones filogenéticas de la especie a nivel global, proporcionando una base genética para entender las variaciones en la sensibilidad de ciertos antígenos utilizados en el diagnóstico de la LV. Por último, pero no menos importante, se ha ampliado la comprensión de la resistencia de *L. infantum* al estibogluconato de sodio, revelando un panorama complejo y multifactorial que incluye eventos no considerados previamente; y aunque, no es posible señalar nuevos blancos terapéuticos con certeza, confiamos en que esta información puede ser crucial para el desarrollo de nuevas propuestas terapéuticas o la mejora de las existentes.

# ABSTRACT

Leishmaniasis, a vector-born disease (VBD), presents various clinical forms including cutaneous (CL), mucosal (LM), and visceral (VL). These parasitic illnesses are collectively categorized as neglected tropical diseases due to their association with poverty and lack of attention. Globally, the main causative agents of VL belong to the *Leishmania donovani* complex, with species of the *L. enrietti* complex playing a lesser role. In regions like Central and South America, the Mediterranean basin, and eastern Africa, *L. infantum* is the predominant species associated. Despite Colombia being one of the endemic countries for VL in South America, there remains limited research on the epidemiology of the disease, the biology of *L. infantum*, and the evolutionary patterns of this parasite within the country.

Currently, VL presents several challenges, including: *i)* emergence of cases in new transmission areas, *ii)* coinfection with HIV and other pathogens, *iii)* lack of tools to identify potential coinfection events and *iv)* increasing therapeutic failures primarily due to the circulation of strains resistant to first-line treatments like Sodium Stibogluconate (Sb), Amphotericin B (AMB), and Miltefosine (MIL). Given the disease's severity, the vulnerability of affected populations, the spread of transmission foci, gaps in resistance knowledge, and the urgent need to identify new therapeutic targets, there's a growing interest in studying *L. infantum* locally. This aims to generate relevant data applicable not only at a regional but also at a global level.

Considering the abovementioned context, our research aimed to address VL from four main perspectives, utilizing biostatistical, epidemiological, molecular, and bioinformatic tools. Firstly, we analyzed official data and scientific literature to describe the demographic characteristics of patients and examine changes in the spatial and temporal distribution patterns of VL cases in Colombia from 2007 to 2018, particularly focusing on incidence rates. Additionally, we investigated the correlation between the distribution of *Lutzomyia* spp. vectors and the incidence of cases nationwide. Secondly, we employed amplicon-based sequencing targeting a fragment of the HSP70 gene, enabling the detection of coinfection events involving various species of *Leishmania* and *Trypanosoma* spp. in samples from vectors, wild and domestic mammals, as well as patients with LC and VL. Moreover, we identified the circulation of previously undescribed *Leishmania* species in certain Colombian regions. Thirdly, through genome comparisons of four *L. infantum* isolates from Colombia with genomes from different global regions, we characterized the genetic diversity within and between geographic groups (America, Europe, Africa, Asia, and Colombia), identifying four distinct populations of the species and their geographic distributions. We also noted a closer phylogenetic relationship between Colombian genomes, Europe, and North Africa, compared to those from the local region and identified intriguing differences in nucleotide sequences of key parasite antigens used in VL diagnosis. Finally, employing high-depth proteomics, we qualitatively and quantitatively characterized protein changes in SbIII-induced resistant *L. infantum*. The resistant line's promastigotes exhibited altered growth kinetics, with a prolonged logarithmic phase compared to the wild type. Furthermore, we observed significant production of proteins related to transcription, translation, lipid and carbohydrate metabolism, energy compensation, and peroxisome biogenesis in the resistant line, alongside a reduction in proteins associated with iron and metal metabolism.

The current thesis underscores the importance of addressing VL from multiple perspectives. Through the utilization of a wide array of methods and tools focusing not only on the parasite but also on its vectors and reservoirs, a better understanding of the disease at local and global levels has been achieved. On one hand, it has examined the impact of various factors on VL over time and space. On the other hand, the development of an effective strategy for the simultaneous identification of different *Leishmania* and *Trypanosoma* species in the same individual has shed light on the occurrence of coinfection events and their impact on disease severity and progression in endemic countries for both leishmaniasis and Chagas disease. Additionally, the study of the genetic diversity of autochthonous parasites has helped to better understand the genetic flow and phylogenetic relationships of the species on a global scale, providing genetic support for understanding the variation in sensitivity of certain antigens used in the diagnosis of this parasitic disease. Finally, but no less important, the understanding of *L. infantum* resistance to sodium stibogluconate has been expanded beyond what was previously known, elucidating a complex and multifactorial landscape that includes biological events not previously considered; while pinpointing new therapeutic targets is not feasible with certainty, we trust that this information can yield crucial insights for the development of new therapeutic proposals or the enhancement of existing ones.

# 1. MARCO TEÓRICO

## 1.1. Epidemiología de la Leishmaniasis visceral

En conjunto, las diferentes formas de leishmaniasis (LV: leishmaniasis visceral, LC: leishmaniasis cutánea y LM: leishmaniasis mucosa) se encuentran en 98 países, afectando aproximadamente a 12 millones de personas cada año [1]. La leishmaniasis visceral (LV) es endémica en 56 países, siendo Brasil en Sudamérica, los países del subcontinente de la India y las naciones del oeste de África las más afectadas por esta forma de la enfermedad (Figura 1). A nivel regional, Brasil, Venezuela, Paraguay y Colombia son los países con mayor incidencia de casos [2]. Para este último país, se reconocen dos focos principales de transmisión: la ribera del río Magdalena, que abarca los departamentos de Huila, Tolima y Cundinamarca; y la zona norte en el departamento de Córdoba y en la subregión de los Montes de María en los departamentos de Sucre y Bolívar. [3].

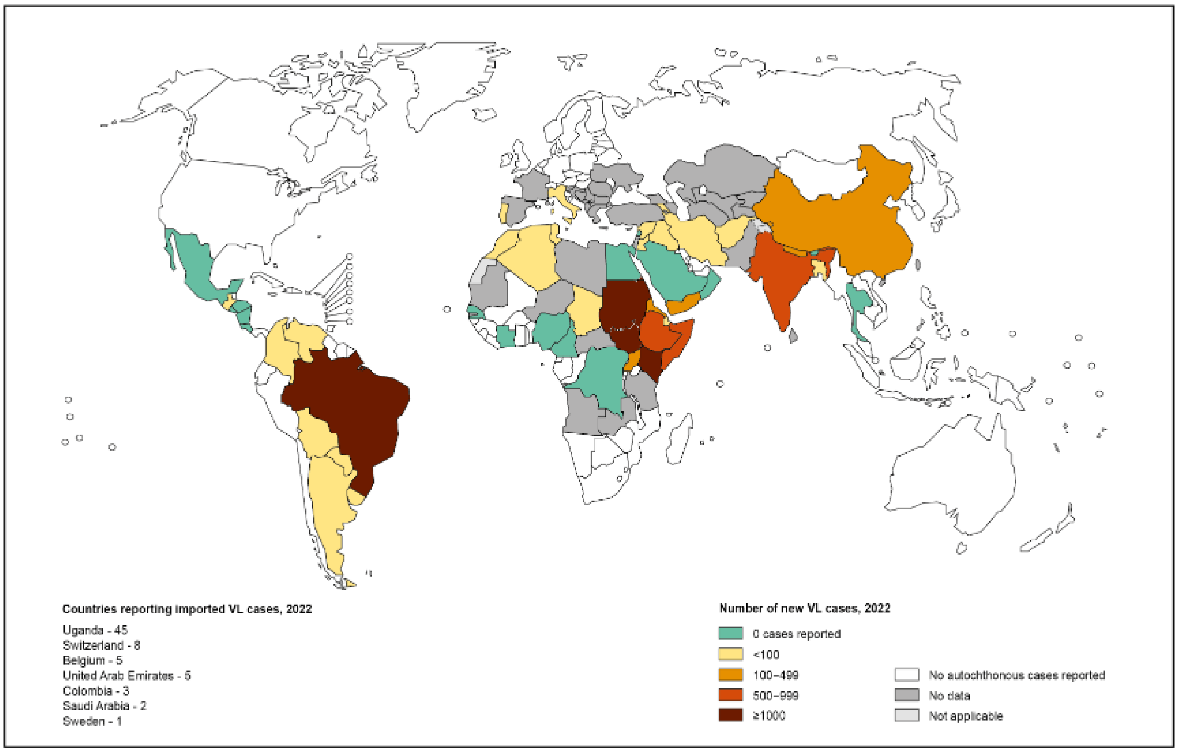


**Figura 1.** Estatus de la endemicidad de la LV a nivel mundial 2022 (reporte noviembre de 2023). Fuente: Organización Mundial de la Salud

A nivel mundial, la OMS reporta una mayor prevalencia de la enfermedad en adultos jóvenes de sexo masculino [4], una estadística que corresponde con los estudios de datos demográficos y meta-análisis realizados en Brasil [5], China [6] e India [7]. Asimismo, se evidencia una mayor tasa de seroprevalencia y seroconversión en individuos de este sexo. En contraste, el rango de edad de los pacientes con LV en los países de Centroamérica, Colombia y Venezuela muestra una mayor tendencia hacia la población pediátrica menor de 5 años [2,3,8], como se puede corroborar en los informes del SIVIGILA y los estudios de reporte de brotes realizados en Huila [9,10] y Bolívar [11,12]. Respecto al sexo, en Colombia no se cuenta con un respaldo estadístico que indique diferencias significativas en los patrones de ocurrencia de la LV en función del sexo.

Como se mencionó anteriormente, la leishmaniasis se considera una enfermedad relacionada con la pobreza y el abandono. Por lo tanto, la situación socioeconómica de las poblaciones en riesgo es un factor determinante para la transmisión, progresión, diagnóstico y tratamiento oportuno de la enfermedad [13]. En Colombia, esta vulnerabilidad se observa principalmente en los casos de LV provenientes de áreas rurales y periurbanas. En estas zonas, las condiciones de las viviendas, la cercanía con áreas boscosas y la falta de acceso adecuado a servicios básicos de saneamiento crean un entorno propicio para la presencia de vectores y el contacto con los reservorios de la enfermedad [9–11,14,15].

En la epidemiología de la enfermedad en Colombia, factores culturales y socioeconómicos también desempeñan un papel crucial. Las poblaciones más vulnerables incluyen a comunidades indígenas, afrodescendientes y mulatas, donde el desconocimiento sobre la etiología de la enfermedad alcanza niveles cercanos al 90%. [15]; dicha situación dificulta enormemente el reporte, diagnóstico y tratamiento oportuno de la enfermedad [3]. Además, los escasos recursos económicos, que para el caso de las comunidades indígenas ascienden al 78% de las familias en focos de transmisión, conllevan directamente a deficiencias nutricionales y a la falta de medidas regulares de protección y autocuidado [14,15].

Otros factores sociales que actúan como moduladores de la LV incluyen los fenómenos migratorios en países de América del Sur. En el caso de Colombia, entre 2019 y 2020 se reportaron al SIVIGILA cuatro casos importados de LV. Además, las crisis políticas pueden desarticular las entidades públicas, afectando los programas de vigilancia en salud pública, la disponibilidad de tratamientos y los recursos para la adecuada atención de pacientes. [16,17]; y finalmente, el conflicto armado interno, especialmente en países como Irak, Etiopía y Colombia, ha representado un gran desafío en el control de la LC. La circulación de grupos armados al margen de la ley entre los territorios, principalmente en los nichos selváticos, complica aún más la situación [18,19].

## 1.2. Agente causal de la leishmaniasis

El género *Leishmania* es parte de los protozoos hemoflagelados, un género parasitario clasificado dentro del orden Kinetoplastida y la familia Trypanosomatidae, se caracterizan por ser digénicos y afectar tanto a humanos y otros mamíferos [20]. Este género se encuentra dividido en cuatro subgéneros: *Leishmania (Leishmania), Leishmania (Viania), Leishmania (Sauroleishmania)* y *Leishmania (Mundina)* (Figura 2) [21]. mamíferos, incluidos los humanos, y 20 son conocidas por causar enfermedad en los seres humanos [22,23].

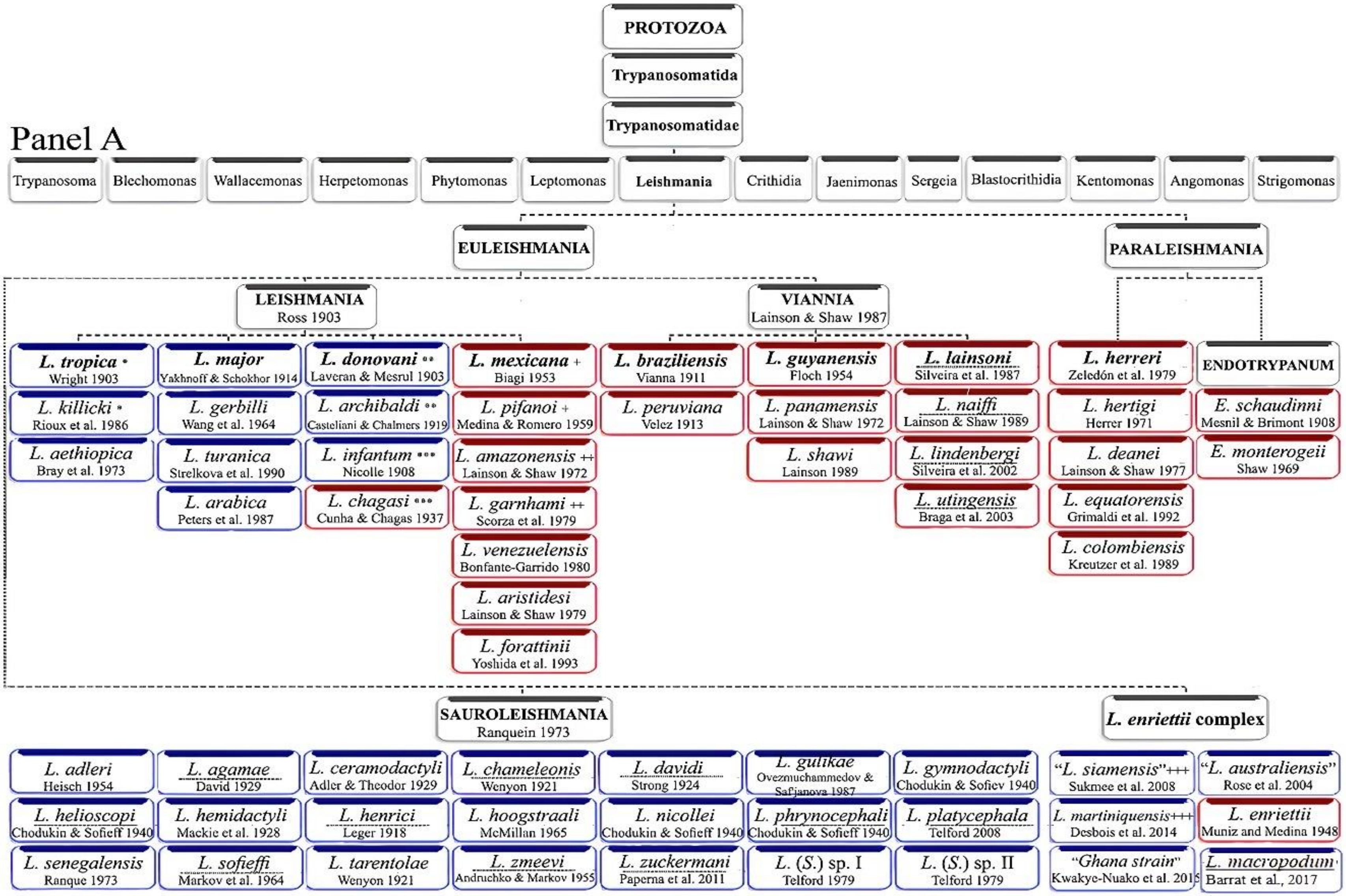


**Figura 2.** Clasificación taxonómica del género *Leishmania*, mostrando los diferentes subgéneros y especies de cada uno de estos. Adaptado de Akhoundi y colaboradores (24)

En el caso de la LV, las especies del complejo *Leishmania donovani*: *L. donovani, L. infantum, L. infantum (chagasi)* y *L. archibaldi* [24] son las principales causantes de la Hasta el momento se han descrito alrededor de 53 especies de *Leishmania*, de estas, 31 especies son patógenas para enfermedad, y en menor medida las especies del complejo *Leishmania enriettii* [25]. En los últimos años se han documentado casos de leishmaniasis visceral humana vinculados a otras especies además de las tradicionalmente asociadas, como *L. colombiensis* [26], *L. amazonensis* [27,28] y *L. tropica* [29]; y casos esporádicos de LV canina asociada a *L. braziliensis* y *L. amazonensis* [30].

## 1.3. Ciclo de vida

El ciclo de vida del parásito *Leishmania* se caracteriza por presentar dos estadios morfológicos y bioquímicos distintivos, adaptados según las demandas del entorno (Figura 3). La forma amastigota se desarrolla intracelularmente en células del sistema retículo endotelial de los mamíferos hospederos, mientras que la forma promastigota se encuentra extracelularmente en el tracto gastrointestinal de los mosquitos vectores, es en este último sitio donde tiene lugar la metaciclogénesis del parásito [31,32].

El ciclo comienza con un evento de hematofagía, cuando el vector de la familia *Psychodidae* ingiere sangre que contiene las formas amastigotes del parásito circulantes en un mamífero infectado (Figura 3). Esta sangre llega al sistema digestivo del vector entre 18 y 48 horas después de la ingestión [33]. Allí los parásitos activan su maquinaria genética e inician procesos de replicación, transcripción y diferenciación celular; pasando por formas morfológicamente diferentes: promastigotes procíclicos, nectomónadas, leptomónadas, haptomónadas, paramastigotes y promastigotes metacíclicos [34–36]. Desde los estadios tempranos, los parásitos deben ser capaces de resistir a las enzimas proteolíticas del sistema digestivo del vector, la matriz periotrópica [37] y las condiciones desfavorables generadas por las enzimas de la inmunidad innata del insecto [38,39].

En el estadio más tardío, los promastigotes metacíclicos se caracterizan por ser más delgados, de menor tamaño y poseer un flagelo de mayor longitud en comparación con el resto de las formas móviles del parásito. Estas características les permiten migrar hacia la parte más anterior del sistema digestivo, cerca de la probóscide. Este proceso migratorio, junto con la obstrucción causada por la acumulación de las formas parasitarias en el intestino, daña estructuralmente la válvula estomodal, interfiriendo con su función y facilitando el reflujo de los parásitos desde el intestino medio durante la hematofagia [40]; esto, a su vez, facilita el ciclo de transmisión durante una nueva picadura.[31,41].

El ciclo continua con una nueva ingestión de sangre [41]. Durante este proceso, se introducen los promastigotes metacíclicos, formas flageladas altamente infectivas, en el cuerpo del reservorio/hospedero mamífero al regurgitar la sangre consumida; una vez dentro del hospedero, los macrófagos y las células del sistema reticuloendotelial fagocitan los parásitos, y posteriormente, estos se desarrollan y replican como formas amastigotas dentro de las vacuolas fagolisosomales,; este cambio morfológico se da debido a los cambios de temperatura y pH [31].

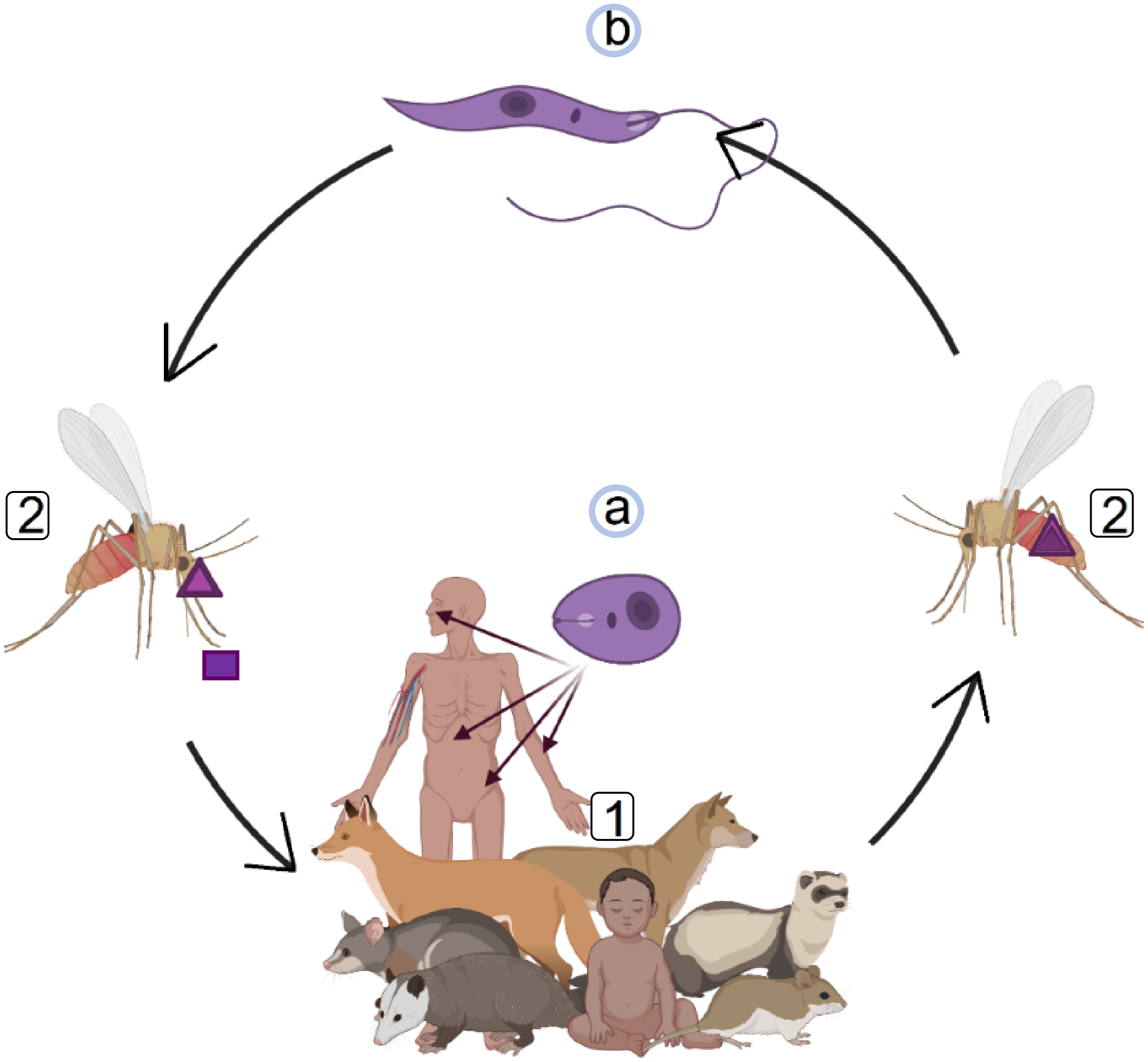


**Figura 3.** Ciclo de vida de *Leishmania* considerando eventos antroponóticos y zoonóticos con reservorios mamíferos silvestres y domésticos. El ciclo inicia cuando un mamífero infectado con *Leishmania* spp. (1) es picado por una hembra de la familia *Pyshodidae* (2). Al ingerir sangre con amastigotes (a), estos llegan al intestino delgado del vector, allí se activa la metaciclogénes, y el parásito madura de promastigotes procíclicos hasta metacíclicos▲; posteriormente estos migran a la parte anterior del sistema digestivo, cerca de la probóscide ■. Cuando el insecto (2) ingiere sangre nuevamente, inocula estos promastigotes (b) al mamífero (1). Los parásitos son fagocitados por células del sistema retículo endotelial, allí se transforman a amastigotes (a). Figura de autoría propia.

Después de la replicación, la alta carga parasitaria dentro de los macrófagos conduce a la lisis celular, liberando nuevos amastigotes que continúan infectando nuevas células del tejido. Esto facilita la invasión celular en el tejido cutáneo, lo que resulta en diferentes formas de leishmaniasis, como la cutánea (LC), mucosa (LM) o visceral (LV), dependiendo del tropismo y la virulencia de la especie involucrada, el estatus inmunológico del individuo y el lugar de picadura del vector.

### 1.3.1. Vectores

Los mosquitos hembra responsables del mantenimiento y la transmisión del parásito pertenecen a la familia Psychodidae, subfamilia Phlebotominae. La clasificación taxonómica de estos flebotomíneos puede resultar compleja, pero para fines prácticos se reconocen seis géneros principales que participan en los ciclos de transmisión de *Leishmania* spp. en el Viejo mundo se encuentran *Phlebotomus*, *Sergentomyia* y *Chinius*; mientras que en el Nuevo Mundo se destacan *Lutzomyia*, *Brumptomyia* y *Warileya* [36,42,43]; siendo las especies más comúnmente asociadas a la LV *Migonemyia migonei*, *Lutzomyia cruzi*, *Lutzomyia longipalpis* y *Pintomyia evansi* (Tabla 1).

Otras especies que han sido consideradas vectores potenciales son aquellas que han sido capturadas e identificadas molecularmente dentro de las viviendas de pacientes diagnosticados con LV, este grupo incluye especies como *Evandromyia cortelezzii*, *Evandromyia sallesi*, *Evandromyia lenti*, *Lutzomyia almerio*, *Lutzomyia ayacuchensis*, *Lutzomyia ischnacantha* y *Nyssomyia whitmani* [36,44]. En Colombia, la amplia variedad de ecosistemas y pisos térmicos influyen en la diversidad de la entomofauna del país, propiciando la ocurrencia de las diferentes formas clínicas de la enfermedad (LC, LM y LV) en una misma área endémica. En la actualidad, se tiene un conocimiento más detallado sobre la distribución de los flebótomos [45–50] y los nichos ecológicos donde se encuentran con mayor prevalencia, así como las especies de *Leishmania* que infectan a estos vectores [51,52] (Tabla 1).

Por otro lado, en relación con la distribución de estos artrópodos en Colombia, el Laboratorio Nacional de Referencia (LNR) de Entomología de la Dirección de Redes en Salud Pública (DRSP) del Instituto Nacional de Salud (INS) ha elaborado mapas de distribución que muestran la presencia de los distintos miembros de la familia Psychodidae y su asociación con cada una de las formas clínicas de la leishmaniasis (Figura 4).

En cuanto a las especies con un papel vectorial destacado para *L. infantum* en Colombia, se han identificado *Lu. longipalpis*, que se distribuye ampliamente a lo largo de la Región Andina y Caribe, y *Pi. evansi*, con una distribución principalmente en la Región Caribe [46] (Figura 5). Sin embargo, investigaciones recientes utilizando técnicas moleculares han revelado la presencia de material genético de *L. infantum* en vectores como *Lu. gomezi*, *Pi. ovallesi* y *Pi. spinicrassa* (Tabla 1) [51]; demás del informe de la circulación de *Pi. evansi* en la Región de la Orinoquía, principalmente en los departamentos de Casanare y Arauca.

**Tabla 1.** Especies de flebótomos involucrados en la transmisión de *Leishmania* spp. en Colombia y las características de sus nichos ecológicos.

| **Especie de vector** | **Especie de *Leishmania*** | **Hábitat** |
|---|---|---|
| *Lu. longipalpis* | *L. infantum* | Rocas, cuevas, vegetación selvática, área peridoméstica. A nivel urbano y rural en gallineros e intradomiciliaria |
| *Lu. (Pintomyia) evansi* | *L. infantum* | Arbustos, troncos ahuecados, peridomicilio, intradomiciliaria a nivel urbano y rural<br>0 a 300 m.s.n.m |
| *Lu. (helcocyrtomyia) hatmanni* | *Leishmania* spp. | Vegetación selvática, madrigueras, troncos de árboles, plantaciones de café, caña de azúcar y praderas |
| *Lu. (Pintomyia) spinicrassa* | *L. braziliensis, L. infantum, L. panamensis* y *L. amazonensis* | Vegetación selvática, huecos en troncos de árboles, plantaciones de café, caña de azúcar y cítricos, praderas y zona peri-urbana<br>218 a 1690 m.s.n.m |
| *Lu. (Nyssomyia) trapidoi* | *L. panamensis* | Troncos de árboles, árboles ahuecados y madrigueras a nivel selvático |
| *Lu. (nyssomyia) umbratilis* | *L. guyanensis* | Troncos de árboles. |
| *Lu. (Lutzomyia) gomezi* | *L. braziliensis, L. infantum* y<br>*L. panamensis* | Urbano y rural en peridomicilio, selvático en troncos ahuecados, madrigueras, plantaciones de caña de azúcar, café y cítricos, y en praderas |
| *Lu. (Pintomyia) ovallesi* | *L. braziliensis, L. amazonensis, L. panamensis* y *L. infantum* | Troncos de árboles, árboles ahuecados a nivel selvático, plantaciones de caña de azúcar, café y cítricos, a nivel rural y praderas<br>3 a 2160 m.s.n.m |
| *Lu. (Psychodopygus) panamensis* | *L. panamensis* y *L. amazonenesis* | Intradomiciliaria, plantaciones de café, a nivel selvático en troncos de árboles, a bajas alturas |
| *Lu. (verrucarum) longiflocosa* | *L. braziliensis* | Plantaciones de café, troncos ahuecados<br>900 a 2110 m.s.n.m |

Por su parte, la influencia del vector en la biología y ciclo de vida de *Leishmania* spp.*,* se ha visto relacionada con el tiempo de desarrollo del parásito dentro del vector, que puede variar según la especie y oscilar entre 4 y 18 días, aumentando o disminuyendo proporcionalmente con los cambios de temperatura ambiental y fenómenos naturales como las precipitaciones y las sequías [53–55]. Adicionalmente, la adaptación de los vectores a las alteraciones del nicho ecológico debido al calentamiento global, los desastres naturales o las actividades antropológicas ha provocado cambios en los ciclos de transmisión urbana, periurbana y silvestre de este patógeno [36,44].

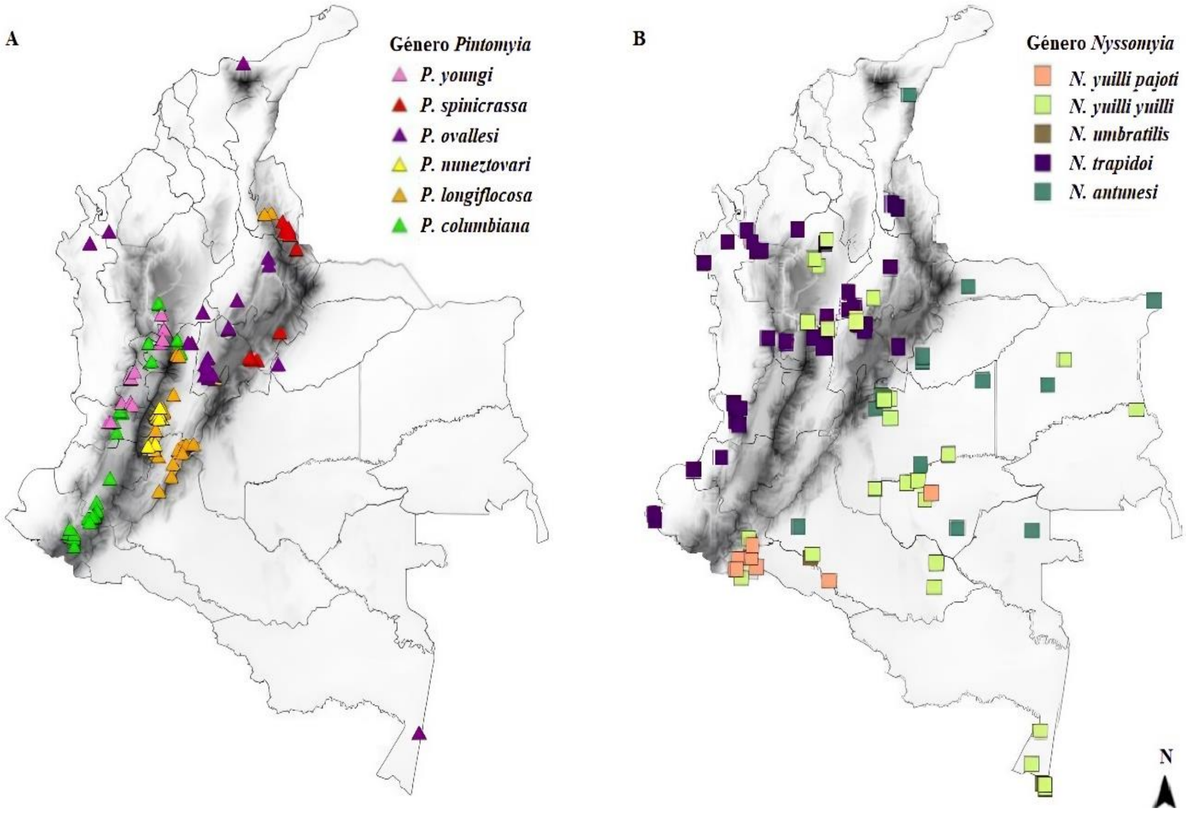


**Figura 4.** Distribución de especies de los géneros *Pintomyia* (A) y *Nyssomyia* (B), vectores de especies causales de Leishmaniasis Cutánea y/o mucocutánea en Colombia principalmente. Adaptado del Informe de vigilancia entomológico de Leishmaniasis del Instituto Nacional de Salud del 2018 [49]

Además de su papel en el tiempo de desarrollo del parásito, el vector juega un papel crucial en la interacción intra e interespecie. Los primeros estudios que demostraron la generación de híbridos intraespecíficos se llevaron a cabo en *L. donovani* [56,57]. Posteriormente en 2014, Calvo-Álvarez y colaboradores reportaron por primera vez el intercambio genético entre clones de *L. infantum* transgénicos para marcadores de resistencia [58]. En el mismo año, Romano y colaboradores demostraron por primera vez que puede ocurrir un intercambio genético interespecífico al inocular simultáneamente *L. major* y *L. infantum* en el vector *Lu. longipalpis*; a partir de estos cruces, se evidenció que los patrones de hibridación influyen en los factores de virulencia y los mecanismos de resistencia entre los clones aislados de los cruces y retrocruces [59].

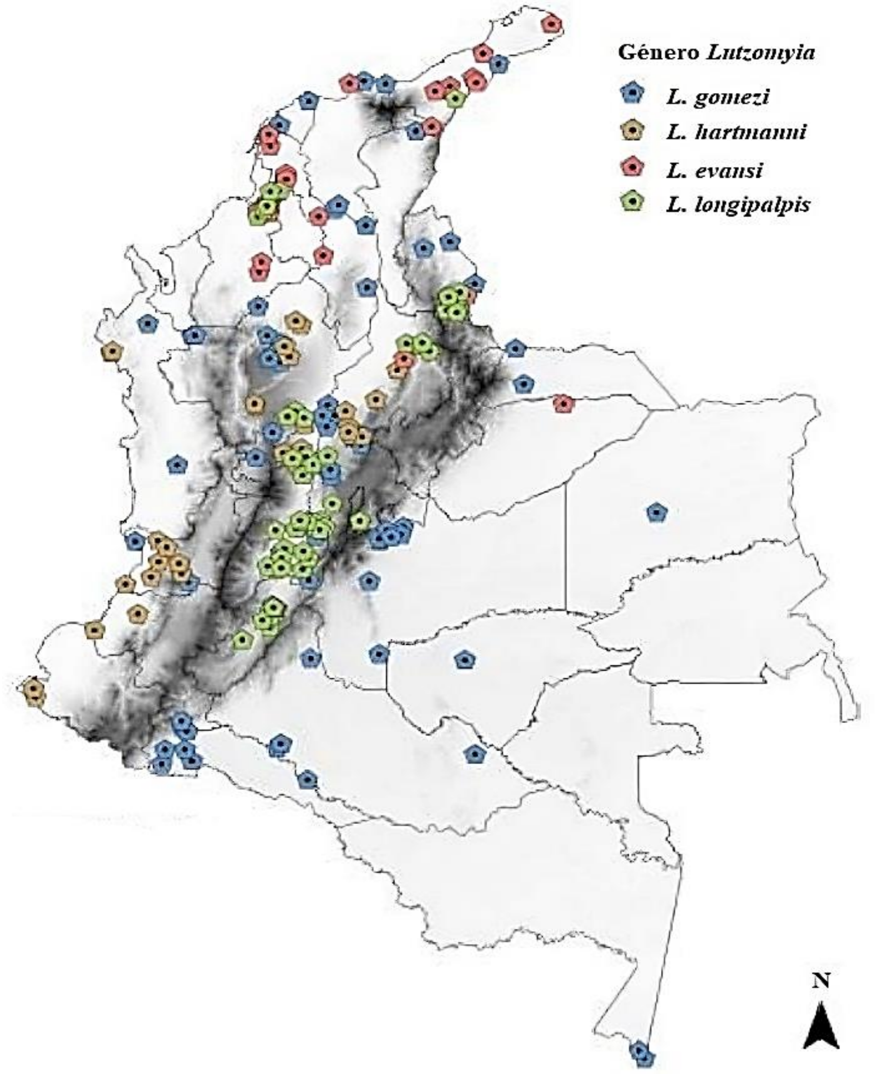


**Figura 5.** Distribución de especies del género *Lutzomyia*, vectores de especies de *Leishmania* spp. en Colombia. Adaptado del Informe de vigilancia entomológico de leishmaniasis del INS, 2018 [49]

La identificación de híbridos circulando naturalmente en países endémicos se ha reportado principalmente en zonas de transmisión de LC y LM. En 2009, se informó en Turquía un foco de LC asociada a *L. infantum*; sin embargo, investigaciones posteriores realizadas en 2014 por Rogers y colaboradores, y en 2015 por Seblova y colaboradores, sobre aislamientos del parásito a partir de muestras de *Phlebotomus tobbi* y pacientes, determinaron que se trataba de híbridos entre *L. donovani*-*L.infantum*, que posteriormente se recombinaron y se establecieron dentro de la población [60,61]. En 2019, Cardoso y colaboradores informaron por primera vez la co-circulación de diferentes especies de

*Leishmania* en *Lu. longipalpis* capturados en la ciudad de Governador Valadares, al sureste de Brasil [62], Estos hallazgos se basaron en análisis moleculares de 616 hembras *de Lu. longipalpis* mediante PCR especie específica, donde encontraron 80 muestras infectadas con *Leishmania*; de las cuales, cinco muestras presentaron amplificación para *L. infantum* y especies del subgénero *Leishmania (Viannia).*

### 1.3.2. Reservorios

Hasta ahora, la infección por *Leishmania* spp. se ha documentado en mamíferos tanto domésticos como silvestres en diversas regiones del mundo [41]. Esto respalda la idea de que los reservorios desempeñan un papel crucial en el ciclo de vida de este parásito. Para identificar una especie como reservorio de *Leishmania* spp., se deben considerar dos criterios importantes: *i*) la carga parasitaria en la sangre y/o la piel debe ser suficiente para permitir una transmisión efectiva del parásito cuando el vector se alimenta y *ii)* la infección debe ser no patógena o asintomática para asegurar la supervivencia del reservorio y la transmisión continua del parásito [63]. En el caso específico de la transmisión de *L. infantum* en América Latina, se establece la necesidad del ciclo zoonótico, mientras que para especies como *L. donovani* y *L. tropica* se ha confirmado la transmisión antroponótica mediada por los vectores [64,65].

Diferentes especies de mamíferos domésticos, silvestres y sinantrópicos han sido reportados a nivel mundial como reservorios u hospederos ocasionales de *Leishmania* spp. Entre los reservorios domésticos se encuentran perros domesticos (*Canis lupus familiaris)*, gatos (*Felis catus domesticus)* y hamsters (*Cricetus cricetus)*; mientras que entre los reservorios silvestres se han descrito especies de los órdenes: *Chiroptera, Rodentia, Didelphimorphia y Primate* [66,51,52,67–69]. Al igual que los vectores, las características comportamentales y la proximidad de estos animales tanto a los vectores como a los humanos pueden influir en la transmisión del parásito y por consiguiente en la ocurrencia de la enfermedad [70].

A nivel mundial, el reservorio doméstico con mayor relevancia en la transmisión de las especies de *Leishmania* es el perro doméstico [71]. Su implicación es tan significativa que en países endémicos se ha recurrido a medidas como la eutanasia y la terapia leishmanicida para controlar los reservorios, con el objetivo de interrumpir el ciclo de transmisión dentro y alrededor del hogar [72,73]. Sin embargo, en países como Brasil, España y Francia, estudios de asociación han demostrado que la efectividad de estas medidas en salud pública no alcanzan las expectativas esperadas, y su impacto es limitado [74]. Lo anterior ha motivado el estudio del papel de reservorios como los felinos domésticos e incluso los propios humanos en la transmisión del parásito, así como la propuesta de enfoques One Health que involucren, además de los perros, otros factores bióticos y abióticos relevantes para la transmisión y el establecimiento de la endemicidad de la enfermedad [75,76].

En Colombia, los estudios relacionados con los reservorios se han centrado principalmente en los perros debido a su conexión con los ciclos de transmisión urbano y periurbana. Se han determinado tasas de infección a nivel de muestra en cuatro departamentos endémicos para la LV: 17,2% en Huila [77], del 12 % al 31,5% en Tolima [14,71], 36% en Bolívar [12] y entre el 33,6% y el 66,9% en Sucre [78,79]. Además, es importante mencionar las ciudades de Cali y Pereira, donde hasta el momento no se han registrado casos de leishmaniasis visceral en humanos; sin embargo, se ha detectado un caso autóctono de leishmaniasis visceral canina en zona urbana para cada ciudad [80,81]. En cuanto a los eventos de coinfección en los reservorios domésticos y silvestres, hasta el momento se ha reportado este fenómeno en Brasil en caballos (*Equus caballus),* ratones *(Mus musculus)* y ratas (*Rattus rattus);* los cuales estaban coinfectados *con L. braziliensis* y *L. infantum* [82]. también se han registrado diversos informes de mamíferos coinfectados por *Leishmania* spp. y *Trypanosoma* spp [83].

En cuanto a los seres humanos humanos es relevante señalar que se han identificado híbridos y casos de coinfección con diferentes especies de *Leishmania*. Recientemente, Cotton y colaboradores en 2019 aplicaron la secuenciación completa del genoma (*Whole-Genome Sequencing*) para investigar los patrones de recombinación y la historia evolutiva de la mezcla genética entre aislamientos de *L. donovani* vinculados a importantes brotes de leishmaniasis visceral en Etiopía [84]. Estos aislamientos habían sido identificados como genotipos híbridos en estudios previos mediante el uso de microsatélites [85] y secuenciación de múltiples loci (multilocus sequencing) [86]. Entre los hallazgos más destacados, se observó que los híbridos en la región presentan una alta polisomía, lo que podría estar asociado con la gravedad de la clínica y los eventos de recaída en pacientes con leishmaniasis visceral, así como en pacientes coinfectados con leishmaniasis visceral y VIH.

En Suramérica, se llevó a cabo un estudio en Ecuador sobre un foco de transmisión de leishmaniasis cutánea y mucocutánea mediante análisis de PCR-RFLP, encontrando una distribución genéticamente compleja de los aislamientos clínicos, con la presencia de híbridos entre *L. guyanensis-L. braziliensis* y *L. guyanensis-L. panamensis,* estos híbridos fueron identificados a partir de patrones de discordancia mitocondrial y nuclear [87]. Además, se reportó la coinfección con *Leishmania (Viannia) braziliensis* y *L. (V.) lainsoni* en un paciente con LC procedente de Perú, utilizando la misma técnica molecular. Los autores de este reporte de caso reconocen que las coinfecciones tienen una alta relevancia clínica, ya que determinan la fisiopatología de la enfermedad, el pronóstico de la respuesta terapéutica y la evolución del paciente hasta su curación [88]. En el mismo país, Nolder y colaboradores identificaron un foco de LC y LM utilizando microsatélites, donde circulaban cerca de siete diferentes híbridos, los cuales mostraron características fenotípicas y genotípicas entre *L. braziliensis-L. peruviana*; y adicionalmente, en cuatro pacientes con LM aislaron un mismo híbrido [89].

## 1.4. Estructura genética

*Leishmania* es un parasito diploide y funcionalmente asexual; sin embargo, en algunas especies se han descrito procesos reproductivos de tipo meiótico y la descripción de híbridos entre diferentes especies, como por ejemplo, entre *L. major–L. donovani* [90], *L. donovani–L. infantum* [60], *L. guyanensis–L. braziliensis* y *L. guyanensis–L. panamensis* [87]. El genoma nuclear de *Leishmania* contiene aproximadamente 3,55 x $10^7$ pb, aunque este número puede variar entre especies en algunos miles de pares de bases. Además, posee una única mitocondria que contiene cerca del 15% del ADN celular, la cual generalmente se encuentra ubicada próxima a la región basal del flagelo y recibe el nombre de kinetoplasto. La información genética contenida en el kinetoplasto se organiza formando de 5.000 a

10.000 copias concatenadas en minicirculos y aproximadamente entre 800 y 1.200 copias que corresponden a los maxicirculos [91–94].

En cuanto a su organización cromosomal, *Leishmania* posee entre 34 y 36 cromosomas, los cuales presentan regiones repetitivas a nivel telomérico. Diversos estudios han demostrado que *Leishmania* exhibe plasticidad genómica, la cual está influenciada por diversos factores estresores. Estos incluyen cambios significativos en la temperatura, pH, ciertos componentes del medio como por ejemplo la presencia de antiparasitarios o reducción de la concentración de suero fetal bovino inactivado; incluso, se ha reportado plasticidad en *L. infantum* durante la infección de *Lu. longipalpis* [95]. Estos cambios a nivel cromosomal pueden implicar alteraciones en el tamaño o el número de copias de cada cromosoma, esta plasticidad genómica puede conducir a la aparición de variantes genéticas que podrían tener implicaciones en la virulencia, resistencia a fármacos y adaptación a diferentes condiciones ambientales [96–98].

González y colaboradores llevaron a cabo la resecuenciación y el ensamblaje de novo del genoma de *L. infantum* utilizando como referencia el genoma JPCMC5. Sus resultados revelaron que el genoma de esta especie consta de 32,1 megapares de bases (Mpb), distribuidos en 36 cromosomas. Además, en esta versión depurada del genoma, identificaron un total de 8.045 genes codificantes. Este análisis condujo a la adición de 495 nuevos genes anotados, así como a la corrección de alrededor de 100 genes y a la discontinuación de 75 genes que habían sido anotados previamente [99].

Actualmente, el uso de la secuenciación de próxima generación (NGS, Next-generation sequencing) ha permitido no solo la secuenciación del genoma de *L. infantum*, sino también la disponibilidad de genomas completos de especies de los subgéneros *Leishmania* como: .*L. major*, *L. mexicana, L. tropica*, *L. amazonensis*, *L. donovani* [99–103]; para el caso del subgénero *Viannia*: *L. panamensis*, *L. braziliensis*, *L. guyanensis*, *L. naiffi*, *L. peruviana*, *L. lainsoni* [104–106]; para el subgénero *Sauroleishmania*: *L. tarentolae*, *L. adleri* [107,108]; y para el subgénero *Mundinia: L. enriettii*, *L. macropodum*, *L. martiniquensis* [109] . Sin embargo, aún existen limitaciones en la genómica de estos parásitos que dificultan la comprensión de la expresión génica y los tropismos característicos entre las especies [110]. Aproximadamente el 50% de los genes codificantes de proteínas aún no tienen una anotación funcional definida. Por otro lado, los estudios de microarreglos indican que la mayoría de los genes muestran una transcripción constitutiva [111]. De los 8.000 a 9.000 genes codificantes de *Leishmania*, alrededor de 6.200 son compartidos entre la familia Trypanosomatidae, aproximadamente 1.000 son específicos de *Leishmania* y menos de 200 son específicos de especie [112,113].

Hasta la fecha, varios estudios han utilizado el conocimiento de la arquitectura genómica de *Leishmania* para dilucidar como ocurren los eventos evolutivos de especiación e hibridación en este parásito [85,114]. Estos eventos, como se mencionó anteriormente, están influenciados por condiciones ambientales, barreras geográficas, biología, distribución de reservorios y vectores, así como por la interacción entre especies y dentro de ellas. La estructura genética particular de algunas especies de *Leishmania*, ha permitido identificar diferencias en la patología, ecología y adaptación a eventos estresores en aislamientos de *L. braziliensis, L. infantum, L. major* y *L. donovani* [97,115–117]. Estudios han demostrado que el aumento del número de copias de cromosomas conlleva paralelamente a la amplificación y aumento en la expresión de genes relacionados con virulencia y resistencia frente a los antiparasitarios [97,118,119].

En el caso particular de *L. infantum* en América, su adaptación a nuevos nichos ecológicos, condiciones ambientales, diversidad y riqueza en entomofauna, así como, la abundancia de reservorios [95,120], encontradas por la especie durante su introducción desde Portugal en la época de la conquista española, explican la baja heterocigosidad de la especie en el continente [121,122]**.** Otro ejemplo que destaca la importancia del estudio de la estructura y arquitectura genética es una investigación realizada en Brasil, donde se analizaron aislamientos de *L. infantum* en pacientes con LV en Teresina [123]. En esta se encontró que los polimorfismos de nucleótido simple (SNP, single nucleotide polymorphism) no estaban distribuidos uniformemente en los cromosomas, generando una gran diversidad nucleotídica en los cromosomas 12, 33, 34, 35 y 36 de *L. infantum.* En contraste, en *L. braziliensis,* los SNPs se distribuyen homogéneamente en los cromosomas y se presentan con una baja densidad [124]. Con respecto a la presencia de mutaciones, se encontró que el cromosoma 22 presentaba la mayor tasa de mutaciones, este cromosoma alberga la familia de genes *A2*, que han sido considerados de gran importancia en *L. donovani* debido a su papel en la virulencia y visceralización [125].

Por otra parte, Franssen y sus colaboradores llevaron a cabo un análisis exhaustivo de la estructura genómica de aislamientos de *L. donovani* relacionados con LV en Etiopía, se enfocaron principalmente en la aneuploidía, la cobertura haploide y los SNP a lo largo de todo el genoma. Su objetivo era determinar la estructura poblacional del parásito y su posible relación con los episodios de recaída de la enfermedad en pacientes con VIH y sin VIH [126]. A partir de estos análisis, se llegó a las siguientes conclusiones: *i)* los casos de LV estaban vinculados a una población parasitaria clonal, *ii)* los episodios recurrentes de LV surgían debido a la reactivación de la población parasitaria inicial después de completar el tratamiento antiparasitario, *iii)* mediante el análisis de SNP y la variación en el número de copias de genes, se observó una marcada pérdida de diversidad genética en los parásitos del primer episodio recurrente en comparación con el aislamiento inicial, posiblemente debido a la presión selectiva y al efecto de cuello de botella generado por el fármaco utilizado en la primera infección; y finalmente, *iv)* la reducción de la heterocigosidad entre el aislamiento del primer episodio recurrente y los posteriores no mostró cambios significativos. En este mismo estudio, en contraste con las expectativas, el número de copias de genes asociados con la resistencia en el locus-H y locus-M (como el gen *MAPK1*, implicado en la resistencia a los antimoniales) [127,128], así como los transportadores de miltefosina y el locus de sensibilidad a la miltefosina (MSL) [129,130], no mostraron cambios significativos a lo largo del tiempo. Sin embargo, se observaron cambios en la somia, como la reducción del número de copias del cromosoma 31, lo que sugiere adaptaciones beneficiosas para el parásito.

## 1.5. Diversidad genética:

En las últimas dos décadas, el empleo de técnicas moleculares como la Tipificación de Secuencias de Múltiples Loci (MLST, Multilocus Sequence Typing) y la Tipificación de Microsatélites de Múltiples

Loci (MLMT, Multilocus Microsatelite Typing) ha sido fundamental para la identificación precisa de especies de *Leishmania* y su clasificación dentro de los diversos subgéneros y complejos [121,131,132].

La metodología de MLST se fundamenta en la utilización de múltiples genes constitutivos (generalmente entre 4 y 6) que se amplifican simultáneamente mediante PCR y luego se analizan en geles de agarosa [133,134]. Actualmente, se han desarrollado y reportado alrededor de 289 cebadores para la amplificación de diversos genes constitutivos. Entre los más empleados se encuentran la aspartato aminotransferasa *(asat)*, la glucosa-6-fosfato deshidrogenasa *(g6pd),* la 6-fosfogluconato deshidrogenasa *(6pgd)*, la manosa fosfato isomerasa *(mpi),* la isocitrato deshidrogenasa y el citocromo B *(cytb)* [135,136]. Estos cebadores fueron utilizados en 2017 para diseñar el primer esquema de MLST para la identificación de especies pertenecientes al complejo *Leishmania* (*Viannia*) en Colombia [136]. Además, en el complejo *L. donovani*, el uso de MLST ha demostrado la capacidad para discriminar aislamientos a nivel intercontinental, revelando la divergencia de linajes de *L. donovani* en una región restringida, permitiendo además asociar SNP con diferentes fenotipos relacionados con resistencia [137].

Se han llevado a cabo otros de gran importancia utilizando MLST y MLMT a nivel mundial con otras especies de *Leishmania* de importancia clínica humana. En el Viejo Mundo se han investigado especies como *L. archibaldi* en África y Eurasia [138], *L. tropica* en África [139], y *L major* en Irán [140]. En el caso del Nuevo Mundo, los estudios se centran en especies del subgénero *Leishmania (Viannia)*: *L. lainsoni, L. lindenbergi, L. brazilienss, L. guyanensis* y *L. naiffi* en Brasil [141], y *L. braziliensis* y *L. peruviana* en Perú [89].

El MLMT, por otro lado, se fundamenta en la amplificación mediante PCR de entre 10 y 20 repeticiones de secuencias simples (SSR, single sequence repeats) o repeticiones cortas en tándem (STR, short tandem repeats), que no están conectadas entre sí y que experimentan eventos de mutación escalonada que alteran el tamaño de los fragmento, estos fragmentos se analizan utilizando geles de agarosa o poliacrilamida, o mediante metodologías automatizadas que emplean sondas marcadas con fluorocromos [131]. En la literatura científica actual, se dispone de un amplio registro de SSR/STR para cada especie, incluyendo, por ejemplo: *L. major* (39*), L. tropica* (29), *L. aethropica* (34), *L. donovani/L.infantum* (102), *L. braziliensis* (30), *L. panamensis* (47), *L. guyanensis* (30), *L. peruviana* (18), *L. shawi* (18), *L. naiffi* (15) y *L. lainsoni* (18) [131].

Varios estudios han empleado MLMT con distintos propósitos para las especies del complejo *L. donovani.* Por ejemplo, se utilizó para determinar la especie del complejo asociada a un foco de LC en Chipre, identificando un grupo monofilético de *L. infantum* como el agente causal y estableciendo que su origen fue en Turquía [142]. En Francia, mediante estudios poblacionales de aislamientos de *L. infantum* en pacientes asintomáticos y con LV se determinó la circulación de dos genotipos en este foco de transmisión [143].

Estudios realizados por Kuhls y colaboradores, que emplearon 15 marcadores MLMT en 91 aislamientos de diferentes especies del complejo *L. donovani)*, permitieron organizar cada uno de estos según su origen geográfico en los principales focos de LV a nivel mundial: Suramérica, la Región del

Mediterráneo, Sudán, Etiopía, Kenia e India. [121,132]; además, permitieron establecer subestructuras poblacionales en diferentes focos de transmisión, lo que consolidó el uso de MLMT como una herramienta valiosa en análisis taxonómicos globales, estudios de población genética y epidemiología. [132].

Sin embargo, a pesar del uso de las técnicas descritas anteriormente, con el paso de los años surgió la necesidad de emplear métodos con mayor resolución y poder discriminatorio para mejorar la comprensión de los eventos evolutivos y la epidemiología molecular de la LV. En las últimas décadas, el uso de las NGS ha cobrado protagonismo, permitiendo asociar y agrupar las especies del complejo *L. donovani* según su procedencia geográfica. Además, se ha evidenciado una baja heterocigosidad entre ellas, como lo demostró Franssen y colaboradores en 2020 mediante el análisis del genoma completo de diferentes aislamientos de especies del complejo y el análisis de SNP [24] (Figura 6).

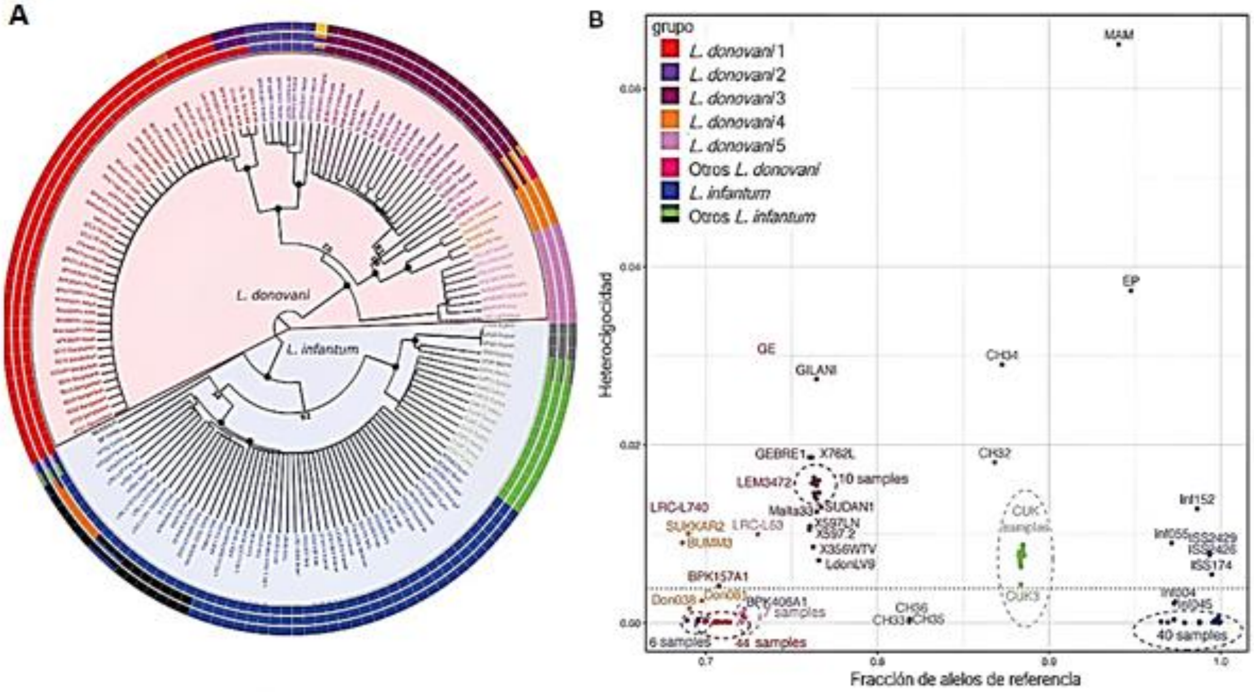


**Figura 6.** Relación filogenética del complejo *L. donovani* alrededor del mundo. Se muestra la heterocigosidad del genoma completo versus fracción de alelos de referencia (A) y la ccomparación entre la variabilidad de la somia y la heterocigosidad de SNPs a través del genoma (B). Adaptado de Franssen y colaboradores [24] .

En este estudio se reveló que los aislamientos de *L. infantum* no exhibieron una distribución geográfica claramente definida. Este grupo se dividió en dos grandes subtipos: Mon-1 y no Mon-1; además de la homocigosidad general de la especie, se observó un patrón de heterocigosidad diferente en dos aislamientos de *L. infantum* en el estudio, en comparación con los demás analizados. Este fenómeno podría atribuirse a eventos aislados de evolución de la especie o a una posible introducción alternativa en la región de las Américas, lo cual requiere una investigación específica para su confirmación.

En América del Sur, se ha evaluado y documentado la heterocigosidad genómica mediante estudios geográficos que han empleado aislamientos de *L. infantum* recolectados de humanos y perros en países como Brasil y Paraguay [144,145]; en general, se observó una baja heterocigosidad y una escasa diversidad en la población de *L. infantum* en diversos escenarios, incluso cuando se presenta variabilidad clínica en la sintomatología, severidad o progresión de la LV. Este fenómeno ha desviado la atención de los rasgos genéticos del parásito que podrían influir en la patofisiología de la LV, llevando a que la enfermedad se asocie generalmente con factores ambientales o del hospedero [146].

Recientemente Schwabl y colaboradores [96] han reportado la existencia de patrones de diversidad que relacionan la deleción de fragmentos de gran tamaño en el genoma de *L. infantum* con ancestros mutantes en diversos estados de Brasil. Esta pérdida significativa de material genético se vincula con alteraciones en la susceptibilidad a los medicamentos. Además, se ha demostrado que este patrón resistente puede revertirse mediante eventos de hibridación con cepas que no portan la mutación. Hallazgos significativos como estos subrayan la importancia de llevar a cabo estudios de secuenciación de alta profundidad, que permitan explicar y asociar los cambios en la virulencia, la susceptibilidad a fármacos y otros patrones que regulan el ciclo de vida y la transmisión de *L. infantum* desde una perspectiva genómica.

## 1.6. Amplicon-Based Sequencing

Como se ha señalado previamente, el uso de NGS es una herramienta altamente sensible para estudiar la genómica poblacional, la arquitectura genómica de los agentes infecciosos y la identificación de mutaciones en todo el genoma, este último aspecto es especialmente relevante en estudios oncogénicos. Sin embargo, aún persiste un costo elevado asociado al uso de NGS, especialmente en estudios genómicos a gran escala [147].

Desde la última década, la secuenciación completa del exoma se ha propuesto como una metodología costo-efectiva para capturar la mayoría de las mutaciones que conducen a cambios fenotípicos y para estudiar regiones específicas del genoma con algún interés particular; inicialmente, se centró en el enriquecimiento del exoma mediante hibridación para capturar estas regiones. [148]. Recientemente, se han desarrollado métodos que se basan en la secuenciación de amplicones de alta profundidad para simplificar la preparación de muestras y permitir el uso de matrices de ADN más cortas [149], Esto ha resultado en una mayor cobertura en la región exómica analizada en comparación con las técnicas de captura basadas en hibridación [147].

Esta metodología de amplicon-based sequencing se basa en la hibridación por medio de PCR con primers específicos del gen blanco a estudiar, acoplados al uso de barcodes e index en la primer corrida y adaptadores para la plataforma de secuenciación en la segunda PCR (151). Esto incluye pasos intermedios de limpieza de los amplicones y cuantificación de calidad y concentración antes de la preparación de las bibliotecas para la secuenciación en plataformas NGS [150]. En el caso de muestras biológicas, esta técnica permite un enriquecimiento sustancial de las secuencias del microorganismo de interés, sin la introducción de sesgos asociados a la amplificación de secuencias contaminantes del huésped eucariota, como mamíferos y vectores en el caso de *Leishmania* spp.[151]. Dentro de las plataformas NGS, la platafoma Illumina ha mostrado una mayor cobertura en la secuenciación de amplicones, sensibilidad en la identificación de SNP y profundidad [152,153].

En los últimos años, la metodología de amplicon-based sequencing ha sido empleada en estudios oncogénicos [153,154] y también metagenómicos bacterianos; principalmente, en muestras de heces humanas, heces murinas y de suelo basándose en el uso del gen *16S rRNA* [150,155]. Además, esta técnica se ha aplicado en enfermedades virales de interés en salud pública, como el Zika y el virus del Nilo Occidental, desarrollando herramientas específicas para los análisis bioinformáticos, como iVar, y para la correcta implementación de protocolos de laboratorio, como PrimalSeq [156]. Además, se han llevado a cabo estudios más completos que amplían el análisis a las comunidades virales, eucariotas y bacterianas en muestras de agua. En estos estudios, se ha utilizado la metodología de amplicon-based sequencing para investigar la distribución y diversidad de estas comunidades, empleando diferentes genes como el *g23* y *RdRp* para los virus, el *16S rRNA* y *cpn60* para las bacterias, y el *18S rRNA* e *ITS* para los organismos eucariotas [157].

Esta metodología también ha sido empleada para caracterizar poblaciones parasitarias, como la biodiversidad de helmintos y protozoos en el tracto digestivo de ratas silvestres y en el agua de riego; estos estudios, se basaron en la secuenciación del *18S rRNA* utilizando la plataforma Illumina MiSeq. En el caso de las heces de roedores, se identificaron secuencias correspondientes a *Trichomonas*, *Giardia* sp., *Trypanosoma* sp. y *Acanthamoeba* spp. [158]; mientras que, en las muestras de agua de riego se identificó *Giardia intestinalis*, *Acanthamoeba castellanii, Toxoplasma gondii*, *Entamoeba histolytica* y *Blastocystis* [159].

Un estudio clínico de 2020 utilizó la detección por NGS de genes ribosomales en raspados de córnea, demostrando ser un método eficaz de cribado para detectar causas no virales de la queratitis infecciosa. Este enfoque permitió la identificación de parásitos del género *Acanthamoeba* [160]. Así mismo, se han llevado a cabo investigaciones mediante la secuenciación del amplicón para el gen codificante de la proteasa de superficie *TcGP63I* en muestras de pacientes con Enfermedad de Chagas crónica y congénita [161].

Para el caso específico de los tripanosomátidos, diversos estudios han empleado esta metodología para obtener datos cualitativos y cuantitativos, mostrando la abundancia de estos microorganismos en muestras obtenidas de los diferentes elementos del circuito epidemiológico de transmisión.[162]. Por ejemplo, en el caso de muestras de vectores en una zona endémica de leishmaniasis, se maximizó la utilización de cada insecto colectado. Se secuenciaron los amplicones de *ITS* para la identificación de *Leishmania* spp., el citocromo oxidasa I (*COI*) para determinar la especie de vector, el citocromo b (*Cytb*) y la subunidad mayor del gen ribulosa-1,5-bisfosfate carboxilasa (*rbcL*) para identificar la fuente alimenticia ya sea de vertebrados o de plantas, respectivamente. Finalmente se analizó el microbioma procariota presente en el vector utilizando el gen *16S rRNA* [162]. Otras investigaciones relacionadas con *Leishmania* spp. han optado por diseñar y utilizar primers específicos para el kDNA de los minicírculos con el fin de detectar el parásito y lograr una identificación más sensible de la especie [163].

En los reservorios de *Trypanosoma* spp., se ha utilizado la secuenciación de amplicones del *18S rDNA* para detectar una amplia variedad de especies en marsupiales [164], koalas [165] y murciélagos [166]; informado tasas de poliparasitismo del 50% y 75% en las muestras de los dos primeros casos e infecciones mixtas complejas en el último. De manera interesante, en una investigación liderada por Cooper y su equipo, se encontraron lecturas para *Trypanosoma avium* y *Trypanosoma* spp. en muestras sanguíneas de marsupiales, especies que infectan aves de corral [164]. En un estudio llevado a cabo por Dario y sus colegas en murciélagos del Neotrópico, se lograron identificar diferentes especies de *Trypanosoma,* como *T. cruzi, T. cruzi marinkellei, T. rangeli* y *T. dionisii,* e incluso una Unidad Operativa Taxonómica relacionada con reptiles [166]. Estos hallazgos sugieren que la secuenciación por Sanger podría estar subestimando la abundancia y diversidad de especies de *Trypanosoma* spp. en los reservorios silvestres de las zonas endémicas estudiadas [164–166].

En América Latina, específicamente en Ecuador, al utilizar esta técnica en muestras de diferentes etapas del desarrollo de los triatominos *Rhodnius ecuadoriensis* y *Panstrongylus chinai*, se ha logrado determinar la coinfección por *T. cruzi* y *T. rangeli* en el 73.5% de los triatominos analizados a través de la secuenciación del mini-exon [167]; así mismo, se observó una mayor abundancia de *T. rangeli* circulante y se informó por primera vez la presencia de *T. cruzi* DTU TcIV en la región de muestreo. Esto evidencia el poder de resolución del amplicon-based sequencing para determinar eventos de coinfección en diferentes muestras biológicas y, a su vez, la identificación de especies con una alta sensibilidad.

## 1.7. Antiparasitarios y falla terapéutica

La falta de respuesta terapéutica ante los diversos compuestos de primera línea (antimoniales pentavalentes y miltefosina) y de rescate (anfotericina B) empleados en el tratamiento de la leishmaniasis ha sido ampliamente informada durante las últimas décadas, especialmente en áreas altamente endémicas [168–172]. Es crucial destacar que, además de los factores intrínsecos del parásito, existen variables relacionadas con el huésped y los medicamentos que pueden influir en la eficacia de los tratamientos contra la leishmaniasis (Figura 7), tal como ocurre en otras enfermedades infecciosas como las bacterianas, fúngicas y parasitarias.

Aspectos intrínsecos del hospedero, en el caso clínico de los humanos, han sido relacionados directa o indirectamente con la falla terapéutica. Estos aspectos incluyen la edad (infantes o población joven), el sexo (masculino), la procedencia geográfica (países de oriente) y comorbilidades como la infección con el VIH. Se ha observado que estos factores también están asociados con mayores tasas de infección, seroconversión y eventos de recaída [173–175].

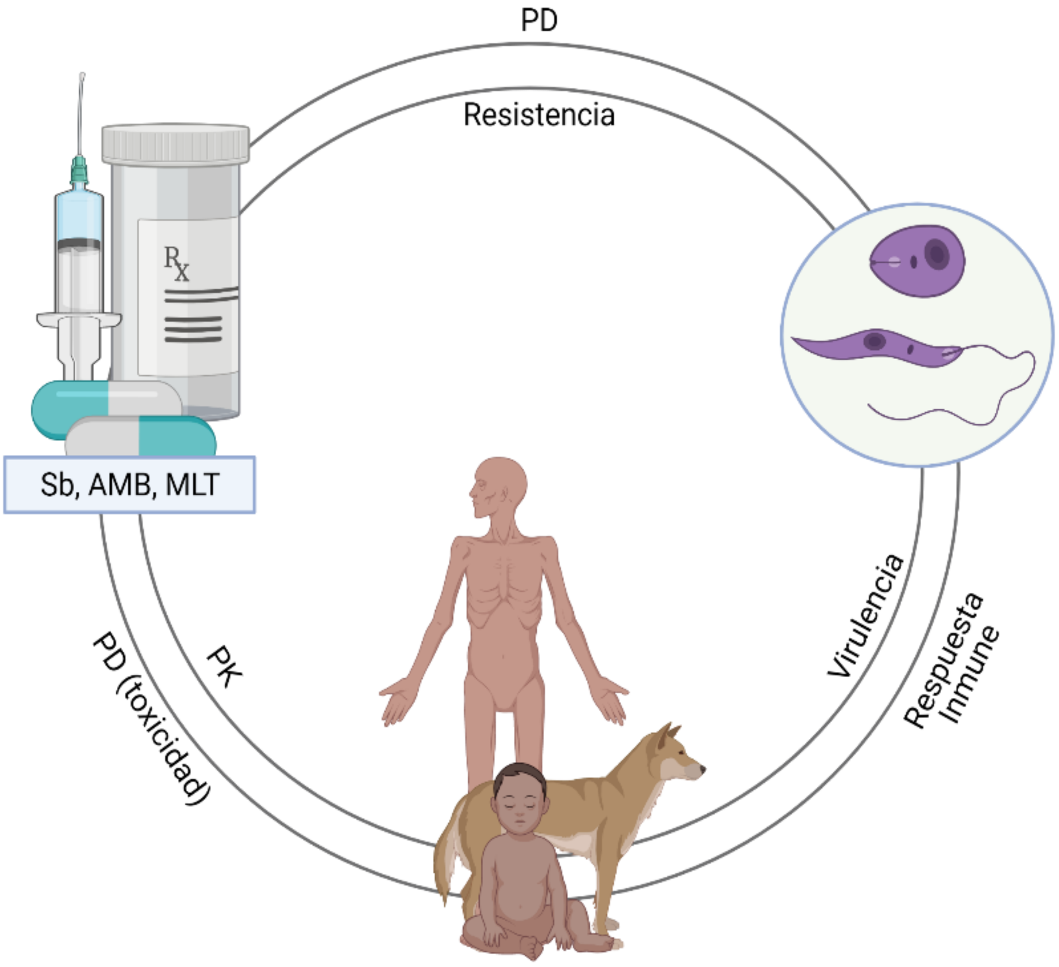


**Figura 7.** Relación entre farmacodinamia (PD; farmacodinámica)/ (PK;farmacocinética), hospedero, fármaco y patógeno. Adaptado de Asín y colaboradores [176]

Por otra parte, entre los factores directamente asociados con los medicamentos, se incluyen la calidad de los mismos [177], la formulación de la droga y su estabilidad ante condiciones ambientales, especialmente en zonas tropicales [110,176].También se consideran características farmacocinéticas como la vida media del compuesto activo, así como la farmacodinamia de los medicamentos, especialmente su actividad en entornos como los macrófagos-fagolisosomas (donde predomina un ambiente ácido) y en las vísceras, donde factores como la tensión de oxígeno, la permeabilidad de las membranas y el flujo sanguíneo son variables importantes [178,179].

En *Leishmania* spp., la resistencia adquirida se relaciona principalmente con ciclos de tratamiento repetitivos con el mismo medicamento, esquemas terapéuticos incompletos, baja adherencia al tratamiento y baja calidad del medicamento, especialmente en áreas de difícil acceso [110]. Entre los cambios principales que se observan en los parásitos en respuesta a los medicamentos Sb, AMB y MIL, ya sea de forma natural o inducida, se incluyen la variación en el número de copias de genes y cromosomas, deleciones y SNPs. También se destaca la activación de genes asociados a bombas de expulsión o transporte activo; así como, otros genes importantes para el adecuado funcionamiento del fármaco, ya sea porque son el blanco de acción o porque ayudan a metabolizar el compuesto a nivel intracelular [110].

Además de los factores genéticos asociados a los perfiles resistentes, recientemente se ha investigado el epi-fenotipo del parásito en los casos de falla terapéutica. Se han encontrado diferentes fenotipos del parásito causando la enfermedad en pacientes con recaídas, infecciones repetidas o baja respuesta al tratamiento. En algunos casos, se identifican subpoblaciones de amastigotes con alteraciones en la tasa metabólica, presencia de factores de virulencia particulares, inmunomodulación aberrante y quiescencia [180–182].

### 1.7.1. Antimoniales pentavalentes

Desde 1940, el estibogluconato de sodio pentavalente (SbV) y el antimoniato de meglumina (AM) han sido los medicamentos de primera línea para el tratamiento de la leishmaniasis.[183]. Sin embargo, en los últimos 20 años, se ha aumentado el reporte de falla terapéutica y la aparición de cepas resistentes, principalmente en lugares de alto impacto como la India [171,184,185]. El metabolismo e internalización de estas moléculas está asociado a componentes enzimáticos necesarios para la reducción del SbV a SbIII como la tiol- reductasa [186] y la arsenato/antimoniato reductasa [187,188]; así como, a componentes estructurales como la acuaporina *AQP1* [189]. Dentro de los mecanismos de acción de los antimoniales se incluye la alteración del metabolismo de los tioles [190], alteración de la ruta de la beta-oxidación y glicolisis [191], y la muerte celular programada debido a la fragmentación del ADN [192].

Los mecanismos de resistencia o fenotipos asociados con susceptibilidad reducida a los antimoniales se han caracterizado principalmente mediante transcriptómica y análisis de tipo qRT-PCR, se han descrito alteración en producción del gen *AQP1* en *L. donovani* y *L. major* [184,193,194], aumento en la síntesis de tripanotion, glutatión y poliaminas [195], bombas de expulsión tipo *ABC* [196,197] como la *MRPA* en el caso de *L. infantum* [198] y la respuesta a estrés oxidativo como peroxidoxina, glutarredoxina, tripanotion sintetasa en *L. amazonensis* [199,200]. Además, se han observado cambios en la expresión de genes relacionados con enzimas biosintéticas del 2-tiol, como la gamma-glutamilcisteina sintetasa *(gcs)* y la ornitina decarboxilasa *(odc)*, en aislamientos de *L. donovani* con resistencia natural al estibogluconato de sodio [201].

En Colombia, Patiño y colaboradores en 2019 caracterizaron líneas de *L. braziliensis y L. panamensis* con resistencia inducida al SbIII. Identificaron una sobreexpresión de transportadores de proteínas y metales como el zinc y el hierro, histonas y proteínas de choque térmico. Por otro lado, observaron una regulación negativa de genes codificantes para superóxido dismutasa *(SOD)*, genes de la familia de las amastinas, y transportadores de proteínas tipo *ABC* [97]. Además, se observó que al comparar la somia de *L. braziliensis* entre la cepa con resistencia inducida al SbIII y las cepas silvestres, se detectaron variaciones en la cantidad de copias de ADN (CNV, copy number variation) en los cromosomas 11, 13, 14, 23 y 26, que mostraron un aumento en la somia. Por otro lado, en el cromosoma 34 se identificó una disminución del CNV a un estado disómico. Estos cambios a nivel cromosómico estuvieron asociados directamente con los CNV de genes, identificándose un total de 467 de ellos. Entre los genes con importancia biológica se encontraron la *alfa-tubulina*, genes relacionados con el transporte de proteínas y zinc, y un gen asociado a la resistencia a los antimoniales. En contraste, al realizar la misma comparación con un aislamiento de *L. panamensis*, se observó una menor cantidad de CNV a nivel cromosómico. Solo los cromosomas 13 y 23 mostraron cambios a un estado disómico desde un estado trisómico y tetrasómico, respectivamente. En cuanto a los CNV de genes, se detectaron variaciones en 31 de ellos, siendo la protein-kinasa *(PTK1)* el gen identificado como el de mayor importancia.

Se han informado cambios similares en la arquitectura genética de especies frente a los antimoniales en *L. tropica* [202]*, L. infantum* [203], *L. donovani* [204] y *L. major* [205]. Hasta la fecha, se cree que la expresión génica en cepas resistentes está regulada principalmente por la dosis de genes y los cambios en el CNV, y que además estos cambios resultan beneficiosos para estas especies y otros tripanosomátidos [120,206]; caso contrario ocurre en agentes infecciosos como las levaduras, para los cuales estos CNVs son desfavorables [207].

## 1.8. Proteómica asociada a la resistencia antiparasitaria en *Leishmania* spp.

En las últimas décadas, la alta sensibilidad y capacidad de adquisición y procesamiento de datos ha consolidado las ómicas como las herramientas tecnológicas avanzadas para el estudio de los agentes infeccioso. Las técnicas en proteómica engloban un amplio espectro de métodos experimentales como la electroforesis en dos dimensiones (2DE), la cromatografía líquida (LC) y la espectrometría de masas (MS). Las técnicas proteómicas se dividen en cualitativas (presencia-ausencia de una proteína o grupo proteico) y en cuantitativas (determinar y comparar cantidad de proteína en la muestra).

Estas técnicas se basan en el uso de técnicas en gel para la separación de proteínas según tamaño, punto isoeléctrico, conformación (2DE, 2D-DIGE), y las técnicas *gel-free* que requieren una separación inicial de las proteínas mediante cromatografía líquida y un posterior análisis mediante MS para la identificación de las proteínas (LC MS/MS). Esta técnica *gel-free* además puede usar marcaje de las muestras isotópico o isobárico, o ser *label-free*. Para este último caso, la cuantificación de proteínas se realiza basado en: *i)* la medición de los cambios de intensidad de los iones (intensidad y/o altura de los picos) en espectrofotometría y *ii)* el recuento de los espectros de las proteínas identificadas por medio de MS/MS [208].

Estudios de proteómica cuantitativa de marcaje con isótopos (SILAC, Stable-isotope labelling by amino acids in cell culture) y posterior espectofotometría de masas de alta resolución han mostrado que cepas de *L. donovani* con resistencia inducida a la paramomicina tienen una mayor concentración de proteínas relacionadas con la traducción y el transporte mediado por vesículas [209,210]. Este último hallazgo es de gran importancia en el género *Leishmania,* pues se ha demostrado que las vesículas y los exosomas juegan un papel importante en la patobiología y fisiología parasitaria; ya que, permiten la comunicación celular y la transferencia de factores de virulencia *(gp63)*, genes asociados a resistencia antiparasitaria y proteínas inmunomoduladoras [211].

Por ejemplo, por medio del uso de electroforesis en gel de poliacrilamida con dodecilsulfato de sodio (SDS-PAGE) en aislamientos clínicos de *L.* donovani con resistencia cruzada a MIL y AMB; se han identificado dos proteínas vinculadas con el fenotipo de muerte celular programado, la proteína de choque térmico HSP83 y la proteína relacionada con la calpaína de kinetoplasto pequeño *(SKCRP14.1)* [212]. En esta misma especie, el uso de la técnica M MALDI-TOF/TOF ha permitido identificar una importante fracción de proteínas enriquecidas en la membrana parasitaria, que incluyen proteínas históricamente relacionadas a resistencia a los antiparasitarios como es el caso de los transportadores tipo *ABC* y proteína de choque térmico *HSP83* [213].

Por otra parte, los análisis proteómicos realizados mediante electroforesis en gel de diferencia bidimensional (2D-DIGE) en *L. amazonensis, L. braziliensis* y *L. infantum* han identificado proteínas asociadas con el transporte mediado por vesículas, la triparedoxina, la respuesta al estrés oxidativo y las interacciones proteína-proteína, incluyendo proteínas de choque térmico [214]. Asimismo, un análisis comparativo de proteómica utilizando electroforesis en gel bidimensional (2-DE) y cromatografía líquida/espectrometría de masas (LC/MS/MS) en líneas de *L. braziliensis* y *L. infantum chagasi* resistentes y susceptibles al antimonio reveló una sobreexpresión de triparedoxina peroxidasa, alfa-tubulina, *HSP70*, *HSP83* y *HSP60* en ambas líneas resistentes, mientras que se observó una reducción en la ciclofilina-A en las líneas resistentes [215].

Saboia-Vahia y colaboradores en 2022 describieron los cambios cualitativos y cuantitativos presentados por una línea de *L. infantum* resistente a MIL; en estos parasito además de evidenciar cambios en la cinética de crecimiento de los promastigotes, se observó un aumento significativo en los complejos de fosforilación oxidativa, principalmente, complejo IV, junto con una mayor actividad en la β-oxidación de ácidos grasos y los transportadores *ABC*; con una reducida concentración de proteínas asociadas a desintoxicación de especies reactivas de oxígeno (ROS) [216]. Recientemente investigadores como Gutiérrez-Guarnizo y colaboradores han realizado estudios de proteómica enfocados a comprender el traductoma en *Leishmania*, generando importantes hipótesis como la adaptación preventiva y la optimización del metabolismo energético de los parásitos con fenotipos de resistencia [217,218].

En *Leishmania*, la proteómica no solo ha ayudado a mejorar la comprensión del fenómeno de la resistencia antiparasitaria, sino que además ha permitido proponer antígenos para el diseño de pruebas diagnósticas, factores de virulencia, posibles nuevos blancos terapéuticos y programas metabólicos especie-específicos asociados con el aprovechamiento y uso energético de diferentes componentes celulares [219,220].

# 2. HIPÓTESIS DEL TRABAJO Y OBJETIVOS

## 2.1. HIPÓTESIS DEL TRABAJO

Históricamente, la leishmaniasis ha sido una enfermedad asociada a la pobreza y al olvido [221–223] y se encuentra dentro de las metas de eliminación y erradicación de la Asamblea de la OMS para las Enfermedades Tropicales Desatendidas (ETD; Neglected tropical diseases) [224]. En India, Nepal, Irán y Brasil, se realizan investigaciones y seguimiento a los cambios en los patrones de distribución de la leishmaniasis, en especial para la forma visceral; cubriendo aspectos epidemiológicos, biológicos, moleculares y evolutivos de los agentes causales [193,146,7,5,96].

En Colombia, los estudios relacionados con la leishmaniasis se centran en la LC, que es la forma más frecuente de la enfermedad; mientras que, la baja prevalencia de la LV ha generado desinterés por parte de organizaciones gubernamentales y privadas frente a la enfermedad, llevando al desconocimiento de la epidemiología de esta enfermedad y sus factores determinantes, principalmente en términos de: *i)* cambios en la distribución de los casos de la LV en Colombia, *ii)* las interacciones del parásito con vectores, reservorios y el ambiente, *iii)* la estructura genómica y relaciones filogenéticas de *L. infantum* en el país, y *iv)* los cambios biológicos y proteómicos de parásitos resistentes al tratamiento de primera línea.

Considerando las problemáticas previamente mencionadas, los vacíos en el conocimiento tanto en la LV como en *L. infantum* en Colombia, y la necesidad de realizar investigaciones centradas en esta enfermedad tropical desatendida en el marco de las metas de erradicación y control de la OMS, surge nuestra propuesta de investigación. Partiendo de la hipótesis de que el uso de herramientas bioestadísticas, bioinformáticas y moleculares de NGS, junto con proteómica de alta profundidad, permitirá una comprensión más amplia y la obtención de datos valiosos para entender mejor la LV en un contexto global e integrado de todos los diferentes actores del ciclo de vida y del parásito mismo. Esta integración analítica generará información actualizada y estadísticamente respaldada sobre la ocurrencia de la enfermedad y los factores que la modelan. Facilitará el análisis clínico de casos y mejorará el abordaje de la enfermedad en áreas donde circulan simultáneamente diferentes tripanosomátidos de interés en salud pública teniendo como premisa la posibilidad identificar eventos de coinfección. Desde la perspectiva de One Health, respaldará y apoyará la toma de decisiones en actividades de promoción y prevención comunitaria mediante la caracterización de la circulación de los agentes infecciosos presentes en humanos, vectores y reservorios en los territorios de forma más sensible. También proporcionará información para el refinamiento de antígenos utilizados en el diagnóstico, basado en la comprensión de la diversidad genética de *L. infantum*. Por último, los datos proteómicos abriran la posibilidad de considerar nuevos blancos terapéuticos asociados a rutas biosintéticas, a partir del análisis de la resistencia inducida a los antimoniales, entendiendo este fenómeno como complejo y multicausal.

## 2.2. OBJETIVO GENERAL

Abordar de manera integral el patógeno *Leishmania infantum* mediante el análisis de la leishmaniasis visceral en Colombia, los eventos de coinfección en los diferentes actores del ciclo de vida, la diversidad genética del parásito a nivel local y global, así como los cambios biológicos y proteómicos asociados a la resistencia antiparasitaria.

## 2.3. OBJETIVOS ESPECÍFICOS

- Describir los cambios en la epidemiología de la leishmaniasis visceral en Colombia y su relación con la distribución de vectores en el país.
- Desarrollar una estrategia de Amplicon-Based Sequencing que permita identificar eventos de coinfección por diferentes especies de *Leishmania* y *Trypanosoma* en humanos, vectores y reservorios de zonas endémicas para leishmaniasis visceral y/o leishmaniasis cutánea en Colombia.
- Describir la diversidad genética de aislamientos autóctonos de *L. infantum* desde un enfoque comparativo con diferentes aislamientos a nivel mundial; así como, las relaciones filogenéticas de los principales antígenos usados en el diagnóstico de la LV.
- Caracterizar los cambios proteómicos y biológicos de *L. infantum* con resistencia inducida al estibogluconato de sodio trivalente (SbIII) en comparación con su cepa originaria silvestre.

# 3. INTRODUCCIÓN A LOS CAPÍTULOS

Comprender y articular la epidemiología clásica y molecular de las enfermedades infecciosas puede fortalecer los sistemas de salud y las alertas tempranas, mejorando las actividades de promoción y prevención. Esto puede evitar la ocurrencia de brotes y la emergencia de las enfermedades en nuevos focos de transmisión, facilitando la detección oportuna de casos y el manejo adecuado de las morbilidades en los pacientes [225], como es el caso de la LV. Para lograr estos resultados, es necesario estudiar y comprender a nivel biológico y genético el agente causal, sus vectores, sus reservorios y, en el caso particular de la LV, al hospedero humano. Dado el interés de la LV en la salud pública, los estudios articulados deben consolidar información relacionada con diferentes aspectos, como lo son:

*i*) Las características demográficas de la población humana en riesgo, su vulnerabilidad socioeconómica y la tasa de seroconversión, como se ha observado en países altamente afectados por la LV como India, China y Brasil [7], son aspectos cruciales a considerar. Además, es importante analizar los cambios a lo largo del tiempo en términos de prevalencia, incidencia, morbilidad y mortalidad [5,6]. Esto cobra aún más relevancia dado los cambios inminentes en los nichos ecológicos, el calentamiento global [55,226,227] y la amplia gama de actividades antropogénicas que pueden influir en las enfermedades infecciosas [228,19,229,17]. Se han reportado casos de emergencia y reemergencia de la LV en todo el mundo, lo que subraya la necesidad de una vigilancia continua y medidas de prevención efectivas [11,230,231].

*ii*) El desarrollo de una metodología que permita identificar de manera simultánea, sensible y costo-efectiva diferentes especies de tripanosomátidos, especialmente *L. infantum*, en todos los componentes del ciclo de vida del parásito en zonas endémicas, así como la distribución de vectores [54,232,233], reservorios domésticos [71,74,75] y reservorios silvestres [66,234,235] en el país. Considerando la posibilidad de la ocurrencia de fenómenos de coinfección por múltiples especies en un mismo actor, lo cual puede tener implicaciones importantes en el flujo genético de los parásitos, el intercambio de material genético relacionado con la resistencia a medicamentos y virulencias; así como, sobre la severidad de la enfermedad, curso de la enfermedad y tratamiento oportuno.

*iii* ) La comprensión de los aspectos genéticos y biológicos propios del parásito ha revelado en varias especies de *Leishmania* cómo la estructura y el flujo genético pueden determinar factores cruciales como la patogenicidad, la virulencia y la resistencia a los antiparasitarios [60,84,90]. Interesantes interrogantes han surgido sobre la posible existencia de una población definida de *L. infantum* en Colombia, Venezuela y Centroamérica, distinta de la que circula en Brasil. Esto podría explicar las discrepancias en la distribución de casos, incidencias y poblaciones afectadas, entre otros aspectos.

*iv)* El estudio de la resistencia a los antiparasitarios, que se considera una de las principales y más urgentes problemáticas en la leishmaniasis. En todo el mundo, se han llevado a cabo numerosas investigaciones centradas en la caracterización de cambios genómicos, transcripcionales y proteómicos en *Leishmania*, especialmente en las especies causantes de CL y VL (como *L. donovani* y *L. infantum* en el Viejo Mundo). Estos estudios han proporcionado información valiosa sobre los cambios estructurales del genoma del parásito y las alteraciones en la síntesis de enzimas que le confieren resistencia natural o adquirida a los leishmanicidas. Sin embargo, dado que la expresión génica en los tripanosomátidos es policistrónica y su regulación se basa principalmente en procesos post-transcripcionales y post-traduccionales, es crucial emplear herramientas de alta profundidad para el análisis proteómico, lipidómico, metabólico, del translatoma y del secretoma de *L. infantum*. Esto permitirá correlacionar e integrar los datos para proponer nuevos blancos terapéuticos o tratamientos compuestos como alternativa frente a la falla terapéutica asociada a la resistencia.

Por todo lo anterior, y reconociendo el impacto de la LV en Colombia y la región, el objetivo de esta tesis doctoral se centró en la integración de diferentes métodos y herramientas de análisis para abordar de manera integral la especie *Leishmania infantum,* desde la caracterización de la epidemiología de la leishmaniasis visceral en Colombia, la identificación de los eventos de coinfección en los diferentes actores del ciclo de vida a partir de la implementación de una metodología basada en NGS, la descripción de la diversidad genética del parásito a nivel local (autóctono) frente a la especie a nivel global y las relaciones filogenéticas de los principales antígenos usados a nivel de diagnóstico de la LV para comprender los cambios en la sensibilidad diagnóstica de las pruebas rápidas alrededor del mundo, y finalmente, los cambios biológicos y proteómicos asociados a la resistencia inducida a uno de los medicamentos de primera línea para el tratamiento oportuno de la enfermedad, el SbIII, generando la posibilidad de considerar nuevos blancos terapéuticos asociados a rutas biosintéticas, a partir del análisis de este fenómeno complejo y multicausal. Por lo que, la actual tesis de investigación se dividió en los siguientes cuatro capítulos :

**Capítulo 1.** Descripción demográfica, espacial y temporal de la leishmaniasis visceral en Colombia.

**Capítulo 2.** Uso de Amplicon-Based Sequencing para la identificación simultánea de *Leishmania infantum* y otros tripanosomátidos en diferentes actores del ciclo de vida del parásito en Colombia.

**Capítulo 3.** Diversidad genética de aislamientos autóctonos de *Leishmania infantum* en Colombia y sus relaciones filogenéticas intra-especie a nivel global.

**Capítulo 4.** Caracterización cualitativa y cuantitativa de la proteómica en una cepa *Leishmania infantum* resistente al estibogluconato de sodio trivalente**.**

## 3.1. CAPÍTULO 1

**Título:** Descripción demográfica, espacial y temporal de la leishmaniasis visceral en Colombia.

El primer capítulo de la tesis tuvo como objetivo principal describir la epidemiología de la leishmaniasis visceral en Colombia y su relación con la distribución de vectores en el país. Para el cumplimiento de este objetivo se recolectó información del SIVIGILA, historias clínicas de pacientes en el Laboratorio Nacional de Referencia-Parasitología del INS y publicaciones científicas de reportes de casos de LV en Colombia desde 2007 hasta 2018; así mismo, reportes de distribución de las especies de *Lutzomyia* y la información histórica disponible en la página de DANE (Departamento Nacional de Estadística) sobre la proyección de habitantes por departamentos y municipios. El análisis de datos se hizo por medio de estadística descriptiva, regresiones Lowes, incidencia espacio-temporal y relación espacial de los casos reportados y las especies de vectores para *Leishmania* spp. a nivel nacional.

Entre 2007 y 2018 se hallaron un total de 306 casos de LV reportados en SIVIGILA y confirmados por laboratorio, con una cobertura de 25.5 casos/año, y una mortalidad de 2.28%; en cuanto a número de casos totales, el 2007 fue el año con mayor número de casos (n=52) y el 2012 el año con el menor reporte de casos (n=9). En cuanto a los pacientes, los casos de LV predominaron en los niños menores de 7 años, sin diferencia significativa de casos entre el sexo femenino y el masculino, afiliados al sistema de salud en el régimen subsidiado. En cuanto a la distribución geográfica, los casos se presentaron en 42 municipios de 10 departamentos, con un interesante reporte de casos en 3 localidades que no habían presentado casos de LV previamente; finalmente, la presencia de los vectores *Lutzomyia longipalpis*, *Lu. evansi* y *Lu. gomezi* pueden estar cambiando el panorama de la LV en el país.

Este estudio permitió la publicación de un artículo científico:

Castillo-Castañeda A, Herrera G, Ayala MS, Fuya P, Ramírez JD. Spatial and Temporal Variability of Visceral Leishmaniasis in Colombia, 2007 to 2018. Am J Trop Med Hyg. 2021 Jul 7;105(1):144-155. doi: 10.4269/ajtmh.21-0103. PMID: 34232908; PMCID: PMC8274786

Am. J. Trop. Med. Hyg., 105(1), 2021, pp. 144–155
doi:10.4269/ajtmh.21-0103

# Spatial and Temporal Variability of Visceral Leishmaniasis in Colombia, 2007 to 2018

Adriana Castillo-Castañeda,[1] Giovanny Herrera,[1] Martha S. Ayala,[2] Patricia Fuya,[3] and Juan David Ramı´rez[1]*

[1]Centro de Investigaciones en Microbiologı´a y Biotecnologı´a-UR (CIMBIUR), Facultad de Ciencias Naturales, Universidad del Rosario, Bogota´, Colombia; [2]Grupo de Parasitologı´a, Instituto Nacional de Salud, Bogota´, Colombia; [3]Grupo de Entomologı´a, Instituto Nacional de Salud, Bogota´, Colombia

Abstract. Visceral leishmaniasis (VL) is a neglected tropical disease associated with poverty and is endemic in 56 countries worldwide. Brazil, Venezuela, and Colombia are the most affected countries in South America. In Colombia, the National Public Health Surveillance System (SIVIGILA) consolidates epidemiological information and monitors all VL cases nationwide. However, to date, no studies have investigated the occurrence of VL in Colombia using metadata analysis. We studied the demographic data, the spatial and temporal distribution of VL cases, and the association with vector distribution of Leishmania species in Colombia from 2007 to 2018. We found 306 VL cases reported to SIVIGILA for this period, with a coverage of 25.5 cases/year, and a mortality of 2.28% (seven deaths). The highest number of confirmed cases (N = 52) occurred in 2007; the lowest (N = 9) occurred in 2012. The cases were reported mainly in children (< 7 years) affiliated with the subsidized health regimen. Regarding the geographic distribution, the cases were reported by 42 municipalities distributed in 10 departments. The occurrence of VL cases toward the northeast of Colombia, and the distribution of vectors, such as Lutzomyia longipalpis and Lu. evansi, may be changing the panorama of VL in the country. We conclude that VL, mainly in recent years, shows a temporal and spatial variability associated with the occurrence of cases in new settings. Our findings increase our understanding and knowledge of this disease, and suggest the need to monitor and prioritize areas with changes in geographic expansion to improve prevention and control actions in the country.

## INTRODUCTION

Globally, vector-borne diseases constitute a major public health problem because of their high incidence and complex control—particularly in endemic areas, where transmission rates are high.[1,2] This group of diseases includes leishmaniasis, which is transmitted primarily by the bite of the female sand fly of the Phlebotominae family that is infected with promastigotes of the protozoan parasite Leishmania.[3] The disease has three main clinical forms, named according to the anatomic location affected in the host: cutaneous leishmaniasis (CL), mucosal leishmaniasis, and visceral leishmaniasis (VL).[4] The presentation and evolution of each clinical form has been attributed to multifactorial characteristics of the parasite and the host.[5]

VL is recognized as the most serious form of leishmaniasis. In VL pathology, the amastigote stage of the parasite invades vital organs, such as the liver and spleen, and tissues such as bone marrow. Physiological and metabolic functions of the body are altered, leading to death if patients do not receive timely treatment.[6] Historically, the etiology of VL has been attributed mainly to species of the Leishmania donovani complex[3,7] and, to a lesser extent, species of the L. enrietti complex[8]; however, in recent years, there have been reported cases of VL associated with other species, including L. colombiensis,[9] L. amazonensis,[10,11] and L. tropica.[12]

According to official reports of the Global Leishmaniasis Program of the WHO for January 2020, 56 countries are endemic for VL, with 90% of the cases occurring in Brazil, Ethiopia, India, Kenya, Somalia, South Sudan, and Sudan.[1] Annually, there are an estimated 50,000 to 90,000 new cases worldwide; however, the disease may be underreported by as much as 55% to 75%.[13] According to reports by the Drugs for Neglected Diseases initiative, about 20,000 to 30,000 deaths are attributed to VL every year.[14] In 2018, the majority of indigenous VL cases in the Americas was reported in Brazil (N = 3,460), Venezuela (N = 43), Paraguay (N = 19), and Colombia (N = 16).[15] However, incidence has been greatly affected by climate change, migratory movements, and internal political crises. This last factor leads to health deficits and the disruption of public health programs.[16–18]

In Colombia, the National Public Health Surveillance System (SIVIGILA) was created with Decree 3518 in 2006 to provide information rapidly and systematically on diseases of public health interest, and thus guide policies and programs aimed at the prevention, control, and monitoring According to information provided by the Colombian government, the occurrence of indigenous VL cases has been limited primarily to two geographically defined areas known as the Magdalena River and the Montes de María subregion, San Andrés de Sotavento (Córdoba).[19] In Magdalena River, there are four VL-endemic departments: Huila, Tolima, Cundinamarca, and Santander. In the Montes de María subregion, the endemic departments are Bolívar and Sucre. In addition, there have been cases reported in the department of Córdoba in the same subregion of the Caribbean coast. However, in recent years, SIVIGILA has recorded indigenous cases occurring in new departments, as well as cases imported from other countries.[20–22]

In endemic municipalities, the public health laboratories perform surveillance and focus studies to understand VL transmission. During entomological studies, occurrences of Lutzomyia longipalpis and Lu. evansi infected with L. infantum have been reported in areas with cases of VL. Similarly, researchers have studied the distribution of vectors involved in the transmission of L. infantum,[23–25] including Lu. evansi in the northwest of the country and Lu. longipalpis in the Andean region, with a high prevalence in Huila, Tolima, and Cundinamarca.[23] In addition, analysis of biological samples from canines has revealed different rates of infection in four endemicdepartments: Bolívar, 36%[26]; Sucre,33.6%[27] to66.9%[28]; Huila, 17.2%[29]; and Tolima, 31.5%.[30] Furthermore, a similar panorama of infection rates has been observed in endemic areas of Venezuela, 24.1%[31]; Spain, > 34%[32]; Brazil, 45.6%[33]; and Bangladesh, 35%.[34]

At the national level, metadata studies have shown changes in the demographic pattern and distribution of the disease and vectors in relation to CL as well as VL.[35–37] However, despite the importance of this disease and the mandatory reporting of all VL cases in SIVIGILA, the combined geospatial and temporal variation of VL in Colombia has not yet been documented, and the full extent of the changes in the national distribution of this parasitic disease remains unknown. Therefore, this study aimed to describe the temporal and spatial distribution of VL in Colombia from 2007 to 2018 and to analyze descriptively the occurrence and distribution of the disease. Using publicly available information from official governmental sites and scientific data, we conducted statistical analyses and made biological associations with the vector distributions. Our results provide a scientific basis in relation to spatial and temporal changes and vector distribution for VL, which will help disease monitoring and deployment of public health strategies and programs aimed at the effective and efficient prevention and control of VL transmission in endemic areas of the country.

---

* Address correspondence to Juan David Ramírez, Centro de Investigaciones en Microbiología y Biotecnología-UR (CIMBIUR), Facultad de Ciencias Naturales, Universidad del Rosario, Bogotá, Colombia. E-mail: juand.ramirez@urosario.edu.co

## METHODS

Datacollectionandprocessing. The information recorded in the SIVIGILA platform are reported by the primary national data-generating units, which are obliged to report events of public health interest in Colombia. These events may be related to infectious diseases (including VL), noncommunicable diseases, or mortality-related events. The primary national data-generating units correspond to institutions providing health services at the municipal level (third sub-national territorial and administrative levels). The registered information was consolidated and checked at the department level (second sub-national territorial and administrative levels) by professionals of the public health surveillance department of INS (National Institute of Health) to verify cases and confirm their status.

We obtained the data available in reports of public health events from 2007 to 2018 and event microdata (for VL) from 2007 to 2017 from the SIVIGILA website (https://www.ins. gov.co/Direcciones/Vigilancia/Paginas/SIVIGILA.aspx). In addition, the 2018 Leishmaniasis Events report by the National Laboratory of Reference-Parasitology Group from the INS was used to corroborate the information. The tables for each year were consolidated with the addition of demographic data from the National Statistics Department (Departamento Administrativo Nacional de Estadı´stica [DANE]) available in the document Estimation and Projection of Total National, Departmental, and Municipal Population by Area 1985–2020 for 2007 to 2017 and Population Projections 2018–2020 for 2018 to take advantage of the data updates by DANE, and thus obtain a more accurate estimate. The data on case occurrences were organized annually and biannually using Microsoft Excel (v. 2018; Microsoft Corp., Redmond, WA) at both the department and municipal levels.

For VL cases where there was no information about the municipality of origin were weighted in the department for the year of occurrence. Thus, the case was added to the municipality that had more cases reported by department during the year of analysis.

Cases of unknown department origin or Venezuelan origin were not used when analyzing incidence. Data on the national distribution of Leishmania vectors (mainly the sand flies associated with species transmitting VL) were taken from the entomological surveillance report for leishmaniasis of the INS[38] and research articles by Ferro et al.[23] and Bejarano et al.[39] The data were consolidated and filtered to construct a map of Colombia showing the geographic distribution of the Leishmania vectors (Lutzomyia spp.) and their relationship to VL cases. The map was made using QGIS software (v. 3.24.1, Washington, DC).

Statistical analysis. Descriptive statistical analysis of the data was performed to determine the demographic characteristics of the population with VL. The variables analyzed were age, gender, department of origin, affiliation to the health system, and number of deaths. The variable "affiliation to the health system" included four categories: contributory regimen (workers and pensioners contributing to the health system for their care and that of their family members), subsidized regimen (unemployed people from socioeconomic strata 1 and 2 who receive many health services free of charge), other (special regimes, such as those for indigenous, military, or state teachers), and none (no affiliation to any health regimen).

To show differences in infection by gender, a normality test was performed for age in both groups (male and female). Because the data were not distributed normally, we proceeded to present the data with median and interquartile range values. Later, a Mann-Whitney U test was performed to evaluate the existence of differences among ages by gender.

These demographic variables were also used to perform a multivariate linear regression to determine whether the cases had an association with the covariates available in the public databases used. Given the small number of cases, we collated all cases in the study period into a single analysis by departments. We calculated standardized incidences based on the total population by department. Standardized incidence ratios (SIRs) were calculated from the total number of cases in Colombia over the study period using empirical Bayesian smoothing as described by Clayton and Kaldor.[40] We constructed probability maps based on the empirical negative binomial distribution for the data. Last, we calculated measures of spatial autocorrelation in several ways. A global Moran's I randomization test on the SIRs was performed, and spatial weight matrices were based on first-order queen contiguity.

Municipal crude incidence (Io) was calculated by dividing the number of VL cases per year by the population, and the result was recorded per 100,000 inhabitants. The same calculation was made at the department level, and the data were plotted on a heat map using ggplot2 (R package, v. 3.6.1). To display the cases and incidences data in a timeline, the number of cases and Io data were smoothed using Lowess regression. To show the geographic evolution of VL cases during the analyzed period, a map of Colombia was drawn showing the VL biannual incidence rates from 2007 to 2018 by department using QGIS software.

## RESULTS

Description of sociodemographic data of patients with VL in Colombia. For the years analyzed, 306 laboratoryconfirmed cases of VL in Colombia were reported to SIVIGILA. Four of these cases were associated with a department of unknown origin and one originated abroad (Venezuela), and thus were discarded from the statistical analyses. In general, most VL cases occurred in children; 75% of the patients were between 0 and 7 years old, with a median of 2 years. The remaining 25% of patients were between 8 and 78 years old (Table 1). There were no statistically significant differences in cases of VL between the sexes at the national level ($\chi^2 = 0.40199$, $P = 0.5261$). Regarding social security affiliation, most of the patients were in the subsidized regimen (73.2%) or they were not affiliated with any health system regimen (15.6%), which can be extrapolated to represent conditions of vulnerability associated with poverty because, in the subsidized regimen of the health system in Colombia, people from socioeconomic strata 1 and 2 are affiliated to this one. Similarly, not being affiliated with any health regimen implies greater vulnerability in relation to the difficulty of accessing health services coupled with poverty. For the years analyzed, seven (2.29%) cases reported to SIVIGILA were individuals who had died.

CharacterizationofVLcasesandincidencebydepartment. Analysis showed the existence of two geographically defined groups for the presentation of VL in the country—one in the north and the other toward the center of the country—in the departments that converge on the Magdalena River (Figure 1). Each area contains three main departments: the North Zone consists of Bolı´var, Sucre, and Co´ rdoba, whereas the Central Zone contains Huila, Tolima, and Cundinamarca. The comparison between observed and expected cases showed SIR values that demonstrated the high level of risk of VL occurrence mainly in the North Zone (Bolı´var, Sucre, and Co´ rdoba), where the transmission of the parasite and the presentation of the disease are historically recorded (Figure 1) and the population is lower in comparison with the departments in the Central Zone.

The clear pattern in the geographic distribution of VL in Colombia had an impact on the occurrence and incidence of cases per 100,000 inhabitants at the department and municipal levels. The departments with the greatest occurrence were Bolı´var (105 cases), Sucre (76 cases), and Co´ rdoba (70 cases), followed by Huila (28 cases) and Tolima (11 cases) (Figure 2A). Since 2016, the number of VL cases in Huila began to exceed those in Sucre and Co´ rdoba. Interestingly, between 2010 and 2012, there was a marked decrease in the number of cases in the three departments that generally have the greatest number of cases per year. In 2012, only nine cases were reported in Colombia, seven in Huila, and the other two in Sucre and Tolima (Supplemental Table 1). We then analyzed annual incidence by department (Figure 2B), and although, until 2015, the department with the greatest number of cases was Bolı´var, Sucre had the greatest annual incidence. As of 2016, both the incidence and number of cases reported in Bolı´var were the greatest in the entire country. However, Huila has also begun to be an important department for VL in recent years; in 2017, the incidence exceeded that of Sucre and Cordoba. In 2018, the number of cases exceeded those of the two regions.

In congruence with the previous analyses, the histograms of observedcases and theirtrends showed that, in Colombia, the presentation of VL is a highly variable phenomenon over time and that it does not follow a certain pattern. It has a tendency to increase or decrease in the number of cases per year (Supplemental Figures 1 and 2). In addition to this finding, it noticed that during the first months of each year, there is a seasonality factor, with an increase in the number of cases reported (Supplemental Figure 2).

CharacterizationofVLcasesandincidencebymunicipality. When we analyzed the crude incidence of VL at the interdepartment and municipal levels, we observed high data variation andcongruenceintheirdistribution(Figure3A).Atalowerlevel ,we found 42 municipalities reporting VL cases in the analyzed period. The municipalities with the greatest historical incidence per year were El Carmen de Bolı´var, Ovejas, and San Andre´ s de Sotavento (Figure 3B), in the departments of Bolı´var, Sucre, and Co´ rdoba, respectively. However, the greatest incidence was reported in Chala´ n (Sucre), with 34.46 and 34.34 cases/inhabitant in 2010 and 2009, respectively. In Huila, the annual VL cases were reported by different municipalities, and the number of cases reported was not constant over time. Notably, in the department of La Guajira, all VL cases were limited to the municipality of Hatonuevo (Figure 3b).

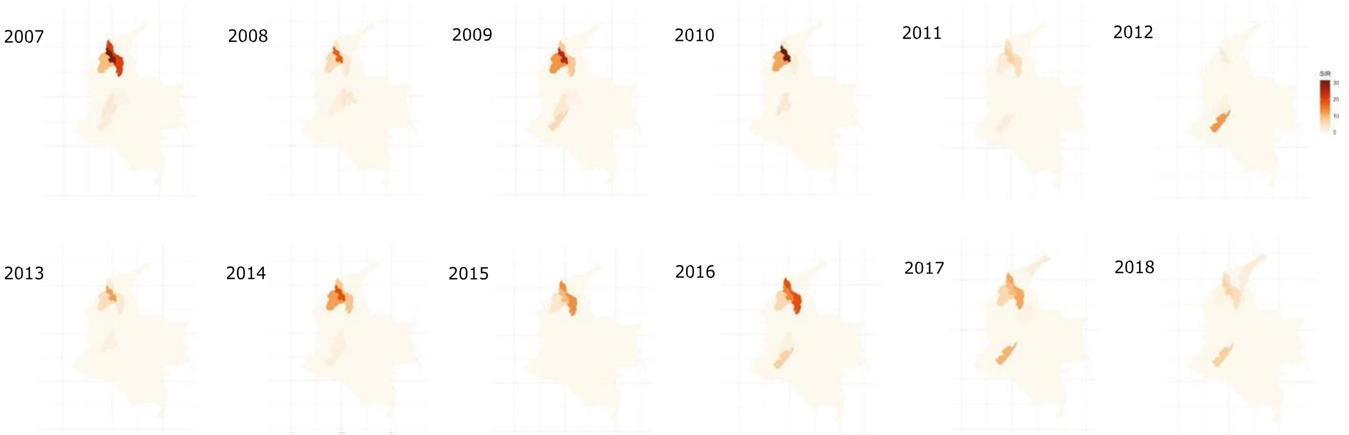
Table 1

Sociodemographic characteristics of patients with visceral leishmaniasis in Colombia reported to the National Public Health Surveillance System from 2007 to 2018

| | | Location in Columbia | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Variable | Bolı´var | Co´ rdoba | Sucre | Cesar | Huila | Tolima | Cundinamarca | Santander | Norte de Santander | La Guajira | Total |
| No. of cases | 105 | 70 | 76 | 1 | 28 | 11 | 6 | 1 | 1 | 2 | 301 |
| No. of deaths | 2 | 2 | 1 | 0 | 2 | 0 | 0 | 0 | 1 | 0 | 8 |
| Age, y; median (IQR) | 3 (1–7) | 2 (1–8) | 2 (1–8) | * | 1 (1–3) | 2 (1–2) | 26 (22.75–77.26) | † | * | ‡ | 2 (1–7) |
| Gender | | | | | | | | | | | |
| Female | 50 | 31 | 32 | 1 | 19 | 9 | 2 | 0 | 0 | 0 | 144 |
| Male | 55 | 39 | 44 | 0 | 9 | 2 | 2 | 1 | 1 | 2 | 155 |
| Regimen in the health system | | | | | | | | | | | |
| Contributive | 4 | 9 | 1 | 0 | 6 | 2 | 4 | 0 | 0 | 0 | 26 |
| Subsidized | 79 | 52 | 62 | 1 | 21 | 5 | 2 | 1 | 0 | 2 | 225 |

|  |  |  |  |  |  |  |  |  |  |  |  |
|---|---|---|---|---|---|---|---|---|---|---|---|
| None | 22 | 8 | 13 | 0 | 0 | 4 | 0 | 0 | 1 | 0 | 48 |
| Other | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 2 |

IQR = interquartile range.
* Only case (15 y).
† Two cases (15 and 34 y).
‡ Two cases (1 and 5 y).



§ FIGURE 1. Exploratory data analysis of the occurrence and incidence of visceral leishmaniasis cases in Colombia. The political map of Colombia divided into departments the occurrence of observed cases (left), the occurrence of expected cases (middle), and standardized incidence ratio (SIR) (right) per year from 2007 to 2018. This figure appears in color at www.ajtmh.org.

Analysis of the changes in VL distribution demonstrated by biannual incidence. By plotting the biannual Io of VL both temporarily and spatially by department, we see that, initially, from 2007 to 2010, there were two geographically defined areas of VL occurrence (Figures 1 and 4). Historically, the northern departments have been mostly affected; for example, incidences of more than 1.2/100,000 inhabitants have occurred in Sucre, compared with the more stable and lower VL incidence in the entire country (0.31–0.60/100,000 inhabitants during the years analyzed) (Figure 4). When we compared the distribution and initial incidence of VL of the 2007–08 biennial data with the 2017 to 2018 data, it was seen that, as VL incidence of departments in central Colombia decreased, sporadic instances of new cases began to be reported in the departments of Santander, Cesar, and La Guajira, shifting the VL distribution toward the northwest.

When we calculated measures of spatial autocorrelation, we did not obtain evidence of significant spatial autocorrelation (Moran's I randomization test on the SIRs, z = –0.082, pseudoP = 0.39). We then constructed a probability map based on the empirical negative binomial distribution of the data (Figure 5). Because we did not have evidence of significant spatial autocorrelation, we chose not to calibrate any models.

Species specificity in the distribution of vectors and VL foci cases. We compared the entomological data registered from various research groups and the data from the Entomology Reference Laboratory of the INS with the national distribution of sandflies in relation to the VL endemic departments (Figure 6). The geographic correlation performed fits a spatial ecological association inference. The distribution of Lu. evansi was related mainly to the distribution of VL cases in northern Colombia (Bolı´var, Sucre, and Co´ rdoba) and in the northeastern departments (Norte de Santander, Cesar, and La Guajira). In these latter departments, VL cases have been reported in the past few years.

In contrast, in Huila, Tolima, and Cundinamarca, Lu. longipalpis was the primary vector distributed in these departments. Lutzomyia longipalpis has also been reported in some foci of the Montes de Marı´a subregion, Norte de Santander and La Guajira. Similar concomitance was observed in the distribution of Lu. gomezi (CL Leishmania spp. vector) with VL cases from the Andean area to northern Colombia. Last, *Lu. columbiana* (CL *Leishmania* spp. vector) was only found in the Andean area, and the absence of vectors associated with the transmission of VL in other departments bordering Venezuela is evident, such as in Arauca, Vichada, and Guaviare (Figure 6).

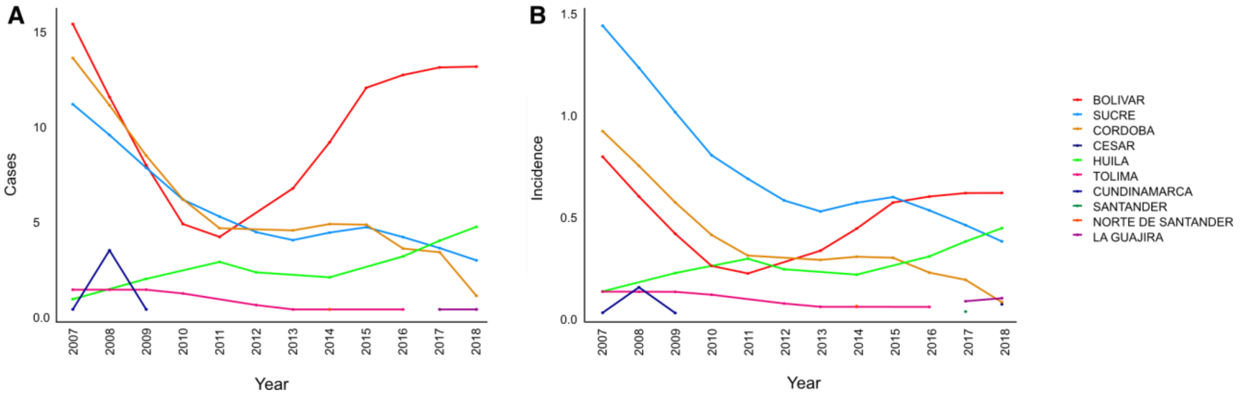


FIGURE 2. (A) Occurrence and (B) incidence per 100,000 inhabitants of visceral leishmaniasis (VL) at the departmental level. Lowess fit was used to smooth the frequency of VL (cases, incidence) by department in a time-series graph. The single point represents a department in which a unique case was reported. This figure appears in color at www.ajtmh.org

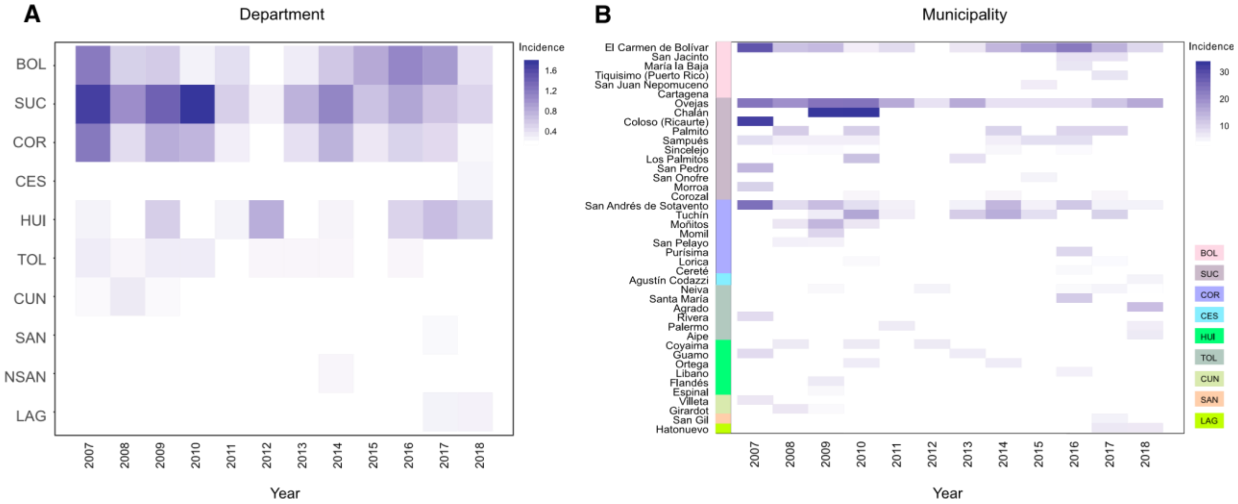


FIGURE 3. Heat map of visceral leishmaniasis incidence variation at the (A) department and (B) municipal levels. Incidence is 100,000 inhabitants/ year.BOL=Bolívar;SUC=Sucre;COR= Córdoba;CES=Cesar;HUI= Huila;TOL=Tolima;CUN= Cundinamarca; SAN=Santander; NSAN=Norte de Santander; LAG = La Guajira. This figure appears in color at www.ajtmh.org.

## DISCUSSION

VL continues to be a major global public health problem in terms of the number of people at risk, related mortality–morbidity rates, andthe increasing emergence ofresistance to commonly used drugs.[41–43] The occurrence of VL in Colombia shows an expansion in its geographic distribution, probably related to the presence of Lu. evansi and Lu. longipalpis on new transmission foci (Figure 6). Likewise, the population most affected by this parasitic disease are the children affiliated with the subsidized regimen of the Colombian health system. The results of our study show that cases of VL have occurred mainly in children in situations of vulnerability associated with poverty (Table 1), considered as one of the greatest risk factors for the development and evolution of the disease.[44,45] In the case of Colombia, not being affiliated with any health system regimen or being in the subsidized regimen is related to belonging to socioeconomic strata 1 and 2, which implies a greater state of economic vulnerability and difficult access to medical and hospital services. According to official reports, in Colombia, people from these socioeconomic strata have a daily income of less than $2.50.

In terms of age, the epidemiological behavior of VL in Colombia is similar to that in Central American countries,[15] Venezuela,[15] Alagoas (Brazil),[46] and western and central China,[47] where children are the most affected by this disease. In contrast, countries with a high prevalence of the disease, such as Brazil (national data), India, and Nepal, show that the adult population is the most affected by VL,[15,48,49] with a growing number of HIV-positive patients.[50] In this sense, the marked difference between the populations affected by VL, according to geographic location of occurrence, may be the result of various factors such as the intraspecific genetic variability and divergence of L. infantum in the New World and the interspecies genetic variability against species of L. donovani complex of the Old World,[7,51,52] in which, for example, L. donovani has shown a large number of polymorphisms and high genetic diversity.[7,51,53] In the human host, susceptibility to VL is associated with some human leukocyte antigen type I and II genes,[54–56] nutritional factors, and immunization rates, which have been found to have protective effects for other infectious diseases,[52] and there are associations at the vector level in relation to the behavior and adaptation to the ecological niche of the main vectors for Leishmania.[25,57–59]

However, concurrent with studies carried out by the WHO in Brazil,[60] the presentation of cases in Colombia was not significantly different between genders. Likewise, this variable in conjunction with the other covariates included in our study did not show a direct association with the occurrence of VL cases in the country. This contrasts with a meta-analysis study by Cloots et al.,[48] who reported males in Bangladesh, India, and Nepal are more prone to developing the disease, and have higher seroprevalence rates and risks for seroconversion. However, population studies in relation to the latter two variables have not been conducted in endemic areas in Colombia. Regarding geographic distribution, VL was found to affect six main departments, with consistent behavior over time, but variability in the number of cases (Figures 1–4), mainly in the municipalities of El Carmen de Bolı´var, Ovejas, San Andre´ s de Sotavento, and Tuchin (Figure 3B). In the other municipalities, the occurrences were usually not sustained over time. In Colombia, there is a trend of an increase of case notifications of VL during the first quarter of each year (Supplemental Figure 2). This fact is possibly associated with the increase in the vector population and its parasite transmission capacity during the rainy season[61] of the last quarter of the previous year in the foci (http://www.ideam.gov.co/web/tiempo-y-climate/ precipitation-monthly-per-year) and the average incubation time of Leishmania in the host, which ranges from 2 to 6 months after infection in the case of VL.[1]

Population abundance and distribution of VL vectors in Latin America, such as L. evansi and L. longipalpis, have been studied in Colombia, Brazil, and Argentina. In general, a direct relationship has been found between increased rainfall and vector abundance, in addition to other environmental factors such as the vegetation richness index.[23,62,63] However, regarding the abundance levels of L. longipalpis, the climatic conditions can affect each focus of VL in a different way.[57,63–66] Therefore, keeping the information on population density and vector distribution updated can help entomological surveillance systems improve vector control activities and thus mitigate the incidence of the disease in the country.

As shown in Figure 3B, there was a significant decrease in reported VL cases in 2012 at the national level, with only nine cases occurring in three locations: Neiva (N = 7), Ovejas (N = 1), and Coyaima (N = 1). This interesting change in the pattern of VL occurrence can be related to climate changes that affect the life cycle of the vector. We inferred that the marked decrease in the number of cases could be related to the El Niño–Southern Oscillation phenomenon and hurricane activity in the Atlantic Ocean during 2012.[67] These meteorological events trigger changes in rainfall, humidity, and continental temperature patterns,[68] and, when rainfall increases above average, there is less organic matter available for the development of sandflies,[69–71] leading to a reduction in leishmaniasis transmission rates.[72] This negative impact on the occurrence of VL has been documented in the Andean region[73] and Venezuela.[74] However, da Silva Neto et al.[68] showed that, in 2012, VL had an increase in incidence (12.37 cases/100,000 inhabitants) in the state of Mato Grosso do Sulen, an area in southern Brazil with particular geographic conditions, and concluded

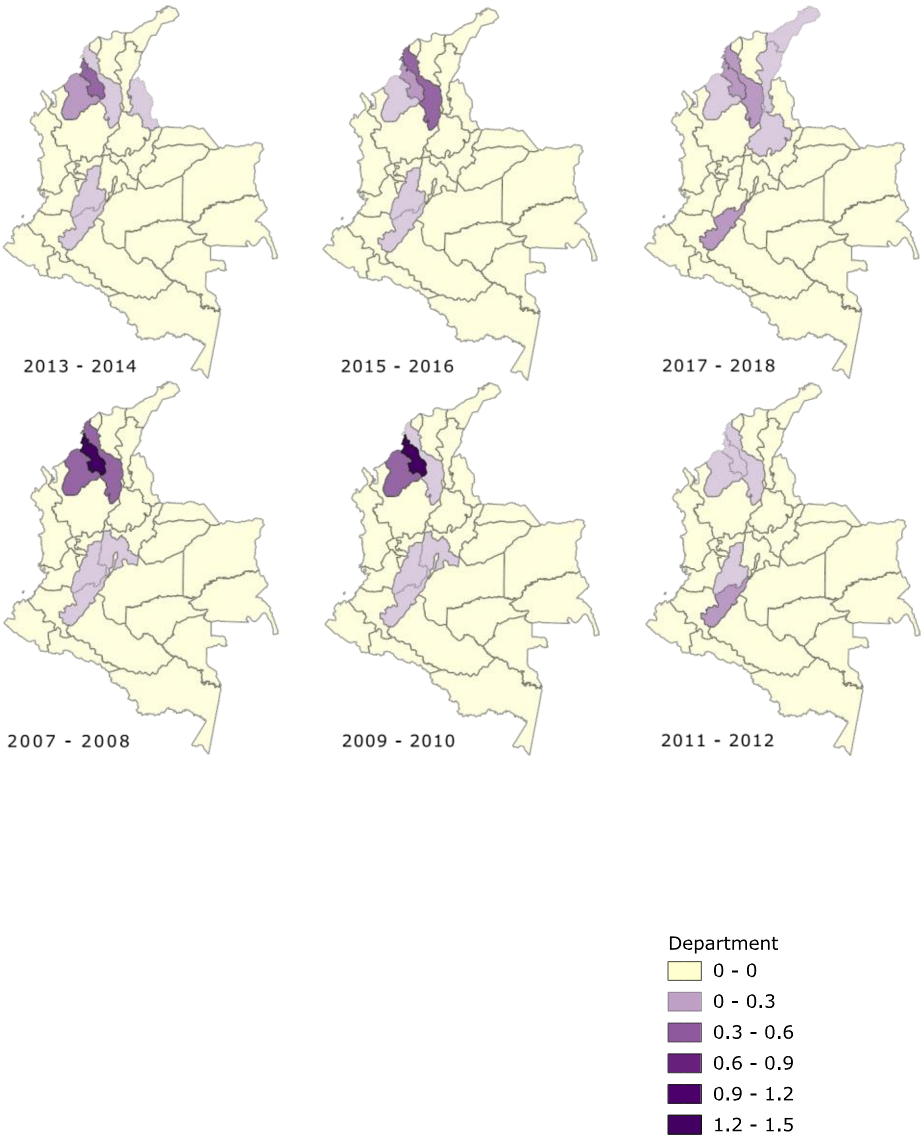


FIGURE 4. Changes in geospatial distribution of visceral leishmaniasis incidence through time by biannual segmentation of department data. Incidence is per 100,000 inhabitants. The map was constructed using QGIS software. This figure appears in color at www.ajtmh.org.

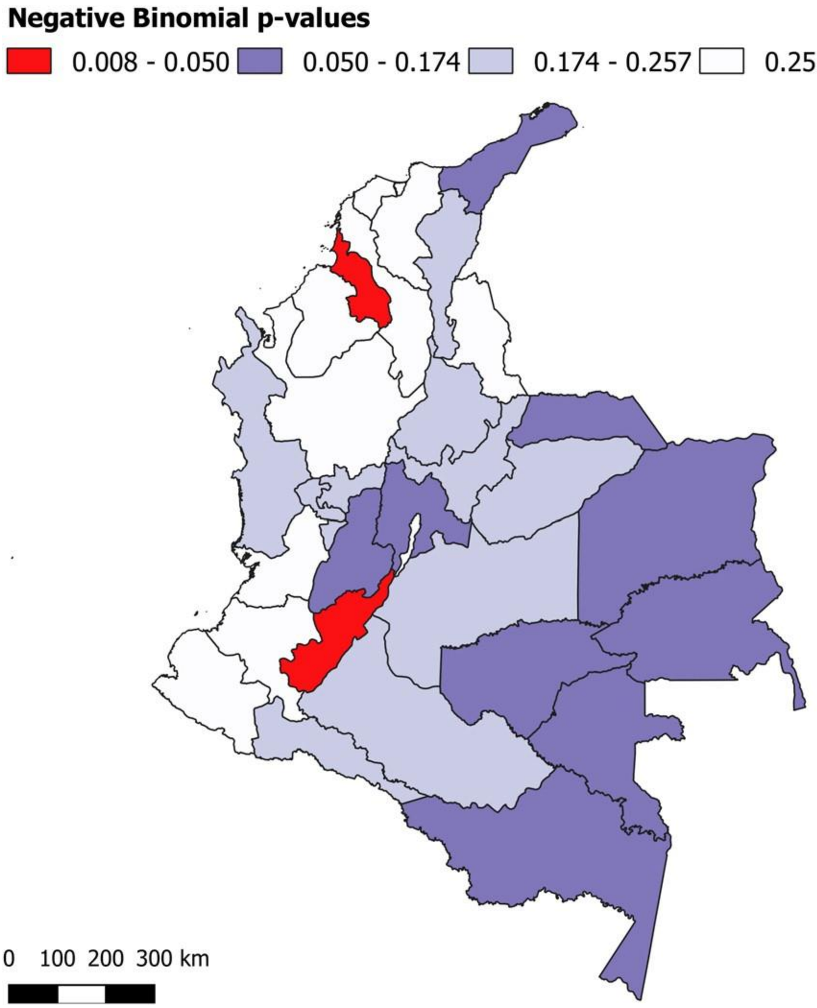


Figure 5.Map of empirical negative binomial distribution for the visceral leishmaniasis data in Colombia from2007 to 2018. This figure appears in color at www.ajtmh.org

this natural phenomena was related to the greatest VL index recorded in this population.

In general, the areas historically endemic for VL are confined to the North (Montes de Marı´a and Co´ rdoba) and Central Zones of the country (Figures 1, 4, and 5). These zones have a wide distribution of Leishmania species-transmitting vectors, such as Lu. longipalpis, Lu. evansi for VL, and Lu. gomezi for CL (Figure 6), and an abundance of reservoir mammals for the parasite,[37,75] which aid the pathogen's epidemiological circuit and, therefore, maintain the transmission rates. Regarding mammalian reservoirs, canines are the main reservoirs for the domestic and peri-domestic transmission of VL[76] in Brazil,[77,78] Spain,[79] and other endemic countries. Studies have been conducted to measure the impact of canines on the incidence of VL, and the effectiveness of euthanasia and other control strategies to interrupt transmission.[80,81] However, to carry out effective control of leishmaniasis in endemic areas, it is necessary to resort to One Health strategies,[82,83] including the concept that the prevention of leishmaniasis requires targeting both canines and the environment in which the insects perpetuate their life cycle.[84]

For VL in Colombia, the epidemiological behavior of the disease in Santander and La Guajira is misleading. By the 19th century, one case of VL had been reported in each of the two departments, in San Vicente de Chucurı´-Santander in 1943 (the first case of VL documented in Colombia)[85] and in Barrancas–La Guajira in 1987,[86] both of which were in infants from rural areas. Curiously, there were reports of cases of CL, but not VL, in various municipalities until VL cases reemerged in 2017 and 2018 (Figure 3B). This panorama, which is similar to that seen in India and Sri Lanka, involving the recent emergence of this clinical form of the disease in new foci,[87] jeopardizes strategies to control and eliminate leishmaniasis in these territories. New studies are needed specifically in these departments to understand more fully the emerging transmission dynamics of the parasite.

It is important to highlight the presence of different vector species for Leishmania in the new foci mentioned in our study and in other border departments, as parasitological and entomological surveillance should be increased in these areas to control and prevent leishmaniasis outbreaks.[3,23] In addition, studies on the rates of infection and coinfection in various Phlebotominae species and domestic/peri-domestic/sylvatic reservoirs by the various Leishmania species are necessary to facilitate the prediction of likely VL-causing species in vulnerable populations. This could increase the effectiveness

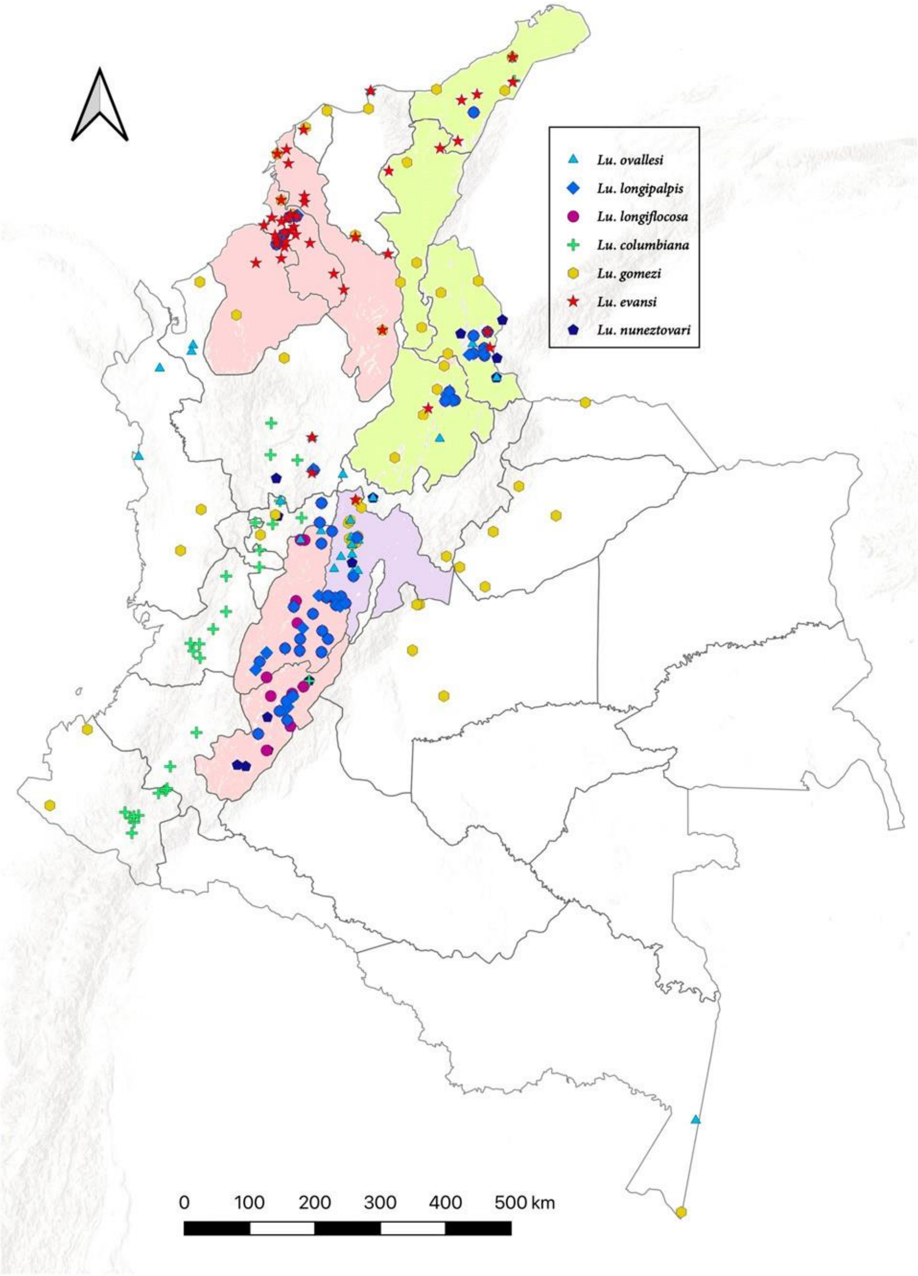


Figure 6. Map of visceral leishmaniasis cases and Leishmania vectors implicated in the transmission of the pathogen. The map shows the overlap between vector diversity and total number of departmental cases across the study periods. Pink shading, endemic department with current cases; violet shading, endemic department with cases until 2010; yellow shading, department with new cases. The map was constructed using QGIS software. This figure appears in color at www.ajtmh.org.

of species-specific therapeutic management strategies, which is crucial considering the different intra- and interspecies susceptibility profiles that have been documented worldwide.[88–90]

However, in addition to the presence of vectors and reservoirs, the emergence of VL in new geographic areas may be associated with environmental factors, such as climate fluctuation[91]; the local intensification of extractive activities[92]; human migration[93,94]; parasite–host interaction factors[95]; host factors, such as nutritional and immunological deficiencies, which are sometimes related to, for example, limited economic resources and limited access to basic sanitation services, education, and self-protection measures[44]; and the intrinsic evolution of parasites, including adaptation to new environments, reduction in life cycle duration, and changes in host immune system evasion. Regarding the immigration of people from Venezuela during the past decade, the cross-border dispersal of Leishmania species has been apparent in both CL and VL cases, as shown in Table 1. In addition to the data used in our study, four cases of VL in the Venezuelan migrant population were reported to SIVIGILA in 2019 and 2020 (http://www.ins. gov.co/buscador-eventos/BoletinEpidemiologico). This phenomenon has been seen for other vector-borne diseases, such as Chagas disease, malaria, and arboviral infections, which implies there is a substantial risk of pathogen transmission to the countries bordering Venezuela.

Likewise, considering that although all the official data for VL was used for this 12-year period, the amount of data and the nature of it do not allow statistical analysis of inferences, as is done in countries such as India and Brazil, where the high prevalence of the disease, availability of data, and population studies have allowed generating these types of associations. The low number of nationwide cases reported to SIVIGILA and confirmed by the laboratory may be associated with technical difficulties or medical staff ignorance of the pathology in some areas. Likewise, this possible under-registration of cases and the precarious demographic information available may be a result of the negative impact of the armed conflict in Colombia experienced during this period, which made it difficult for the community to access health services and timely diagnosis and treatment, and affected indirectly the realtime reporting of information in SIVIGILA. Negative effects on war-associated leishmaniasis have been seen in different countries worldwide.[47,50,96–98] This situation can lead to underreporting of the disease, overestimating the effectiveness of the current VL promotion and prevention programs, and generating possible biases in our data analyses.

Considering the this scenario along with the high variability of ecological niches, different geographic profiles of VL foci in Colombia, and the various characteristics of the population at risk, we consider that, at this time, to make predictions about the transmission rate and detail the risk factors associated with the acquisition of infection and development of VL could lead to fallacies and erroneous hypotheses at the national and even department levels. Therefore, we suggest that, in addition to using the data on changes in the spatial–temporal distribution of infectious diseases generated by our study, researchers must carry out eco-epidemiological analyses that involve the main actors of VL transmission (parasite/vector/reservoir/human) and the relationship among with the different determinants and risk factors associated with them.[48,99] One of the shortcomings of our study is the lack of information related to domestic canines, because they are an important host for the transmission of this zoonotic parasite.[100] Unfortunately, there are no population data that show the incidence of this parasitosis in canines in a sustained manner over time, nor with the coverage of all transmission foci in the country, which represents a knowledge gap for the country.

## CONCLUSION

Because ours is the first study to analyze the spatial and temporal evolution of VL from 2007 to 2018 in Colombia, it is highly important that both the scientific community and national regulatory institutions consider the findings of this study regarding the current situation of the disease. Furthermore, population studies and robust predictive models of the disease should be integrated with epidemiological, sociodemographic, zoonotic, and environmental studies to facilitate effective decision making in relation to the surveillance, prevention, and control of these parasites at the national and regional levels, focusing on those populations with a greater risk for infection.

Received January 26, 2021. Accepted for publication March 26, 2021.

Note: Supplemental tables and figure appear at www.ajtmh.org.

Acknowledgments: We thank Direccio´ n de Investigaci ´on e Innovacio´ n from Universidad del Rosario for providing the English edit of this manuscript. We also thank Ivan Pradilla for his help with the statistical analyses.

Funding support: The publication fee was provided by Direccio´ n de Investigacio´ n e Innovaci ´on from Universidad del Rosario.

Authors' addresses: Adriana Castillo-Castañeda, Giovanny Herrera, and Juan David Ramı´rez, Centro de Investigaciones en Microbiologı´a y Biotecnologı´a, UR (CIMBIUR), Facultad de Ciencias Naturales, Universidad del Rosario, Bogota´ , Colombia, E-mails: adrianac.castillo @urosario.edu.co, giovannya.herrera@urosario.edu.co, and juand. ramirez@urosario.edu.co. Martha S. Ayala, Grupo de Parasitologı´a, Instituto Nacional de Salud, Bogota´ , Colombia, E-mail: mayalas@ ins.gov.co. Patricia Fuya, Grupo de Entomologı´a, Instituto Nacional de Salud, Bogota´ , Colombia, E-mail: pfuya@ins.gov.co.

## REFERENCES

1. PAHO W, 2019. Manual de Procedimientos para Vigilancia y Control de las Leishmaniasis en las Ame´ ricas. Washington, DC: Panamerican Health Organization (PAHO).
2. World Health Organization, 2015. Abordar las Enfermedades Tropicales Desatendidas con el Enfoque de los Derechos Humanos. Geneva, Switzerland: WHO.
3. Akhoundi M, Kuhls K, Cannet A, Voty´pka J, Marty P, Delaunay P,Sereno D, 2016. A historical overview of the classification, evolution, and dispersion of Leishmania parasites and sandflies. PLoS Negl Trop Dis 10: e0004349.
4. Alemayehu B, Alemayehu M, 2017. Leishmaniasis: a review onparasite, vector and reservoir host. Health Sci J 11. Available at: https://www.hsj.gr/medicine/leishmaniasis-a-review-onparasite-vector-and-reservoir-host.php?aid=20131.
5. Akuffo H, Costa C, van Griensven J, Burza S, Moreno J, HerreroM, 2018. New insights into leishmaniasis in the immunosuppressed. PLoS Negl Trop Dis 12: e0006375.
6. McCall L-I, Zhang W-W, Matlashewski G, 2013. Determinants forthe development of visceral leishmaniasis disease. PLoS Pathog 9: e1003053.
7. Franssen SU et al., 2020. Global genome diversity of the Leishmania donovani complex. eLife 9: e51243.
8. Paranaiba LF, Pinheiro LJ, Torrecilhas AC, Macedo DH, Menezes-Neto A, Tafuri WL, Soares RP, 2017. Leishmania enriettii (Muniz & Medina, 1948): a highly diverse parasite is here to stay. PLoS Pathog 13: e1006303.
9. Rodriguez-Bonfante C, Bonfante-Garrido R, Grimaldi G, MomenH, Cupolillo E, 2003. Genotypically distinct Leishmania colombiensis isolates from Venezuela cause both cutaneous and visceral leishmaniasis in humans. Infect Genet Evol 3: 119–124.
10. Aleixo JA, Nascimento ET, Monteiro GR, Fernandes MZ, RamosAMO, Wilson ME, Pearson RD, Jeronimo SMB, 2006. Atypical American visceral leishmaniasis caused by disseminated Leishmania amazonensis infection presenting with hepatitis and adenopathy. Trans R Soc Trop Med Hyg 100: 79–82.
11. Carvalho EM, Momem H, Grimaldi G, Barral-Netto M, Badaro´ R,Barral A, 1986. Isolation of Leishmania mexicana amazonensis from the bone marrow in a case of American visceral leishmaniasis. Am J Trop Med Hyg 35: 732–734.
12. Fakhar M, Pouladfar GR, Alborzi A, Kadivar MR, Hatam GR,Motazedian MH, 2008. Isolation of Leishmania tropica from a patient with visceral leishmaniasis and disseminated cutaneous leishmaniasis, southern Iran. Am J Trop Med Hyg 79: 435–437.
13. World Health Organization, 2020. Leishmaniasis: Newsroom. Geneva, Switzerland: WHO. Available at: https://www.who.int/neglected_diseases/resources/leishmaniasis/en/.
14. Drugs for Neglected Diseases Initiative, 2020. Visceral Leishmaniasis Disease Background. Available at: https://dndi.org/ diseases/visceral-leishmaniasis/.
15. Panamerican Health Organization, 2019. Epidemiological Report of the Americas: Leishmaniases. Washington, DC: Pan American Health Organization. Available at: https://iris.paho. org/handle/10665.2/50505.
16. Afonso MM dos S, Chaves SA de M, Magalhães M de AFM,Gracie R, Azevedo C, de Carvalho BM, Rangel EF, 2017. Ecoepidemiology of American visceral leishmaniasis in Tocantins State, Brazil: factors associated with the occurrence and spreading of the vector Lutzomyia (Lutzomyia) longipalpis (Lutz & Neiva, 1912) (Diptera: Psychodidae: Phlebotominae). ClabornD,ed.TheEpidemiologyandEcologyof Leishmaniasis. InTech. Available at: https://www.intechopen.com/books/the-epidemiology-and-ecology-of-leishmaniasis/ecoepidemiologyof-american-visceral-leishmaniasis-in-tocantins-state-brazilfactors-associated-with.
17. Norman FF, Comeche B, Chamorro S, Lo´ pez-V´elez R, 2020. Overcoming challenges in the diagnosis and treatment of parasitic infectious diseases in migrants. Expert Rev Anti Infect Ther 18: 127–143.
18. Valero NNH, Uriarte M, 2020. Environmental and socioeconomicrisk factors associated with visceral and cutaneous leishmaniasis: a systematic review. Parasitol Res 119: 365–384.
19. Palau MT et al., 1990. Distribution and etiology of leishmaniasisin Colombia. Am J Trop Med Hyg 42: 206–214.
20. INS, 2018. Informe de evento: Leishmaniasis 2017. Bogota, Columbia: Instituto Nacional de Salud.
21. INS, 2017. Informe de evento: Leishmaniasis 2016. Bogota, Columbia: Instituto Nacional de Salud.
22. Zambrano P, Ayala MS, Fuya P, Barraza O, Rodrı´guez Toro G, 2016. Cartagena: new urban focus of visceral leishmaniasis in Colombia. Scielo 7: 83–91.
23. Ferro C, Lo´ pez M, Fuya P, Lugo L, Cordovez JM, Gonz´alez C, 2015. Spatial distribution of sand fly vectors and ecoepidemiology of cutaneous leishmaniasis transmission in Colombia. PLoS One 10: e0139391.
24. Gonza´ lez C et al., 2018. Diversity patterns, Leishmania DNA detection, and bloodmeal identification of Phlebotominae sand flies in villages in northern Colombia. PLoS One 13: e0190686.
25. Paternina LE, Verbel-Vergara D, Romero-Ricardo L, Pe´ rez-DoriaA, Paternina-Go´ mez M, Martı´nez L, Bejarano EE, 2016. Evidence for anthropophily in five species of phlebotomine sand flies (Diptera: Psychodidae) from northern Colombia, revealed by molecular identification of bloodmeals. Acta Trop 153: 86–92.
26. Corte´ s LA, 2012. Foco de leishmaniasis en El Hobo, municipiode El Carmen de Bolı´var, Bolı´var, Colombia. Biomedica 26: 236.
27. Paternina-Go´ mez M, Dı´az-Olmos Y, Paternina LE, Bejarano EE, 2013. Alta prevalencia de infeccio´ n por Leishmania (Kinetoplastidae: Trypanosomatidae) en caninos del norte de Colombia. Biomedica 33. Available at: https://revistabiomedica.org/index.php/biomedica/article/view/780.
28. Paternina Tuira´ n LE, Dı´az-Olmos YA, Paternina-Go´ mez M, Carrillo-Bonilla LM, Ve´ lez ID, Bejarano Martı´nez EE, 2015. Deteccio´ n de anticuerpos anti-Leishmania (Trypanosomatidae) en poblaciones caninas del Departamento de Sucre, Colombia. Acta Biol Colomb 21. Available at: https://www.redalyc.org/articulo.oa?id=319043374018.
29. Ferna´ ndez Manrique J, C T, Bello F, Escovar J, Lozano C, AyalaM, Nicholls R, Vargas J, Moncada L, A A, Lo´ pez M, 2002. Prevalence of canine visceral leishmaniasis in municipalities of Huila, Colombia. Rev Salud Publica (Bogota) 4: 278–285. Available at: http://www.scielo.org.co/scielo.php?script=sci_arttext&pid=S0124-00642002000300005.
30. Romero MH, Lo´ pez MC, Sanchez JA, 2009. B ´usqueda activa de casos de leishmaniasis visceral zoono´ tica en poblaci ´on infantil indı´gena y canina colombiana. Rev Salud Publica (Bogota) 11: 944–951.

31. Cannova DC, Aguilar CM, Guevara H, Delgado O, FeliciangeliMD, 2017. Visceral leishmaniasis in low-income urban areas of Carabobo State, Venezuela: I. Human and canine prevalence, risk of infection. Bol Malariol Salud Ambient 57: 58–68.
32. Dujardin J-C, Campino L, Cañavate C, Dedet J-P, Gradoni L,Soteriadou K, Mazeris A, Ozbel Y, Boelaert M, 2008. Spread of vector-borne diseases and neglect of leishmaniasis, Europe. Emerg Infect Dis 14: 1013–1018.
33. Lopes EG et al., 2017. Serological and molecular diagnostictests for canine visceral leishmaniasis in Brazilian endemic area: one out of five seronegative dogs are infected. Epidemiol Infect 145: 2436–2444.
34. Islam A, Rahman M, Islam S, Debnath P, Alam M, Hassan M,2017. Sero-prevalence of visceral leishmaniasis (VL) among dogs in VL endemic areas of Mymensingh District, Bangladesh. J Adv Vet Anim Res 4: 241.
35. Herrera G, Tehera´ n A, Pradilla I, Vera M, Ramı´rez JD, 2018. Geospatial–temporal distribution of tegumentary leishmaniasis in Colombia (2007–2016). PLoS Negl Trop Dis 12: e0006419.
36. Ovalle-Bracho C, Londoño-Barbosa D, Salgado-Almario J,Gonza´ lez C, 2019. Evaluating the spatial distribution of Leishmania parasites in Colombia from clinical samples and human isolates (1999 to 2016). PLoS One 14: e0214124.
37. Ramı´rez JD, Herna´ ndez C, Le ´on CM, Ayala MS, Flo´ rez C, Gonza´ lez C, 2016. Taxonomy, diversity, temporal and geographical distribution of cutaneous leishmaniasis in Colombia: a retrospective study. Sci Rep 6. Available at: https://www.nature. com/articles/srep28266.
38. INS,2018. Informede Vigilancia Entomolo´ gicadeLeishmaniasis, Colombia2018.Bogota,Colombia:InstitutoNacionaldeSalud.
39. Bejarano EE, Sierra D, Ve´ lez ID, 2003. Novedades en la distribucio´ n geogr´afica del grupo verrucarum (Diptera: Psychodidae) en Colombia. Biomedica 23: 341.
40. Clayton D, Kaldor J, 1987. Empirical Bayes estimates of agestandardized relative risks for use in disease mapping. Biometrics 43: 671–681.
41. Cohen BE, 2016. The role of signaling via aqueous pore formation in resistance responses to amphotericin B. Antimicrob Agents Chemother 60: 5122–5129.
42. Dumetz F, Cuypers B, Imamura H, Zander D, D'Haenens E, Maes I, Domagalska MA, Clos J, Dujardin J-C, De Muylder G, 2018. Molecular preadaptation to antimony resistance in Leishmania donovani on the Indian subcontinent. MSphere 3. Available at: https://pubmed.ncbi.nlm.nih.gov/29669889/.
43. Fre´ zard F, Monte-Neto R, Reis PG, 2014. Antimony transportmechanisms in resistant Leishmania parasites. Biophys Rev 6: 119–132.
44. Alvar J, Yactayo S, Bern C, 2006. Leishmaniasis and poverty.Trends Parasitol 22: 552–557.
45. Hotez P, Ottesen E, Fenwick A, Molyneux D, 2006. Theneglected tropical diseases: the ancient afflictions of stigma and poverty and the prospects for their control and elimination. Pollard AJ, Finn A, eds. Hot Topics in Infection and Immunity in Children III. Springer, 23–33. Available at: https://digitalcommons. library.tmc.edu/childrenatrisk/vol4/iss2/10/.
46. Braz BM de A, Silva RBS, Lins SC, Silva DRX, Ramalho WM, deMelo MA, 2021. Demographic and spatial study of visceral leishmaniasis in the state of Alagoas, Brazil, during 2007–2018. Rev Soc Bras Med Trop 54: e06102020. Available at: https://www. scielo.br/scielo.php?pid=S0037-86822021000100310&script= sci_arttext.
47. Jiang D et al., 2021. Spatiotemporal patterns and spatial riskfactors for visceral leishmaniasis from 2007 to 2017 in western and central China: a modelling analysis. Sci Total Environ 764: 144275.
48. Cloots K et al., 2020. Male predominance in reported visceralleishmaniasis cases: nature or nurture? A comparison of population-based with health facility-reported data. PLoS Negl Trop Dis 14: e0007995.
49. Jervis S et al., 2017. Variations in visceral leishmaniasis burden,mortality and the pathway to care within Bihar, India. Parasit Vectors 10. Available at: https://www.ncbi.nlm.nih.gov/pmc/ articles/PMC5719561/.
50. World Health Organization, 2020. Global Leishmaniasis Surveillance, 2017–2018, and First Report on 5 Additional Indicators. Geneva, Switzerland: WHO.
51. Lypaczewski P, Hoshizaki J, Zhang W-W, McCall L-I, TorciviaRodriguez J, Simonyan V, Kaur A, Dewar K, Matlashewski G, 2018. A complete Leishmania donovani reference genome identifies novelgeneticvariationsassociatedwithvirulence.Sci Rep 8: 16549.
52. Boite´ MC, Sp¨ath GF, Bussotti G, Porrozzi R, Morgado FN, Llewellyn M, Schwabl P, Cupolillo E, 2019. Trans-Atlantic spillover: deconstructing the ecological adaptation of Leishmania infantum in the Americas. Genes (Basel) 11: 4.
53. Downing T et al., 2011. Whole genome sequencing of multipleLeishmania donovani clinical isolates provides insights into population structure and mechanisms of drug resistance. Genome Res 21: 2143–2156.
54. Samaranayake N, Fernando SD, Neththikumara NF, Rodrigo C,Karunaweera ND, Dissanayake VHW, 2016. Association of HLA class I and II genes with cutaneous leishmaniasis: a case control study from Sri Lanka and a systematic review. BMC Infect Dis 16: 292.
55. Singh B, Fakiola M, Sudarshan M, Oommen J, Singh SS, SundarS, Blackwell JM, 2019. HLA-DR class II expression on myeloid and lymphoid cells in relation to HLA-DRB1 as a genetic risk factor for visceral leishmaniasis. Immunology 156: 174–186.
56. Singh T, Fakiola M, Oommen J, Singh AP, Singh AK, Smith N,Chakravarty J, Sundar S, Blackwell JM, 2018. Epitope-binding characteristics for risk versus protective DRB1 alleles for visceral leishmaniasis. J Immunol 200: 2727–2737.
57. de Santana Martins Rodgers M, Bavia ME, Fonseca EOL, CovaBO, Silva MMN, Carneiro DDMT, Cardim LL, Malone JB, 2019. Ecological niche models for sand fly species and predicted distribution of Lutzomyia longipalpis (Diptera: Psychodidae) and visceral leishmaniasis in Bahia State, Brazil. Environ Monit Assess 191: 331.
58. Hoyos-Lo´ pez R, Bolaños R, Contreras-Gutierrez M, CarreroSarmiento D, 2016. Phlebotomine sandflies (Diptera: Psychodidae) in a sub-Andean forest from the Norte de Santander, Colombia. J Vector Borne Dis 53: 70–76.
59. Bates PA et al., 2015. Recent advances in phlebotomine sand fly research related to leishmaniasis control. Parasit Vectors 8: 131.
60. Oliveira-Sena IV, Werneck GL, 2019. Risk factors for in-hospitalmortality from visceral leishmaniasis: a case-control study. J Infect Public Health S1876034119303181. Available at: https://pubmed.ncbi.nlm.nih.gov/31718991/.
61. Mota TF et al., 2019. Natural infection by Leishmania infantum in the Lutzomyia longipalpis population of an endemic coastal area to visceral leishmaniasis in Brazil is not

associated with bioclimatic factors. PLoS Negl Trop Dis 13: e0007626.

62. Estrada LG, Ortega E, Vivero RJ, Bejarano EE, Cadena H, 2020. Development of Lutzomyia evansi immature stages in peridomiciliary environment in a leishmaniasis urban focus in the Colombian Caribbean. Acta Trop 208: 105523.
63. Salomon OD 2020. Lutzomyia longipalpis: gone with the wind and other variables. Neotrop Entomol. Available at: https://www.scielo.br/scielo.php?script=sci_arttext&pid=S007402762015000700831.
64. Go´ mez-Bravo A, German A, Abril M, Scavuzzo M, Salom ´on OD, 2017. Spatial population dynamics and temporal analysis of the distribution of Lutzomyia longipalpis (Lutz & Neiva, 1912) (Diptera: Psychodidae: Phlebotominae) in the city of Clorinda, Formosa, Argentina. Parasit Vectors 10: 352.
65. Berrozpe PE, Lamattina D, Santini MS, Araujo AV, Torrusio SE,Salomo´ n OD, 2019. Spatiotemporal dynamics of Lutzomyia longipalpis and macro-habitat characterization using satellite images in a leishmaniasis-endemic city in Argentina. Med Vet Entomol 33: 89–98.
66. Ribeiro da Silva RC, Guimarães e Silva AS, da Silva Sousa SS,Bezerra JMT, Maca´ rio Rebelo JM, Pinheiro VCS, 2019. Oc-ˆ currence of Phlebotominae (Diptera: Psychodidae) in urban leishmaniasis transmission foci in north-eastern Brazil. J Med Entomol 56: 247–253.
67. Meyer RJ, Baker J, Broad K, Czajkowski J, Orlove B, 2014. Thedynamics of hurricane risk perception: real-time evidence from the 2012 Atlantic hurricane season. Bull Am Meteorol Soc 95: 1389–1404.
68. da Silva Neto AB, de Oliveira EF, Encina CCC, de Figueiredo HR,Paranhos Filho AC, de Oliveira AG, 2020. Effects of El Niño– Southern Oscillation on human visceral leishmaniasis in the Brazilian State of Mato Grosso do Sul. Mem Inst Oswaldo Cruz 115: e190298.
69. Carme B et al., 2013. Climate and leishmaniasis in French Guiana. Am J Trop Med Hyg 89: 564–569.
70. Chowell G, Mizumoto K, Banda JM, Poccia S, Perrings C, 2019. Assessing the potential impact of vector-borne disease transmission following heavy rainfall events: a mathematical framework. Phil Trans R Soc Lond B Biol Sci 374: 20180272.
71. Naqvi Z, 2009. Using Remote Sensing to Assess Potential Impacts of Hurricanes on Mosquito Habitat Formation: Investigating the Mechanisms for Interrelationship between Climate and the Incidence of Vector-Borne Diseases. Available at: https://baylor-ir.tdl.org/handle/2104/5530.
72. WatsonJT,Gayer M, ConnollyMA, 2007.Epidemicsafter naturaldisasters. Emerg Infect Dis 13: 1–5.
73. Pe´ rez-Fl ´orez M, Ocampo CB, Valderrama-Ardila C, Alexander N, 2016. Spatial modeling of cutaneous leishmaniasis in the Andean region of Colombia. Mem Inst Oswaldo Cruz 111: 433–442.
74. Cabaniel G, Rada L, Blanco J, Rodriguez-Morales A, EscaleraAntezanaJP, 2005. Impacto delos eventosde ElNiño Southern Oscillation (ENSO) sobre la leishmaniosis cuta´ nea en Sucre, Venezuela, a trave´ s del uso de informaci´on satelital, 1994–2003. Rev Peru Med Exp Salud Publica 22: 32–37.
75. Alexander B, Lozano C, Barker DC, McCann SHE, Adler GH, 1998. Detection of Leishmania (Viannia) braziliensis complex in wild mammals from Colombian coffee plantations by PCR and DNA hybridization. Acta Trop 69: 41–50.
76. Dantas-Torres F, Solano-Gallego L, Baneth G, Ribeiro VM, dePaiva-Cavalcanti M, Otranto D, 2012. Canine leishmaniosis in the Old and New Worlds: unveiled similarities and differences. Trends Parasitol 28: 531–538.
77. Gomez SA, Chapman LAC, Dilger E, Courtenay O, Picado A,2018. Estimating the efficacy of community-wide use of systemic insecticides in dogs to control zoonotic visceral leishmaniasis: a modelling study in a Brazilian scenario. PLoS Negl Trop Dis 12: e0006797.
78. Seva´ AP, Ovallos FG, Amaku M, Carrillo E, Moreno J, Galati EAB,Lopes EG, Soares RM, Ferreira F, 2016. Canine-based strategies for prevention and control of visceral leishmaniasis in Brazil. PLoS One 11: e0160058.
79. Le Rutte EA, van Straten R, Overgaauw PAM, 2018. Awarenessand control of canine leishmaniosis: a survey among Spanish and French veterinarians. Vet Parasitol 253: 87–93.
80. Lopes EGP, Oviedo-Pastrana ME, Borges LFNM, Freitas ACP,Dias ES, Silva SR, Haddad JPA, França-Silva JC, Soares DFM, 2016. Transmission of visceral leishmaniasis in dogs in a risk area of the metropolitan region of Belo Horizonte, MinasGerais, Brazil. Arq Bras Med Vet Zootec 68: 1403–1412.
81. Dantas-Torres F, Miro´ G, Bowman DD, Gradoni L, Otranto D,2019. Culling dogs for zoonotic visceral leishmaniasis control: the wind of change. Trends Parasitol 35: 97–101.
82. Dantas-Torres F et al., 2019. Canine leishmaniasis control in thecontext of One Health. Emerg Infect Dis 25: 1–4.
83. Vilas VJDR, Maia-Elkhoury ANS, Yadon ZE, Cosivi O, SanchezVazquez MJ, 2014. Visceral leishmaniasis: a One Health approach. Vet Rec 175: 42–44.
84. Otranto D, Dantas-Torres F, 2013. The prevention of canineleishmaniasis and its impact on public health. Trends Parasitol 29: 339–345.
85. Gast A, Renginfo S, 1944. Leishmaniosis visceral: estudio epidemiolo´ gico del primer caso diagnosticado en Colombia. Anales Soc Biol 1: 5–12.
86. Morales A, Ferro C, Isaza de Rodrı´guez C, Cura E, 1987. Encuesta sobre artropodos de intere´ s m ´edico en La Guajira, Colombia, Surame´ rica. Biomedica 7: 89.
87. Sandhya R, Rakesh PS, Dev S, 2019. Emergence of visceralleishmaniasis in Kollam District, Kerala, southern India. Int J Community Med Public Health 6: 1350.
88. Couto DV, Hans Filho G, Medeiros MZ, Vicari CFS, Barbosa AB,Takita LC, 2014. American tegumentary leishmaniasis: a case of therapeutic challenge. An Bras Dermatol 89: 974–976.
89. Herrera G, Herna´ ndez C, Ayala MS, Fl´orez C, Tehera´ n AA, Ramı´rez JD, 2017. Evaluation of a multilocus sequence typing (MLST) scheme for Leishmania (Viannia) braziliensis and Leishmania (Viannia) panamensis in Colombia. Parasit Vectors 10: 236.
90. SilvaSC,Guimarães LH,SilvaJA,MagalhãesV,MedinaL,QueirozA, Machado PRL, Schriefer A, 2018. Molecular epidemiology and in vitro evidence suggest that Leishmania braziliensis strain helps determine antimony response among American tegumentary leishmaniasis patients. Acta Trop 178: 34–39.
91. Ceccato P, Ramirez B, Manyangadze T, Gwakisa P, ThomsonMC, 2018. Data and tools to integrate climate and environmental information into public health. Infect Dis Poverty 7: 126. 92. Salah I, Abbasi I, Warburg A, Davidovitch N, Kotler B, 2020. Ecology of leishmaniasis in an urbanized landscape: relationship of sand fly densities, and Leishmaniatropica infectionrates with reservoir host colonies. Acta Trop 204: 105332.
93. Hotez PJ, 2018. The rise of leishmaniasis in the twenty-first century. Trans R Soc Trop Med Hyg 112: 421–422.

94. Purse BV, Masante D, Golding N, Pigott D, Day JC, IbañezBernal S, Kolb M, Jones L, 2017. How will climate change pathways and mitigation options alter incidence of vectorborne diseases? A framework for leishmaniasis in South and Meso-America. PLoS One 12: e0183583.
95. Afrin F, Khan I, Hemeg HA, 2019. Leishmania–host interactions: an epigenetic paradigm. Front Immunol 10: 492.
96. Berry I, Berrang-Ford L, 2016. Leishmaniasis, conflict, and political terror: a spatio-temporal analysis. Soc Sci Med 167: 140–149.
97. MurshedSM, 2002.Conflict, civilwar andunderdevelopment:an introduction. J Peace Res 39: 387–393.
98. Rehman K, Walochnik J, Mischlinger J, Alassil B, Allan R,Ramharter M, 2018. Leishmaniasis in northern Syria during civil war. Emerg Infect Dis 24: 1973–1981.
99. da Rocha ICM, dos Santos LHM, Coura-Vital W, da Cunha GMR,Magalhães F do C, da Silva TAM, Morais MHF, Oliveira E, Reis IA, Carneiro M, 2018. Effectiveness of the Brazilian Visceral Leishmaniasis Surveillance and Control Programme in reducing the prevalence and incidence of Leishmania infantum infection. Parasit Vectors 11: 586. Available at: https://pubmed.ncbi.nlm.nih.gov/30419944/.
100. Klohe K, Amuasi J, Kaducu JM, Haavardsson I, Bogatyreva E,Onarheim KH, Harrison W, Kristensen F, Prazeres da Costa C, Winkler AS, 2019. The 2017 Oslo conference report on neglected tropical diseases and emerging/re-emerging infectious diseases: focus on populations underserved. Infect Dis Poverty 8: 40.

## 3.2. CAPÍTULO 2

**Título:** Uso de Amplicon-Based Sequencing para la identificación simultánea de *Leishmania infantum* y otros tripanosomátidos en diferentes actores del ciclo de vida del parásito en Colombia.

Dada la amplia diversidad de vectores y mamíferos reservorios, es crucial mantener actualizada la información sobre la distribución espacial de *Leishmania* spp. Además, estudios previos a nivel mundial han señalado la coexistencia de diferentes especies de *Leishmania* e incluso *Trypanosoma* spp. en un mismo individuo, detectadas mediante técnicas como RFLP, serología y aislamiento parasitario. Aunque estas técnicas han proporcionado datos valiosos, presentan varias limitaciones.Por lo que, considerando la costo-efectividad, acceso, sensibilidad y aporte de información a gran escala de las NGS, nos propusimos adoptar la técnica de amplicon-based sequencing para desarrollar una metodología basada en un fragmento de la proteína de choque térmico *HSP70* de los triapnosomátidos, acoplando a su vez el análisis de datos bioinformáticos a una base de datos local curada con la secuencia de nucleótidos disponibles en NCBI para todos los tripanosomátidos. El flujo de trabajo inició con la identificación de la infección por *Leishmania* spp. por medio de PCR convencional y secuenciación Sanger en diferentes muestras y matríces; posteriormente, las muestras positivas fueron procesadas y analizadas por la metodología desarrollada.

La validación inicial de la metodología se realizó a partir del ADN obtenido de cultivos de dos especies de *Leishmania*, las cuales fueron analizadas de forma individual y combinada. Luego, a partir de un total de 231 muestras (8 vectores, 83 perros, 73 mamíferos silvestres, 49 pacientes con LC y 18 pacientes con LV) se realizó PCR convencional del gen HSP70 para identificar la infección por *Leishmania* spp.; posteriormente, según el origen de la muestra analizada, se realizaron PCR convencionales para el codificante de la *Citocromo oxidasa I – COI* (identificar especie de vector analizado) y una subregión del gen *12s* (identificar la especie de mamífero analizado), todos los amplicones fueron secuenciados por sanger y analizados por medio de Blastn.

Las muestras positivas para *Leishmania* fueron seleccionadas respecto a su calidad y cantidad para ser procesadas y analizadas por medio de amplicon-based sequencing del *HSP70* en la plataforma Illumina (8 vectores, 11 caninos domésticos, mamíferos silvestres: 9, pacientes con LC: 49 y con LV: 7); las lecturas fueron filtradas por su calidad y analizadas por medio de Blastn frente a una base de datos local construida para el fragmento de 337pb del gen *HSP70* a partir de las secuencias de tripanosomátidos disponibles en NCBI, analizando posteriormente los índices de diversidad alfa y beta en cada grupo. En el caso particular de las muestras obtenidas de pacientes previamente diagnosticados con LV mediante microscopía (aspirado de médula ósea) o inmunofluorescencia indirecta (suero), se llevó a cabo una correlación con la historia clínica, considerando la sintomatología y la evolución de la enfermedad.

Los resultados obtenidos mostraron una importante distribución de *Leishmania* spp. en reservorios y vectores en diferentes departamentos de Colombia; así mismo, se evidenció un importante panorama al permitir la identificación de coinfecciones en múltiples muestras en los diferentes grupos de análisis, la coinfección por *Leishmania-Trypanosoma* fue identificada en un paciente con LV, varios caninos y reservorios silvestres; así mismo la presencia de ADN de estos dos géneros parasitarios en vectores. La estrategia de secuenciación puede ser útil para el análisis de muestras provenientes de zonas endémicas para LC, LV, y Enfermedad de Chagas.

Finalmente, se propuso diseñar una nueva estrategia de secuenciación basada en amplicones del gen *HSP70*, con dos variaciones importantes con respecto a la metodología previamente implementada (*HSP70-corto,* secuenciado en la plataforma Illumina). Se utilizó un fragmento de 771 pares de bases (*HSP70-largo*) y la plataforma de secuenciación fue MinION™. Esta nueva estrategia se puso a prueba con 28 muestras de ADN de pacientes con LV o LC. En este estudio, se logró una alta especificidad del amplicón *HSP70-largo*, pero se observó una sensibilidad menor en comparación con el *HSP70-corto*. A pesar de esto, ambas estrategias destacaron por su especificidad y eficacia para identificar una amplia variedad de especies de *Leishmania*, así como los eventos de coinfección.

Estos estudios permitieron la publicación de cuatro artículos científicos:

Patiño LH, Castillo-Castañeda AC, Muñoz M, Jaimes JE, Luna-Niño N, Hernández C, Ayala MS, Fuya P, Mendez C, Hernández-Pereira CE, Delgado L, Sandoval-Ramírez CM, Urbano P, Paniz-Mondolfi A, Ramírez JD. Development of an Amplicon-Based Next-Generation Sequencing Protocol to Identify *Leishmania* Species and Other Trypanosomatids in Leishmaniasis Endemic Areas. Microbiol Spectr. 2021 Oct 31;9(2):e0065221. doi: 10.1128/Spectrum.00652-21. Epub 2021 Oct 13. PMID: 34643453; PMCID: PMC8515931.

Castillo-Castañeda A, Patiño LH, Muñoz M, Ayala MS, Segura M, Bautista J, Shaban MV, Paniz-Mondolfi A, Ramírez JD. Amplicon-based next-generation sequencing reveals the co-existence of multiple *Leishmania* species in patients with visceral leishmaniasis. Int J Infect Dis. 2022 Feb;115:35-38. doi: 10.1016/j.ijid.2021.11.029. Epub 2021 Dec 1. PMID: 34863923.

Castillo-Castañeda AC, Patiño LH, Zuñiga MF, Cantillo-Barraza O, Ayala MS, Segura M, Bautista J, Urbano P, Jaimes-Dueñez J, Ramírez JD. An overview of the trypanosomatid (Kinetoplastida: Trypanosomatidae) parasites infecting several mammal species in Colombia. Parasit Vectors. 2022 Dec 16;15(1):471. doi: 10.1186/s13071-022-05595-y. PMID: 36522757; PMCID: PMC9756507.

Patiño LH, Ballesteros N, Muñoz M, Jaimes J, Castillo-Castañeda AC, Madigan R, Paniz-Mondolfi A, Ramírez JD. Validation of Oxford nanopore sequencing for improved New World *Leishmania* species identification via analysis of 70-kDA heat shock protein. Parasit Vectors. 2023 Dec 18;16(1):458. doi: 10.1186/s13071-023-06073-9. PMID: 38111024; PMCID: PMC10726620.

AMERICAN SOCIETY FOR MICROBIOLOGY
Microbiology Spectrum

RESEARCH ARTICLE

# Development of an Amplicon-Based Next-Generation Sequencing Protocol to Identify Leishmania Species and Other Trypanosomatids in Leishmaniasis Endemic Areas

Luz H. Patiño,[a] Adriana C. Castillo-Castañeda,[a] Marina Muñoz,[a] Jesus E. Jaimes,[a] Nicolas Luna-Niño,[a] Carolina Hernández,[a] Martha S. Ayala,[b] Patricia Fuya,[b] Claudia Mendez,[c] Carlos E. Hernández-Pereira,[d] Lourdes Delgado,[d] Claudia M. Sandoval-Ramírez,[e] Plutarco Urbano,[f] Alberto Paniz-Mondolfi,[d,g] Juan David Ramírez[a]

[a]Centro de Investigaciones en Microbiología y Biotecnología-UR (CIMBIUR), Facultad de Ciencias Naturales, Universidad del Rosario, Bogotá, Colombia [b]Grupo de Parasitología, Instituto Nacional de Salud, Bogotá, Colombia

[c]Grupo de Investigación en Enfermedades Tropicales del Ejército (GINETEJ), Laboratorio de Referencia e Investigación, Dirección de Sanidad Ejército, Bogotá, Colombia

[d]Instituto de Investigaciones Biomédicas IDB/Incubadora Venezolana de la Ciencia, Barquisimeto, Venezuela

[e]Grupo de Investigaciones en Ciencias Básicas y Aplicadas para la Sostenibilidad (CIBAS), Facultad de Ciencias Exactas, Naturales y Agropecuarias, Universidad de Santander, Bucaramanga, Colombia

[f]Grupo de Investigaciones Biológicas de la Orinoquia, Universidad Internacional del Trópico Americano (Unitropico), Yopal, Colombia [g]Icahn School of Medicine at Mount Sinai, New York, New York, USA

Luz H. Patiño and Adriana C. Castillo-Castañeda contributed equally to this article. The author order was determined by their equal but gradated contributions for this paper.

ABSTRACT Trypanosomatid infections are an important public health threat affecting many low-income countries across the tropics, particularly in the Americas. Trypanosomatids can infect many vertebrate, invertebrate, and plant species and play an important role as human pathogens. Among these clinically relevant pathogens are species from the genera Leishmania and Trypanosoma. Mixed trypanosomatid infections remain a largely unexplored phenomenon. Herein, we describe the application of an amplicon-based next-generation sequencing (NGS) assay to detect and identify trypanosomatid species in mammalian reservoirs, human patients, and sand fly vectors throughout regions of Leishmania endemicity. Sixty-five samples from different departments of Colombia, including two samples from Venezuela, were analyzed: 49 samples from cutaneous leishmaniasis (CL) patients, 8 from sand flies, 2 from domestic reservoirs (Canis familiaris), and 6 from wild reservoirs (Phyllostomus hastatus). DNA from each sample served to identify the presence of trypanosomatids through conventional PCR using heat shock protein 70 (HSP70) gene as the target. PCR products underwent sequencing by Sanger sequencing and NGS, and trypanosomatid species were identified by using BLASTn against a reference database built from trypanosomatid-derived HSP70 sequences. The alpha and beta diversity indexes of amplicon sequence variants were calculated for each group. The results revealed the presence of mixed infections with more than two Leishmania species in 34% of CL samples analyzed. Trypanosoma

Citation Patiño LH, Castillo-Castañeda AC, Muñoz M, Jaimes JE, Luna-Niño N, Hernández C, Ayala MS, Fuya P, Mendez C, HernándezPereira CE, Delgado L, Sandoval-Ramírez CM, Urbano P, Paniz-Mondolfi A, Ramírez JD. 2021. Development of an amplicon-based nextgeneration sequencing protocol to identify Leishmania species and other trypanosomatids in Leishmaniasis endemic areas. Microbiol Spectr 9:e00652-21. https://doi.org/10.1128/Spectrum.00652-21.

Editor Tim Downing, Dublin City University

Address correspondence to Juan David Ramírez, juand.ramirez@urosario.edu.co.

jdramirez24

Received 22 June 2021
Accepted 27 August 2021
Published 13 October 2021

cruzi was identified in samples from wild reservoirs, as well as in sand fly vectors. Coinfection events with three different Leishmania species were identified in domestic reservoirs. These findings depose the traditional paradigm of leishmaniasis as being a single-species-driven infection and redraw the choreography of host-pathogen interaction in the context of multiparasitism. Further research is needed to decipher how coinfections may influence disease progression. This knowledge is key to developing an integrated approach for diagnosis and treatment.

IMPORTANCE Traditionally, there has been a frequent, yet incorrect assumption that phlebotomine vectors, animal reservoirs, and human hosts are susceptible to Leishmania infection by a single parasite species. However, current evidence supports that these new vector-parasite-reservoir associations lend vectors and reservoirs greater permissiveness to certain Leishmania species, thus promoting the appearance of coinfection events, particularly in disease-endemic regions. The present study describes the application of an amplicon-based next-generation sequencing (NGS) assay to detect and identify trypanosomatid species in mammalian reservoirs, human patients, and sand fly vectors from regions of endemicity for leishmaniasis. This changes our understanding of the clinical course of leishmaniasis in areas of endemicity.

KEYWORDS amplicon-based NGS, Leishmania, NGS, species

Leishmaniasis is a complex vector-borne infectious disease caused by parasites of the genus Leishmania, which are transmitted by insect vectors of the Psychodidae family. The disease exhibits a wide clinical spectrum, driven not only by the host's immune response but also by the infecting parasite species. To date, three main clinical forms of the disease are recognized, cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), and mucocutaneous leishmaniasis (MCL), with CL being the mildest and most common form of the disease. According to WHO, about 1.2 million CL cases have been reported worldwide, with nearly 75% of them occurring in 10 countries: Afghanistan, Algeria, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica, Peru, and Colombia (1).

Current knowledge suggests that different demographic and geographical factors may promote substantial variation in the ecoepidemiological patterns associated with Leishmania transmission cycles. Such variations not only reflect the increased spatial distributions and habitat ranges of different sand fly vector species (2) and reservoirs (3) but also appear to influence the emergence of new vector-parasite-reservoir associations (4).

Traditionally, there has been a frequent yet incorrect assumption that phlebotomine vectors, animal reservoirs, and human hosts are susceptible to infection by a single parasite species. However, current evidence supports that these new vector-parasite-reservoir associations lend vectors and reservoirs greater permissiveness to certain Leishmania species, thus promoting the appearance of coinfection events, particularly in regions where the disease is endemic (5, 6).

So far, several natural human cooccurring infectious events involving diverse Leishmania species have been reported worldwide. In Old World countries, such as Sudan and Iran, cases of Leishmania donovani and Leishmania major (CL patients) (7) and Leishmania tropica and L. major (in an MCL patient) (8) coinfection have been documented. Moreover, several coinfections have also been described in the New World. For example, in Bolivia, a case of Leishmania amazonensis and Leishmania infantum (Leishmania chagasi) coinfection was described in a patient with severe diffuse CL (5). In Mexico, Monroy-Ostria et al. reported on a patient with synchronous Leishmania mexicana and Leishmania braziliensis infection (9). Veland et al. also documented a case of simultaneous infection by L. braziliensis and Leishmania lainsoni in a Peruvian patient with CL (6). More interestingly, the presence of Leishmania guyanensis and Leishmania panamensis hybrid strains causing both CL and MCL has been described in Ecuador (10). Furthermore, studies from Brazil have revealed the presence of double and triple infections caused by several Leishmania species, both in mammals and humans (11, 12).

Likewise, natural coinfections in sand fly vectors and reservoirs have been reported, as in the case of dogs (Canis lupus familiaris) and rodents (Mus musculus and Rattus rattus) infected with L. braziliensis and L. infantum from Brazil (13) or the case of naturally occurring Leishmania and Trypanosoma coinfections observed across different genera of sand flies and mammals (11, 14, 15). Despite the well-documented occurrence of coinfection events among mammalian reservoirs and sand fly vectors in the Americas, the significance of this phenomenon remains largely unexplored.

To date, the most common molecular approaches to detect coinfection events in both Leishmania-infected vectors and mammalian reservoirs are PCR targeting the kinetoplast DNA (kDNA) (6) or small-subunit (SSU) rRNA gene (13), PCR-restriction fragment length polymorphism (PCR-RFLP) (11, 16), and species-specific PCR. Although fast and sensitive, these methods are often expensive and time consuming, particularly when screening a large number of specimens. On the other hand, the targeted nature of species-specific PCR further precludes recognition of rare and new species, as well as the detection of multiple coamplified genetic variants, thus obscuring the presence of lower-abundance genotypes in the context of coinfections (17).

Next-generation sequencing (NGS), particularly amplicon-based sequencing, has emerged as an alternative option to circumvent such limitations. Various studies capitalizing on the advantages of this technology have been conducted for different purposes, for example, determining the prevalence and diversity of bacterial communities (18, 19) and intestinal protozoa (Blastocystis) (20) or characterizing single-nucleotide polymorphisms in viruses (21). Moreover, NGS has allowed the identification of bloodfeeding sources of triatomines (22), detection of Leishmania parasites, blood sources, and plant meals, and deciphering of the intestinal microbiome of phlebotomine sand flies (23), in addition to assessment of the genetic diversity of trypanosomatids in humans (Trypanosoma cruzi and Trypanosoma rangeli) (24, 25) and mammalian reservoir hosts (17, 26, 27). However, relatively few studies have applied this technique to identify and detect coinfection events associated with Leishmania in different biological samples (28).

Here, we designed and implemented a novel amplicon-based NGS assay targeting heat shock protein 70 (HSP70) in order to detect and identify trypanosomatid species infecting not only humans but also mammalian reservoirs and sand fly vectors collected across different geographic regions of endemicity in Colombia and Venezuela. Identifying coinfection events can improve our understanding of pathogen-host interactions and their influence on the epidemiology, pathophysiology, and clinical course of the disease and, additionally, assist in diagnosis and guidance for appropriate therapeutic interventions.

## RESULTS

HSP70 gene is useful to identify trypanosomatid species. The specificity of the HSP70 primers used in this study, both intra- and interspecies, was evaluated. At the intraspecies level, the results obtained showed that, in the evaluated species, the primers only annealed in the different copies of the HSP70 gene of the same chromosome. Additionally, it was observed that those sequences recognized by the primers were totally identical to each other (percent identity, 100%), demonstrating that there was no loss of identity between copies in the different chromosomes of the same species. Regarding the interspecies analysis, we performed a phylogenetic analysis with those copies of the HSP70 gene recognized by the primers for each species, and the results demonstrated the high discriminatory power of the primers across the different species evaluated (Fig. S1 in the supplemental material).

Once the specificity of the HSP70 primers was confirmed, the next step was to determine their ability to identify different species of trypanosomatids. The 385 sequences that constituted the database built as a reference, the 113 sequences from 65 samples included in this study, and the 48 haplotypes were used. The results obtained from the phylogenetic analysis showed, both for the complete data set (385 and 113 sequences) and for the haplotypes, the presence of two populations grouped in well-supported nodes (bootstrap support of .90%) (Fig. 1 and 2A). One was represented by species belonging to the genus Trypanosoma (Fig. 1 and 2, highlighted in purple) and the second by different species of the genus Leishmania. Within the genus Leishmania, the tree topology showed a clear distinction between species of the subgenus Viannia (Fig. 1 and 2, highlighted in green) and Leishmania species (Fig. 1 and 2, highlighted in red). Four groups were recognized within the Viannia subgenus: L. lainsoni, Leishmania naiffi, the L. guyanensis complex, and the L. braziliensis complex. Regarding the Leishmania subgenus, three groups were identified, one of them represented by the L. mexicana complex, the second by sequences of L. major and the L. donovani and L. tropica complex, and the third by L. infantum (L. chagasi). These findings were confirmed by analyzing the paired comparison between the haplotypes (Fig. 2B) and supported by the tree topology obtained in SplitsTree5, where the members of the groups were consistently grouped (Fig. 2C). Additionally, the results obtained evidenced correct clustering among the 113 sequences analyzed in this study and the sequences from the reference database (Fig. 1). Finally, we evaluated and compared the nucleotide diversity between the sequences used as reference sequences; the results identified 132 variable sites among the 337 bp that constitute the HSP70 gene fragment, which represents 39% variability among the sequences.

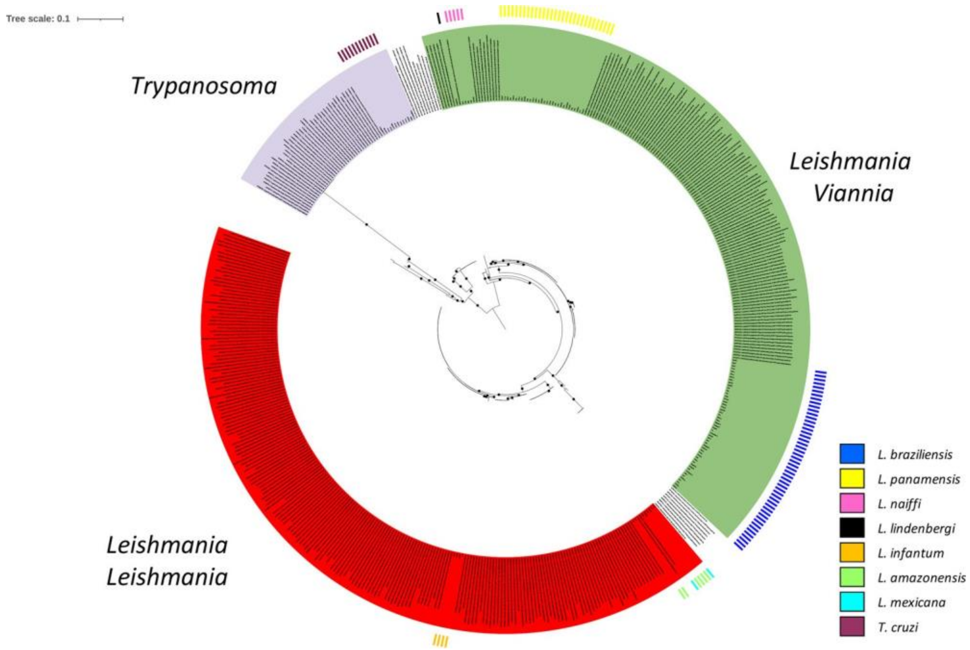


FIG 1 Phylogenetic relationship among the HSP70 sequences used for the reference database and the HSP70 sequences analyzed in this study. The tree represents the phylogenetic analysis based on 385 HSP70 sequences used for the reference database and the 113 HSP70 sequences obtained from 65 samples analyzed in this study. The colors of the inner circle represent the genera, and the outside colors represent each of the species identified in the 113 sequences obtained in this study. The black dots in the tree represent well-supported nodes (bootstrap support of $90).

Identification of Leishmania species by Sanger sequencing. An analysis from CL patients' samples revealed that the most commonly identified Leishmania species was L. braziliensis (63%), followed by L. panamensis (29%), L. amazonensis (4%), L. naiffi (2%), and Leishmania lindenbergi (2%). In domestic reservoirs (Canis familiaris), we identified infection by L. braziliensis (50%) and L. panamensis (50%). Finally, samples from sand fly vectors and wild reservoirs (Phyllostomus hastatus) revealed the presence of L. braziliensis (100%) and T. cruzi (100%), respectively. In none of the samples analyzed the presence of more than one species was identified, (Table S3).

Sequence analysis by the HSP70 amplicon-based NGS. A minimum of 134,316 and a maximum of 179,347 paired-end reads were generated after performing the Illumina sequencing of HSP70 amplicons. Subsequently, we analyzed the quality of the reads obtained, observing that in the samples from patients with CL, between 84 and 95% of the reads passed the quality filter (minimal average quality score of 20). In the vectors, the percentages were between 84 and 94%, except for the R95 sample (domestic reservoir), where only 41% of reads passed the control, and in the reservoirs, the range was between 90 and 95%. Finally, we evaluated the number of high-quality reads that were taxonomically assigned by BLASTn. In the samples from patients with CL, we observed that between 71 and 100% of the reads were assigned, in vectors between 60 and 100% were assigned, and finally, in the reservoirs, assignments were made in 88 to 97% of the reads (Fig. S2 and Table S4).

Method comparisons. Once we determined that the results obtained for HSP70 amplicon-based sequencing matched those obtained from Sanger sequencing, we

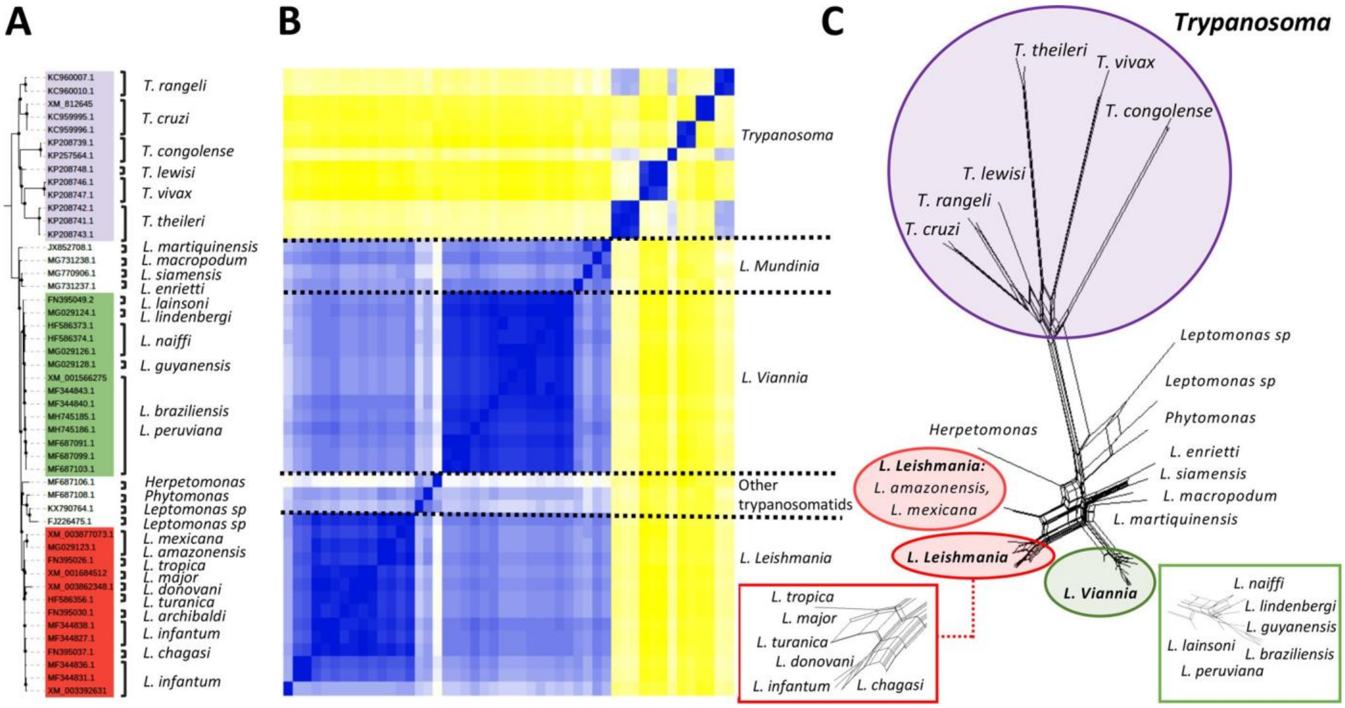


FIG 2 Phylogenetic relationships between the haplotypes from HSP70 sequences used for the reference database. (A) Phylogenetic analysis based on 48 haplotypes from HSP70 reference sequences. The species are listed on the right; the black dots represent well-supported nodes (bootstrap support of $90). (B) The heatmap represents the pairwise comparison of the 48 reconstructed haplotypes. The subgenera are listed on the right. (C) Phylogenetic network (Neighbor-Net) constructed in SplitsTree 5. The squares show enlargements of the clusters represented by Viannia and Leishmania subgenera.

performed an agreement analysis between the two methods. The results demonstrated a global agreement of 53% and agreement ranging from 76.9% to 100% with a Kappa coefficient between 0.31 and 1.0 (Fig. S3) when comparing identified species. We hypothesize that the lower global agreement could be due to multispecies identification in the same samples.

Detection of mixed infections using HSP70 amplicon-based NGS. To determine the capacity of HSP70 amplicon-based NGS, discriminate mixed infections, and estimate the limit of detection of the method, we mixed (1/1) L. braziliensis and L. amazonensis promastigotes at different concentrations in the laboratory and included concentrations of 1 $10^6$ (mix-1) and 1 $10^3$ (mix-2) in the analysis. The results obtained demonstrate detection of L. braziliensis and L. amazonensis in the analyzed samples at concentrations proportional to those expected. Additionally, we observed that detection of the relative abundance was maintained at a concentration of up to 10 parasites/sample (Fig. 3A).

Frequency and diversity of Leishmania species in CL patients using ampliconbased NGS. The NGS analysis revealed infection and coinfection events in CL patient samples. Sixty-six percent of samples revealed single-species infection, with L. braziliensis being the predominant species (72%), followed by L. panamensis (28%). An interesting finding was the identification of L. mexicana and L. infantum in 4% and 6% of the samples analyzed, previously not identified by Sanger sequencing (Fig. 3A). Additionally, multiple coinfection events were recorded in 34% of samples, which were also not visualized through Sanger sequencing. In these coinfection events, the frequency of infection by L. braziliensis was 80%, followed by L. panamensis in 42% of cases and L. mexicana in 4%. L. amazonensis, L. naiffi, and L. infantum were each present in 6% of cases.

L. lindenbergi was present in 2% (Fig. 3A). Double infections were detected in 11 samples, 9 of them, from the departments of Meta, Guaviare, Nariño, and Cundinamarca, showing L. braziliensis/L. panamensis coinfection. Two samples from Guaviare revealed coinfections with L. naiffi/L. panamensis and L. naiffi/L. braziliensis (L. naiffi with a significant prevalence). Triple coinfections (L. lindenbergi/L. braziliensis/L. amazonensis, L. braziliensis/L. panamensis/L. infantum, and L. naiffi/L. braziliensis/L. panamensis) were recorded in three samples from Nariño and Guaviare, and finally, two samples from Venezuela presented multiple coinfection with four Leishmania species (L. amazonensis/L. mexicana/L. infantum/L. braziliensis) (Fig. 3A and 4B).

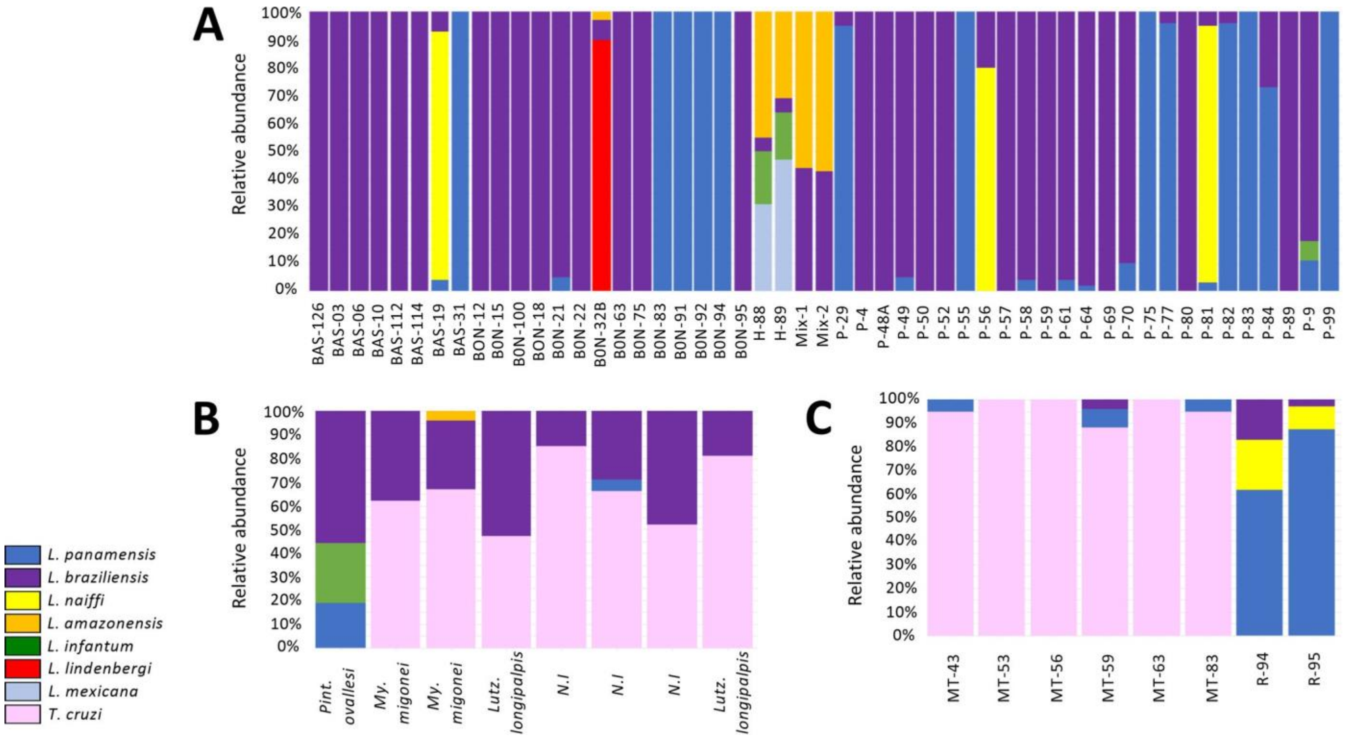


FIG 3 Relative abundances of species identified using the novel amplicon-based NGS assay. The figure represents the relative abundances of Leishmania and Trypanosoma species found in CL patients (A), sand fly vectors (B), and reservoirs (C). The mixtures of L. braziliensis and L. amazonensis made in the laboratory are identified as mix-1 (1  10$^6$) and mix-2 (1  10$^3$). The colors in the bars represent the Leishmania and Trypanosoma species found.

Frequency and diversity of trypanosomatids in sand fly vectors and reservoirs using amplicon-based NGS. Coinfection events with two or more Leishmania species and/or with Trypanosoma cruzi were observed in sand fly vectors and reservoirs. The results obtained from sand flies allowed us to identify coinfection events in all specimens analyzed. A remarkably high proportion of the reads (.60%) identified T. cruzi (present in 87% of all vectors). For the remaining samples, reads from three different Leishmania species (L. panamensis, L. braziliensis, and L. infantum) were recorded (Fig. 3B). The frequency of infection with T. cruzi and L. braziliensis was 87.5% for each, followed by L. panamensis with 25% and L. amazonensis and L. infantum with 12.5% each.

On the other hand, we observed the presence of T. cruzi in all samples from wild reservoirs (Phyllostomus hastatus), where 50% of samples evidenced T.

cruzi at 100% of reads, while the remaining 50% evidenced low proportions of reads (between 4 and 8%) of L. panamensis and L. braziliensis, with infection frequencies of 50% and 16%, respectively (Fig. 3C). Regarding domestic reservoirs (Canis familiaris), we observed coinfection events with three different Leishmania species, L. braziliensis/L. panamensis/L. naiffi, in the two samples analyzed, with a frequency of infection of 100% for each species.

Statistical analysis. The nonparametric Mann-Whitney-Wilcoxon test was performed to explore potential differences between single and coinfection groups, and the Kruskal-Wallis test was applied to explore the differences between the several coinfection groups. The results did not reveal differences related to the continuous variables (age, disease evolution in months, and number of lesions) between single and coinfection groups (Mann-Whitney-Wilcoxon test, with P values of 0.08 for age, 0.87 for disease evolution in months, and 0.437 for number of lesions) or between the coinfection groups (Kruskal-Wallis test, P values of 0.23 for age, 0.181 for disease evolution in months, and 0.617 for number of lesions).

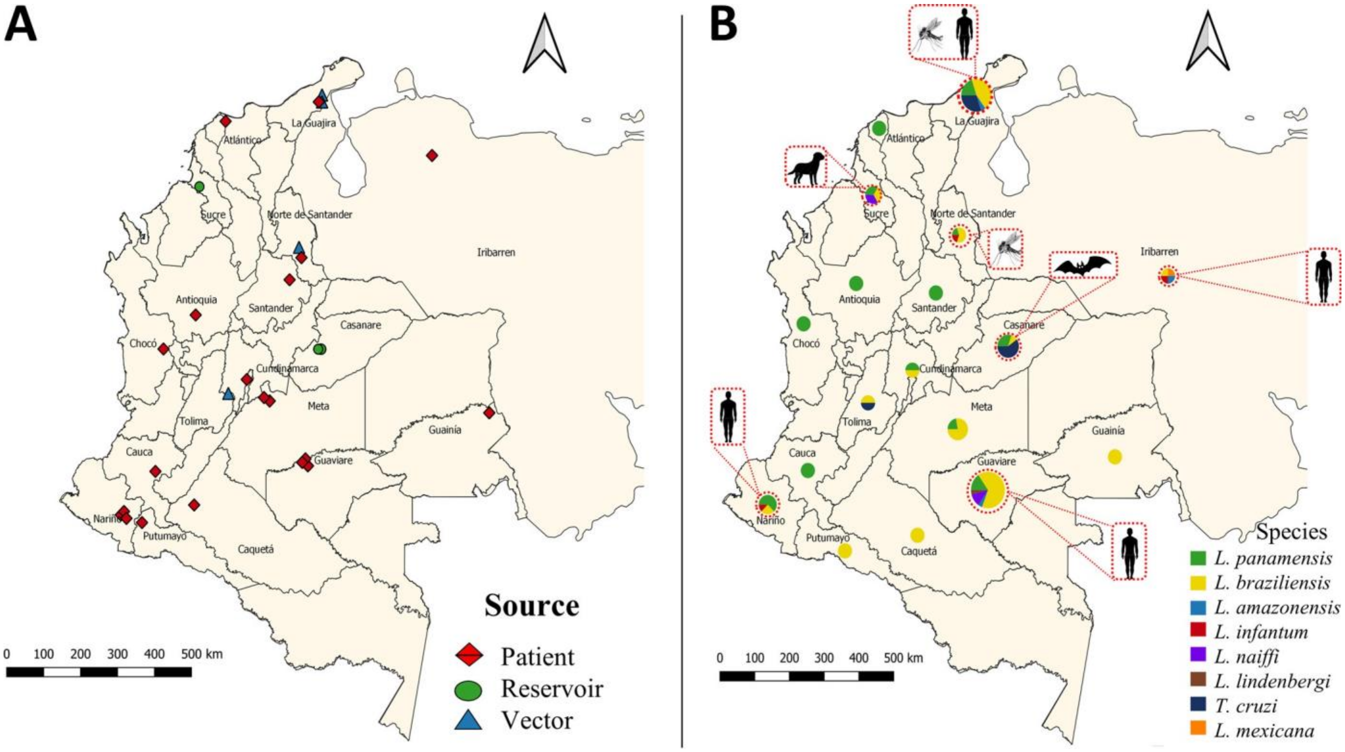


FIG 4 Geographical distribution of samples analyzed in the study and of the species identified through HSP70 amplicon-based NGS. GPS coordinates were used to build georeferenced maps of sampled locations. (A) Geographical localization of 65 samples included in the study. (B) Relative abundances of Leishmania and Trypanosoma species found in each Colombian department and one state of Venezuela (Lara). The size of the circle refers to the number of samples collected by the department.

Chi-square tests and Fisher exact tests were performed to identify potential associations between the categorical variables and species groups. The results showed a relationship between the departments evaluated and the species identified (chi-square test, P = 0.03); however, post hoc comparisons did not have sufficient statistical power due to low or no availability of data for certain departments. In a descriptive approach to reveal the potential differences between the presence of species in the different departments, it was found that in the department of Guaviare, where the largest proportion of samples were obtained (37.5%), L. braziliensis was the most predominant species (66.6%), followed by L. braziliensis/L. panamensis (11.1%) L. naiffi/L. braziliensis (5.5%), L. naiffi/L. panamensis (5.5%) L. lindenbergi/L. braziliensis/L. amazonensis (5.5%), and L. naiffi/L. braziliensis/L. panamensis (5.5%). In Atlantico, Antioquia, Cauca, Choco, Nariño, and Santander, we identified L. panamensis. In Caqueta, Guanía, Meta, Norte de Santander, and Putumayo, we found only L. braziliensis (Fig. 4B and Table S5). We did not identify statistical differences between biological, ecological, and epidemiological variables and species groups in sand fly vectors and reservoirs.

Diversity analysis. The alpha diversity of amplicon sequence variants (ASVs) (Simpson index [dominance] and Shannon index [diversity]) revealed statistically significant differences between the groups analyzed (Kruskal-Wallis test, P = 1.734e206 and P = 1.979e206, respectively). We performed a pairwise post hoc comparison based on the DunnBonferroni test to determine the groups among which these differences were present.

Domestic reservoirs and sand flies exhibited higher diversity (Shannon) indices of 1.3 and 0.75, respectively, than did humans and wild reservoirs, where the median values obtained for both were close to 0 (Kruskal-Wallis test, P = 1.979e206, and post hoc Dunn-Bonferroni, P = 0.031 and P = 0.002, respectively) (Fig. 5A). Correspondingly, the dominance (Simpson) indices were higher in domestic reservoirs and sand flies (0.6 and 0.48, respectively) than in humans and wild reservoirs (Kruskal-Wallis test, P = 1.734e206, and post hoc Dunn-Bonferroni, P = 0.00001 and P = 0.0021, respectively) (Fig. 5A). Regarding pairwise comparisons among groups, the Shannon index analysis revealed statistically significant differences between domestic reservoirs and humans, sand flies and humans, domestic reservoirs and wild reservoirs, and sand flies and wild reservoirs (Table S6); however, the Simpson index analysis revealed statistically significant differences only between humans and sand flies and between sand flies and wild reservoirs (Table S6).

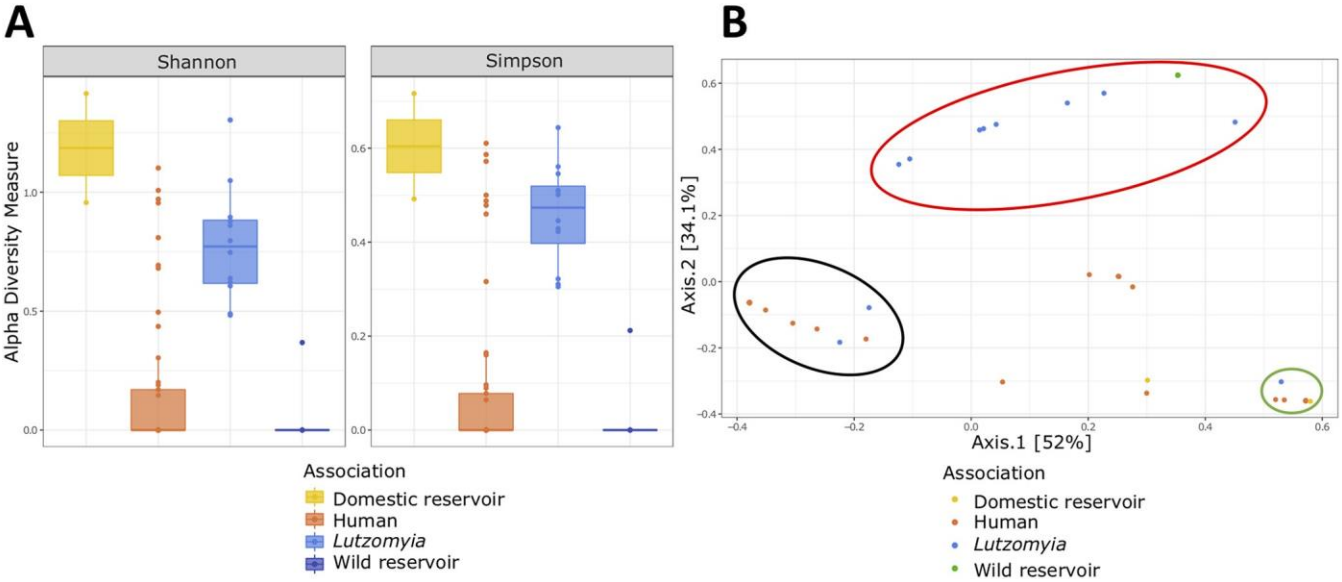


FIG 5 Sequence diversity analysis of HSP70 reads. (A) Boxplots displaying Shannon and Simpson index results for each group evaluated (human CL, Lutzomyia, and wild and domestic reservoirs). (B) Principal coordinate analysis (PCoA) plots between the groups evaluated. The ovals encompass those individuals that had the same diversity of species of trypanosomatids.

Permutational multivariate analysis of variance (PERMANOVA) showed statistically significant differences of beta diversity among the populations analyzed (PERMANOVA, P , 0.05). Similarity percentage (SIMPER) analysis revealed that ASVs corresponding to L. panamensis, L. braziliensis, and T. cruzi contributed significantly to the dissimilarity among clustering. Three groups were observed; the first comprised 15 human and 2 sand fly samples (Fig. 5B), where L. braziliensis (PERMANOVA, P = 0.0094) and T. cruzi (PERMANOVA, P = 0.0002) were the genera that mainly contributed to the differentiation, with contributions of 35.84 and 36.82%, respectively (Table S7). The second group included all the wild reservoir samples and nine sand fly samples (Fig. 5B). However, the results revealed that none of the ASVs identified showed statistically significant differences, indicating that they did not contribute to the differentiation of the grouping (PERMANOVA, P . 0.05) (Table S7). Finally, the last group was represented by human, sand fly, and domestic reservoir samples, where L. panamensis, L. braziliensis, and T. cruzi contributed to the dissimilarity. L. braziliensis (PERMANOVA, P = 00026) and L. panamensis (PERMANOVA, P = 0.048) were the Leishmania species that mostly contributed to the differentiation between humans and domestic reservoirs, with contributions of 43 and 41.57%, respectively, and L. panamensis (PERMANOVA, P = 0.038) and T. cruzi (PERMANOVA, P = 0.042) were the genera that contributed the most to the differentiation between sand flies and domestic reservoirs, with contributions of 38.63 and
33.63%, respectively (Table S7).

## DISCUSSION

Mixed trypanosomatid infections remain a largely unexplored phenomenon. However, given the marked heterogeneity in ecoepidemiological patterns associated with trypanosomatid transmission and the ever-increasing trends in spatial distribution of different vector species (2) and reservoirs, it seems reasonable to infer that the prevalence of trypanosomatid polyparasitism may be higher than previously thought. Deciphering the true prevalence of coinfections would help to improve our understanding of the interactions between these pathogens and the implications for host health.

Herein, we designed an NGS-based method for the detection of mixed trypanosomatid infections in CL patients (Fig. 3A) in order to assess the prevalence and genetic diversity not only in human hosts but also in sand fly vectors and mammalian reservoirs. Our data analysis revealed that the most commonly observed coinfection events were caused by L. braziliensis/L. panamensis (Fig. 3A and 4B). This was predictable given the high prevalence and overlapping occurrence of these species across most regions of endemicity in Colombia (29, 30).

Currently, little is known about the effects of coinfecting pathogen interactions and how this may influence disease outcomes. Previous studies have suggested that preceding Leishmania infections may confer protection against reinfection with other homologous or heterologous species (31, 32). Multiple studies from Mexico, Perú, Ecuador, Bolivia, Brazil, and more recently Colombia have demonstrated the presence of mixed infection with different Leishmania species in CL patients (5, 6, 9, 10, 12). Such findings also raise questions regarding possible synergistic or antagonizing effects between species in the context of coinfections. Further research looking into potential synergistic interactions among species is of utmost importance in order to evaluate aspects such as virulence enhancement, immune response modulation, individual risk for infection, and differential response to antileishmanial therapy (33) in multispecies parasitism. Understanding the effects of polyparasitism will also undoubtedly improve our knowledge in the management of CL, as well as refine our diagnostic approach and determine the true prevalences of mixed infections, particularly in areas of endemicity.

Another interesting observation was uncovering the presence of coinfection in sand fly vectors and the close ecological interaction between Leishmania species, T. cruzi, and insect vectors (Fig. 3B). During the analysis, two interesting scenarios drew our attention. The first was the capacity of Pintomyia ovallesi to harbor two different Leishmania species. These findings coincide with previous reports depicting the intimate relationship of this vector with the parasite (34, 35), as well as the fundamental role that P. ovallesi plays in the transmission cycle of the parasite and progression across the clinical spectrum. Second, our results underscore the close relationship of Migonemyia migonei and Lutzomyia longipalpis with T. cruzi (Fig. 3B). Even though the presence of Trypanosoma in sand flies has not been described in Colombia, reports from other localities, including Italy (15, 36), Madrid (37), Thailand (14), and Brazil (38), have documented this association. Our findings suggest that sand flies may be acting as potential vectors of Leishmania and Trypanosoma parasites. However, the detection of T. cruzi DNA in seven of the sand flies analyzed is not sufficient evidence to incriminate these species as competent vectors, given that the genomic source could have originated from a blood meal, particularly in overlapping areas of endemicity where Trypanosoma and Leishmania species cocirculate, potentially causing mixed infections on both host and vectors. These results emphasize the need to prioritize vector competence studies of sand flies and Trypanosoma species.

The amplicon-based NGS analysis of Phyllostomus hastatus samples also revealed two interesting findings. The first was the identification of T. cruzi as the predominant parasite in this bat species (Fig. 3C), and the second was the presence of three bats coinfected with T. cruzi, L. braziliensis, and/or L. panamensis (Fig. 3C). Previous studies from Brazil (27, 39) and Colombia (40) have also recorded the presence of T. cruziinfected Phyllostomus hastatus, as well as trypanosomatid polyparasitism in bats. So far, various studies have demonstrated mixed Leishmania species infections (L. braziliensis, L. amazonensis, L. chagasi, L. mexicana, and L. infantum) in bats, mainly from Brazil and Mexico (41–44), while other studies have recorded coinfection events involving Trypanosoma and Leishmania (45, 46). Still, no previous study has reported the presence of Leishmania DNA from bats in Colombia. To the best of our knowledge, this is the first study to demonstrate the presence of Leishmania and other trypanosomatids in wild reservoirs from areas of endemicity of Colombia using an amplicon-basedNGS methodology. From an ecological standpoint, we consider that T. cruzi and Leishmania coinfection in Phyllostomus hastatus may be linked to its feeding habits, given that this bat species is known to feed on wild mammals and a significant number of triatomines and other insects (sand flies) found in areas of endemicity of leishmaniasis and Chagas disease. In addition, it is important to highlight that these food sources occupy the same habitats (inside or around human dwellings) while sharing nighttime activity patterns with these bats (39, 41, 47), thus increasing the chances for consumption and infection. These findings demonstrate the advantages of using an ampliconbased NGS approach to detect mixed infections and provide further insights into the interaction of bats and different Leishmania species in order to design novel strategies for prevention and control of the disease.

When analyzing the results obtained from samples of domestic reservoirs (Canis familiaris), we observed the occurrence of coinfection events involving three different Leishmania species (L. panamensis/L. brazilienis/L. naiffi) (Fig. 3C). This was not surprising given that all these species have previously been associated with canine Leishmaniasis throughout different countries of South America (48–50). Despite the fact that canine leishmaniasis involving mixed species has been reported in both Brazil (51, 52) and Venezuela (11), to date, no cases of coinfection have been documented in Colombia. Therefore, this is the first report of Leishmania polyparasitism in naturally infected dogs from Colombia, showing triple-infection events. Interestingly, two of the canines showed coinfection with L. naiffi, a recently identified species in Colombia (53) that is known to cause human cutaneous disease in Panama, French Guiana, and the Brazilian Amazon (53, 54). Furthermore, the facts that L. naiffi had not been previously identified in Sincelejo, where the sample was obtained, and that one of its vectors (Lutzomyia gomezi) is endemic across neighboring departments (Cordoba, Atlantico, and La Guajira) (30, 55, 56) where cutaneous leishmaniasis is endemic strongly suggest a possible expansion of the ecological range of the sand fly vector, thus highlighting the introduction of new species and/or genotypes to certain geographical areas where they had not been previously reported, increasing the possibility that a vector/reservoir could be infected with more than one species. Identifying canines coinfected with different Leishmania species living in close proximity to humans has important public health implications for the design and implementation of epidemiological control programs of VL and CL in Colombia. In fact, assessing the true prevalence of coinfections is essential to improving surveillance and prevention strategies, particularly in at-risk populations from endemic rural and urban areas. Although we identified mixed infections in both vectors and reservoirs, we consider that a much deeper sampling with individuals from different geographic regions should be performed to confirm these findings. Finally, the diversity indices estimated from our results are revealing not only given the high diversity of species present in domestic reservoirs and sand flies (Fig. 5A) (57, 58) but also based on the fact that L. panamensis, L. braziliensis, and T. cruzi were the main species contributing to the dissimilarity between the groups (Fig. 5B). This was anticipated due to the wide geographic distribution of these species across the sampling areas, which signals an excellent power of adaptation of these species to different mammalian hosts and vectors. We consider that a deeper sampling of both sand fly vectors and domestic reservoirs is necessary to confirm these findings and determine the zoonotic contributions of trypanosomatids to clinical disease.

In summary, this study describes findings showing that HSP70 amplicon-based NGS, unlike Sanger sequencing, is a useful tool to detect mixed trypanosomatid infections. This is because amplicon-based NGS allows greater coverage of a specific region of interest, thus detecting new variants, rare species, or genotypes that are present in low abundance, as can occur in cases of mixed infection, and cannot be detected or unveiled by Sanger sequencing. Our findings showed this to be a reliable and efficient molecular method to screen for the presence and assess the genetic diversity of trypanosomatids in humans, mammalian (reservoir) hosts, and insect vectors. Our study showed a high level of concordance between our amplicon-based NGS method and Sanger sequencing, offering the additional advantage over Sanger sequencing of interrogating for mixed trypanosome infections, capturing lower-abundance genotypes, and identifying rare or previously unknown species commonly overlooked by Sanger sequencing. In addition, this assay could reduce costs and response times compared to broader approaches like whole-genome sequencing. In addition, the results presented herein depose the traditional paradigm of leishmaniasis as being a singlespecies-driven infection and redraw the choreography of host-pathogen interactions in the context of multiparasitism. Further research is needed to decipher how coinfections may influence disease progression. This knowledge is key to developing an integrated approach for diagnosis and treatment. Although the results obtained in this study describe the presence of mixed infections in the three groups analyzed (humans/vectors/ reservoirs), it is also important to consider the possibility that some of the samples evaluated may contain hybrid strains, as previously described (59, 60), a hypothesis that would provide an additional advantage to our amplicon-based NGS method, since identifying hybrid strains circulating in certain geographic regions would not only change the epidemiological panorama of leishmaniasis but would also allow the techniques used for diagnosis and treatment of this pathology to be redesigned. Future studies should evaluate the feasibility of HSP70 to detect hybrid strains of Leishmania.

## MATERIALS AND METHODS

Ethical statement. This study was approved by the Ethics Committee of the Central Military Hospital of Colombia, following the principles established in the Declaration of Helsinki under Act No. 2043 of 22 March 2017. The procedures employed for collecting clinical material from reservoirs and sand fly vectors were carried out by the Universidad de Santander (UDES), which provided the collection permit from ANLA (Autoridad Nacional de Licencias Ambientales) (permit no. 01749).

Sampling. A total of 65 samples from humans, sand fly vectors, and reservoirs (wild and domestic) were included in the study. The samples included 49 samples obtained from direct smears of lesions from CL patients, 2 from Lara State (Venezuela) and 47 who attended the Dirección de Sanidad Militar, Ejercito Nacional de Colombia, Bogotá. As inclusion criteria, patients had to be male, over 18 years of age, to have had clinical and parasitological diagnosis of CL, with lesions of a minimum of 1 month and a maximum of 3 months of evolution, and to have been without antileishmanial treatment for at least 2 months prior to sampling. Only patients with a positive result for at least one direct smear or PCR of a skin biopsy specimen were included in the study. Those patients with lesions on the face, genitals, or mucosa and secondary infections of lesions were not sampled (61). Eight sand flies were collected across three different departments of Colombia (Norte de Santander, La Guajira, and Tolima). The collection, manipulation, and taxonomic identification of these specimens was performed according to previously reported methodology (34). Serum from two domestic reservoirs (Canis familiaris) with previous diagnosis of CL collected in the Sucre Department were also provided by the Laboratorio Nacional de Referencia del Instituto Nacional de Salud (INS), Colombia. Six blood spot samples from wild reservoirs (Phyllostomus hastatus) were collected on FTA cards in the Casanare Department. For blood collection, the animals were noninjuriously captured with a mist net and anesthetized with 20 mg/kg of body weight ketamine (Ketalar1; Parke-Davis, Morris Plains, NJ, USA). Approximately 0.3 ml of whole blood obtained by cardiac puncture was collected and placed on FTA cards. FTA cards were adequately preserved to maintain low humidity and avoid contamination. All metadata on the 65 samples included in the study is summarized in Table S1. The geographic localization of samples is represented in Fig. 4A. Finally, as control of the sequencing and with the aim of having an estimate of the limit of detection and reliability of the test, we made mixtures (1/1) of promastigotes of L. amazonensis and L. braziliensis at different concentrations, ranging from 1 $10^{10}$ to 1 $10^{1}$ cells/ml. For our analysis, we sequenced two concentrations: 1 $10^{6}$ (mix-1) and 1 $10^{3}$ (mix-2) were selected for further analysis.

DNA extraction. Once the samples were obtained, the next step was to extract the DNA from each of them. Two commercial kits were used. The ZR tissue/insect miniprep DNA Zymo kit (Zymo Research, Irvine, CA, USA) was used to extract the DNA from whole bodies of sand flies, and the High Pure PCR template preparation kit (Roche Life Science, Mannheim, Germany) was used to extract the DNA from samples collected from humans, reservoirs, and mixtures made for this study. Each kit was used following the protocol described by the manufacturer. Subsequently, the concentration, quality, and integrity of DNA obtained from each of the samples was determined. For this we used the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA), which allowed us to measure the DNA concentration, and in addition, a 1% agarose gel electrophoresis was performed to verify the quality and integrity of products obtained. Finally, the DNA obtained was used for species identification by Sanger sequencing as reported elsewhere (62) and for amplicon-based NGS as developed herein.

Specificity of the HSP70 primers employed in the assay. In order to determine the intra- and interspecies specificity of the primers used in this study, we downloaded the annotation of the main Leishmania and Trypanosoma species from tritrypdb, a publicly available database (https://tritrypdb.org/ common/downloads/Current_Release), selecting by species only those sequences corresponding to the HSP70 gene. Each of these sequences per chromosome, per species was aligned to the primers under study. Those sequences recognized by the primers were used to build a maximum-likelihood-based phylogenetic tree using FastTree double precision version 2.1.10 with 1,000 bootstrap replicates (63). The final tree obtained was visualized using the interactive tool Interactive Tree Of Life v4 (http://itol .embl.de) (64).

Identification of Leishmania species by Sanger sequencing. We carried out direct Sanger sequencing to identify the trypanosomatid species associated with infection in CL patients, sand fly vectors, and reservoirs. For this, the gene encoding heat shock protein (HSP70) was amplified. HSP70F (59 AGG TGA AGG CGA CGA ACG 39) and HSP70R (59 CGC TTG TCC ATC TTT GCG TC 39) primers were used to amplify a 337- bp region of the HSP70 gene (65). The thermal profile was as follows: denaturation at 94°C for 5 min, followed by 40 cycles with denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and elongation at 72°C for 1 min and a final extension at 72°C for 10 min (29, 65). EXOSAP (Affymetrix, Santa Clara, CA, USA), was used to purify the amplified products, and the sequencing was performed using the dideoxy-terminal method (AB3730; Applied Biosystem, Foster City, CA, USA). The sequences obtained were submitted to BLASTn (using the webserver) for a similarity search against trypanosomatid sequences deposited in GenBank (29). The best matches of E value and percentage of identity were selected, thus allowing the assignment of species to each of the analyzed samples. Once a trypanosomatid species was identified, the results were compared with those obtained using the amplicon-based NGS.

Amplicon-based next-generation sequencing. Genomic DNA ($200 ng/ml) from CL patients, sand fly vectors, and reservoirs (wild and domestic) was used for sequencing, which was conducted by Novogen (Beijing, China). Briefly, primers F (59 AGGTGAAGGCGACGAACG) and R (59 CGCTTGTCCATCTTTGCGTC) were used to amplify the HSP70 gene using the Phusion high-fidelity PCR master mix (New England Biolabs). The quality, integrity, and size of the amplicon (337 bp) were determined by 2% agarose gel electrophoresis. The amplicons from each sample were purified using the Qiagen gel extraction kit (Qiagen, Germany) according to the manufacturer's protocol. Later, the sequencing libraries were generated using the NEBNext ultra DNA library prep kit for Illumina, following the manufacturer's recommendations, and index codes were added (Table S2). The libraries were purified using Agencourt AMPure XP beads (to remove excess primers, nucleotides, salts, and enzymes produced during the reactions), quantified using the Qubit 2.0 fluorometer (Thermo Scientific), and qualified using the Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an Illumina platform, and 250-bp paired-end reads were generated following the manufacturer's instructions.

Bioinformatics analysis. FASTQ files (forward and reverse) for every sample were obtained from Illumina sequencing of HSP70 amplicons. The sequences were initially filtered for quality using the QIIME software (66), following the quality parameters described previously (22), and subsequently merged. Those sequences that passed the quality filtering were compared with an in-house-built database (see "Reference data set" below) for trypanosomatid species detection.

The taxonomic assignation of high-quality reads was conducted using BLASTn locally against the built database, considering as the threshold a minimum 95% identity and an E value of 10. Those matches that had a relative abundance (number of reads assigned per sample divided by the total amount of reads of the sample) greater than 3% were considered in the analysis in order to rule out potential sequencing errors; additionally, we adjusted the relative abundance of reads of the Leishmania species identified to the number of copies of the HSP70 gene present in each of them to have a better approximation of the abundance of the species per individual. All quantitative results were graphed using R software version 3.6.1.

Reference data set. An in-house database was built considering all the HSP70 sequences of trypanosomatids in the NCBI Nucleotide database (https://www.ncbi.nlm.nih.gov/nucleotide/). We conducted a search considering two criteria: the gene name "Heat shock protein 70" and the organism name "Kinetoplastids," obtaining a total of 990 sequences. Later, the search was filtered considering the molecule type (genomic DNA/RNA), the source database (GenBank), and the sequence length (330 to 5,000). Additionally, we included six HSP70 sequences from reference genomes, including those of L. braziliensis MHOM/BR/75/M2904 (XM_001566275), L. major strain Friedlin (XM_001684512), L. infantum JPCM5 (XM_003392631), L. mexicana

MHOM/GT/2001/U1103 (XM_003877073.1), L. donovani LDBPK_283000 (XM_003862348.1), and T. cruzi strain CL Brener (XM_812645), which were downloaded from the Reference Sequence (RefSeq) database. We excluded those sequences of Leishmania that did not have a correct taxonomic assignment to species level (Leishmania sp.). The sequences obtained were aligned in Clustal W and trimmed according to the HSP70 forward/reverse primer sequence in UGENE version 33.0 software (67). Sequences with high levels of gaps in content and low quality were excluded. A total of 385 sequences constituted our final reference database, which is publicly available at https://github.com/gimur/Amplicon-Base-Next-Generation-Sequencing-.git. To confirm the utility of HSP70 for identifying trypanosomatid species, we recovered sequences by haplotype from the 385 sequences obtained, using the DNAsp software version 5.0, obtaining a total of 48 haplotypes. Considering the total number of single-nucleotide polymorphisms (SNPs) between the haplotypes identified and using the snp-dists SNP distance program (https://github.com/tseemann/snp-dists), we generated a distance matrix. Additionally, we built two maximum-likelihood-based phylogenetic trees using FastTree double precision version 2.1.10 (63). One of them was constructed to explore the clustering among the 385 sequences used as a reference database and the 113 sequences obtained from 65 samples included in this study, and the second was performed to observe the clustering among the 48 haplotypes from 385 sequences used as the reference database. The robustness of the nodes was evaluated using the bootstrap test (BT, with 1,000 replicates). The tree obtained was visualized using the interactive tool Interactive Tree Of Life v4 (http://itol.embl.de) (64). Finally, phylogenetic networks were also built in SplitsTree5 (68) using the Neighbor-Net method.

Statistical analysis. A descriptive analysis of the clinical and epidemiological variables was performed. For continuous values, the distribution of the data and the hypothesis of normality were initially evaluated using the Shapiro-Wilk test. Because the data did not have a normal distribution, continuous variables were summarized in terms of medians and interquartile ranges (IQRs). Qualitative variables were summarized in frequencies and proportions according to the infectious species and single- or coinfection patterns. Due to the distribution of the quantitative data, to identify the statistical significance of possible differences between the single-infection and coinfection groups, the nonparametric MannWhitney-Wilcoxon test was applied (69).

Additionally, to compare infection species corresponding to several groups, we applied the KruskalWallis test and the corresponding test for pairwise multiple comparisons between groups using the Dunn-Bonferroni post hoc method. The chi-square test and Fisher exact test were performed to identify potential associations between the categorical variables and species groups. R software (RStudio Team 2019) was used to perform the statistical analysis. A P value of ,0.05 was considered statistically significant. Finally, we used STATA 11 software to calculate the agreement between the two methods analyzed here (Sanger sequencing and HSP70 amplicon-based NGS), considering the agreement percentages and the Kappa coefficients (k). A 0.05 significance level was fixed (70).

Diversity analysis. The diversity analyses were done using the R package phyloseq (71) version 4.0.2. The alpha diversity of amplicon sequence variant (ASVs) of each group analyzed (humans, sand flies, and wild and domestic reservoirs) was calculated using Simpson dominance and Shannon diversity. The Kolmogorov-Smirnov test was implemented to verify the normality of data obtained from the diversity analysis. The nonparametric Kruskal-Wallis test compared the alpha diversity metrics for the four groups. Finally, we conducted multiple pairwise comparisons of alpha diversity between groups using the Dunn-Bonferroni post hoc method, to determine the groups among which these differences were present. A P value of ,0.05 was considered statistically significant.

Likewise, using the phyloseq package in R, principal coordinates analysis (PCoA) and principal-component analysis (PCA) diagrams were produced, in which Bray-Curtis distances were used between the samples to visualize the behavior of the groups. We performed permutational multivariate analysis of variance (PERMANOVA) to identify differences in the distribution and dispersion of the groups. Additionally, similarity percentage (SIMPER) analysis was implemented to establish which species were likely to be the main contributors to any differences between groups detected by PERMANOVA.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

## ACKNOWLEDGMENTS

We thank Giovanny Herrera for the support in the construction of the maps. This work was funded by Dirección de Investigación e Innovación from Universidad del Rosario. Juan David Ramírez González is a Latin American fellow in the biomedical sciences, supported by The Pew Charitable Trusts.

## REFERENCES

1. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den BoerM, WHO Leishmaniasis Control Team. 2012. Leishmaniasis worldwide and global estimates of its incidence. PLoS One 7:e35671. https://doi.org/10 .1371/journal.pone.0035671.
2. Ramirez JD, Hernandez C, Leon CM, Ayala MS, Florez C, Gonzalez C. 2016. Taxonomy, diversity, temporal and geographical distribution of Cutaneous Leishmaniasis in Colombia: a retrospective study. Sci Rep 6:28266. https://doi.org/10.1038/srep28266.
3. Dutari LC, Loaiza JR. 2014. American cutaneous leishmaniasis in Panama: a historical review of entomological studies on anthropophilic Lutzomyia sand fly species. Parasit Vectors 7:218. https://doi.org/10.1186/1756-3305 -7-218.
4. Araujo-Pereira T, Pita-Pereira D, Boite MC, Melo M, Costa-Rego TA, FuzariAA, Brazil RP, Britto C. 2017. First description of Leishmania (Viannia) infection in Evandromyia saulensis, Pressatia sp. and Trichophoromyia auraensis (Psychodidae: Phlebotominae) in a transmission area of cutaneous leishmaniasis in Acre state, Amazon Basin, Brazil. Mem Inst Oswaldo Cruz 112:75–78. https://doi.org/10.1590/0074-02760160283.
5. Martinez E, Mollinedo S, Torrez M, Munoz M, Banuls AL, Le Pont F. 2002. Co-infection by Leishmania amazonensis and L. infantum/L. chagasi in a case of diffuse cutaneous leishmaniasis in Bolivia. Trans R Soc Trop Med Hyg 96:529–532. https://doi.org/10.1016/S0035-9203(02)90428-1.
6. Veland N, Valencia BM, Alba M, Adaui V, Llanos-Cuentas A, Arevalo J,Boggild AK. 2013. Simultaneous infection with Leishmania (Viannia) braziliensis and L. (V.) lainsoni in a Peruvian patient with cutaneous leishmaniasis. Am J Trop Med Hyg 88:774–777. https://doi.org/10.4269/ ajtmh.12-0594.
7. Babiker AM, Ravagnan S, Fusaro A, Hassan MM, Bakheit SM, Mukhtar MM,Cattoli G, Capelli G. 2014. Concomitant infection with Leishmania donovani and L. major in single ulcers of cutaneous leishmaniasis patients from Sudan. J Trop Med 2014:170859. https://doi.org/10.1155/2014/170859.
8. Shirian S, Oryan A, Hatam GR, Daneshbod Y. 2012. Mixed mucosal leishmaniasis infection caused by Leishmania tropica and Leishmania major. J Clin Microbiol 50:3805–3808. https://doi.org/10.1128/JCM.01469-12.
9. Monroy-Ostria A, Nasereddin A, Monteon VM, Guzman-Bracho C, JaffeCL. 2014. ITS1 PCR-RFLP diagnosis and characterization of Leishmania in clinical samples and strains from cases of human cutaneous leishmaniasis in states of the Mexican southeast. Interdiscip Perspect Infect Dis 2014:607287. https://doi.org/10.1155/2014/607287.
10. Kato H, Gomez EA, Seki C, Furumoto H, Martini-Robles L, Muzzio J, CalvopinaM, Velez L, Kubo M, Tabbabi A, Yamamoto DS, Hashiguchi Y. 2019. PCR-RFLP analyses of Leishmania species causing cutaneous and mucocutaneous leishmaniasis revealed distribution of genetically complex strains with hybrid and mito-nuclear discordance in Ecuador. PLoS Negl Trop Dis 13:e0007403. https://doi.org/10.1371/journal.pntd.0007403.
11. Viettri M, Herrera L, Aguilar CM, Morocoima A, Reyes J, Lares M, LozanoArias D, Garcia-Alzate R, Chacon T, Feliciangeli MD, Ferrer E. 2019. Molecular characterization of Trypanosoma cruzi and Leishmania spp. coinfection in mammals of Venezuelan coendemic areas. J Vector Borne Dis 56: 252–262. https://doi.org/10.4103/0972-9062.289394.
12. Gosch CS, Resende BS, Amorim CB, Marques CP, Pereira LIA, Pinto SA, UlianaSRB, Coelho AC, Ribeiro-Dias F, Dorta ML. 2018. Case report: atypical cutaneous leishmaniasis in a patient with mixed Leishmania guyanensis and Leishmania amazonensis infection. Am J Trop Med Hyg 99:1165–1169. https://doi .org/10.4269/ajtmh.17-0760.
13. de Castro Ferreira E, Cruz I, Cañavate C, de Melo LA, Pereira AAS, MadeiraFAM, Valério SAN, Cunha HM, Paglia AP, Gontijo CMF. 2015. Mixed infection of Le ishmania infantum and Leishmania braziliensis in rodents from endemic urban area of the New World. BMC Vet Res 11:71. https://doi .org/10.1186/s12917-015-0392-y.
14. Srisuton P, Phumee A, Sunantaraporn S, Boonserm R, Sor-Suwan S, BrownellN, Pengsakul T, Siriyasatien P. 2019. Detection of Leishmania and Trypanosoma DNA in field-caught sand flies from endemic and non-endemic areas of leishmaniasis in southern Thailand. Insects 10:238. https://doi.org/10 .3390/insects10080238.
15. Abbate JM, Maia C, Pereira A, Arfuso F, Gaglio G, Rizzo M, Caracappa G,Marino G, Pollmeier M, Giannetto S, Brianti E. 2020. Identification of trypanosomatids and blood feeding preferences of phlebotomine sand fly species common in Sicily, Southern Italy. PLoS One 15:e0229536. https://doi .org/10.1371/journal.pone.0229536.
16. Saraiva L, Andrade Filho JD, Silva SDO, Silva Ribeiro de Andrade A, MeloMN. 2010. The molecular detection of different Leishmania species within sand flies from a cutaneous and visceral leishmaniasis sympatric area in Southeastern Brazil. Mem Inst Oswaldo Cruz 105:1033–1039. https://doi .org/10.1590/s0074-02762010000800013.
17. Barbosa AD, Gofton AW, Paparini A, Codello A, Greay T, Gillett A, WarrenK, Irwin P, Ryan U. 2017. Increased genetic diversity and prevalence of coinfection with Trypanosoma spp. in koalas (Phascolarctos cinereus) and their ticks identified using next-generation sequencing (NGS). PLoS One 12:e0181279. https://doi.org/10.1371/journal.pone.0181279.
18. Chaorattanakawee S, Korkusol A, Tippayachai B, Promsathaporn S, PooleSmith BK, Takhampunya R. 2021. Amplicon-based next generation sequencing for rapid identification of Rickettsia and ectoparasite species from entomological surveillance in Thailand. Pathogens 10:215. https:// doi.org/10.3390/pathogens10020215.
19. Wen C, Wu L, Qin Y, Van Nostrand JD, Ning D, Sun B, Xue K, Liu F, Deng Y,Liang Y, Zhou J. 2017. Evaluation of the reproducibility of amplicon sequencing with Illumina MiSeq platform. PLoS One 12:e0176716. https://doi.org/10.1371/journal.pone.0176716.
20. Stensvold CR, Lebbad M, Hansen A, Beser J, Belkessa S, O'Brien Andersen L, Clark CG. 2020. Differentiation of Blastocystis and parasitic archamoebids encountered in untreated wastewater samples by amplicon-based next-generation sequencing. Parasite Epidemiol Control 9:e00131. https://doi.org/10 .1016/j.parepi.2019.e00131.
21. Taylor MK, Williams EP, Wongsurawat T, Jenjaroenpun P, Nookaew I, JonssonCB. 2020. Amplicon-based, next-generation sequencing approaches to characterize single nucleotide polymorphisms of Orthohantavirus species. Front Cell Infect Microbiol 10:565591. https://doi.org/10.3389/fcimb.2020.565591.
22. Arias-Giraldo LM, Munoz M, Hernandez C, Herrera G, Velasquez-Ortiz N,Cantillo-Barraza O, Urbano P, Cuervo A, Ramirez JD. 2020. Identification of blood-feeding sources in Panstrongylus, Psammolestes, Rhodnius and Triatoma using amplicon-based next-generation sequencing. Parasit Vectors 13:434. https://doi.org/10.1186/s13071-020-04310-z.
23. Abbasi I, Nasereddin A, Warburg A. 2019. Development of a next generation DNA sequencing-based multi detection assay for detecting and identifying Leishmania parasites, blood sources, plant meals and intestinal microbiome in phlebotomine sand flies. Acta Trop 199:105101. https:// doi.org/10.1016/j.actatropica.2019.105101.
24. Llewellyn MS, Messenger LA, Luquetti AO, Garcia L, Torrico F, Tavares SB,Cheaib B, Derome N, Delepine M, Baulard C, Deleuze JF, Sauer S, Miles MA. 2015. Deep sequencing of the Trypanosoma cruzi GP63 surface proteases reveals diversity and diversifying selection among chronic and congenital Chagas disease patients. PLoS Negl Trop Dis 9:e0003458. https://doi.org/10.1371/journal.pntd.0003458.
25. Maiguashca SJ, Sueto SOB, Schwabl P, Grijalva MJ, Llewellyn MS, CostalesJA. 2020. Remarkable genetic diversity of Trypanosoma

cruzi and Trypanosoma rangeli in two localities of southern Ecuador identified via deep sequencing of mini-exon gene amplicons. Parasit Vectors 13:252. https://doi.org/10.1186/s13071-020-04079-1.

26. Cooper C, Keatley S, Northover A, Gofton AW, Brigg F, Lymbery AJ, PallantL, Clode PL, Thompson RCA. 2018. Next generation sequencing reveals widespread trypanosome diversity and polyparasitism in marsupials from Western Australia. Int J Parasitol Parasites Wildl 7:58–67. https://doi.org/10.1016/j.ijppaw.2018.01.005.
27. Dario MA, Moratelli R, Schwabl P, Jansen AM, Llewellyn MS. 2017. Smallsubunit ribosomal metabarcoding reveals extraordinary trypanosomatid diversity in Brazilian bats. PLoS Negl Trop Dis 11:e0005790. https://doi .org/10.1371/journal.pntd.0005790.
28. Flaherty BR, Barratt J, Lane M, Talundzic E, Bradbury RS. 2021. Sensitiveuniversal detection of blood parasites by selective pathogen-DNA enrichment and deep amplicon sequencing. Microbiome 9:1. https://doi.org/10 .1186/s40168-020-00939-1.
29. Patino LH, Mendez C, Rodriguez O, Romero Y, Velandia D, Alvarado M, PerezJ, Duque MC, Ramirez JD. 2017. Spatial distribution, Leishmania species and clinical traits of cutaneous leishmaniasis cases in the Colombian army. PLoS Negl Trop Dis 11:e0005876. https://doi.org/10.1371/journal.pntd.0005876.
30. Ovalle-Bracho C, Londono-Barbosa D, Salgado-Almario J, Gonzalez C. 2019. Evaluating the spatial distribution of Leishmania parasites in Colombia from clinical samples and human isolates (1999 to 2016). PLoS One 14:e0214124. https://doi.org/10.1371/journal.pone.0214124.
31. Falcão SC, de Moura TR, Clarencio J, Brodskyn C, Barral A, de Oliveira CI.2012. The presence of Tregs does not preclude immunity to reinfection with Leishmania braziliensis. Int J Parasitol 42:771–780. https://doi.org/10 .1016/j.ijpara.2012.05.006.
32. Osorio Y, Gonzalez SJ, Gama VL, Travi BL. 1998. Reinfection in Americancutaneous leishmaniasis: evaluation of clinical outcomes in the hamster model. Mem Inst Oswaldo Cruz 93:353–356. https://doi.org/10 .1590/S0074-02761998000300015.
33. Patino LH, Imamura H, Cruz-Saavedra L, Pavia P, Muskus C, Mendez C,Dujardin JC, Ramirez JD. 2019. Major changes in chromosomal somy, gene expression and gene dosage driven by Sb(III) in Leishmania braziliensis and Leishmania panamensis. Sci Rep 9:9485. https://doi.org/10 .1038/s41598-019-45538-9.
34. Sandoval-Ramirez CM, Hernandez C, Teheran AA, Gutierrez-Marin R, Martinez-Vega RA, Morales D, Hoyos-Lopez R, Araque-Mogollon A, Ramirez JD. 2020. Complex ecological interactions across a focus of cutaneous leishmaniasis in Eastern Colombia: novel description of Leishmania species, hosts and phlebotomine fauna. R Soc Open Sci 7:200266. https://doi.org/10.1098/ rsos.200266.
35. Veasey JV, Zampieri RA, Lellis RF, Freitas THP, Winter LMF. 2020. Identification of Leishmania species by high-resolution DNA dissociation in cases of American cutaneous leishmaniasis. An Bras Dermatol 95:459–468. https://doi.org/10.1016/j.abd.2020.02.003.
36. Calzolari M, Rugna G, Clementi E, Carra E, Pinna M, Bergamini F, Fabbi M,Dottori M, Sacchi L, Votypka J. 2018. Isolation of a trypanosome related to Trypanosoma theileri (Kinetoplastea: Trypanosomatidae) from Phlebotomus perfiliewi (Diptera: Psychodidae). Biomed Res Int 2018:2597074. https://doi.org/10.1155/2018/2597074.
37. Gonzalez E, Molina R, Jimenez M. 2018. Rabbit trypanosome detection in Phlebotomus perniciosus sand flies from the leishmaniasis outbreak in Madrid, Spain. Acta Trop 187:201–206. https://doi.org/10.1016/j.actatropica.2018.08 .011.
38. Ferreira TDS, Minuzzi-Souza TTC, de Andrade AJ, Coelho TO, de AlmeidaRocha D, Obara MT, Hecht M, Nitz N, Gurgel-Gonçalves R. 2015. Molecular detection of Trypanosoma sp. and Blastocrithidia sp. (Trypanosomatidae) in phlebotomine sand flies (Psychodidae) in the Federal District of Brazil. Rev Soc Bras Med Trop 48:776–779. https://doi.org/10.1590/0037-8682 -0076-2015.
39. Lisboa CV, Pinho AP, Herrera HM, Gerhardt M, Cupolillo E, Jansen AM.2008. Trypanosoma cruzi (Kinetoplastida , Trypanosomatidae) genotypes in neotropical bats in Brazil. Vet Parasitol 156:314–318. https://doi.org/10 .1016/j.vetpar.2008.06.004.
40. Thomas ME, Rasweiler JJ, IV, D'Alessandro A. 2007. Experimental transmission of the parasitic flagellates Trypanosoma cruzi and Trypanosoma rangeli between triatomine bugs or mice and captive neotropical bats. Mem Inst Oswaldo Cruz 102:559–565. https://doi.org/10 .1590/s0074-02762007005000068.
41. de Rezende MB, Herrera HM, Carvalho CME, Carvalho Anjos EA, RamosCAN, de Araujo FR, Torres JM, de Oliveira CE. 2017. Detection of Leishmania spp. in bats from an area of Brazil endemic for visceral Leishmaniasis. Transbound Emerg Dis 64:e36–e42. https://doi.org/10.1111/tbed.12597.
42. Shapiro JT, da Costa Lima MS, Jr, Dorval MEC, de Oliveira França A, CepaMatos MDF, Bordignon MO. 2013. First record of Leishmania braziliensis presence detected in bats, Mato Grosso do Sul, southwest Brazil. Acta Trop 128:171–174. https://doi.org/10.1016/j.actatropica.2013.07.004.
43. Savani ES, de Almeida MF, de Oliveira Camargo MC, D'Auria SR, Silva MM, de Oliveira ML, Sacramento D. 2010. Detection of Leishmania (Leishmania) amazonensis and Leishmania (Leishmania) infantum chagasi in Brazilian bats. Vet Parasitol 168:5–10. https://doi.org/10.1016/j.vetpar .2009.10.019.
44. Berzunza-Cruz M, Rodriguez-Moreno A, Gutierrez-Granados G, GonzalezSalazar C, Stephens CR, Hidalgo-Mihart M, Marina CF, Rebollar-Tellez EA, Bailon-Martinez D, Balcells CD, Ibarra-Cerdena CN, Sanchez-Cordero V, Becker I. 2015. Leishmania (L.) mexicana infected bats in Mexico: novel potential reservoirs. PLoS Negl Trop Dis 9:e0003438. https://doi.org/10.1371/ journal.pntd.0003438.
45. da Costa AP, Costa FB, Soares HS, Ramirez DG, Mesquita ET, Gennari SM,Marcili A. 2015. Trypanosoma cruzi and Leishmania infantum chagasi infection in wild mammals from Maranhao State, Brazil. Vector Borne Zoonotic Dis 15:656–666. https://doi.org/10.1089/vbz.2015.1771.
46. Lourenco JLM, Minuzzi-Souza TTC, Silva LR, Oliveira AC, Mendonca VJ,Nitz N, Aguiar LMS, Gurgel-Goncalves R. 2018. High frequency of trypanosomatids in gallery forest bats of a Neotropical savanna. Acta Trop 177: 200–206. https://doi.org/10.1016/j.actatropica.2017.10.012.
47. Zortea M. 2003. Reproductive patterns and feeding habits of three nectarivorous bats (Phyllostomidae: Glossophaginae) from the Brazilian Cerrado. Braz J Biol 63:159–168. https://doi.org/10.1590/S1519-69842003000100020.
48. Dantas-Torres F. 2009. Canine leishmaniosis in South America. ParasitVectors 2(Suppl 1):S1. https://doi.org/10.1186/1756-3305-2-S1-S1.
49. Santaella J, Ocampo CB, Saravia NG, Mendez F, Gongora R, Gomez MA,Munstermann LE, Quinnell RJ. 2011. Leishmania (Viannia) infection in the domestic dog in Chaparral, Colombia. Am J Trop Med Hyg 84:674–680. https://doi.org/10.4269/ajtmh.2011.10-0159.
50. Brilhante AF, Lima L, Zampieri RA, Nunes VLB, Dorval MEC, Malavazi P,Melchior LAK, Ishikawa EAY, Cardoso CO, Floeter-Winter LM, Teixeira MMG, Galati EAB. 2019. Leishmania (Viannia) braziliensis type 2 as probable etiological agent of canine cutaneous leishmaniasis in Brazilian Amazon. PLoS One 14:e0216291. https://doi.org/10.1371/journal.pone.0216291.
51. Madeira MF, Schubach A, Schubach TM, Pacheco RS, Oliveira FS, PereiraSA, Figueiredo FB, Baptista C, Marzochi MC. 2006. Mixed infection with Leishmania (Viannia) braziliensis and Leishmania (Leishmania) chagasi in a naturally infected dog from Rio de Janeiro, Brazil. Trans R Soc Trop Med Hyg 100:442–445. https://doi.org/10.1016/j.trstmh.2005.07.011.
52. Pires MQ, Madeira MDF, Bittencourt VREP, Pacheco RDS. 2014. Cutaneousand visceral leishmaniasis co-infection in dogs from Rio de Janeiro, Brazil: evaluation by specific PCR and RFLP-PCR assays.

Rev Soc Bras Med Trop 47:243–246. https://doi.org/10.1590/0037-8682-0007-2013.

53. Correa-Cardenas CA, Perez J, Patino LH, Ramirez JD, Duque MC, RomeroY, Cantillo-Barraza O, Rodriguez O, Alvarado MT, Cruz C, Mendez C. 2020. Distribution, treatment outcome and genetic diversity of Leishmania species in military personnel from Colombia with cutaneous leishmaniasis. BMC Infect Dis 20:938. https://doi.org/10.1186/s12879-020-05529-y.
54. Ducharme O, Simon S, Ginouves M, Prevot G, Couppie P, Demar M, Blaizot R. 2020. Leishmania naiffi and lainsoni in French Guiana: clinical features and phylogenetic variability. PLoS Negl Trop Dis 14:e0008380. https://doi.org/10.1371/journal.pntd.0008380.
55. Ferro C, Lopez M, Fuya P, Lugo L, Cordovez JM, Gonzalez C. 2015. Spatialdistribution of sand fly vectors and eco-epidemiology of cutaneous leishmaniasis transmission in Colombia. PLoS One 10:e0139391. https://doi .org/10.1371/journal.pone.0139391.
56. Gonzalez C, Leon C, Paz A, Lopez M, Molina G, Toro D, Ortiz M, CordovezJM, Atencia MC, Aguilera G, Tovar C. 2018. Diversity patterns, Leishmania DNA detection, and bloodmeal identification of Phlebotominae sand flies in villages in northern Colombia. PLoS One 13:e0190686. https://doi.org/ 10.1371/journal.pone.0190686.
57. Brandao EMV, Xavier SCC, Rocha FL, Lima CFM, Candeias IZ, Lemos FG,Azevedo FC, Jansen AM, Roque ALR. 2020. Wild and domestic canids and their interactions in the transmission cycles of Trypanosoma Cruzi and Leishmania spp. in an area of the Brazilian Cerrado. Pathogens 9:818. https://doi.org/10.3390/pathogens9100818.
58. Chargui N, Slama D, Haouas N, Rmadi L, Babba H. 2018. Transmission cycleanalysis in a Leishmania infantum focus: infection rates and blood meal origins in sand flies (Diptera: Psychodidae). J Vector Ecol 43:321–327. https://doi.org/10.1111/jvec.12316.
59. Inbar E, Shaik J, Iantorno SA, Romano A, Nzelu CO, Owens K, Sanders MJ,Dobson D, Cotton JA, Grigg ME, Beverley SM, Sacks D. 2019. Whole genome sequencing of experimental hybrids supports meiosis-like sexual recombination in Leishmania. PLoS Genet 15:e1008042. https://doi.org/ 10.1371/journal.pgen.1008042.
60. Van den Broeck F, Savill NJ, Imamura H, Sanders M, Maes I, Cooper S,Mateus D, Jara M, Adaui V, Arevalo J, Llanos-Cuentas A, Garcia L, Cupolillo E, Miles M, Berriman M, Schnaufer A, Cotton JA, Dujardin JC. 2020. Ecological divergence and hybridization of Neotropical Leishmania parasites. Proc Natl Acad Sci U S A 117:25159–25168. https://doi.org/10.1073/pnas .1920136117.
61. Patino LH, Muñoz M, Cruz-Saavedra L, Muskus C, Ramírez JD. 2020. Genomic diversification, structural plasticity, and hybridization in Leishmania (Viannia) braziliensis. Front Cell Infect Microbiol 10:582192. https:// doi.org/10.3389/fcimb.2020.582192.
62. Patino LH, Munoz M, Muskus C, Mendez C, Ramirez JD. 2020. Intraspecific genomic divergence and minor structural variations in Leishmania (Viannia) panamensis. Genes (Basel) 11:252. https://doi.org/10.3390/genes11030252.
63. Price MN, Dehal PS, Arkin AP. 2009. FastTree: computing large minimumevolution trees with profiles instead of a distance matrix. Mol Biol Evol 26: 1641–1650. https://doi.org/10.1093/molbev/msp077.
64. Letunic I, Bork P. 2019. Interactive Tree Of Life (iTOL) v4: recent updatesand new developments. Nucleic Acids Res 47:W256–W259. https://doi .org/10.1093/nar/gkz239.
65. Hernandez C, Alvarez C, Gonzalez C, Ayala MS, Leon CM, Ramirez JD.2014. Identification of six New World Leishmania species through the implementation of a high-resolution melting (HRM) genotyping assay. Parasit Vectors 7:501. https://doi.org/10.1186/s13071-014-0501-y.
66. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, CostelloEK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights

D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M,

Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336. https://doi.org/10.1038/ nmeth.f.303.

67. Okonechnikov K, Golosova O, Fursov M, UGENE Team. 2012. UniproUGENE: a unified bioinformatics toolkit. Bioinformatics 28:1166–1167. https://doi.org/10.1093/bioinformatics/bts091.
68. Huson DH, Bryant D. 2006. Application of phylogenetic networks in evolutionary studies. Mol Biol Evol 23:254–267. https://doi.org/10.1093/molbev/ msj030.
69. Quiroga SA, Hernandez C, Castaneda S, Jimenez P, Vega L, Gomez M,Martinez D, Ballesteros N, Munoz M, Cifuentes C, Sierra N, Florez C, PanizMondolfi A, Ramirez JD. 2021. Contrasting SARS-CoV-2 RNA copies and clinical symptoms in a large cohort of Colombian patients during the first wave of the COVID-19 pandemic. Ann Clin Microbiol Antimicrob 20:39. https://doi.org/10.1186/s12941-021-00445-8.
70. Munoz M, Rios-Chaparro DI, Herrera G, Soto-De Leon SC, Birchenall C,Pinilla D, Pardo-Oviedo JM, Josa DF, Patarroyo MA, Ramirez JD. 2018. New insights into Clostridium difficile (CD) infection in Latin America: novel description of toxigenic profiles of diarrhea-associated to CD in Bogota. Front Microbiol 9:74. https://doi.org/10.3389/fmicb.2018.00074.
71. McMurdie PJ, Holmes S. 2013. phyloseq: an R package for reproducibleinteractive analysis and graphics of microbiome census data. PLoS One 8: e61217. https://doi.org/10.1371/journal.pone.0061217.

International Journal of Infectious Diseases 115 (2022) 35–38

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

# Amplicon-based next-generation sequencing reveals the co-existence of multiple *Leishmania* species in patients with visceral leishmaniasis

Adriana Castillo-Castañeda [a], Luz H. Patiño [a], Marina Muñoz [a], Martha S. Ayala [b], Maryi Segura [b], Jessica Bautista [b], Maryia V. Shaban [c], Alberto Paniz-Mondolfi [d], Juan David Ramírez [a], *

[a] *Centro de Investigaciones en Microbiología y Biotecnología-UR, Facultad de Ciencias Naturales, Universidad del Rosario, Bogotá, Colombia* [b] *Grupo de Parasitología, Instituto Nacional de Salud, Bogotá, Colombia*

[c] *Incubadora Venezolana de la Ciencia/Centro de Investigaciones Biomédicas IDB, Barquisimeto, Venezuela*

[d] *Department of Pathology, Molecular and Cell-Based Medicine, Division of Microbiology, Icahn School of Medicine at Mount Sinai, New York, NY, USA*

## article info

*Article history:*
Received 19 October 2021
Revised 17 November 2021 Accepted 20 November 2021

*Keywords:*
Visceral leishmaniasis
Amplicon-based NGS
*HSP70*
Co-infection
*Leishmania*
Trypanosomatid

## abstract

Visceral leishmaniasis (VL) is a mammalian protozoal disease propagated in the Americas by female phlebotomine sandflies, mainly caused by *Leishmania infantum* . However, in recent years, cases of VL caused by different *Leishmania* species, such as *L. amazonensis* and *L. colombiensis,* have been reported in the continent. This study used an amplicon-based next-generation sequencing approach to identify VL aetiologic species using high-depth sequencing targeting a region on the *Heat Shock Protein 70* gene. In this first approach, six samples from five patients diagnosed with VL were selected and analysed to identify DNA of *Leishmania* spp. All samples harboured DNA of *L. infantum* ; five samples were found to be co-infected with other *Leishmania* spp. or with *Trypanosoma cruzi* , and just one sample was mono-infected with *L. infantum* . This study demonstrates the usefulness of this methodology to identify trypanosomatid co-infections in clinical samples, which presents an interesting study panorama considering their biological, clinical and epidemiological implications.

Visceral leishmaniasis (VL) is a neglected tropical disease affecting at least 57 countries around the tropical and subtropical equatorial belt. VL is associated with *Leishmania donovani* species complex ( Franssen et al., 2020 ) and, to a lesser extent, *Leishmania enriettii* ( Paranaiba et al., 2017 ). In addition, sporadic cases of human VL have been associated with *Leishmania colombiensis* ( Rodriguez-Bonfante et al., 2003 ), *Leishmania amazonensis* ( Barral et al., 1986 ; Aleixo et al., 2006 ), *Leishmania major* and *Leishmania tropica* ( Fakhar et al., 2008 ; Al Balushi et al., 2018 ).

Recently, trypanosomatid co-infections have been reported from asymptomatic patients in areas endemic for both leishmaniasis and trypanosomiasis in Brazil ( Mendes et al., 2007 ); from symptomatic patients in Bolivia ( Bastrenta et al., 2003 ); and from patients with cutaneous leishmaniasis in Bolivia ( Bastrenta et al., 2003 ), Peru ( Veland et al., 2013 ) and Mexico ( Monroy-Ostria et al., 2014 ).

As there are increasing reports of co-infections with *Leishmania* species, and capitalizing on the advantages of a metagenomics approach for detection of a wide range of pathogens, the objective of this study was to demonstrate co-infections in patients with VL using amplicon-based next-generation sequencing (NGS).

Three serum and three bone marrow aspirate smears were selected from five patients with parasitological or serological diagnosis of VL ( Table 1 ). DNA extracted from each sample was used for *Leishmania* spp. identification by Sanger sequencing and *HSP70* - amplicon-based NGS as per protocol ( Patiño et al., 2021 ). *Leishmania* spp. were identified in all samples (Table S1, see online supplementary material). As expected, considering the nature of each sequencing method ( Voelkerding et al., 2009 ), Sanger sequencing detected one species per sample *: Leishmania infantum* or *L. amazonensis* . In contrast, with amplicon-based NGS, only one sample was found to be solely infected with *L. infantum* , and the remaining five samples were co-infected by two or more species ( Figure 1 ), including *L. infantum, L. amazonensis, Leishmania braziliensis, Leishmania panamensis, Leishmania lindenbergi, Leishmania naiffi* and *Trypanosoma cruzi* .

---

* Corresponding author. Facultad de Ciencias Naturales, Centro de Investigaciones en Microbiología y Biotecnología-UR, Universidad del Rosario, Carrera 26 # 63B – 48, Bogotá, Colombia. Tel.: + 571 297 0200 Ext. 4033.
*E-mail address:* juand.ramirez@urosario.edu.co (J.D. Ramírez).

https://doi.org/10.1016/j.ijid.2021.11.029

**Table 1**
Demographic and clinical characteristics of patients with visceral leishmaniasis selected for the study.

| Sample ID | Sample type | Department of occurrence | Municipality of occurrence | Rural zone | Sex | Age | Weight | Comorbidities | Evolution time (days) | Signs | Treatment |
|---|---|---|---|---|---|---|---|---|---|---|---|
| H78 | BMA smear | Sucre | Ovejas | Almagra | Male | 1 year | 10 kg |  | 15 | Fever, splenomegaly, anaemia, thrombocytopenia | N-methyl glucamine |
| H83 | BMA smear | Cesar | La Jagua de Ibirico |  | Female | 10 years | 25 kg |  | 40 | Fever, splenomegaly, anaemia, leukopenia. | Amphotericin B |
| H79<br>H85 | BMA smear<br>Serum | Tolima | Chaparral | Risaralda-Calarma | Male | 37 years | 45 kg | HIV positive, on antiretroviral treatment since 2011 | 5 | Skin lesions on upper limbs, hepatomegaly, splenomegaly, anaemia, pancytopenia | N-methyl glucamine |
| H86 | Serum | Santander | Girón | Palogordo | Female | 25 years | 23.3 kg | Immunocompromised secondary to inspection of intestinal malabsorption syndrome | 76 | Hepatomegaly, splenomegaly, anaemia, pancytopenia, 1-year history of chronic diarrhoea, cachexia, depression | Amphotericin B |
| H720 | Serum | Venezuela, Aragua [a] | Maracay |  | Female | 11 months | 8 kg | Differential diagnosis with Chagas disease. Total antigens (TestELISA Chagas Grupo Bios III), recombinant antigens (Vircell), IIF *T. cruzi* NR 1/16 IIF *L. infantum* R 1/256 | 15 | Fever, anaemia, pancytopenia, hepatomegaly, splenomegaly, cough | N-methyl glucamine |

BMA, bone marrow aspirate; IIF, indirect immunofluorescence; NR, non-reactive; R, reactive; *T. cruzi, Trypanosoma cruzi; L. infantum, Leishmania infantum.* [a] Attended Herasmo Meoz Hospital, reported by Public Health Laboratory of Norte de Santander (Colombia).

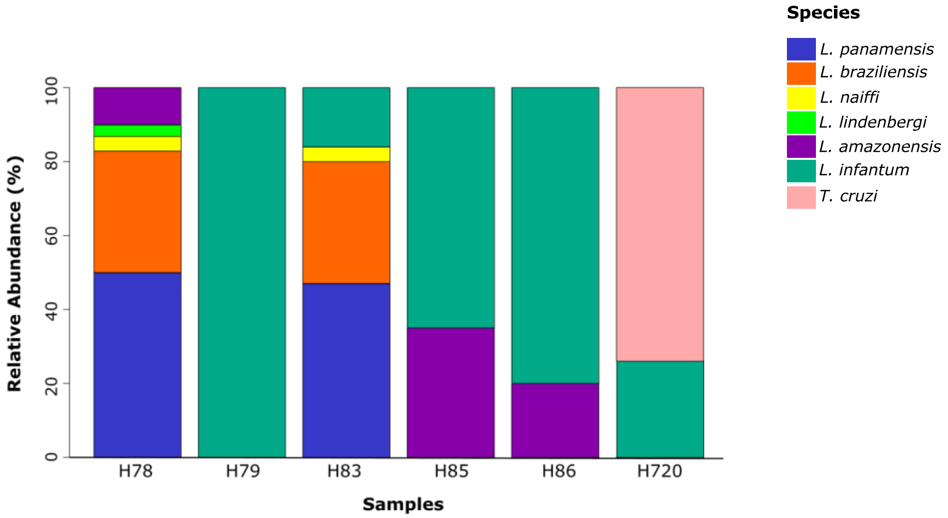


**Figure 1.** Relative abundance of *Leishmania* spp. identified in patients with visceral leishmaniasis. Relative abundance of *Leishmania* spp. and *Trypanosoma cruzi* found in the analysed samples based on *HSP70* gene amplicon-based next-generation sequencing. Bar colours represent the *Leishmania* and *Trypanosoma* species detected.

These findings reflect the epidemiological complexities of *Leishmania* spp. infection. Samples H78 and H83 presented the highest abundance of *Leishmania* spp. (Table S1, see online supplementary material), highlighting the presence of *L. naiffi* in both samples, as well as *L. lindenbergi* in Sample H78. Interestingly, Sample H78 was from a child from Almagra, a rural zone where the proximity with domestic ecotope is an ecological niche, availability of vectors ( Paternina et al., 2016 ; González et al., 2018 ) and reservoirs ( López et al., 2021 ; Medkour et al., 2019 ; Roque and Jansen, 2014 ) play an important role in the VL ecoepidemiology. Furthermore, this is a historical armed conflict zone, so the high flow of militaries, illegal groups and displacement due to violence may have generated an unusual pattern in the distribution of *Leishmania* spp. related to sylvatic and forest niches ( Berry and Berrang-Ford, 2016 ; Patiño et al., 2017 ). As such, one can hypothesize that the diversity of *Leishmania* spp. in Colombia is underestimated.

*L. infantum–L. amazonensis* co-infection was recorded in two immunocompromised patients ( Figure 1 ) – one with human immunodeficiency virus (HIV, Sample H85) and one with an unspecified malabsorption syndrome (Sample H86) (see Table 1 ). *L. infantum* alone was identified in the serum sample (Sample H75) from the same patient with HIV . In patients with HIV, the reduction of CD4 + T lymphocytes favours replication, spread and visceralization of *Leishmania* spp., and the coexistence of this pathogen accelerates progress towards acquired immunodeficiency syndrome ( Okwor and Uzonna, 2013 ), therapeutic failure and relapse ( Lindoso et al., 2018 ). Therefore, the implementation of NGS diagnostic approaches facilitates understanding the prognosis of the disease.

An important finding was the co-infection with *Leishmania* and *Trypanosoma* spp. in one patient (Sample H720). Despite having a negative serological diagnosis for Chagas disease ( Table 1 ), determinant sequences were retrieved for both *T. cruzi* (74%) and *L. infantum* (26%) (Table S1, see online supplementary material). From a clinical standpoint, it is hypothesized that initial infection and establishment of *L. infantum* in bone marrow affected haematopoietic stem cells ( McCall et al., 2013 ). In theory, a subsequent infection with *T. cruzi* could have a false-negative serological diagnosis associated with a short period of evolution and a limited immune response. In this clinical context, therapy with pentavalent antimonials could potentially exacerbate Chagasic cardiomyopathy in symptomatic patients due to their well-known cardiotoxicity, potentially leading to death ( Bastrenta et al., 2003 ).

As such, the clinical utility of metagenomics as depicted in these scenarios is relevant for being advantageous beyond mere species identification from direct clinical samples, but also to characterize virulence determinants and drug susceptibility profiles, giving information about co-infection with multiple *Leishmania* spp. in VL, particularly in endemic regions. These findings recalibrate the classical understanding of VL, in which mono-infection by any viscerotropic *Leishmania* sp. ( *L. donovani* complex) is the standard description in disease pathogenesis.

## Acknowledgements

The authors wish to thank Dirección de Investigación e Innovación from Universidad del Rosario for supporting open access of this manuscript.

**Conflict of interest statement** None declared.

## Funding

This work was supported by Dirección de Investigación e Innovación from Universidad del Rosario.

## Ethical approval

The patient samples analysed in this study were authorized for use by, and belong to, the cryo-bank of Grupo de Parasitología, Instituto Nacional de Salud-INS (National Reference Laboratory in Colombia, charged with confirming cases of VL and holding custody of collected samples). The samples were de-identified to protect the identity of patients. The Colombian National Institute of Health (INS) is defined as the reference laboratory in Colombia. When a public health emergency occurs, such as VL, national law 9–1979, decrees 786–1990 and 2323–2006, authorizes the INS to use biological material and associated epidemiological information without informed consent, including the anonymous disclosure of results.

This study was performed in accordance with the Declaration of Helsinki and its later amendments. Patient data were anonymized.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi: 10.1016/j.ijid.2021.11.029 .

## References

Al Balushi A , Khamis F , Klaassen CHW , Gangneux J-P , Van Hellemond JJ , Petersen E . Double infection with *Leishmania tropica* and *L. major* in an HIV patient controlled with high doses of amphotericin B. Open Forum Infect Dis 2018;5 ofy323 .

Aleixo JA , Nascimento ET , Monteiro GR , Fernandes MZ , Ramos AMO , Wilson ME , et al . Atypical American visceral leishmaniasis caused by disseminated *Leishmania amazonensis* infection presenting with hepatitis and adenopathy. Trans R Soc Trop Med Hyg 20 06;10 0:79–82 .

Barral A , Badaró R , Barral-Netto M , Grimaldi G , Momem H , Carvalho EM . Isolation of *Leishmania mexicana amazonensis* from the bone marrow in a case of American visceral leishmaniasis. Am J Trop Med Hyg 1986;35:732–4 .

Bastrenta B , Mita N , Buitrago R , Vargas F , Flores M , Machane M , et al . Human mixed infections of *Leishmania* spp. and *Leishmania–Trypanosoma cruzi* in a sub Andean Bolivian area: identification by polymerase chain reaction/hybridization and isoenzyme. Mem Inst Oswaldo Cruz 2003;98:255–64 .

Berry I , Berrang-Ford L . Leishmaniasis, conflict, and political terror: a spatio-temporal analysis. Soc Sci Med 2016;167:140–9 .

Fakhar M , Pouladfar GR , Alborzi A , Kadivar MR , Hatam GR , Motazedian MH . Isolation of *Leishmania tropica* from a patient with visceral leishmaniasis and disseminated cutaneous leishmaniasis, Southern Iran. Am J Trop Med Hyg 2008;79:435–7 .

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 .

González C , León C , Paz A , López M , Molina G , Toro D , et al . Diversity patterns, leishmania DNA detection, and bloodmeal identification of Phlebotominae sand flies in villages in northern Colombia. PLoS One 2018;13 .

Lindoso J , Moreira C , Cunha M , Queiroz IT . Visceral leishmaniasis and HIV coinfection: current perspectives. HIVAIDS Res Palliat Care 2018;10:193–201 .

López M , Erazo D , Hoyos J , León C , Fuya P , Lugo L , et al . Measuring spatial co-occurrences of species potentially involved in leishmania transmission cycles through a predictive and fieldwork approach. Sci Rep 2021;11:6789 .

McCall L-I , Zhang W-W , Matlashewski G . Determinants for the development of visceral leishmaniasis disease. PLoS Pathog 2013;9 .

Medkour H , Davoust B , Dulieu F , Maurizi L , Lamour T , Marié J-L , et al . Potential animal reservoirs (dogs and bats) of human visceral leishmaniasis due to *Leishmania infantum* in French Guiana. PLoS Negl Trop Dis 2019;13 .

Mendes DG , Lauria-Pires L , Nitz N , Lozzi SP , Nascimento RJ , Monteiro PS , et al . Exposure to mixed asymptomatic infections with *Trypanosoma cruzi, Leishmania braziliensis* and *Leishmania chagasi* in the human population of the greater Amazon: human exposure to mixed kinetoplastid infections. Trop Med Int Health 2007;12:629–36 .

Monroy-Ostria A , Nasereddin A , Monteon VM , Guzmán-Bracho C , Jaffe CL . ITS1 PCR-RFLP diagnosis and characterization of leishmania in clinical samples and strains from cases of human cutaneous leishmaniasis in states of the Mexican Southeast. Interdiscip Perspect Infect Dis 2014;2014:1–6 .

Okwor I , Uzonna JE . The immunology of leishmania/HIV co-infection. Immunol Res 2013;56:163–71 .

Paranaiba LF , Pinheiro LJ , Torrecilhas AC , Macedo DH , Menezes-Neto A , Tafuri WL , et al . *Leishmania enriettii* (Muniz & Medina, 1948): a highly diverse parasite is here to stay. PLoS Pathog 2017;13 .

Paternina LE , Verbel-Vergara D , Romero-Ricardo L , Pérez-Doria A , Paternina-Gómez M , Martínez L , et al . Evidence for anthropophily in five species of phlebotomine sand flies (Diptera: Psychodidae) from northern Colombia, revealed by molecular identification of bloodmeals. Acta Trop 2016;153:86–92 .

Patiño LH , Mendez C , Rodriguez O , Romero Y , Velandia D , Alvarado M , et al . Spatial distribution, *Leishmania* species and clinical traits of cutaneous leishmaniasis cases in the Colombian army. PLoS Negl Trop Dis 2017;11 .

Patiño LH , Castillo-Castañeda AC , Muñoz M , Jaimes JE , Luna-Niño N , Hernández C , et al . Development of an amplicon-based next-generation sequencing protocol to identify *Leishmania* species and other trypanosomatids in leishmaniasis endemic areas. Microbiol Spectr 2021;9 e00652-21 .

Rodriguez-Bonfante C , Bonfante-Garrido R , Grimaldi G , Momen H , Cupolillo E . Genotypically distinct *Leishmania colombiensis* isolates from Venezuela cause both cutaneous and visceral leishmaniasis in humans. Infect Genet Evol 2003;3:119–24 .

Roque ALR , Jansen AM . Wild and synanthropic reservoirs of *Leishmania* species in the Americas. Int J Parasitol Parasites Wildl 2014;3:251–62 .

Veland N , Adaui V , Llanos-Cuentas A , Arevalo J , Boggild AK , Valencia BM , et al . Simultaneous infection with *Leishmania (Viannia) braziliensis* and *L. (V.) lainsoni* in a Peruvian patient with cutaneous leishmaniasis. Am J Trop Med Hyg 2013;88:774–7 .

Voelkerding KV , Dames SA , Durtschi JD . Next-generation sequencing: from basic research to diagnostics. Clin Chem 2009;55:641–58

Castillo-Castañeda et al. Parasites & Vectors (2022) 15:471
https://doi.org/10.1186/s13071-022-05595-y

Parasites & Vectors

RESEARCH Open Access

# An overview of the trypanosomatid (Kinetoplastida: Trypanosomatidae) parasites infecting several mammal species in Colombia

Adriana C. Castillo-Castañeda[1], Luz H. Patiño[1], Maria Fernanda Zuñiga[1], Omar Cantillo-Barraza[1,2], Martha S. Ayala[3], Maryi Segura[3], Jessica Bautista[3], Plutarco Urbano[4], Jeiczon Jaimes-Dueñez[5] and Juan David Ramírez[1,6*]

## Abstract

**Background:** Trypanosomatids are among the most critical parasites for public health due to their impact on human, animal, and plant health. Diseases associated with these pathogens manifest mainly in poor and vulnerable populations, where social, environmental, and biological factors modulate the case incidence and geographical distribution. **Methods:** We used Sanger and amplicon-based next-generation sequencing (NGS) in samples from different mammals to identify trypanosomatid infections in several departments in Colombia. A total of 174 DNA samples (18 humans, 83 dogs, and 73 wild mammals) were analyzed by conventional PCR using a fragment of the heat shock protein 70 (*Hsp70*) gene and Sanger sequenced the positive samples. Twenty-seven samples were sent for ampliconbased NGS using the same gene fragment. Data obtained were used to perform diversity analyses.

**Results:** One hundred and thirteen samples were positive for PCR by *Hsp70* fragment; these corresponded to 22.1% *Leishmania* spp., 18.6% *L. amazonensis*, 9.7% *L. braziliensis*, 14.2% *L. infantum*, 8% *L. panamensis*, and 27.4% *Trypanosoma cruzi*. Comparison of the identified species by the two sequencing technologies used resulted in 97% concordance. Alpha and beta diversity indices were significant, mainly for dogs; there was an interesting index of coinfection events in the analyzed samples: different *Leishmania* species and the simultaneous presence of *T. cruzi* and even *T. rangeli* in one of the samples analyzed. Moreover, a low presence of *L. braziliensis* was observed in samples from wild mammals. Interestingly, to our knowledge, this is the first report of *Leishmania* detection in *Hydrochaeris hydrochaeris* (capybara) in Colombia.

**Conclusions:** The *Hsp70* fragment used in this study is an optimal molecular marker for trypanosomatid identification in many hosts and allows the identification of different species in the same sample when amplicon-based sequencing is used. However, the use of this fragment for molecular diagnosis through conventional PCR should be carefully interpreted because of this same capacity to identify several parasites. This point is of pivotal importance in highly endemic countries across South America because of the co-circulation of different genera from the Trypanosomatidae family. The findings show an interesting starting point for One Health approaches in which coevolution and vector-host interactions can be studied.

**Keywords:** Amplicon-based NGS, Sanger, Mammals, Trypanosomatids, Coinfection, Diversity

---

*Correspondence: juand.ramirez@urosario.edu.co

[1] Centro de Investigaciones en Microbiología y Biotecnología-UR (CIMBIUR), Facultad de Ciencias Naturales, Universidad del Rosario, Bogotá, Colombia
Full list of author information is available at the end of the article

## Background

Kinetoplastid parasites have been a primary worldwide concern for centuries, where *Leishmania* and *Trypanosoma* stand out as the most critical genera [1]. These have tremendous importance for public health because of their impact on human and animal diseases, reflected in economic losses associated with morbidity, mortality, cost overrun in health systems, and investment in prevention programs, among others [2]. Furthermore, for plants, *Phytomonas* spp. is associated with damage to coffee, oil palm, and coconut plantations, with economic effects due to crop failures, pesticides use, loss of cultivable fields, and biodiversity, leading to ecological imbalance [3–5].

Human and animal diseases associated with these pathogens have great significance for the World Health Organization (WHO), considering that they are included in the 2030 agenda for the elimination of neglected tropical diseases (NTDs) [6]. For both leishmaniasis and trypanosomiasis, poverty, vulnerability [7], environmental [8], social [9–13], and biological factors [1] modulate the geographic distribution of the pathogens, their vectors, and consequently the incidence of human cases. In mammals, trypanosomatids are transmitted mainly by vectors; however, oral infections represent a vital infection route in the wild transmission cycle. For *Leishmania* spp., transmission is by the bite of infected female phlebotomine sand flies [14], having three clinical manifestations in humans: cutaneous, mucocutaneous, and visceral leishmaniasis (VL) [15]. In the case of *Trypanosoma* spp., the vectorial transmission is mediated by triatomines for *T. cruzi* and *T. rangeli* and tsetse flies for *T. brucei*, causing asymptomatic infections or acute disease that can evolve to a chronic phase in humans [1]. The severity of these parasitic diseases has been related to the infecting species, infection route, patient's immunological response, comorbidities, and treatment opportunities [16, 17].

Sanger sequencing has helped the study of *Leishmania* spp., *Trypanosoma* spp., their vectors, and their feeding preferences [18–24]. Indeed, Asia and the Mediterranean basin have reported the presence of *Trypanosoma* spp. DNA in phlebotomines [25–27]. Also, Sanger technology has helped determine the causal agents of leishmaniasis and trypanosomiasis in urban and periurban transmission cycles [24, 28–32]. Likewise, the DNA of trypanosomatids has also been identified in several mammals of the sylvatic cycle, such as rodents [33–35], didelphids [36, 37], marsupials [38], bats [39–43], and primates [44, 45]. Such analyses in vectors and reservoirs are highly relevant for public health, considering they allow determining the incidence of parasitic species in the transmission hotspots and their geographical distribution as well as the study of the genetic diversity of *Leishmania* spp.

[46–49] and *Trypanosoma* spp. [50–52] worldwide.

Easy access to next-generation sequencing (NGS) technologies and methodologies, such as amplicon-based NGS, has allowed generating and analyzing large and complete amounts of data on the parasites [53–57]. For leishmaniasis expressly, this methodology has provided numerous highlights, for instance, the identification of *Leishmania* species in new geographic regions [58], infection indices and feeding preferences in vectors [25, 59], and identification of the most influential reservoirs in the transmission cycles [30, 40, 60, 61]. Regarding trypanosomiasis, NGS has facilitated the study of *T. cruzi* and *T. rangeli* genetic diversity [56], lineage associations in asymptomatic, acute, and chronic cases of Chagas disease [62], and identification of multiple feeding preferences in triatomines [53], among others, and detected coinfection events by different parasitic species in a single host [58, 63]. Although leishmaniasis and Chagas disease are important because of their incidence and wide geographical distribution [18, 64], there are few investigations related to the study of these agents in mammals, especially in different transmission cycles in Colombian departments, with an active circulation of the parasites. Therefore, using NGS (amplicon-based) and Sanger, we aimed to study and improve the understanding of the transmission cycles of trypanosomatids in samples obtained from different wild and domestic mammals in many departments in Colombia. This study has the additional purpose of encouraging the use of this type of research on different players in the life cycle of parasites in endemic countries, hence generating updated data useful for government stakeholders for the promotion and prevention of these diseases using a One Health context.

## Methods

### Samples

A total of 174 samples were included by convenience in this study: 18 from humans with VL diagnosis from the departments of Bolívar, Córdoba, Huila, La Guajira, Norte de Santander, Santander, Sucre, and Tolima; 83 of domestic dogs (*Canis lupus familiaris*) from Antioquia, Santander, La Guajira, Cesar, Córdoba, Huila, Norte de Santander, Santander, Sucre, and Tolima; 73 of wild mammals (*Callicebus cupreus*, *Carollia brevicauda*, *Carollia perspicillata*, *Chinchilla lanigera*, *Choloepus didactylus*, *Coendou bicolor*, *Desmodus rotundus*, *Didelphis marsupialis*, *Glossophaga soricina*, *Hydrochaeris hydrochaeris*, *Myotis brandtii*, *Myotis martiniquensis*, *Odocoileus virginianus*, *Pecari tajacu*, *Phyllostomus elongatus*, *Phyllostomus hastatus*, *Proechimys roberti*, and

*Tapirus terrestris*) from Antioquia and Casanare (Fig. 1; Additional file 1: Table S1). Most departments are endemic for leishmaniasis and Chagas disease. The geographic distribution by department in Colombia is shown in the supplementary information (Additional file 2: Fig. S1).

Human samples were obtained from two sources: serum via venipuncture and bone marrow aspirate smear slides. From canines, these were obtained by anticoagulated total blood with EDTA (venipuncture) or serum in a dry tube. The samples were anticoagulated whole blood with EDTA or collected in FTA cards for wild mammals. All animals were captured with the minimum damage possible. The wild mammals were anesthetized with 20 mg/kg body weight ketamine (Ketalar, Parke Davis, Morris Plains, NJ, USA), and blood was obtained via venipuncture. For bats, only 300 µl whole blood was collected. All the plasma and serum samples were conserved at –80 °C until their processing; FTA cards and slides were stored at environmental temperature and humidity for optimal storage conditions.

### DNA extraction

All the biological samples collected were processed using the High Pure PCR Template Preparation Kit (Roche Life Science, Mannheim, Germany) following the protocol described by the manufacturer. The slide samples were submerged in xylol to clean the immersion oil traces; next, 200 µl lysis buffer was added for 10 min, and the smear was carefully removed and put into a microtube to start the DNA extraction. DNA concentration was determined using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the DNA quality and integrity were checked through gel electrophoresis in agarose 1%. Samples were conserved at -80 °C until processing.

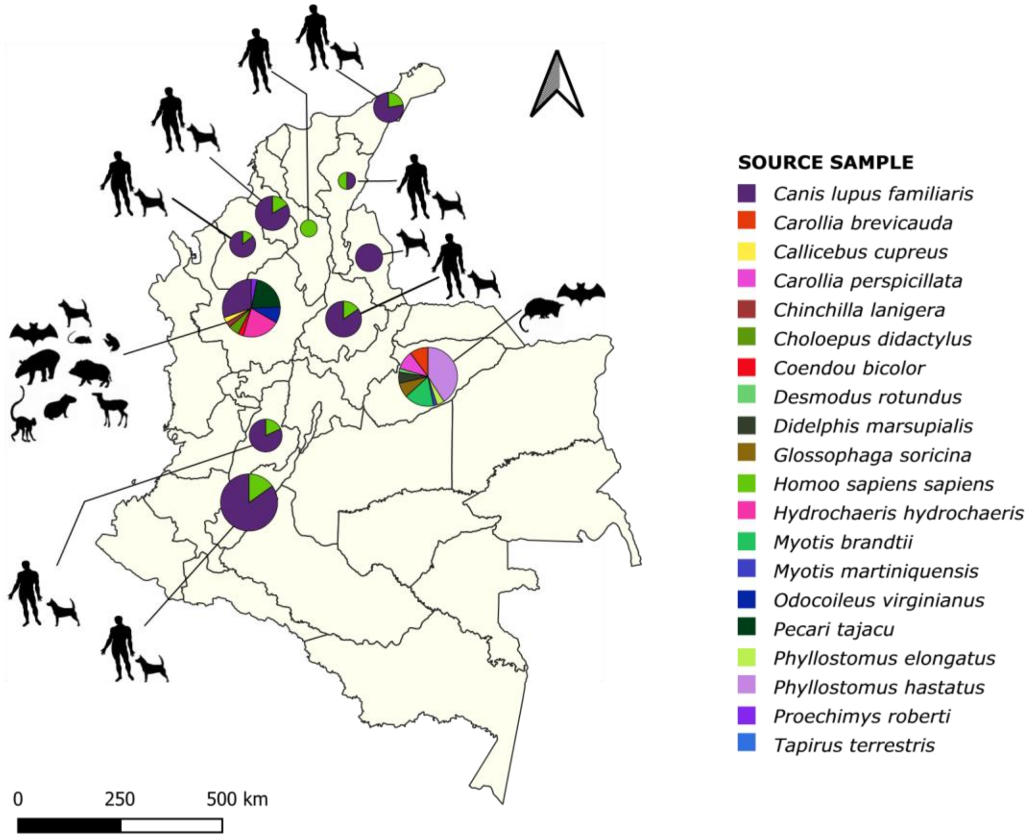


**Fig. 1** Geographical and biological distribution of the different samples analyzed in this study. Plot size is proportional to the total number of samples per department, and each mammal species is represented by a single color

### Molecular test and *Trypanosoma* species identification by Sanger sequencing

As previously reported, a 337-bp region of the *Hsp70* gene for both *Trypanosoma* and *Leishmania* was amplified by conventional PCR [65, 66]. Amplicon products were analyzed by gel electrophoresis in 2% agarose. Those products with gel band presence (positive for *Hsp70*) were purified with EXOSAP (Affymetrix, Santa Clara, CA, USA) and sent for sequencing by the dideoxyterminal method in an automated capillary sequencer (AB3730; Applied Biosystem, Foster City, CA, USA). The sequences were submitted to BLASTn using the NCBI platform [65]. Subsequently, the DNA of all the samples identified with some species of *Leishmania* that met the quality requirements for Novogene were sent for amplicon-based sequencing by Illumina. Additionally, 60% of the samples were BLAST-identified as *T. cruzi*, and ten samples from canines with visceral leishmaniasis diagnosis were also sent for sequencing. In the first scenario to assess the co-infection, when Sanger sequencing identified *T. cruzi* as the main infecting parasite, the second validated the possibility that amplicon-based NGS had more power to detect target reads.

### Amplicon-based next-generation sequencing

Genomic DNA (> 200 ng/μl) from humans, canines, and wild mammals was sent to amplicon-based sequencing by Illumina (Novogene, Beijing, China). The primers used were the same for the conventional PCR, forward (5’AGG TGA AGG CGA CGA ACG ) and reverse (5’CGC TTG TCC ATC TTT GCG TC), following the protocol, as reported before [58].

### Bioinformatics analysis

The FASTA files from the *Hsp70* raw sequences were filtered using QIIME software [67], considering the parameters described before [53]. Then, barcode trimming and forward and reverse sequence merging were made. Then, another quality filter was made for the merged files. The reads that passed the quality filters were compared against an in-house database, which contains sequences for the *Hsp70* 337-bp fragment of kinetoplastids available in GenBank [58]. The database includes species of *Leishmania*, *Trypanosoma*, and *Leptomonas*. The local BLASTn was made with a threshold of 95% identity and an e-value of 10. Of those species that matched, only the ones with abundance of the total reads per sample of > 3% significance were considered. Quantitative results were plotted using R software version 3.6.2 and the Sankey diagram package available at www. online. visualpar adigm. com.

### Statistical analysis

The qualitative variables were clustered by frequency and proportions according to the parasite species and coinfection patterns depending on the data obtained from amplicon-based sequencing. Considering the normality of the data, a Chi-square test ($\chi^2$) was made to analyze the relation between mammal-parasite and origin (department)-parasites. The statistical analysis was executed in R software (RStudio Team 2019). Two-sided significance tests and *P*-value < 0.05 were established. Moreover, to analyze the correspondence among the parasites reported by *hsp70* sequencing by Sanger and the amplicon-based sequencing, a kappa (κ) coefficient was calculated using STATA11 with 0.05 significance.

## Results

### Trypanosomatid identification by Sanger sequencing

Overall, 64.9% of the total samples used in the study (113/174) had amplification for *Hsp70* by conventional PCR (Additional file 1: Table S1), of which 12.2% (14/18) were from humans, 40.4% (46/83) from canines, and 47.4% (54/73) from wild mammals. Results obtained from BLASTn (Fig. 2; Additional file 3: Table S2) show that Colombia has a wide variety of *Leishmania* species, mainly in the departments with co-circulation of *T. cruzi*. For mammal species, *L. infantum* (71.4%) and *L. amazonensis* (21.4%) were the most frequent species in human samples with VL diagnosis; for canines, they were *L. amazonensis* (26.1%), *L. braziliensis* (17.4%), and *Leishmania* spp. (21.8%). For wild mammals, they were *T. cruzi* (47.2%) and *Leishmania* spp. (26.4%), *L. amazonensis* (11.3%), and *L. braziliensis* (5.7%) (Fig. 2). Furthermore, considering the origin of the samples, a high diversity of parasitic species was found for each animal, *T. cruzi* and *Leishmania* spp. being the most prevalent, with 27.4% and 22.1%, respectively (Fig. 2). The former was more frequent in Casanare, where the samples were collected mostly from bats.

### *Hsp70* sequencing by amplicon-based NGS analysis

Only 118 samples met the requirement of the DNA concentration (≥ 200 ng/μl) for Illumina, of which 22.9% (27/118) were optimal for analysis. Subsequent sequencing of the 337-bp *Hsp70* fragment by Illumina generated between 134,316 and 179,347 paired-end reads. The bioinformatic analysis revealed that for 96.2% of the samples (26/27), > 85% of the reads had a minimum coverage > q20 (initial quality filter). The exception was a canine from Sucre (R95), in which only 41% of the reads successfully passed the initial quality control. Furthermore, the taxonomic assignment made with local BLASTn was performed with

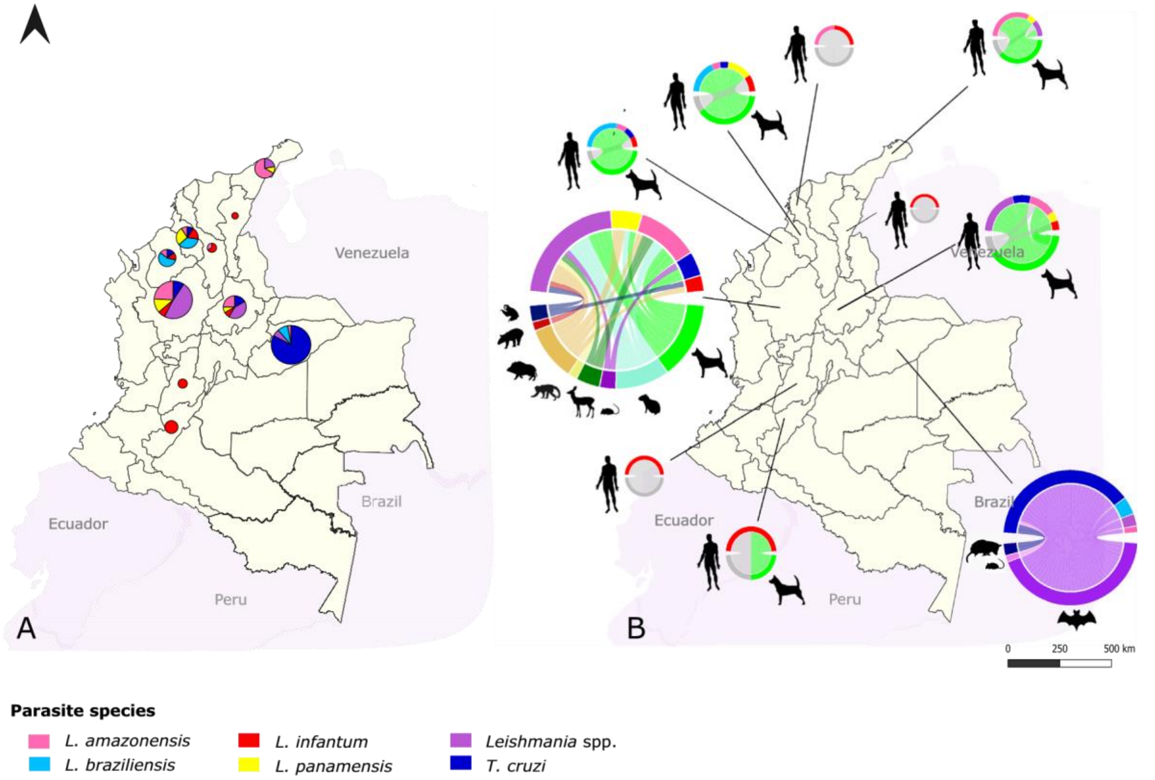


**Fig. 2** Results of the biological and geographical distribution of trypanosomatid species identified by Sanger sequencing. **A** Parasite species found for each animal analyzed per department (**B**). Plot size is proportional to the total number of samples analyzed per department. For each plot from panel (**B**), the size of the inner joining line among mammals (lower half) and parasite species (upper half) is proportional to sample number. Each parasite species is represented by a single color

high-quality reads per sample using > 38% of the reads generated at the beginning for almost all cases.

Unexpectedly, the taxonomic assignation for animal samples C334, C335, PUC07, and SAC382 (Fig. 3) resulted in individual matches for 35, 108, 608, and 234 reads with the species included in the database used.

### Concordance between Sanger and amplicon-based NGS results

It is known that the two sequencing methods used in this study have different methodological principles, scope, and output. However, we compared whether the species (unique) obtained with Sanger were included or not in the unique or multiple species obtained with ampliconbased NGS. We showed a general concordance between the two sequencing techniques of 97% and a kappa coefficient of 0.8–1.0 by comparing the identified species.

### In amplicon-based NGS analysis, coinfection events in VL patients' samples and canines were frequent

The coinfection events were more frequent in human and canine samples compared to samples from wild mammals. Coinfection was identified from the human samples (5/7); infection frequency by *L. infantum* was 85.8%, *L. amazonensis* 42.6%, *L. braziliensis, L. panamensis*, and *L. naiffi* 28.6%, with 14.3% for *L. lindenbergi* along with *T. cruzi*. Double infection events were detected: *T. cruzi/L. infantum* (1 sample) and *L. amazonensis/L. infantum* (2 samples) and multiple infection by *L. infantum/L. braziliensis/L. panamensis/L. naiffi* and *L. amazonensis/L. braziliensis/L. panamensis/L.naiffi/L. lindenbergi* in the same patient (Fig. 3A); a single infection by *L. infantum* in humans was present in 28.6% (2/7) of the samples, in concordance with Sanger reports**.** Canine samples presented a wide diversity of *Leishmania* species, with a single infection in around 50%, *T. cruzi* infection in three samples, *L. infantum* and *L. mexicana* in one sample each, and triple infection by *T. cruzi/T. rangeli/L. infantum* in a canine from Santander; the samples from Sucre showed multiple infections: *L. braziliensis/L. panamensis/L. naiffi* and two canines by *T. cruzi/L. amazonensis/L. braziliensis* and *T. cruzi/L. infantum/L. braziliensis*, respectively (Fig. 3B).

### *Trypanosoma cruzi* prevails in wild mammals

One hundred percent of wild mammals had reads for *T. cruzi* in the amplicon-based NGS. Double infection by *T. cruzi* with *L. panamensis* or *L. braziliensis* was present in three samples and triple infection by *T. cruzi* along with *L.*

*braziliensis/L. panamensis* in one sample. Single infection by *T. cruzi* was present in 44.4% of the samples (Fig. 3C).

### Statistical analysis

The statistical analysis did not reveal statistically significant differences between the coinfection and singleinfection groups analyzed (Mann-Whitney-Wilcoxon test, $P = 0.07$, 0.87, and 0.566) or among species (KruskalWallis test, $P = 0.31$, 0.195 and 0.567).

Chi-squared tests and Fisher exact tests were performed to evaluate a potential association between the categorical variables and for the relation between department and species ($P = 0.038$) and chi-squared test ($P = 0.022$) for parasite species vs. mammal.

### Diversity analysis

Analyzing the alpha diversity of the samples by ampliconbased NGS, statistically significant differences among the three groups were analyzed (Shannon index: $P < 0.0001$; Simpson index $P = 0.0001$). Humans and dogs presented the most diversity (Shannon index 1.14, 1.17 for humans and 1.04 for canines) and dominance (Simpson index 0.64 and 0.62, respectively) compared with the wild mammals where the obtained values were close to zero (Additional file 4: Table S3). This comparison revealed statistically significant differences in alpha diversity between humans and wild mammals and dogs and wild mammals.

## Discussion

The diversity of *Leishmania* species found using *Hsp70* amplicons by Sanger sequencing agree with those expected for patients with VL diagnosis (Additional file 3: Table S2), which historically have been *L. infantum* for the Americas [68–70]. We also found *L. amazonensis* in patient samples from La Guajira, Santander, and Bolívar (Fig. 2), an atypical event that has been previously reported in humans [71] and dogs [72, 73]. For humans, *L. major* and *L. tropica* have been the principal non-*L. donovani* complex species reported in the Old World, while in the Americas they have been *L. braziliensis, L. mexicana*, and *L. amazonensis*. In both geographical contexts, HIV is the main factor described for coinfection events in immunocompromised patients [74]. In La Guajira, the samples collected came from a new hotspot of VL in Hatonuevo municipality, in which *L. amazonensis* was detected in both humans and canines (Additional file 3: Table S2). This allowed us to consider the potential existence of a new VL hotspot solely associated with *L. amazonensis*, even though further investigations must include more comprehensive sampling, vectors, and parasite isolation to prove *L. amazonensis*'s capacity for visceral tropism.

We also found a high diversity of *Leishmania* species in dogs and wild mammals, besides the presence of *T. cruzi* in animals from the different departments of Colombia (Fig. 2). The latter has high prevalence in regions highly endemic for Chagas disease [75, 76] such as Casanare. These findings showed that, regarding the NTD elimination programs focused on vector control and diagnosis/treatment in humans, the pathogen transmission remains enzootic [77]. The above is alarming considering the increasing sylvatic niche fragmentation, which also increases the risk of outbreaks, sylvatic parasite species circulation in urban transmission cycles, and the adaptation of the pathogens according to the availability of vectors and hosts [78, 79]. This problem has been acknowledged and studied in endemic regions in the Brazilian Amazon, keeping in mind their local context and associated variables to strengthen One Health intervention programs [80].

Furthermore, the presence of different *Leishmania* species is related to Colombia's high biodiversity [81], where the vectors' diversity and ecological niches [44, 82] allow the maintenance of *L. panamensis*, *L. amazonensis*, and *L. braziliensis* in sylvatic, urban, and periurban transmission cycles. Additionally, differently than expected for *L. braziliensis* and *L. panamensis* (the most prevalent species of cutaneous leishmaniasis in active military populations) [58, 83], we found a low number of wild mammals infected with these species in Antioquia. On the other hand, *L. amazonensis* predominates, and *L. infantum* was identified in *Pecari tajacu* (collared peccary) and *Choloepus didactylus* (Linnaeus's two-toed sloth). In all *Hydrochaeris hydrochaeris* (Capybara) samples, *L. amazonensis, L. panamensis*, and *Leishmania* spp. were identified, as previously reported in other countries [84, 85]. This represents the first report in Colombia highlighting the need to conduct studies on this species, which represents an exotic source of consumable meat in the region.

For Casanare, *T. cruzi* was the main trypanosomatid detected. *Leishmania* was identified in four animals (3 *L. braziliensis* and 1 *L. amazonensis*) (Fig. 2A). These findings suggest that the vector species distribution could determine the patterns according to specific environmental conditions, their feeding preferences, and the availability of specific reservoirs [86–89]. Therefore, by analyzing the data for Antioquia (Fig. 2; Additional file 3: Table S2), the possibility can be suggested that the lack of identification of *L. braziliensis* was determined by the

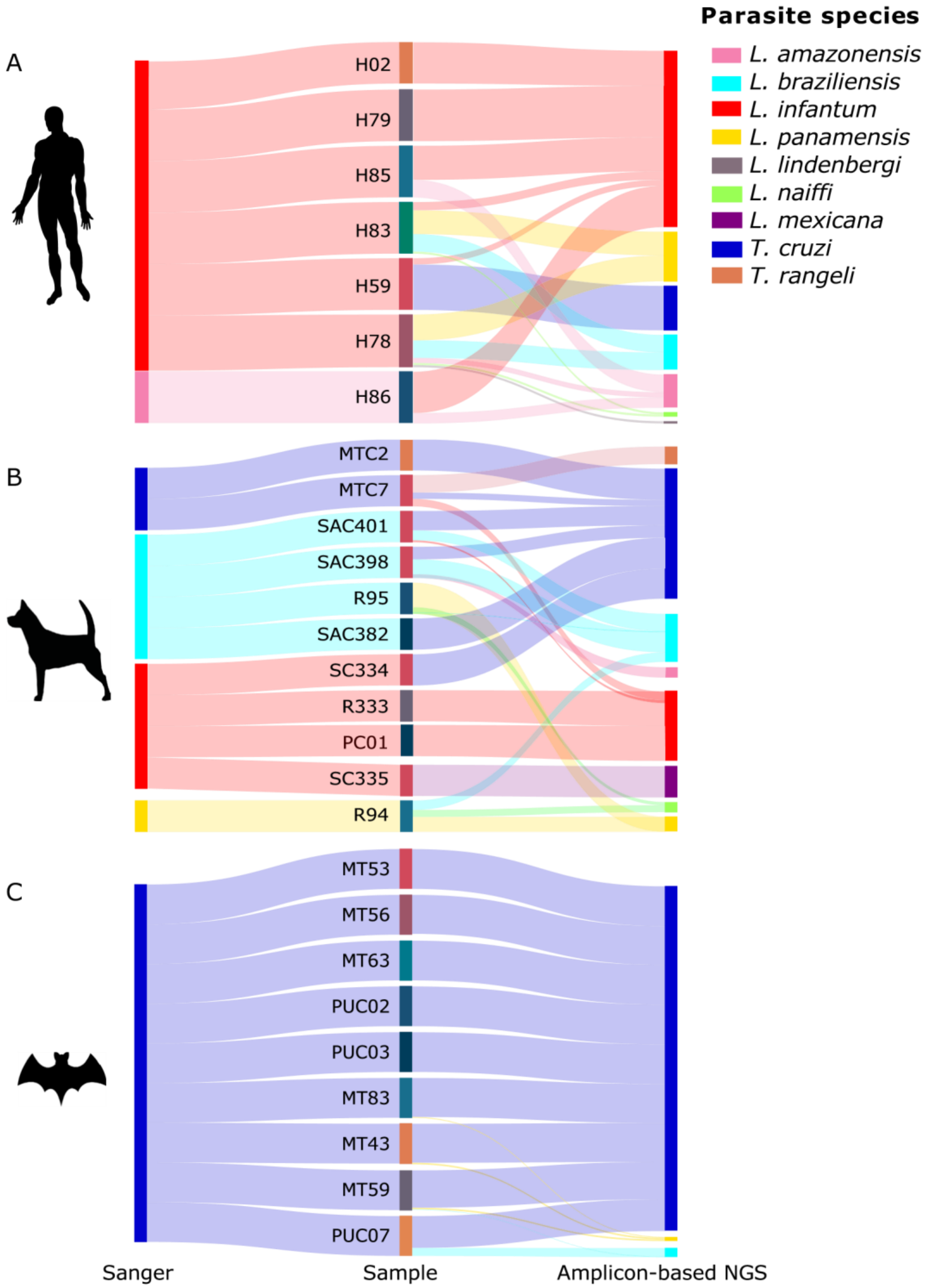


**Fig. 3** *Leishmania* and *Trypanosoma* species correspondence reported by Sanger sequencing and reads obtained by amplicon-based NGS from human samples (**A**), dogs (**B**), and wild mammals (**C**). The size of the inner joining line among samples and parasites is proportional to the reads percentage found in the analyzed sample. Each parasite species is represented by a single color

mammal species sampled in this study, while for Casanare, the hypothesis could explain how, despite finding bats infected with *L. braziliensis* and *L. amazonensis* (Figs. 2, 3C) and the presence of the circulation of *Lutzomyia gomezi* in the department [90], no autochthonous cases of leishmaniasis have been reported according to official data from the Colombian Disease Surveillance System (SIVIGILA). It can be assumed that the phlebotomines play an essential role in disease modeling in humans and the maintenance of the parasite's enzootic cycle, as has been demonstrated in endemic regions for cutaneous and visceral leishmaniasis in Brazil, Spain, and Iran [91–93]. However, a broader sampling and inclusion of more mammals, as well as the parallel study of phlebotomine circulation, distribution, and feeding preferences, are needed.

Additionally, two human and canine samples from Sucre presented the highest diversity index (Additional file 4: Table S3), where we identified coinfection events with *L. naiffi* and *L. lindenbergi* (Fig. 3A, B), as recently reported [58, 63]. The high species richness of *Leishmania* in a single individual could be associated with the proximity of dwellings to forests, with a circulation of different vector species such as *Lu. longipalpis, Lu. evansi*, and *Lu. gomezi* [90, 94], human and canine mobilization to the forests to search for natural resources, and military and illegal groups in this country zone [9, 63, 65, 83]. All these factors make the vector-human-reservoir-pathogen interaction more accessible, maintaining the zoonotic and enzootic transmission cycles of *Leishmania* spp. Some authors have concluded, for instance, that the circulating phlebotomine sand fly species are critical for the vectorial transmission of *Leishmania* spp. [95]; likewise, the mammals' role in parasite transmission concerns the vector, their meal preferences, and feeding behavior [96, 97].

Considering the identified parasite species versus those expected in wild mammals and coinfection events, a new scenario is opening showing the need for research on the following topics: (i) the role of domestic/wild mammals and vectors in the maintenance of transmission cycles, which has been studied and proposed in mathematical models for different vector-borne diseases [98–100]; (ii) the domiciliation transition of vectors in specific areas, phenomena highly relevant for American trypanosomiasis and VL in recent years [101–103]; (iii) the possibility of the genetic recombination of the different actors implicated in the parasites' life cycle, not just for the vector context [104–106]. The latter must transcend the world view of human diseases and recognize their importance and the veterinary diseases that must be equally prioritized in the public health systems [107]. Therefore, considering our results, we highlight the relevance and usefulness of transmission scenarios in Casanare, Antioquia, and Sucre to understand these phenomena's ecological dynamics better.

On the other hand, we found coinfection by *L. infantum, T. rangeli*, and *T. cruzi* in a canine in Santander (Fig. 3B), a department with a high incidence of Chagas disease. It is known that *T. cruzi* and *T. rangeli* share mammal hosts, and their geographical distribution overlaps with the finding of infected mammals and triatomines [108, 109]. This triple coinfection was previously reported in *Tamandua tetradactyla*, a wild mammal [110]. In humans, even though *T. cruzi-T. rangeli* coinfection affects Chagas disease diagnosis, the cases are underestimated as *T. rangeli* has been detected in primates, bats, rodents, marsupials, and dogs in Brazil, Colombia, and Venezuela [110, 113–118].

It is relevant to discuss the benefits and limitations of amplicon-based sequencing and the specificity of the *Hsp70* gene fragment used. In the first place, NGS technologies and the inclusion of new methodologies, such as amplicon-based ones, offer benefits in cost reduction and obtain quick and sensitive high-throughput data [117, 118], allowing the use of different target genes simultaneously [119, 120]. The time between sample processing and data collection is less than for conventional PCR and Sanger sequencing [121]. One of the critical points in sequencing success is the pre-analytics phase; therefore, the samples used in this study (serum, total blood, and bone marrow aspirate smear) determined the DNA integrity and concentration, which directly influence the success of NGS sequencing [122, 123]. Moreover, the biological influence of parasitic load in mammals and the copy number of the *Hsp70* gene should be considered. This explains the sample percentage that could be evaluated by amplicon-based sequencing (Fig. 3; Additional file 3: Table S2). Second, the *Hsp70* gene allows the identification of *Leishmania* and *Trypanosoma* [124], which is an advantage for studying samples from endemic regions for both parasites.

Nevertheless, the *Hsp70* gene sensitivity is not optimal for use as a diagnostic marker. One of our limitations was not being able to include more sensitive markers, such as satellite DNA for *T. cruzi* [125] or 18S for *Leishmania* [126], to determine whether those 60 samples were indeed negative. However, we want to emphasize that the main objective was to depict the infective species, so we chose the *Hsp70* marker. Finally, considering the rising availability of data from outstanding databases such as NCBI, searching for a more sensitive genetic marker to discriminate among these trypanosomatid species through Illumina sequencing or even Oxford Nanopore should be prioritized.

## Conclusions

The present study describes the infection by trypanosomatids in samples from humans, dogs, and wild mammals, using Sanger and amplicon-based sequencing of a coding fragment of the *Hsp70* gene. We confirmed the vast diversity of *Leishmania* species found in the different samples obtained in many departments of Colombia, the presence of *T. cruzi* in bats and dogs, and the occurrence of coinfections.

### Abbreviations

ANLA: Autoridad Nacional de Licencias Ambientales (National Authority for Environmental Licenses); INS: Instituto Nacional de Salud (National Health Institute); NGS: Next-generation sequencing; NTD: Neglected tropical diseases; VBD: Vector-borne diseases; VL: Visceral leishmaniasis; WHO: World Health Organization.

## Supplementary Information

The online version contains supplementary material available at https:// doi. org/ 10. 1186/ s13071- 022- 05595-y.

**Additional file 1: Fig. S1.** Map showing the different departments and the capital district of Colombia.

**Additional file 2: Table S1.** Information on collected samples per department, sample code, and mammal species.

**Additional file 3: Table S2.** BLASTn results for the 337bp *Hsp70* gene fragment of the samples analyzed herein.

**Additional file 4: Table S3.** Shannon and Simpson index values from the different species found in the analyzed samples by amplicon-based NGS.

### Acknowledgements

We thank Dirección de Investigación e Innovación from Universidad del Rosario for providing the publication fees fo this manuscript. We also thank Natalia Velasquez-Ortiz for editing the English of the manuscript.

### Author contributions

ACC and JDR: designed the study, conducted the analysis, and drafted the manuscript. ACC: extracted the DNA, performed the PCR, sequencing and analyzed the data. LHP: analyzed the data. OC: Provided samples and analyzed the data. MSA: Provided samples and analyzed the data. JB, JJ, MS, PU: Provided samples. All authors read and approved the final manuscript.

### Funding

Open Access funding provided by Colombia Consortium. This work was funded by Dirección de Investigación e Innovación and the Research Fund for Undergraduate Students from the Faculty of Natural Sciences, Universidad del Rosario.

### Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files. Sanger sequence data to GenBank code BankIt2630585: OP611209—OP611320. Amplicon-based NGS data to ENA, project accession: PRJEB56730 and submission accession: ERA18523751.

## Declarations

### Ethics approval and consent to participate

The samples of patients and some domestic dogs analyzed in this study belong to the cryobank of the Parasitology Group, Instituto Nacional de SaludINS (National Reference Laboratory in Colombia), and were authorized for use according to National Law 9–1979, decrees 786–1990 and 2323–2006. This study was conducted in accordance with the Declaration of Helsinki and its subsequent amendments. ANLA (Autoridad Nacional de Licencias Ambientales) permit no. 01749 and ethical approval from the Animal Ethics Committee from the Universidad de Antioquia, act No. 113 of 2017.

**Consent for publication** Not applicable.

### Competing interests

The authors declare that they have no competing interests.

### Author details

[1] Centro de Investigaciones en Microbiología y Biotecnología-UR (CIMBIUR), Facultad de Ciencias Naturales, Universidad del Rosario, Bogotá, Colombia. [2] Grupo de Biología y Control de Enfermedades Infecciosas (BCEI), Universidad de Antioquia, Medellín, Colombia. [3] Grupo de Parasitología, Instituto Nacional de Salud, Bogotá, Colombia. [4] Grupo de Investigaciones Biológicas de La Orinoquía, Universidad Internacional del Trópico Americano (Unitropico), Yopal, Colombia. [5] Grupo de Investigación en Ciencias Animales GRICA, Facultad de Medicina Veterinaria y Zootecnia, Universidad Cooperativa de Colombia UCC , Bucaramanga, Colombia. [6] Department of Pathology, Molecular and Cell-Based Medicine, Molecular Microbiology Laboratory, Icahn School of Medicine at Mount Sinai, New York, NY, USA.

Received: 24 June 2022 Accepted: 18 October 2022

### References

1. Rodrigues JCF, Godinho JLP, de Souza W. Biology of human pathogenic trypanosomatids: epidemiology, lifecycle and ultrastructure. In: Santos ALS, Branquinha MH, d'Avila-Levy CM, Kneipp LF, Sodré CL, editors. Proteins and proteomics in leishmania and trypanosoma. Dordrecht: Springer Netherlands; 2014.
2. WHO. WHO Factsheet Vector-borne diseases. 2014. https:// www. who. int/ campa igns/ world-h ealth-d ay/ 2014/ fact-s heets/ en/
3. Bartolomé C, Buendía M, Benito M, De la Rúa P, Ornosa C, MartínHernández R, et al. A new multiplex PCR protocol to detect mixd trypanosomatid infections in species of Apis and Bombus. J Invertebr Pathol. 2018;154:37–41.
4. Ganyukova AI, Frolov AO, Malysheva MN, Spodareva VV, Yurchenko V, Kostygov AY. A novel endosymbiont-containing trypanosomatid *Phytomonas borealis* sp. n. from the predatory bug Picromerus bidens (Heteroptera: Pentatomidae). Folia Parasitol. 2019. https:// doi. org/ 10. 14411/ fp. 2020. 004.
5. Onchuru TO, Martinez AJ, Kaltenpoth M. The cotton stainer's gut microbiota suppresses infection of a cotransmitted trypanosomatid parasite. Mol Ecol. 2018;27:3408–19.
6. World Health Organization. Ending the neglect to attain the Sustainable Development Goals: a road map for neglected tropical diseases 2021–2030. Overview. Washintong; 2021 p. 7. https:// www. who. int/ publi catio ns/i/ item/ 97892 40010 352.
7. Abras A, Ballart C, Fernández-Arévalo A, Pinazo M-J, Gascón J, Muñoz C, et al. Worldwide control and management of Chagas disease in a new era of globalization: a close look at congenital *Trypanosoma cruzi* infection. Clin Microbiol Rev. 2022;35:e00152-e221.
8. da Silva NAB, de Oliveira EF, Encina CCC, de Figueiredo HR, Paranhos FAC, de Oliveira AG. Effects of El Niño-Southern oscillation on human visceral leishmaniasis in the Brazilian State of Mato Grosso do Sul. Mem Inst Oswaldo Cruz. 2020;115:e190298.

9. Berry I, Berrang-Ford L. Leishmaniasis, conflict, and political terror: a spatio-temporal analysis. Soc Sci Med. 2016;167:140–9.
10. Grillet ME, Hernández-Villena JV, Llewellyn MS, Paniz-Mondolfi AE, Tami A, Vincenti-Gonzalez MF, et al. Venezuela's humanitarian crisis, resurgence of vector-borne diseases, and implications for spillover in the region. Lancet Infect Dis. 2019;19:e149–61.
11. Rodríguez-Morales AJ, Suárez JA, Risquez A, Villamil-Gómez WE, PanizMondolfi A. Consequences of Venezuela's massive migration crisis on imported malaria in Colombia, 2016–2018. Travel Med Infect Dis. 2019;28:98–9.
12. Suarez J, Carreño L, Paniz-Mondolfi A, Marco-Canosa F, Freilij H, Riera J, et al. Infectious diseases, social, economic and political crises, anthropogenic disasters and beyond: Venezuela 2019—implications for public health and travel medicine. 2018;1:73–93.
13. Valero NNH, Uriarte M. Environmental and socioeconomic risk factors associated with visceral and cutaneous leishmaniasis: a systematic review. Parasitol Res. 2020;119:365–84.
14. Afrin F, Khan I, Hemeg HA. Leishmania-host interactions—an epigenetic paradigm. Front Immunol. 2019;10:492.
15. Alemayehu B, Alemayehu M. Leishmaniasis: A review on parasite, vector and reservoir host. Health Sci J. 2017. http:// www. hsj. gr/ medic ine/ leish 201mania sis-a- review-o 31. Accessed on 21 Oct 2019n- paras ite- vector-and- reser voir- host. php? ai.d=
16. de Castro Neto AL, da Silveira JF, Mortara RA. Comparative analysis of virulence mechanisms of trypanosomatids pathogenic to humans. Front Cell Infect Microbiol. 2021;11:669079.
17. Elmahallawy EK, Alkhaldi AAM, Saleh AA. Host immune response against leishmaniasis and parasite persistence strategies: a review and assessment of recent research. Biomed Pharmacother. 2021;139:111671. 18. Ferro C, López M, Fuya P, Lugo L, Cordovez JM, González C. Spatial Distribution of Sand Fly Vectors and Eco-Epidemiology of Cutaneous Leishmaniasis Transmission in Colombia. PLOS ONE. 2015;10:e0139391.
19. González C, Cabrera OL, Munstermann LE, Ferro C. Distribución de los vectores de Leishmania infantum (Kinetoplastida: Trypanosomatidae) en Colombia. Biomedica. 2012;26:64.
20. Paternina-Gómez M, Díaz-Olmos Y, Paternina LE, Bejarano EE. Alta prevalencia de infección por Leishmania (Kinetoplastidae: Trypanosomatidae) en caninos del norte de Colombia. Biomédica. 2013. http:// www. revis tabio medica. org/ index. php/ biome dica/ artic le/ view/ 780. Accessed on 26 Apr 2020.
21. Gürtler RE, Cecere MC, Lauricella MA, Cardinal MV, Kitron U, Cohen JE. Domestic dogs and cats as sources of *Trypanosoma cruzi* infection in rural northwestern Argentina. Parasitology. 2007;134:69–82.
22. Kamdem CN, Tiofack AAZ, Mewamba EM, Ofon EA, Gomseu EBD, Simo G. Molecular identification of different trypanosome species in tsetse flies caught in the wildlife reserve of Santchou in the western region of Cameroon. Parasitol Res. 2020;119:805–13.
23. Ribeiro G, dos Santos CGS, Lanza F, Reis J, Vaccarezza F, Diniz C, et al. Wide distribution of *Trypanosoma cruzi*-infected triatomines in the State of Bahia. Brazil Parasit Vectors. 2019;12:604.
24. Rodriguez F, Luna BS, Calderon O, Manriquez-Roman C, AmezcuaWinter K, Cedillo J, et al. Surveillance of *Trypanosoma cruzi* infection in triatomine vectors, feral dogs and cats, and wild animals in and around El Paso county Texas, and New Mexico. PLoS Negl Trop Dis. 2021;15:e0009147.
25. Abbate JM, Maia C, Pereira A, Arfuso F, Gaglio G, Rizzo M, et al. Identification of trypanosomatids and blood feeding preferences of phlebotomine sand fly species common in Sicily Southern Italy. PLOS ONE. 2020;15:e0229536.
26. Buatong J, Dvorak V, Thepparat A, Thongkhao K, Koyadun S, Siriyasatien P, et al. Phlebotomine sand flies in Southern Thailand: entomological survey, identification of blood meals and molecular detection of Trypanosoma spp. Insects. 2022;13:197.
27. Srisuton P, Sunantaraporn B, Sor-suwan B, et al. Detection of Leishmania and Trypanosoma DNA in field-caught sand flies from endemic and non-endemic areas of leishmaniasis in Southern Thailand. Insects. 2019;10:238.
28. Castelli G, Bruno F, Reale S, Catanzaro S, Valenza V, Vitale F. Molecular Diagnosis of Leishmaniasis: Quantification of Parasite Load by a RealTime PCR Assay with High Sensitivity. Pathogens. 2021;10:865.
29. Dantas-Torres F, Solano-Gallego L, Baneth G, Ribeiro VM, de Paiva-Cavalcanti M, Otranto D. Canine leishmaniosis in the old and new worlds: unveiled similarities and differences. Trends Parasitol. 2012;28:531–8.
30. Dantas-Torres F, Miró G, Baneth G, Bourdeau P, Breitschwerdt E, Capelli G, et al. Canine Leishmaniasis control in the context of one health. Emerg Infect Dis. 2019;25:1–4.
31. Eloy LJ, Lucheis SB. Hemoculture and polymerase chain reaction using primers TCZ1/TCZ2 for the diagnosis of canine and feline trypanosomiasis. ISRN Vet Sci. 2012;2012:1–6.
32. Hassan-Kadle AA, Ibrahim AM, Nyingilili HS, Yusuf AA, Vieira RFC. Parasitological and molecular detection of Trypanosoma spp. in cattle, goats and sheep in Somalia. Parasitology. 2020;147:1786–91.
33. Cássia-Pires R, Boité MC, D'Andrea PS, Herrera HM, Cupolillo E, Jansen AM, et al. Distinct Leishmania Species Infecting Wild Caviomorph Rodents (Rodentia: Hystricognathi) from Brazil. PLoS Negl Trop Dis. 2014;8:e3389.
34. de Ferreira EC, Cruz I, Cañavate C, de Melo LA, Pereira AAS, Madeira FAM, et al. Mixed infection of *Leishmania infantum* and *Leishmania braziliensis* in rodents from endemic urban area of the New World. BMC Vet Res. 2015;11:71.
35. Kassahun A, Sadlova J, Dvorak V, Kostalova T, Rohousova I, Frynta D, et al. Detection of *Leishmania donovani* and *L. tropica* in Ethiopian wild rodents. Acta Trop. 2015;145:39–44.
36. Nantes WAG, Santos FM, de Macedo GC, Barreto WTG, Gonçalves LR, Rodrigues MS, et al. Trypanosomatid species in *Didelphis albiventris* from urban forest fragments. Parasitol Res. 2021;120:223–31.
37. Velez ID, Travi BL, Jaramillo C, Montoya J, Segura I, Zea A, et al. *Didelphis marsupialis*, an Important Reservoir of Trypanosoma (Schizotrypanum) cruzi and Leishmania (Leishmania) chagasi in Colombia. Am J Trop Med Hyg. 1994;50:557–65.
38. Keatley S, Botero A, Fosu-Nyarko J, Pallant L, Northover A, Thompson RCA. Species-level identification of trypanosomes infecting Australian wildlife by high-resolution melting—real time quantitative polymerase chain reaction (HRM-qPCR). Int J Parasitol Parasites Wildl. 2020;13:261–8.
39. Kassahun A, Sadlova J, Benda P, Kostalova T, Warburg A, Hailu A, et al. Natural infection of bats with Leishmania in Ethiopia. Acta Trop. 2015;150:166–70.
40. Medkour H, Davoust B, Dulieu F, Maurizi L, Lamour T, Marié J-L, et al. Potential animal reservoirs (dogs and bats) of human visceral leishmaniasis due to *Leishmania infantum* in French Guiana. PLoS Negl Trop Dis. 2019;13:e0007456.
41. de Oliveira FM, Costa LHC, de Barros TL, Rauschkolb KIPK, Colombo FA, de Carvalho C, et al. First detection of Leishmania spp DNA in Brazilian bats captured strictly in urban areas. Acta Trop. 2015;150:176–81.
42. Ardila MM, Carrillo-Bonilla L, Pabón A, Robledo SM. Surveillance of phlebotomine fauna and *Didelphis marsupialis* (Didelphimorphia: Didelphidae) infection in an area highly endemic for visceral leishmaniasis in Colombia. Biomedica. 2019;39:252–64.
43. Godfrey SS, Keatley S, Botero A, Thompson CK, Wayne AF, Lymbery AJ, et al. Trypanosome co-infections increase in a declining marsupial population. Int J Parasitol Parasit Wildl. 2018;7:221–7.
44. González C, León C, Paz A, López M, Molina G, Toro D, et al. Diversity patterns, Leishmania DNA detection, and bloodmeal identification of Phlebotominae sand flies in villages in northern Colombia. PLoS ONE. 2018;13:e0190686.
45. López M, Erazo D, Hoyos J, León C, Fuya P, Lugo L, et al. Measuring spatial co-occurrences of species potentially involved in Leishmania transmission cycles through a predictive and fieldwork approach. Sci Rep. 2021;11:6789.
46. Carvalho FS, Albuquerque GR, Carneiro PLS, Wenceslau AA. Genetic variability of *Leishmania infantum* in naturally infected dogs in the state of Bahia. Brazil Rev Bras Parasitol Veterinária. 2017;26:389–94.
47. Gouzelou E, Haralambous C, Amro A, Mentis A, Pratlong F, Dedet J-P, et al. Multilocus microsatellite typing (MLMT) of strains from Turkey and cyprus

reveals a novel monophyletic *L. donovani* sensu lato group. PLoS Negl Trop Dis. 2012;6:e1507.

48. Nemati S, Fazaeli A, Hajjaran H, Khamesipour A, Anbaran MF, Bozorgomid A, et al. Genetic diversity and phylogenetic analysis of the Iranian Leishmania parasites based on HSP70 gene PCR-RFLP and sequence analysis. Korean J Parasitol. 2017;55:367–74.
49. Ramírez JD, Hernández C, León CM, Ayala MS, Flórez C, González C. Taxonomy, diversity, temporal and geographical distribution of cutaneous leishmaniasis in Colombia: a retrospective study. Sci Rep. 2016;6:28266.
50. Calzada JE, Samudio F, de Juncá C, Pineda V, Burleigh BA, Saldaña A. Genetic diversity of *Trypanosoma cruzi* in Panama inferred by multilocus sequence typing of mitochondrial genes. Microorganisms. 2022;10:287.
51. Velásquez-Ortiz N, Hernández C, Herrera G, Cruz-Saavedra L, Higuera A, Arias-Giraldo LM, et al. *Trypanosoma cruzi* infection, discrete typing units and feeding sources among *Psammolestes arthuri* (Reduviidae: Triatominae) collected in eastern Colombia. Parasit Vectors. 2019;12:157.
52. Weber JS, Ngomtcho SCH, Shaida SS, Chechet GD, Gbem TT, Nok JA, et al. Genetic diversity of trypanosome species in tsetse flies (Glossina spp.) in Nigeria. Parasit Vectors. 2019;12:481.
53. Arias-Giraldo LM, Muñoz M, Hernández C, Herrera G, Velásquez-Ortiz N, Cantillo-Barraza O, et al. Identification of blood-feeding sources in panstrongylus, psammolestes, rhodnius and triatoma using ampliconbased next-generation sequencing. Parasit Vectors. 2020;13:434.
54. Flaherty BR, Barratt J, Lane M, Talundzic E, Bradbury RS. Sensitive universal detection of blood parasites by selective pathogen-DNA enrichment and deep amplicon sequencing. Microbiome. 2021;9:1.
55. Grubaugh ND, Gangavarapu K, Quick J, Matteson NL, De Jesus JG, Main BJ, et al. An amplicon-based sequencing framework for accurately measuring intrahost virus diversity using PrimalSeq and iVar. Genome Biol. 2019;20:8.
56. Maiguashca Sánchez J, Sueto SOB, Schwabl P, Grijalva MJ, Llewellyn MS, Costales JA. Remarkable genetic diversity of *Trypanosoma cruzi* and *Trypanosoma rangeli* in two localities of southern Ecuador identified via deep sequencing of mini-exon gene amplicons. Parasit Vectors. 2020;13:252.
57. Moreno Y, Moreno-Mesonero L, Amorós I, Pérez R, Morillo JA, Alonso JL. Multiple identification of most important waterborne protozoa in surface water used for irrigation purposes by 18S rRNA amplicon-based metagenomics. Int J Hyg Environ Health. 2018;221:102–11.
58. Patiño LH, Castillo-Castañeda AC, Muñoz M, Jaimes JE, Luna-Niño N, Hernández C, et al. Development of an amplicon-based next-generation sequencing protocol to identify leishmania species and other trypanosomatids in Leishmaniasis endemic areas. Microbiol Spectr. 2021;9:e00652-e721.
59. Abbasi I, Nasereddin A, Warburg A. Development of a next-generation DNA sequencing-based multi detection assay for detecting and identifying Leishmania parasites, blood sources, plant meals and intestinal microbiome in phlebotomine sand flies. Acta Trop. 2019;199:105101. 60. de Oliveira GE, Filipe M, de Macedo GC, Barreto WTG, Campos JBV, Meyers AC, et al. Maintenance of *Trypanosoma cruzi*, *T evansi* and *Leishmania* spp by domestic dogs and wild mammals in a rural settlement in Brazil-Bolivian border. Int J Parasitol Parasites Wildl. 2018;7:398–404.
61. Sevá AP, Ovallos FG, Amaku M, Carrillo E, Moreno J, Galati EAB, et al. Canine-Based Strategies for Prevention and Control of Visceral Leishmaniasis in Brazil. PLoS ONE. 2016;11:e0160058.
62. Llewellyn MS, Messenger LA, Luquetti AO, Garcia L, Torrico F, Tavares SBN, et al. Deep sequencing of the *Trypanosoma cruzi* GP63 surface proteases reveals diversity and diversifying selection among chronic and congenital Chagas disease patients. PLoS Negl Trop Dis. 2015;9:e0003458.
63. Castillo-Castañeda A, Patiño LH, Muñoz M, Ayala MS, Segura M, Bautista J, et al. Amplicon-based next-generation sequencing reveals the co-existence of multiple Leishmania species in patients with visceral leishmaniasis. Int J Infect Dis. 2022;115:35–8.
64. PAHO W. Epidemiological Report of the Americas. Leishmaniases . 2019 Mar p. 8. Report No.: 7. http:// iris. paho. org/ xmlui/ handle/ 12345 6789/ 50505.
65. Patino LH, Mendez C, Rodriguez O, Romero Y, Velandia D, Alvarado M, et al. Spatial distribution, Leishmania species and clinical traits of Cutaneous Leishmaniasis cases in the Colombian army. PLoS Negl Trop Dis. 2017;11:e0005876.
66. Sandoval-Ramírez CM, Hernández C, Teherán AA, Gutierrez-Marin R, Martínez-Vega RA, Morales D, et al. Complex ecological interactions across a focus of cutaneous leishmaniasis in Eastern Colombia: novel description of *Leishmania* species, hosts and phlebotomine fauna. R Soc Open Sci. 2020;7:200266.
67. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 2010;7:335–6.
68. 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.
69. Kuhls K, Alam MZ, Cupolillo E, Ferreira GEM, Mauricio IL, Oddone R, et al. Comparative microsatellite typing of new world *Leishmania infantum* reveals low heterogeneity among populations and its recent old world origin. PLoS Negl Trop Dis. 2011;5:e1155.
70. Leblois R, Kuhls K, François O, Schönian G, Wirth T. Guns, germs and dogs: on the origin of *Leishmania chagasi*. Infect Genet Evol. 2011;11:1091–5.
71. Aleixo JA, Nascimento ET, Monteiro GR, Fernandes MZ, Ramos AMO, Wilson ME, et al. Atypical American visceral leishmaniasis caused by disseminated *Leishmania amazonensis* infection presenting with hepatitis and adenopathy. Trans R Soc Trop Med Hyg. 2006;100:79–82.
72. Tolezano JE, Uliana SRB, Taniguchi HH, Araújo MFL, Barbosa JAR, Barbosa JER, et al. The first records of *Leishmania (Leishmania) amazonensis* in dogs (*Canis familiaris*) diagnosed clinically as having canine visceral leishmaniasis from Araçatuba county, São Paulo state. Brazil Vet Parasitol. 2007;149:280–4.
73. Valdivia HO, Zorrilla VO, Espada LJ, Perez JG, Razuri HR, Vera H, et al. Diversity, distribution and natural Leishmania infection of sand flies from communities along the interoceanic highway in the Southeastern Peruvian Amazon. PLoS Negl Trop Dis. 2021;15:e0009000.
74. Abdoli A, Maspi N, Ghaffarifar F, Nasiri V. Viscerotropic leishmaniasis: a systematic review of the case reports to highlight spectrum of the infection in endemic countries. Parasitol Open. 2018;4:e11.
75. Hernández C, Cucunubá Z, Flórez C, Olivera M, Valencia C, Zambrano P, et al. Molecular diagnosis of Chagas disease in Colombia: parasitic loads and discrete typing units in patients from acute and chronic phases. PLoS Negl Trop Dis. 2016;10:e0004997.
76. INS. Informe de evento: Enfermedad de Chagas 2018. Bogotá: Instituto Nacional de Salud; 2019.
77. Jansen AM, das Xavier SCC, Roque ALR. Trypanosoma cruzi transmission in the wild and its most important reservoir hosts in Brazil. Parasit Vectors. 2018;11:502.
78. Bilheiro AB, da Rosa JA, de Oliveira J, Belintani T, Fontes G, Medeiros JF, et al. First Report of Natural Infection with *Trypanosoma cruzi* in *Rhodnius montenegrensis* (Hemiptera, Reduviidae, Triatominae) in Western Amazon. Brazil Vector-Borne Zoonotic Dis. 2018;18:605–10.
79. Bilheiro AB, da Costa GS, da Araújo MS, Ribeiro WAR, Medeiros JF, Camargo LMA. Identification of blood meal sources in species of genus Rhodnius in four different environments in the Brazilian Amazon. Acta Trop. 2022;232:106486.
80. de Sousa PH, Scofield A, Júnior PSB, dos Lira SD, de Sousa SJ, Chaves JF, et al. Chagas disease in urban and peri-urban environment in the Amazon: Sentinel hosts, vectors, and the environment. Acta Trop. 2021;217:105858.
81. Ramírez-Chaves H, Suárez Castro A, González-Maya J. Cambios recientes a la lista de mamíferos de Colombia. Mammal Notes. 2016;3:1–9.
82. Gómez-Bravo A, German A, Abril M, Scavuzzo M, Salomón OD. Spatial population dynamics and temporal analysis of the distribution of Lutzomyia longipalpis (Lutz & Neiva, 1912) (Diptera: Psychodidae: Phlebotominae) in the city of Clorinda, Formosa. Argentina Parasit Vectors. 2017;10:352.
83. Correa-Cárdenas CA, Pérez J, Patino LH, Ramírez JD, Duque MC, Romero Y, et al. Distribution, treatment outcome and genetic diversity of Leishmania species in military personnel from Colombia with cutaneous leishmaniasis. BMC Infect Dis. 2020;20:938.

84. Ferrer E, García H, Bolivar A, Cañizales I, Guerrero R, Herrera L. First molecular detection of *Trypanosoma cruzi*, *T. rangeli* and *Leishmania* spp. in capybaras. Vet Parasitol Reg Stud Rep. 2021;23:100516.
85. da Silva YSGN, da Silva e SD, da Silva SAC, Silva RBS, de Oliveira PRF, Mota RA, et al. Molecular and serological detection of *Leishmania infantum*, *Toxoplasma gondii*, and *Leptospira* spp in free-ranging capybaras (*Hydrochoerus hydrochaeris*) from the Atlantic forest. Eur J Wildl Res. 2021;67:13.
86. Kumari D, Singh K. Exploring the paradox of defense between host and *Leishmania* parasite. Int Immunopharmacol. 2022;102:108400.
87. Mirzaei A, Rouhani S, Kazerooni P, Farahmand M, Parvizi P. Molecular detection and conventional identification of leishmania species in reservoir hosts of zoonotic cutaneous leishmaniasis in Fars Province, South of Iran. Iran J Parasitol. 2013;8:280–8.
88. Palatnik-de-Sousa CB, Day MJ. One Health: the global challenge of epidemic and endemic leishmaniasis. Parasit Vectors. 2011;4:197.
89. Tsokana CN, Sokos C, Giannakopoulos A, Mamuris Z, Birtsas P, Papaspyropoulos K, et al. First evidence of Leishmania infection in European brown hare (*Lepus europaeus*) in Greece: GIS analysis and phylogenetic position within the *Leishmania* spp. Parasitol Res. 2016;115:313–21.
90. Castillo-Castañeda A, Herrera G, Ayala MS, Fuya P, Ramírez JD. Spatial and temporal variability of visceral leishmaniasis in Colombia, 2007 to 2018. Am J Trop Med Hyg. 2021;105:144–55.
91. Golpayegani AA, Moslem AR, Akhavan AA, Zeydabadi A, Mahvi AH, Allah-Abadi A. Modeling of environmental factors affecting the prevalence of zoonotic and anthroponotic cutaneous, and zoonotic visceral leishmaniasis in foci of Iran: a remote sensing and GIS based study. J Arthropod-Borne Dis. 2018;12:41–66.
92. Risueño J, Ortuño M, Pérez-Cutillas P, Goyena E, Maia C, Cortes S, et al. Epidemiological and genetic studies suggest a common Leishmania infantum transmission cycle in wildlife, dogs and humans associated to vector abundance in Southeast Spain. Vet Parasitol. 2018;259:61–7.
93. Salgueiro MM, Pimentel MIF, Miranda LFC, Cunha SRR, Oliveira LFA, Lyra MR, et al. Parasite species variation and impact of spatial displacement of the population on cutaneous leishmaniasis in Rio de Janeiro, Brazil. Trans R Soc Trop Med Hyg. 2022;116:70–9.
94. Estrada LG, Ortega E, Vivero RJ, Bejarano EE, Cadena H. Development of *Lutzomyia evansi* immature stages in peridomiciliary environment in a leishmaniasis urban focus in the Colombian Caribbean. Acta Trop. 2020;208:105523.
95. Chajbullinova A, Votypka J, Sadlova J, Kvapilova K, Seblova V, Kreisinger J, et al. The development of *Leishmania turanica* in sand flies and competition with *L. major*. Parasit Vectors. 2012;5:219.
96. de Rodrigues OA, Pinheiro GRG, Tinoco HP, Loyola ME, Coelho CM, Dias ES, et al. Competence of non-human primates to transmit *Leishmania infantum* to the invertebrate vector *Lutzomyia longipalpis*. PLoS Negl Trop Dis. 2019;13:e0007313.
97. Serafim TD, Coutinho-Abreu IV, Oliveira F, Meneses C, Kamhawi S, Valenzuela JG. Sequential blood meals promote Leishmania replication and reverse metacyclogenesis augmenting vector infectivity. Nat Microbiol. 2018;3:548–55.
98. Funk S, Nishiura H, Heesterbeek H, Edmunds WJ, Checchi F. Identifying transmission cycles at the human-animal interface: the role of animal reservoirs in maintaining gambiense human African trypanosomiasis. PLoS Comput Biol. 2013;9:e1002855.
99. Lambin EF, Tran A, Vanwambeke SO, Linard C, Soti V. Pathogenic landscapes: Interactions between land, people, disease vectors, and their animal hosts. Int J Health Geogr. 2010;9:54.
100. Veeresha P, Malagi NS, Prakasha DG, Baskonus HM. An efficient technique to analyze the fractional model of vector-borne diseases. Phys Scr. 2022;97:054004.
101. Lago R de JM do, Sousa IDB de, Albuquerque LP de A, Moraes FC, Aquino DMC de. Aspectos de uma área endêmica para leishmaniose visceral em um município no Maranhão, Brasil. Rev Epidemiol E Controle Infecção. 2020. https:// online. unisc. br/ seer/ index. php/ epide miolo gia/ artic le/ view/ 15109. Accessed on 6 Jun 2022.
102. de Montes ACOA, González-Martínez A, Chan-González R, Ibarra-López P, Smith-Ávila S, Córdoba-Aguilar A, et al. Signs of urban evolution? Morpho-functional traits co-variation along a nature-urban gradient in a Chagas disease vector. Front Ecol Evol. 2022;10:805040.
103. Ocaña-Mayorga S, Bustillos JJ, Villacís AG, Pinto CM, Brenière SF, Grijalva MJ. Triatomine feeding profiles and *Trypanosoma cruzi* Infection, Implications in Domestic and Sylvatic Transmission Cycles in Ecuador. Pathogens. 2021;10:42.
104. Lypaczewski P, Matlashewski G. Leishmania donovani hybridisation and introgression in nature: a comparative genomic investigation. Lancet Microbe. 2021;2:e250–8.
105. Rogozin IB, Charyyeva A, Sidorenko IA, Babenko VN, Yurchenko V. frequent recombination events in *Leishmania donovani*: mining population data. Pathogens. 2020;9:572.
106. Van den Broeck F, Savill NJ, Imamura H, Sanders M, Maes I, Cooper S, et al. Ecological divergence and hybridization of Neotropical *Leishmania* parasites. Proc Natl Acad Sci. 2020;117:25159–68.
107. World Health Organization. Multisectoral approach to the prevention and control of vector-borne diseases: a conceptual framework [Internet]. Geneva: World Health Organization; 2020. https:// apps. who. int/ iris/ handle/ 10665/ 331861
108. Dario MA, Pavan MG, Rodrigues MS, Lisboa CV, Kluyber D, Desbiez ALJ, et al. *Trypanosoma rangeli* genetic, mammalian hosts, and geographical diversity from five Brazilian biomes. Pathogens. 2021;10:736.
109. Ramirez LE, Lages-Silva E, Alvarenga-Franco F, Matos A, Vargas N, Fernandes O, et al. High prevalence of *Trypanosoma rangeli* and *Trypanosoma cruzi* in opossums and triatomids in a formerly-endemic area of Chagas disease in Southeast Brazil. Acta Trop. 2002;84:189–98.
110. De Araújo VAL, Boité MC, Cupolillo E, Jansen AM, Roque ALR. Mixed infection in the anteater *Tamandua tetradactyla* (Mammalia: Pilosa) from Pará State, Brazil: *Trypanosoma cruzi T. rangeli* and *Leishmania infantum*. Parasitol. 2013;140:455–60.
111. Da Maia SF, Junqueira ACV, Campaner M, Rodrigues AC, Crisante G, Ramirez LE, et al. Comparative phylogeography of *Trypanosoma rangeli* and Rhodnius (Hemiptera: Reduviidae) supports a long coexistence of parasite lineages and their sympatric vectors. Mol Ecol. 2007;16:3361–73.
112. Vallejo GA, Guhl F, Carranza JC, Lozano LE, Sánchez JL, Jaramillo JC, et al. kDNA markers define two major *Trypanosoma rangeli* lineages in LatinAmerica. Acta Trop. 2002;81:77–82.
113. das Xavier SCC, Roque ALR, Bilac D, de Araújo VAL, da Neto SFC, Lorosa ES, et al. Distantiae transmission of *Trypanosoma cruzi*: a new epidemiological feature of acute chagas disease in Brazil. PLoS Negl Trop Dis. 2014;8:e2878.
114. Espinosa-Álvarez O, Ortiz PA, Lima L, Costa-Martins AG, Serrano MG, Herder S, et al. Trypanosoma rangeli is phylogenetically closer to old world trypanosomes than to *Trypanosoma cruzi*. Int J Parasitol. 2018;48:569–84.
115. Saldaña A, Santamaría AM, Pineda V, Vásquez V, Gottdenker NL, Calzada JE. A darker chromatic variation of *Rhodnius pallescens* infected by specific genetic groups of *Trypanosoma rangeli* and *Trypanosoma cruzi* from Panama. Parasit Vectors. 2018;11:423.
116. Vallejo G, Guhl F, Carranza JC, Herrera C, Urrea D, Falla A, et al. Trypanosoma cruzi population variability in Colombia: possible coevolution in different vector species. Rev Soc Bras Med Trop. 2009;42:38–45.
117. Chihi A, O'Brien Andersen L, Aoun K, Bouratbine A, Stensvold CR. Amplicon-based next-generation sequencing of eukaryotic nuclear ribosomal genes (metabarcoding) for the detection of single-celled parasites in human faecal samples. Parasite Epidemiol Control. 2022;17:e00242.
118. Gupta S, Mortensen MS, Schjørring S, Trivedi U, Vestergaard G, Stokholm J, et al. Amplicon sequencing provides more accurate microbiome information in healthy children compared to culturing. Commun Biol. 2019;2:291.
119. Chaorattanakawee S, Korkusol A, Tippayachai B, Promsathaporn S, Poole-Smith BK, Takhampunya R. Amplicon-based next generation

sequencing for rapid identification of rickettsia and ectoparasite species from entomological surveillance in Thailand. Pathogens. 2021;10:215.
120. Tonge DP, Pashley CH, Gant TW. Amplicon –based metagenomic analysis of mixed fungal samples using proton release amplicon sequencing. PLoS ONE. 2014;9:e93849.
121. Ma L, Jakobiec FA, Dryja TP. A review of next-generation sequencing (NGS): applications to the diagnosis of ocular infectious diseases. Semin Ophthalmol. 2019;34:223–31.
122. Cravero K, Medford A, Pallavajjala A, Canzoniero J, Hunter N, Chu D, et al. Biotinylated amplicon sequencing: a method for preserving DNA samples of limited quantity. Pract Lab Med. 2018;12:e00108.
123. Medinger R, Nolte V, Pandey RV, Jost S, Ottenwälder B, Schlötterer C, et al. diversity in a hidden world: potential and limitation of next-generation sequencing for surveys of molecular diversity of eukaryotic microorganisms. Mol Ecol. 2010;19:32–40.
124. Rodrigues DC, Silva R, Rondinelli E, Ürményi TP. Trypanosoma cruzi: modulation of HSP70 mRNA stability by untranslated regions during heat shock. Exp Parasitol. 2010;126:245–53.
125. Hernández C, Salazar C, Brochero H, Teherán A, Buitrago LS, Vera M, et al. Untangling the transmission dynamics of primary and secondary vectors of *Trypanosoma cruzi* in Colombia: parasite infection, feeding sources and discrete typing units. Parasit Vectors. 2016;9:620.
126. Filgueira CPB, Moreira OC, Cantanhêde LM, de Farias HMT, Porrozzi R, Britto C, et al. Comparison and clinical validation of qPCR assays targeting Leishmania 18S rDNA and HSP70 genes in patients with American tegumentary leishmaniasis. PLoS Negl Trop Dis. 2020;14:e0008750.

## Publisher’s Note

*et al. Parasites & Vectors (2023) 16:458* https://doi.org/10.1186/s13071-023-06073-9

Parasites & Vectors

**RESEARCH** **Open Access**

# Validation of Oxford nanopore sequencing for improved New World *Leishmania* species identification via analysis of 70-kDA heat shock protein

Luz Helena Patiño[1], Nathalia Ballesteros[1], Marina Muñoz[1], Jesús Jaimes[1], Adriana C. Castillo-Castañeda[1], Roy Madigan[2], Alberto Paniz-Mondolfi[3] and Juan David Ramírez[1,3*]

## Abstract

**Background** Leishmaniasis is a parasitic disease caused by obligate intracellular protozoa of the genus *Leishmania*. This infection is characterized by a wide range of clinical manifestations, with symptoms greatly dependent on the causal parasitic species. Here we present the design and application of a new 70-kDa heat shock protein gene (*hsp70*)-based marker of 771 bp (HSP70-Long). We evaluated its sensitivity, specificity and diagnostic performance ™ employing an amplicon-based MinION DNA sequencing assay to identify different *Leishmania* species in clinical samples from humans and reservoirs with cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL). We also conducted a comparative analysis between our novel marker and a previously published HSP70 marker known as HSP70Short, which spans 330 bp.

**Methods** A dataset of 27 samples from Colombia, Venezuela and the USA was assembled, of which 26 samples were collected from humans, dogs and cats affected by CL and one sample was collected from a dog with VL in the USA (but originally from Greece). DNA was extracted from each sample and underwent conventional PCR amplification utilizing two distinct HSP70 markers: HSP70-Short and HSP70-Long. The subsequent products were then sequenced ™ using the MinION sequencing platform.

**Results** The results highlight the distinct characteristics of the newly devised HSP70-Long primer, showcasing the notable specificity of this primer, although its sensitivity is lower than that of the HSP70-Short marker. Notably, both markers demonstrated strong discriminatory capabilities, not only in distinguishing between different species within the *Leishmania* genus but also in identifying instances of coinfection.

**Conclusions** This study underscores the outstanding specificity and effectiveness of HSP70-based MinION$^{TM}$ sequencing, in successfully discriminating between diverse *Leishmania* species and identifying coinfection events within samples sourced from leishmaniasis cases.

**Keywords** *Leishmania*, MinION$^{TM}$ sequencing, HSP70-Long, HSP70-Short, Cutaneous Leishmaniasis

---

*Correspondence: Juan David Ramírez
juand.ramirez@urosario.edu.co; juan.ramirezgonzalez@mssm.edu Full list of author information is available at the end of the article

## Background

Leishmaniases comprise a cluster of vector-borne ulcerative skin diseases triggered by parasites belonging to the genus *Leishmania*. The disease manifests in six distinct clinical forms, each characterized by the specific location of the parasite within the affected tissue. Among these forms, cutaneous leishmaniasis (CL) stands out as the most prevalent form of the infection, and visceral leishmaniasis (VL) has the distinction of being the most severe, pathogenic and clinically relevant manifestation across the disease spectrum [1]. To date, 53 distinct *Leishmania* species have been identified, of which 31 are considered to be as parasites affecting mammals, including humans. Among these latter 31 species, 20 are recognized as pathogenic to humans [2]. Several species of *Leishmania* are the causative agents of various clinical forms of the disease [3].

To date, various molecular techniques have been developed for the identification and differentiation of *Leishmania* species. These techniques rely on different DNA sequences as targets, including molecular elements such as minicircles kinetoplast DNA (kDNA) [4, 5], 18S ribosomal DNA (rDNA) [6], internal transcribed spacers (ITS1 and ITS2) [1, 7, 8], glucose-6-phosphate dehydrogenase (G6PD) [9], mannose phosphate isomerase genes (MPI) [8, 9], the miniexon (spliced leader) gene repeat [10], heat-shock proteins (HSPs) [1, 8] and cysteine proteinase B (CPB) [8]. The most widely used and prominent molecular methods currently available involve utilization of species-specific restriction sites via restriction fragment length polymorphism analysis (RFLPs) [11], the random amplified polymorphic DNA (RAPD) technique [12], high-resolution melting (HRM) [3, 13], quantitative PCR (qPCR) [5], multilocus sequence typing (MLST) [1], loop-mediated isothermal amplification (LAMP) [14] and Sanger sequencing. However, it is important to note that while these methods effectively differentiate *Leishmania* species, they can be costly, time-consuming and laborintensive and require specialized laboratory techniques, equipment and robust infrastructure [15]. Moreover, some of these techniques might not be sensitive enough to detect low-level parasite DNA or identify coinfections involving multiple *Leishmania* species.

In recent years, next-generation sequencing (NGS), particularly in the form of amplicon-based sequencing, has emerged as a promising solution to overcome these limitations. Recent investigations have harnessed amplicon-based Illumina sequencing to detect and classify trypanosomatid species across various hosts, including mammals, human patients with CL and VL, as well as sand fly vectors. These studies have also delved into the identification of mixed trypanosomatid infections [16, 17]. Technologies (Oxford, UK) MinION$^{TM}$. Conversely, the capabilities of the Oxford Nanopore$^{TM}$ in following text) have DNA sequencing system (referred to as MinION$^{TM}$ been subjected to extensive scrutiny across numerous publications. These studies have illustrated the capac to produce high-quality, single-contig ity of MinION$^{TM}$ microbial genomes [18], enhance eukaryotic genome assembly [19] and detect a wide array of pathogens, including SARS-CoV-2, Chikungunya virus, Hepatitis and virus C, *Salmonella typhimuriumOrthohantavirus* [20 , 22, 21, 23] *Salmonella enterica*. MinION™ has also proven instrumental in pinpointing drug resistance and pathogenicity biomarkers [Nevertheless, the use of MinION24]. ™ specifically within the context of amplicon-based analysis, with a focus on identifying distinct *Leishmania* species from both cultured and biological samples, and the detection of coinfection events, remains relatively limited. In a recent study, a novel protocol was developed that employed a 234-to identify bp marker of the heat-shock protein 70 gene *Leishmania* species using MinION™(*hsp70* [15]).

While this technique exhibited high sensitivity and proficiency in discriminating reference strains and clinical samples, concerns arose regarding whether the marker's length adequately covered the extent of nucleotide variations present among diverse *Leishmania* species.

The 70-kDa heat-shock proteins (HSP70) exhibit a high degree of conservation both in sequence and function across prokaryotes and eukaryotes. They hold significant importance as molecular chaperones, playing essential roles in protein folding and transport processes [25]. Over time, *hsp70* has emerged as the predominant target for identifying and distinguishing *Leishmania* species [26]. In addition, this gene has been instrumental in studies related to taxonomy and phylogeny [27–29], demonstrating remarkable congruence with multilocus enzyme electrophoresis (MLEE) typing, a widely acknowledged gold standard in *Leishmania* typing [30].

Given the widespread prevalence of leishmaniasis cases across the globe, coupled with the extensive array of vectors implicated in parasite transmission and the various species linked to distinct clinical presentations [31–33], it is imperative to develop rapid, precise and highly sensitive methods for detecting and identifying *Leishmania* species. Moreover, increases in the occurrences of coinfections among humans, reservoirs and vectors have been documented in recent times [16, 17, 34], accentuating the pressing need to optimize these efforts. The insights gleaned from such studies will not only bolster accurate diagnoses but also play a pivotal role in guiding therapeutic decisions. This, in turn, will aid in the selection of suitable antileishmanial agents and optimal treatment courses for patients.

In this context, we developed a 771-bp marker (HSP70Long) that specifically targets *hsp70*. We meticulously evaluated its sensitivity, specificity and diagnostic efficacy by employing an amplicon-based MinION™ sequencing approach. This method was instrumental in identifying *Leishmania* species within clinical samples sourced from both humans and reservoirs afflicted by CL and VL. Additionally, we conducted a comprehensive performance comparison between our newly devised marker and a previously published HSP70 marker [35].

## Methods

### Design of HSP70-Long primers

To develop primers for the HSP70-Long, we established an in-house database leveraging prior research [16]. In brief, we systematically queried the NCBI Nucleotide database (https:// www. ncbi. nlm. nih.g ov/ nucco re? term) to assemble all available HSP70 sequences pertaining to trypanosomatids. This targeted approach resulted in the retrieval of a comprehensive dataset comprising 1460 distinct sequences.

Subsequently, we refined our search outcomes by implementing specific filters. These filters encompassed molecule type categorization (genomic DNA/RNA), source database selection (GenBank) and sequence length delineation (ranging from 700 to 5000). We took care to exclude sequences that did not correspond to the *Leishmania* genus or lacked accurate taxonomic classification at the species level (designated as *Leishmania* spp.). Additionally, sequences exhibiting an excessive abundance of gaps within their content and those of low quality were omitted from consideration. The resultant pool of sequences that met these criteria was then subjected to alignment using the Clustal W algorithm, employing the UGENE version 33.0 software platform [36].

Our final reference database consisted of a total of 414 meticulously selected sequences. This comprehensive dataset can be readily accessed by the public through the following link: https:// github. com/ gimur/- Enhan cing-Leish mania- Speci es- Ident ifica tion- from- Clini cal- Sampl es. Next, employing DnaSP software version 5.0, we diligently recovered sequences by isolating their respective haplotypes from the aforementioned pool of 414 sequences. This meticulous process yielded a total of 34 distinct haplotypes. With the intention of primer design, we harnessed the PRIMER BLAST software package (http:// www. ncbi. nlm. nih. gov/ tools/ primer- blast/) to work on the 34 derived haplotypes. The outcome was an amplicon size uniformly spanning 771 bp across all sequences. To ensure precision, we pinpointed the specific annealing sites for the primers across different *Leishmania* species. This information is visually presented in Additional file 1: Figure S1. Further validation ensued as the designed primers underwent scrutiny through the NCBI BLAST tool software. Moreover, primer physicochemical attributes, including parameters such as Gibbs free energy (ΔG), melting temperature (Tm), enthalpy (ΔH) and hairpin assessment, underwent thorough evaluation. This assessment was conducted using the online tool Oligoanalyzer 3.1 (https:// www. idtdna. com/ pages/ tools/ oligo analy zer? retur nurl=% 2Fcalc% 2Fana lyzer).

### Analytical sensitivity and specificity of HSP70-Long and HSP70-Short primers

To comprehensively assess the sensitivity and specificity of the primers examined in this study (330-bp HSP70 marker [HSP70-Short] and HSP70-Long), we conducted distinct evaluations: analytical sensitivity, analytical specificity, interference-based specificity and diagnostic performance.

#### *Analytical sensitivity*

To scale the analytical sensitivity of the HSP70-Long primers, as well as that of the previously published primers, referred to in the following text as HSP70-Short [35], we employed a method involving the determination of the limit of detection (LoD). This method involved conducting a series of sequential DNA dilutions sourced from the reference strain *Leishmania infantum* UA1664 MHOM/CO/87. The dilution series encompassed a spectrum starting from an initial concentration of 1 × 1 $0^6$ parasites/ml and progressively tapering down to a concentration of merely 1 parasite/ml. Subsequently, these diverse dilutions were subjected to amplification using conventional PCR in which both sets of primers (HSP70Long and HSP70-Short) were employed.

Additionally, as control for sequencing, we prepared mixtures of promastigotes from two different *Leishmania* strains: the reference strain *L. infantum* UA1664 MHOM/CO/87 and *L. amazonensis* strain UA130 [37]. These mixtures consisted of an equal 1:1 ratio of *L. amazonensis* and *L. infantum* at varying concentrations ranging from 1 × $10^6$ to 1 × $10^{-1}$ parasites/ml. For sequencing purposes, we specifically chose concentrations of 1 × 1 $0^6$ parasites/ml of *L. infantum* and 1 × $10^3$ parasites/ml of *L. amazonensis* (Mix-1), as well as 1 × $10^3$ parasites/ml of *L. infantum* and 1 × $10^6$ parasites/ml of *L. amazonensis* (Mix-2). Both the *L. infantum* and *L. amazonensis* strains were cultivated in Schneider's insect medium supplemented with 10% (v/v) fetal bovine serum. The cultures were maintained at 26 °C and $CO_2$ concentration of 5%. The parasites were collected during the late logarithmic growth phase and then subjected to DNA extraction and sequencing.

### *Analytical specificity*

The analytical specificity of the HSP70-Short primers were studied in a previous investigation [16] and, therefore, our analysis centered on the HSP70-Long primers. To thoroughly investigate the intra- and interspecies specificity of the HSP70-Long primers, we collected annotation data for *Leishmania* and *Trypanosoma* species which we sourced from the publicly accessible TriTrypDB database (https:// tritr ypdb. org/ common/ downl oads/ Curre nt_ Relea se). Our approach involved the meticulous selection of sequences corresponding to the HSP70 gene for each individual species. Subsequently, each of these sequences per chromosome underwent alignment with the primers that were the subject of our analysis in this study. The sequences obtained were used to build a maximum-likelihood-based phylogenetic tree which we accomplished using FastTree version 2.1.10 Double precision [38]. The robustness of the nodes was rigorously evaluated through the implementation of 1000 bootstrap replicates. The resulting tree was visualized using the Interactive Tree Of Life v4 online tool (http:// itol. embl. de) [39]. In addition, we used the Neighbor-Net method in SplitsTree5 [40] to build phylogenetic networks. A Nexus matrix was constructed for haplotype network analysis in Network 3.0 based on a medianjoining model with default parameters and 1000 iterations. Finally, we obtained a dissimilarity matrix through sequence alignment using UGENE software. This matrix was then utilized to create a heatmap using the online tool Heatmapper [41]. To construct the heatmap, we employed the average linkage and Pearson distance method.

### *Interference-based specificity*

This assay included other trypanosomatids, including *Trypanosoma cruzi* and *Trypanosoma brucei*, as well as a diverse assortment of pathogenic bacteria, fungi and viruses (see Additional file 2: Table S1 for a comprehensive list). DNA from these various pathogens was extracted and provided by the Molecular Microbiology Laboratory (MML) at Mount Sinai Hospital (New York City, NY, USA). These DNA samples were subsequently subjected to amplification via conventional PCR using both sets of primers (HSP70-Long and HSP70-Short).

### Diagnostic performance evaluation with samples collected from hosts with CL and VL

Twenty-seven samples tested in this study, including 26 samples procured from individuals diagnosed with CL. Of these latter 26 samples, 12 were acquired through direct smears of lesions taken from human patients treated at the Dirección de Sanidad Militar, Ejercito Nacional de Colombia in Bogotá; four samples were derived from blood and deposited on FTA cards (3 samples from humans and 1 sample from a domestic dog [*Canis lupus familiaris*]); and 10 samples were extracted from felines (*Felis catus*) residing in various municipalities across Central-Western Venezuela. An additional specimen was examined from a dog in the USA (Texas) exhibiting clinical findings suggestive of VL; this dog was originally from Greece where he presumably was infected.

Samples from felines were collected by carefully scraping lesional tissues and then depositing the scrapings onto FTA classic cards (Whatman Inc., Newton Center, MA, USA). For the case of the dog afflicted with VL, a blood sample was procured by venipuncture using EDTA tubes. This blood sample was meticulously preserved at − 20 °C until the subsequent DNA extraction process was conducted. Additional file 3: Table S2 provides a comprehensive overview of the metadata corresponding to all 27 samples used in the study.

### DNA extraction

Genomic DNA was extracted from serial dilutions of the reference strain *L. infantum* UA1664 MHOM/ CO/87, clinical samples and mixtures, using the High Pure PCR Template Preparation Kit (Roche Life Science, Mannheim, Germany) according to the manufacturer's instructions. The ChemagicTM Viral DNA/RNA 300 Kit H96 (CMG-1033-S; PerkinElmer Inc., Waltham, MA, USA) was used for DNA extraction from FTA cards and the blood sample, and the extraction was carried out using the automated ChemagicTM 360 instrument (2024-0020; PerkinElmer Inc.) in accordance with the manufacturer's guidelines. Well-established methods, as previously outlined [42], were applied throughout. Finally, the concentration, quality and integrity of the extracted DNA were evaluated by 1% agarose gel electrophoresis and spectrometry (Nanodrop ND-1000 spectrophotometer; Thermo Fisher Scientific, Waltham, MA, USA).

### PCR amplification

To amplify the four distinct DNA sample types featured in this study, we utilized four specific primers: HSP70-Short-F (5′AGG TGA AGG CGA CGA ACG 3′) and HSP70-Short-R (5′ CGC TTG TCC ATC TTT GCG TC 3′) [35], and HSP70-Long-F (5′-GAC TTY CAG GCC AMCAT CAC -3′) and HSP70-Long-R (3′-CGA GTA CGC GTA GTT CTC CA-5′). These primers were used to amplify: (i) DNA stemming from serial dilutions of the reference strain *L. infantum*, which was integral to the sensitivity assay; (ii) DNA extracted from the mixtures that included both the reference strain *L. infantum* and the *L. amazonensis* strain (UA130); (iii) DNA sourced from clinical samples; and (iv) DNA obtained from other pathogens, a vital component of the specificity assays.

The PCR reaction and cycling conditions for the HSP70-Short primers followed an established protocol reported previously [35]. For the HSP70-Long primers, the reaction mixture was prepared to achieve a final volume of 15 µl (6.25 µl of Platinum Master Mix [Invitrogen, Thermo Fisher Scientific] at 1× concentration, 0.5 µM solution [0.625 µl] of each primer, 2.5 µl of enhancer [Invitrogen, Thermo Fisher Scientific], 3.5 µl of water, 10 ng/µl of DNA template). The thermal cycling profile consisted of an initial denaturation at 94 °C for 5 min (1 cycle); followed by 35 cycles of denaturation (94 °C, 1 min), annealing (52 °C for 30 s) and extension (72 °C for 1 min); and a final extension at 72 °C for 10 min. The quality, integrity and size of the obtained amplicon was assessed by 2% agarose gel electrophoresis, following which the amplicons from each sample were purified using the EXOSAP method (Affymetrix, Santa Clara, CA, USA) following the manufacturer's instructions. Manual assessment of the gels was carried out to identify the presence of a unique band in each case.

#### Amplicon-based MinION™ sequencing

The amplicons derived from both clinical samples and mixtures underwent sequencing using the MinION™ platform. To prepare the DNA for this DNA sequencing platform, we performed a series of steps, including end repair, dA tail insertion, barcoding and adapter binding using the EXP-NBD196 kit from Oxford Nanopore Technologies, followed by cleaning of the library using AMPure XP beads (Beckman Coulter Life Sciences, Brea, CA, USA) and finally preparation for load-ing onto a MinION™ flow cell using the SQK-LSK109 sequencing ligation kit (Oxford Nanopore Technologies). The sequencing process was performed on the miniON platform, utilizing the MinKNOW program (version 20.10.3), in accordance with an established protocol, spanning a 48-h run.

#### Bioinformatic analysis

The initial step involved base-calling the raw Fast5 files and demultiplexing the data using the Guppy barcoder. Then, a quality and length filtering process was applied to the reads, which served to eliminate low-quality and potentially chimeric reads. Following filtering, the reads were merged for further analysis. The sequences obtained from HSP70-Long were subjected to comparison against an in-house database constructed during this study, and the sequences derived from HSP70-Short were compared against a previously published database [16]. The taxonomic assignment was conducted locally using BLASTn against the respective databases, with a threshold set at a minimum of 95% identity and an E value of 10. For the analysis, matches exhibiting a relative abundance > 3% were taken into account, a precautionary measure to mitigate potential sequencing errors. Additionally, the relative abundance of *Leishmania* species reads was normalized based on the number of copies of the HSP70 gene present in each species. This adjustment aimed to achieve a more precise estimation of species abundance per individual. Quantitative results were graphically presented using R software version 3.6.1 [16].

## Results

### Analytical sensitivity and specificity of HSP70-Long and HSP70-Short primers

#### *Analytical sensitivity*

The sensitivity assessment clearly indicated that the HSP70-Long primers possessed a dependable detection capacity of at least 100 parasite-equivalents/ml. In contrast, the HSP70-Short primers showed greater sensitivity, successfully detecting levels as low as 1 equivalent-parasite/ml, as shown in Additional file 4: Figure S2. We also evaluated the capacity of both the HSP70-Long and HSP70-Short primers to identify mixed infections. The outcomes from the assays using the laboratorydesigned Mix-1 and Mix-2 samples demonstrated the presence of *L. infantum* and *L. amazonensis* at the anticipated concentrations.

#### *Analytical specificity*

The specificity of each primer was then assessed in both intra- and interspecies analysis. Since HSP70-Short primers had been evaluated previously [16], we focused on the HSP70-Long primers. The outcomes showed that the HSP70-Long primers annealed to distinct copies of the HSP70 gene within the same *Leishmania* species, thereby demonstrating a notable level of intraspecies specificity.

Furthermore, these primers showcased a robust ability to discriminate between various *Leishmania* species, as illustrated in Additional file 5: Figure S3. To assess the ability of the HSP70-Long primers to differentiate between various *Leishmania* species, we conducted an in-depth analysis of their discriminatory potential. Phylogenetic analysis of the acquired sequences revealed three distinct, well-supported clusters, (bootstrap values of ≥ 90.0), with the first cluster associated with *Leishmania* subgenus species, the second cluster associated with *Mundinia* subgenus species and the third cluster represented by species belonging to the *Viannia* subgenus (Fig. 1a). Within the *Leishmania* subgenus cluster, three distinct groups were identified, with the first group (Fig. 1a, highlighted in green) encompassing sequences belonging to the *L. mexicana* complex, the second group (Fig. 1a, highlighted in purple) consisting

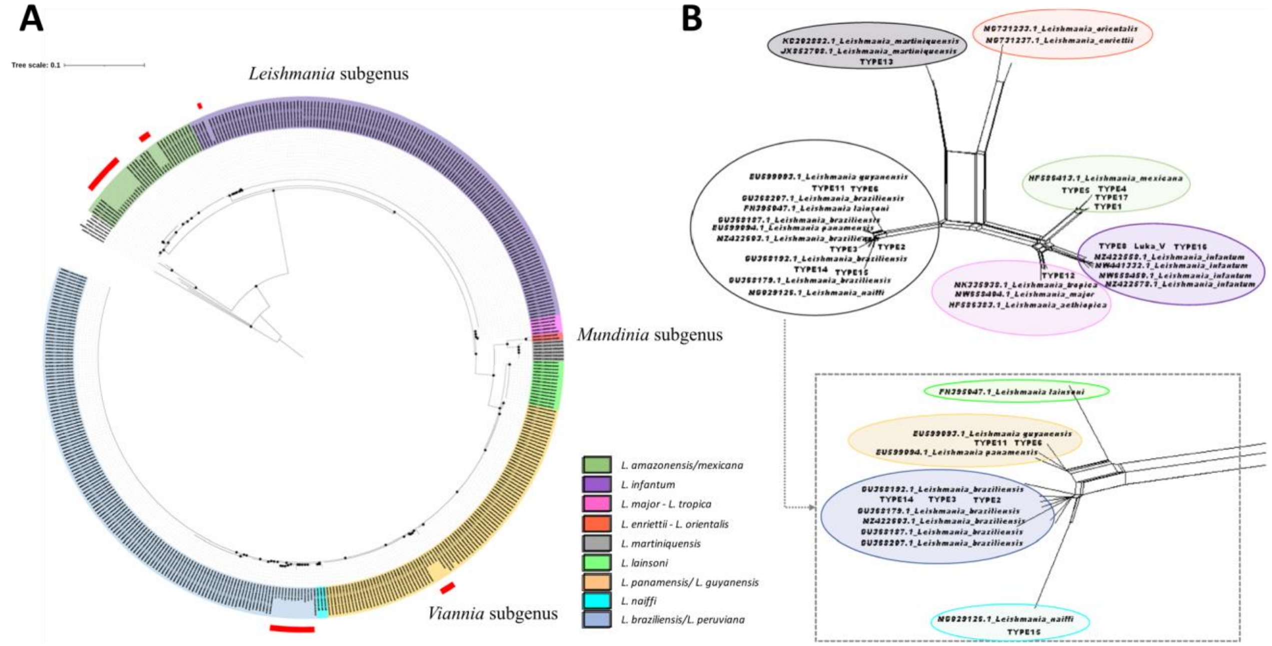


**Fig. 1** Phylogenetic analysis based on HSP70-Long sequences used for the reference database. **A** The tree represents the phylogenetic analysis based on 414 HSP70-Long sequences used for the reference database and the 27 sequences included in this study. The colors of the inner circle represent the different *Leishmania* species, and the red color of the external circle indicates the samples analyzed in this study. Black dots represent well-supported nodes (Bootstrap ≥ 90). **B** Phylogenetic network (Neighbor-Net) constructed in SplitsTree 5, based on 34 haplotypes from HSP70-Long reference sequences. The square shows enlargements of the clusters represented by *Viannia* subgenera species. HSP70-Long, 771-bp 70-kDa heat shock protein gene-based marker

of sequences from *L. infantum* and the third group (Fig. 1a, highlighted in fuchsia) comprising sequences from the *Leishmania major* and *Leishmania tropica* complexes.

For the *Mundinia* subgenus, two distinct groups were discerned. The first group (Fig. 1a, highlighted in light red) encompassed sequences associated with *Leishmania enrietti* and *Leishmania orientalis*, and the second group (Fig. 1a, highlighted in gray) comprised sequences from *Leishmania martiquinensis* (Fig. 1a).

Within the *Viannia* subgenus, four distinct groups were identified. The first group (Fig. 1a, highlighted in lime) included sequences of *Leishmania lainsoni*, the second group (Fig. 1a, highlighted in light yellow) comprised sequences from the *Leishmania guyanensis* complex, the third group (Fig. 1a, highlighted in cyan) was composed of sequences from *Leishmania naiffi* and the fourth group (Fig. 1a, highlighted in light blue) featured sequences from the *Leishmania braziliensis* complex species (Fig. 1a). These findings were further validated by the phylogenetic tree topologies obtained through SplitsTree5 (Fig. 1b). Finally, the haplotype network showcased 34 distinct sequences (Additional file 6: Figure S4), underscoring the robust discriminatory prowess of the designed primers.

### *Interference-based specificity*

The outcomes of assays utilizing the HSP70-Short primers revealed amplification solely for *T. cruzi* DNA, with no observable amplification detected for any other DNA tested. In contrast, the outcomes of assays employing the HSP70-Long primers demonstrated a consistent absence of amplification for all the tested DNA samples.

### Reads analysis by HSP70 amplicon-based MinION™ sequencing

This analysis focused on the reads acquired following the MinKNOW platform's implementation of reading filters for quality and length. The outcomes of the HSP70-Short amplicon-based MinION™ sequencing revealed a spectrum of reads ranging from 947 to 678,302, and those of the HSP70-Long amplicon-based MinION™ sequencing yielded a range of reads from 137 to 204,869 (see Additional file 7: Table S3).

### Diagnostic performance

By employing gene amplicon-based NGS targeting the HSP70-Long and HSP70-Short genes, we not only successfully detected a range of *Leishmania* species within the human, feline and canine samples but also established a strong agreement between the two primer sets studied. Infection events were recognized in 85% (*n* = 23/27) of the analyzed samples, while coinfection events were noted in the remaining 15% (*n* = 4) of samples.

Among the detected species, *L. braziliensis* emerged as the predominant species in the human samples collected from the Colombian army. In comparison, in samples from Venezuela, *L. amazonensis* emerged as the prevailing *Leishmania* species and, notably, *L. mexicana* was predominant present in the feline samples while *L. infantum* was exclusively detected in the canine sample affected by VL. The distinct distributions of these across different host populations are graphically represented in Fig. 2.

We also identified double coinfection events between *Leishmania panamensis* and *L. braziliensis* (Fig. 2a; Additional file 8: Table S4). These findings were in agreement with those obtained for the HSP70-Short gene ampliconbased NGS (Fig. 2b). The most relevant difference was in the relative species abundance identified in two analyzed samples. For example, in the case of sample BON-L11, the relative proportion between *L. braziliensis* and *L. panamensis* was 30/70 with the HSP70-Long approach, and 15/85 with the HSP70-Short approach. A similar pattern emerged in the BON-L16 sample, where the relative abundances were 42/58 (HSP70-Long) and 15/85 (HSP70-Short) for *L. braziliensis* and *L. panamensis*, respectively (Fig. 2).

Subsequently, we aimed to evaluate the intraspecies genetic variability by analyzing the clinical samples collected during this study. The outcomes revealed a moderate level of nucleotide diversity within *L. braziliensis*. Among the 12 sequences analyzed, we identified two different clusters. A total of five polymorphic sites and three distinct haplotypes were identified. The dissimilarity heatmap further illustrated the differentiation between these clusters (Additional file 9: Figure S5).

## Discussion

Numerous studies performed to date have highlighted the potential of the HSP70 marker to distinguish various *Leishmania* species [43–47]. This marker has also been instrumental in reconstructing phylogenetic relationships and establishing taxonomy classifications [27, 29]. Such studies have employed diverse molecular techniques and sequencing methods. Notably, some studies have leveraged the HSP70-based NGS to distinguish mixtures of sequences within a single sample [16, 17, 48], while others have effectively employed the great accuracy of the MinION™ long-read sequencing technology to detect and identify *Leishmania* species in different clinical forms [15].

However, many of these earlier studies used short markers, which could potentially limit the ability to capture nucleotide-level differences among different *Leishmania* species. In the present study, we report the design of a large marker of 771 bp based on *hsp70* (HSP70-Long) and the results of a pioneering evaluation assessment of its

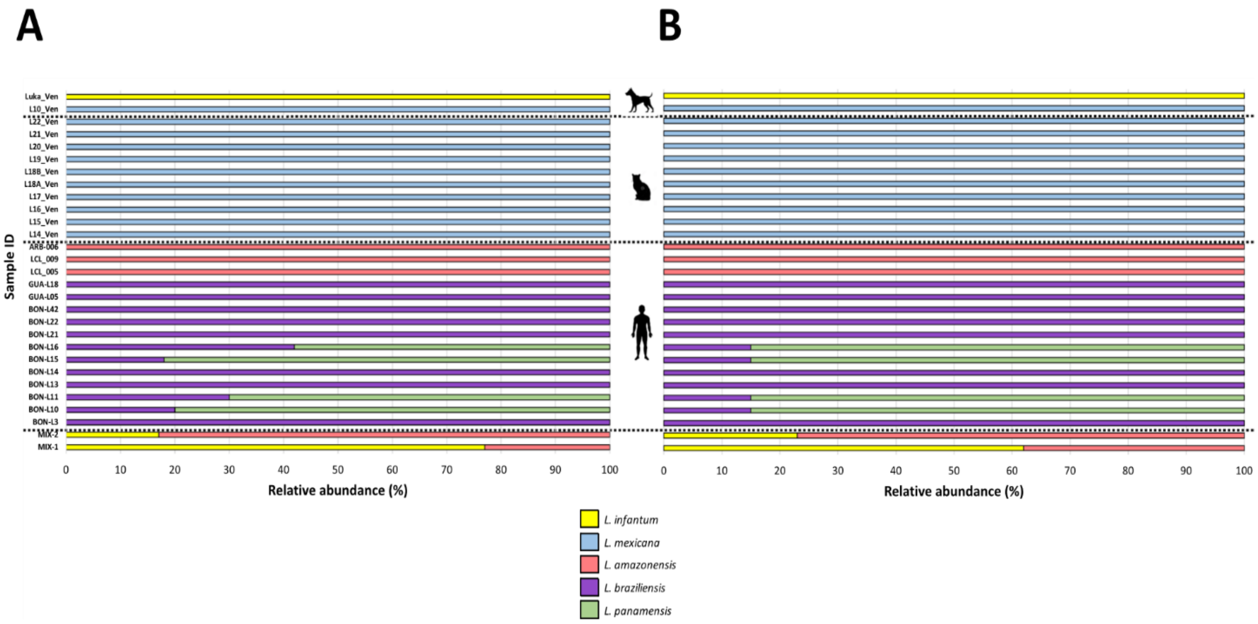


**Fig 2**. Relative abundances of *Leishmania* species identified in the samples in the samples analyzed using HSP70-Long MinION sequencing (**A**) and HSP70-Short MinION$^{TM}$ sequencing (**B**). Mix refers to the mixtures of *L. infantum* and *L. amazonensis* made in the laboratory: Mix-1 contained $1 \times 10^6$ parasites/ml of *L. infantum* and $1 \times 10^3$ parasites/ml of *L. amazonensis*; Mix-2 contained $1 \times 10^3$ parasites/ml of *L. infantum* and 1 × 1 0$^6$ parasites/ml of *L. amazonensis*. Color bars represent the *Leishmania* species found in the sample, HSP70-Long, 771-bp 70-kDa heat shock protein gene-based marker; HSP70-Short, previously published heat shock protein gene-based marker spanning 330 bp.

sensitivity, specificity and diagnostic prowess to identify diverse *Leishmania* species using the MinION™ sequencing technology. We also undertook a comparative analysis, pitting this newly designed marker against a previously established one (HSP70-Short) [35] in a comparison of performance.

The findings of the present study underscore that the HSP70-Short PCR marker exhibited the highest sensitivity in detecting *L. infantum* DNA, achieving a remarkable LoD as low as 1 parasite-equivalent/ml. In contrast, the HSP70-Long PCR marker showed a higher LoD of 1 × $10^2$ parasite-equivalents/ml. These findings are in agreement with prior research that consistently emphasized the heightened sensitivity of shorter segments of the HSP70 gene [49, 50]. The enhanced sensitivity of the short fragment likely accounts for its superior performance in detecting *L. infantum* DNA, as revealed in our study. Incorporating this shorter fragment into PCR assays has the potential to substantially enhance the precision and dependability of *Leishmania* species detection.

When comparing the LoD achieved for the HSP70Short marker with findings from earlier reports, we noted distinct differences. Notably, our results contrast with those of Leon et al., who previously documented an LoD of 1 × 1 $0^1$ parasite-equivalents/ml [51]. Similarly, the LoD reported by Filgueira et al. was $10^2$ parasite-equivalents/reaction [52]. This contrast in LoD values might be explained by the specific *Leishmania* species being studied. It is important to highlight that while the LOD in the present study was determined specifically for *L. infantum*, this value could exhibit variations for other species due to the copy number of the HSP70 gene, which varies depending on the *Leishmania* subgenera analyzed [50, 53, 54].

When we compared the LoD achieved with conventional PCR with those from other *Leishmania* species detection and quantification techniques, particularly those targeting the HSP70 gene, such as qPCR and PCRRFLP, we noted significant differences. Various studies have outlined the range of LoDs obtained by qPCR LoDs which LoDs ranging from 1 × $10^{-1}$ parasite-equivalents/ ml to 10 parasite-equivalents/ml [35, 51, 52]. In contrast, previous investigations centered around PCR–RFLP have shown a remarkable increase in sensitivity, with a detection capability ranging from 0.1 ng to 100 fg of DNA [49, 50]. These findings are extremely relevant in the context of analyzing clinical and biological samples, where the sensitivity of the PCRs is crucial due to samples often containing a small number of parasites.

In terms of primer specificity, our observations revealed that the HSP70-Short primers were not exclusive to *Leishmania* DNA, as they also amplified *T. cruzi* DNA. This cross-reactivity indicates a potential limitation in the application of these primers for precise *Leishmania* detection. In contrast, the HSP70-Long primers demonstrated complete exclusivity in detecting *Leishmania* DNA without any interference from the DNA of other trypanosomatids or other non-target organisms. This remarkable specificity enhances the reliability and accuracy of HSP70-Long primers for specific *Leishmania* identification, making them a valuable tool for diagnostic applications and research purposes. Taking into account the co-endemism of *T. cruzi* and *Leishmania* in many regions, infecting both animals [55, 56] and humans [57, 58], emphasis must be placed on the importance of using genus-specific PCRs to avoid misdiagnosis or missed detection of mixed infections involving both *T. cruzi* and *Leishmania*. By utilizing the HSP70-Long primers, which are designed exclusively for *Leishmania* species, it is possible to effectively tackle this concern and achieve more dependable outcomes, particularly in regions endemic for leishmaniasis. Nonetheless, it is worth noting that this marker might not be efficacious in areas with a high incidence of Chagas Disease or in regions where both parasites coexist sympatrically. In such instances, the HSP70-Short marker could certainly provide higher resolution, given its capability to reliably differentiate among various trypanosomatids and consistently detect coinfection events within a single host [16, 59].

To assess clinical performance, we conducted Min™ ION sequencing using both HSP70-Short and HSP70Long markers on samples from both humans and animals. The outcomes from human samples consistently indicated two significant findings. Firstly *L. braziliensis* emerged as the predominant *Leishmania* species within the Colombian military population. Secondly, exclusive detection of *L. amazonensis* occurred in samples collected from central-western Venezuela (Figs. 1 and 2). Despite the relatively limited sample size scrutinized in this study, our results concur with earlier studies [43, 60–62]. These results underline not only the prevalence of these species in vulnerable populations that frequently deploy to high-endemic regions, exhibiting continuous movement between urban and rural areas, where numerous vectors and reservoirs coexist, thereby contributing to the spread of leishmaniasis [63, 64], but also spotlight the predominance of species linked to different forms of Leishmaniasis [65–68], which exhibit a notable capacity to induce a low immune response [68, 69] while displaying variable response to anti-leishmanial therapy [68, 70].

Our findings revealed the dominance of *L. mexicana* as the primary infecting species in the feline samples (Figs. 1, 2). These results are in agreement with the findings obtained from studies conducted in Texas (USA) [71, 72] as well as observations reported in Venezuela [73–75]. In these regions, *L. mexicana* has been identified as the primary causative agent responsible for feline leishmaniasis. This stands in contrast to observations made in the Old World where *L. infantum*, *L. tropica* and *L. major* are the most prevalent species associated with this disease. The active circulation of *L. mexicana* in cats carries significant clinical implications for the health of the infected animal due to the diverse range of clinical lesions it causes (such as ulcers, verrucous lesions, papules and scaly plaques) and the limited availability of preventive measures and therapeutic options for treating feline leishmaniasis [75]. It also has repercussions for human and canine health, given the ongoing coexistence of cats and humans and the potential role of cats as potential sentinel reservoir hosts in endemic areas of zoonotic VL [76]. Given the widespread occurrence of feline leishmaniasis globally, spanning regions in Europe, the Middle East (Egypt, Iran and Turkey), Thailand, Texas (USA) [77, 78], as well as several countries across the Americas (Mexico, Brazil and Venezuela) [73, 79], it is imperative to emphasize the need to include leishmaniasis in the differential diagnosis of cutaneous lesions for both dogs and cats.On the other hand, HSP70-based MinION™ sequencing revealed coinfection events in 15% of the CL samples analyzed, all of which were attributed to *L. braziliensis/L. panamensis* coinfection (Fig. 2a). These findings consistently align with those obtained with the HSP70-Short marker (Fig. 2b; Additional file 8: Table S4). The detection of coinfection events underscores the importance of accurately identifying the responsible *Leishmania* species in infections. Such events can significantly impact the natural course of the leishmaniasis, as previously documented [80], and add complexity to both the diagnosis of and therapeutic approach to the disease.

Moreover, these findings revealed that coinfections events are more prevalent than previously thought. Coinfection is not limited solely to humans, as indicated by our current study and previous research [16, 17, 34, 80, 81], but is also evident in sand fly vectors [16, 17, 82] and reservoirs [16, 17, 83, 84]. The identification of these coinfection events holds paramount importance in comprehending the intricate eco-epidemiology of the disease. Furthermore, this knowledge assumes a pivotal role in shaping novel and enhanced strategies for the control, prevention and diagnosis of Leishmaniasis.

Both this study and earlier research demonstrated that HSP70 markers are a reliable and efficient molecular approach not only in screening for the presence of *Leishmania* species, but also for assessing their inter- and intraspecies genetic diversity [16]. The HSP70Long primers analyzed in this study exhibited a robust ability to discriminate between different species within the *Leishmania* genus (Fig. 1). Furthermore, this marker facilitated the identification of distinct haplotypes within the analyzed sequences of *L. braziliensis* (Additional file 9: Fig. S5), further confirming the substantial intraspecific genetic diversity within this species—diversity that might contribute to its survival and adaptation to various ecological niches.

There are a number of limitations to this study. First, the number of samples analyzed was relatively small number, particularly those associated with VL. Second, the geographical distribution of the samples evaluated was limited, with the main focus on regions where cocirculation of different *Leishmania* species exists [43, 85] and the inclusion of more *Leishmania* species to analyze the sensitivity of both primer sets. Therefore, future studies with more extensive sampling should be conducted to further enhance our understanding.

## Conclusions

In conclusion, this study is the first to demonstrate the value of HSP70-based MinIO™ sequencing as a potent technology for not only identifying and distinguishing *Leishmania* species but also for detecting mixed infections within clinical samples. This technology stands out for its exceptional specificity and cost-effectiveness, when compared to conventional sequencing platforms, as previously demonstrated [15–17, 86]™. In contrast with NGS Illumina sequencing, the MinION™ DNA sequencing system from Oxford Nanopore Technologies offers the advantage of remarkably low sequencing costs (approximately US$26 per sample vs. US$100 with paired-end short read technology) and a streamlined preparation process, and also eliminates the need for extensive equipment maintenance. Moreover, MinION™ presents operational time benefits, which have been documented to be as short as 6 h from sample collection [20, 87]. This system also capitalizes on the benefit of generating long reads [88]. It is crucial to emphasize that this study was designed for validation purposes, with a sample size we believe is well-suited for this objective (it is recommended to have 20–30 samples for validation purposes in the literature). It is also worth noting that obtaining the required number of samples to meet the objective of this study was a challenging endeavor. Finally, we anticipate that future epidemiological studies will included larger sample sizes and provide important information on *Leishmania* species worldwide. Despite the excellent specificity of HSP70-Long primers, this study revealed their lower sensitivity compared to HSP70-Short primers, thereby highlighting the trade-off between sensitivity levels in the two primer sets, with the HSP70 Short primers exhibiting superior performance in detecting lower parasite concentrations.

## Supplementary Information

The online version contains supplementary material available at https:// doi. org/ 10. 1186/ s13071- 023- 06073-9.

**Additional file 1: Figure S1.** Multiple alignment of 34 haplotypes from HSP70-Long reference sequences and annealing sites of primers. **A** Annealing sites for forward primer, **B** annealing sites for reverse primer. **Additional file 2: Table S1.** Panel of organisms used in the specificity assay, including bacteria, fungi, viruses and parasites.

**Additional file 3: Table S2.** Metadata of samples included in the study.

**Additional file 4: Figure S2.** Analytical sensitivity analysis. The figure illustrates the results of conventional PCR amplification, initiated at a concentration of 1 × $10^6$ parasites/ml and reducing to 1 × $10^{-1}$ parasite/m, employing HSP70-Long (**A**) and HSP70-Short (**B**) primers. Line 1, PPM; lines 2–9, 1 × $10^6$ to 1 × $10^{-1}$ parasites/ml; line 10, negative control.

**Additional file 5: Figure S3.** Phylogenetic relationship between the different HSP70 gene copies in trypanosomatids. The figure represents the phylogenetic analysis of HSP70-Long sequences in trypanosomatids, as recognized by the primers used in the study. The black dots represent well-supported nodes (Bootstrap ≥ 90).

**Additional file 6: Figure S4.** Network analysis of 34 haplotypes from HSP70-Long reference sequences. Each sequenced haplotype is represented by a circle. Black lines on the branches indicate the mutational changes between the different haplotypes.

**Additional file 7: Table S3.** Number of reads obtained from HSP70-Long- ™ and HSP70-Short amplicon-based MinION sequencing for each sample included in the study

**Additional file 8: Table S4.** Comparison of the results obtained between ™ the HSP70-Long- and HSP70-Short amplicon-based MinION sequencing, in each sample included in the study.

**Additional file 9: Figure S5.** Analysis of intraspecies diversity among *L. braziliensis* sequences. **A** Phylogenetic relationship among *L. braziliensis hsp70* sequences. **B** Haplotype of *L. braziliensis* network based on *hsp70* sequences. Each haplotype is denoted by a circle and mutational steps between haplotypes are depicted by the number of lines. **C** Heatmap illustrating pairwise comparison of *L. braziliensis* sequences.

**Acknowledgements**

We thank the Direccion de Investigación e Innovación from Universidad del Rosario for covering the Article Processing Charge of this manuscript.

**Author contributions**

LHP conceived and designed the study, analyzed and interpreted the data and prepared the manuscript. NB analyzed and interpreted the data. CM, JJ, AC, RM and AP critically revised the manuscript and made important suggestions. JDR conceived and designed the study and revised the manuscript. All authors have reviewed and approved the manuscript.

**Funding**

We thank the Dirección de Investigación e Innovación from Universidad del Rosario for funding this study.

**Availability of data and materials**

The dataset generated during the study was deposited at the European Nucleotide Archive (ENA) under the bioproject code PRJNA855474.

## Declarations

**Ethical approval and consent to participate**

This study was approved by the Ethics Committee of the Central Military Hospital of Colombia, following the principles established in the Declaration of Helsinki under Act No. 2043 of 22 March 2017.

**Consent for publication** Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**Author details**

[1] Centro de Investigaciones en Microbiología y Biotecnología-UR (CIMBIUR), Facultad de Ciencias Naturales, Universidad del Rosario, Bogotá, Colombia.

[2] Animal Hospital of Smithson Valley, 286 Singing Oaks, Ste 113, Spring Branch, TX 78070, USA. [3] Molecular Microbiology Laboratory, Department of Pathology, Molecular and Cell-Based Medicine, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA.

Received: 25 September 2023 Accepted: 29 November 2023

**References**

1. Akhoundi M, Downing T, Votypka J, Kuhls K, Lukes J, Cannet A, et al. *Leishmania* infections: molecular targets and diagnosis. Mol Aspects Med. 2017;57:1–29.
2. Akhoundi M, Kuhls K, Cannet A, Votypka J, Marty P, Delaunay P, et al. A historical overview of the classification, evolution, and dispersion of *Leishmania* parasites and sandflies. PLoS Negl Trop Dis. 2016;10:e0004349.
3. Fayaz S, Fard-Esfahani P, Bahrami F, Parvizi P, Ajdary S. High resolution melting assay in discrimination of the main etiologic agents of leishmaniasis in Iran. Iran J Microbiol. 2021;13:137–44.
4. Nicolas L, Prina E, Lang T, Milon G. Real-time PCR for detection and quantitation of *Leishmania* in mouse tissues. J Clin Microbiol. 2002;40:1666–9.
5. Galluzzi L, Ceccarelli M, Diotallevi A, Menotta M, Magnani M. Realtime PCR applications for diagnosis of leishmaniasis. Parasit Vectors. 2018;11:273.
6. Kuhls K, Mauricio IL, Pratlong F, Presber W, Schonian G. Analysis of ribosomal DNA internal transcribed spacer sequences of the *Leishmania donovani* complex. Microbes Infect. 2005;7:1224–34.
7. Reithinger R, Dujardin JC. Molecular diagnosis of leishmaniasis: current status and future applications. J Clin Microbiol. 2007;45:21–5.
8. Lau R, Mukkala AN, Kariyawasam R, Clarke S, Valencia BM, Llanos-Cuentas A, et al. Comparison of whole genome sequencing versus standard molecular diagnostics for species identification in the *Leishmania viannia* subgenus. Am J Trop Med Hyg. 2021;105:660–9.
9. Boite MC, Mauricio IL, Miles MA, Cupolillo E. New insights on taxonomy, phylogeny and population genetics of *Leishmania* (*Viannia*) parasites based on multilocus sequence analysis. PLoS Negl Trop Dis. 2012;6:e1888.
10. Marfurt J, Nasereddin A, Niederwieser I, Jaffe CL, Beck HP, Felger I. Identification and differentiation of *Leishmania* species in clinical samples by PCR amplification of the miniexon sequence and subsequent restriction fragment length polymorphism analysis. J Clin Microbiol. 2003;41:3147–53. 11. Schonian G, Schnur L, el Fari M, Oskam L, Kolesnikov AA, SokolowskaKohler W, et al. Genetic heterogeneity in the species *Leishmania tropica* revealed by different PCR-based methods. Trans R Soc Trop Med Hyg. 2001;95:217–24.
12. Montalvo AM, Monzote L, Fraga J, Montano I, Muskus C, Marin M, et al. PCR-RFLP and RAPD for typing neotropical *Leishmania*. Biomedica. 2008;28:597–606.
13. Talmi-Frank D, Nasereddin A, Schnur LF, Schonian G, Toz SO, Jaffe CL, et al. Detection and identification of old world *Leishmania* by high resolution melt analysis. PLoS Negl Trop Dis. 2010;4:e581.
14. Sriworarat C, Phumee A, Mungthin M, Leelayoova S, Siriyasatien P. Development of loop-mediated isothermal amplification (LAMP) for simple detection of *Leishmania* infection. Parasit Vectors. 2015;8:591.
15. Maia de Souza R, Ruedas Martins RC, Moyses Franco LA, Tuon FF, de Oliveira IG Junior, Maia da Silva CA, et al. Identification of *Leishmania* species by next generation sequencing of *hsp70* gene. Mol Cell Probes. 2022;61:101791.
16. Patino LH, Castillo-Castaneda AC, Munoz M, Jaimes JE, Luna-Nino N, Hernandez C, et al. Development of an amplicon-based next-generation sequencing protocol to identify *Leishmania* species and other trypanosomatids in leishmaniasis endemic areas. Microbiol Spectr. 2021;9:e0065221.
17. Castillo-Castaneda A, Patino LH, Munoz M, Ayala MS, Segura M, Bautista J, et al. Amplicon-based next-generation sequencing reveals the coexistence

of multiple *Leishmania* species in patients with visceral leishmaniasis. Int J Infect Dis. 2022;115:35–8.

18. Loman NJ, Quick J, Simpson JT. A complete bacterial genome assembled de novo using only nanopore sequencing data. Nat Methods. 2015;12:733–5.
19. Michael TP, Jupe F, Bemm F, Motley ST, Sandoval JP, Lanz C, et al. High contiguity *Arabidopsis thaliana* genome assembly with a single nanopore flow cell. Nat Commun. 2018;9:541.
20. Greninger AL, Naccache SN, Federman S, Yu G, Mbala P, Bres V, et al. Rapid metagenomic identification of viral pathogens in clinical samples by realtime nanopore sequencing analysis. Genome Med. 2015;7:99.
21. Taylor MK, Williams EP, Wongsurawat T, Jenjaroenpun P, Nookaew I, Jonsson CB. Amplicon-based, next-generation sequencing approaches to characterize single nucleotide polymorphisms of *Orthohantavirus* species. Front Cell Infect Microbiol. 2020;10:565591.
22. Quick J, Ashton P, Calus S, Chatt C, Gossain S, Hawker J, et al. Rapid draft sequencing and real-time nanopore sequencing in a hospital outbreak of Salmonella. Genome Biol. 2015;16:114.
23. Ashton PM, Nair S, Dallman T, Rubino S, Rabsch W, Mwaigwisya S, et al. MinION nanopore sequencing identifies the position and structure of a bacterial antibiotic resistance island. Nat Biotechnol. 2015;33:296–300.
24. Marti-Carreras J, Carrasco M, Gomez-Ponce M, Noguera-Julian M, Fisa R, Riera C, et al. Identification of *Leishmania infantum* epidemiology, drug resistance and pathogenicity biomarkers with nanopore sequencing. Microorganisms. 2022;10:34.
25. Hartl FU, Hayer-Hartl M. Molecular chaperones in the cytosol: from nascent chain to folded protein. Science. 2002;295:1852–8.
26. Van der Auwera G, Ravel C, Verweij JJ, Bart A, Schonian G, Felger I. Evaluation of four single-locus markers for *Leishmania* species discrimination by sequencing. J Clin Microbiol. 2014;52:1098–104.
27. Fraga J, Montalvo AM, De Doncker S, Dujardin JC, Van der Auwera G. Phylogeny of *Leishmania* species based on the heat-shock protein 70 gene. Infect Genet Evol. 2010;10:238–45.
28. Espinosa OA, Serrano MG, Camargo EP, Teixeira MMG, Shaw JJ. An appraisal of the taxonomy and nomenclature of trypanosomatids presently classified as *Leishmania* and *Endotrypanum*. Parasitology. 2018;145:430–42.
29. Yuan D, Qin H, Zhang J, Liao L, Chen Q, Chen D, et al. Phylogenetic analysis of HSP70 and cyt b gene sequences for Chinese *Leishmania* isolates and ultrastructural characteristics of Chinese *Leishmania* sp. Parasitol Res. 2017;116:693–702.
30. Van der Auwera G, Dujardin JC. Species typing in dermal leishmaniasis. Clin Microbiol Rev. 2015;28:265–94.
31. Ovalle-Bracho C, Londono-Barbosa D, Salgado-Almario J, Gonzalez C. Evaluating the spatial distribution of *Leishmania* parasites in Colombia from clinical samples and human isolates (1999 to 2016). PLoS ONE. 2019;14:e0214124.
32. Sandoval-Ramirez CM, Hernandez C, Teheran AA, Gutierrez-Marin R, Martinez-Vega RA, Morales D, et al. Complex ecological interactions across a focus of cutaneous leishmaniasis in Eastern Colombia: novel description of *Leishmania* species, hosts and phlebotomine fauna. R Soc Open Sci. 2020;7:200266.
33. Herrera G, Teheran A, Pradilla I, Vera M, Ramirez JD. Geospatial-temporal distribution of tegumentary Leishmaniasis in Colombia (2007–2016). PLoS Negl Trop Dis. 2018;12:e0006419.
34. Veland N, Valencia BM, Alba M, Adaui V, Llanos-Cuentas A, Arevalo J, et al. Simultaneous infection with *Leishmania* (*Viannia*) *braziliensis* and *L.* (*V.*) *lainsoni* in a Peruvian patient with cutaneous leishmaniasis. Am J Trop Med Hyg. 2013;88:774–7.
35. Hernandez C, Alvarez C, Gonzalez C, Ayala MS, Leon CM, Ramirez JD. Identification of six New World *Leishmania* species through the implementation of a high-resolution melting (HRM) genotyping assay. Parasit Vectors. 2014;7:501.
36. Okonechnikov K, Golosova O, Fursov M. team U: Unipro UGENE: a unified bioinformatics toolkit. Bioinformatics. 2012;28:1166–7.
37. Patino LH, Muskus C, Munoz M, Ramirez JD. Genomic analyses reveal moderate levels of ploidy, high heterozygosity and structural variations in a Colombian isolate of *Leishmania* (*Leishmania*) *amazonensis*. Acta Trop. 2019;203:105296.
38. Price MN, Dehal PS, Arkin AP. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. Mol Biol Evol. 2009;26:1641–50.
39. Letunic I, Bork P. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Res. 2019;47:W256–9.
40. Huson DH, Bryant D. Application of phylogenetic networks in evolutionary studies. Mol Biol Evol. 2006;23:254–67.
41. Babicki S, Arndt D, Marcu A, Liang Y, Grant JR, Maciejewski A, et al. Heatmapper: web-enabled heat mapping for all. Nucleic Acids Res. 2016;44:W147-153.
42. Paniz-Mondolfi A, Guerra S, Munoz M, Luna N, Hernandez MM, Patino LH, et al. Evaluation and validation of an RT-PCR assay for specific detection of monkeypox virus (MPXV). J Med Virol. 2023;95:e28247.
43. Correa-Cardenas CA, Perez J, Patino LH, Ramirez JD, Duque MC, Romero Y, et al. Distribution, treatment outcome and genetic diversity of *Leishmania* species in military personnel from Colombia with cutaneous leishmaniasis. BMC Infect Dis. 2020;20:938.
44. Montalvo AM, Fraga J, Montano I, Monzote L, Van der Auwera G, Marin M, et al. Molecular identification of *Leishmania* spp clinical isolates from Colombia based on *hsp70* gene. Biomedica. 2016;36:37–44.
45. Hoyos J, Rosales-Chilama M, Leon C, Gonzalez C, Gomez MA. Sequencing of hsp70 for discernment of species from the *Leishmania* (*Viannia*) *guyanensis* complex from endemic areas in Colombia. Parasit Vectors. 2022;15:406.
46. Espada CR, Ortiz PA, Shaw JJ, Barral AMP, Costa JML, Uliana SRB, et al. Identification of *Leishmania* (*Viannia*) species and clinical isolates of *Leishmania* (*Leishmania*) *amazonensis* from Brazil using PCR-RFLP of the heat-shock protein 70 gene reveals some unexpected observations. Diagn Microbiol Infect Dis. 2018;91:312–8.
47. Miranda ADC, Gonzalez KA, Samudio F, Pineda VJ, Calzada JE, CapitanBarrios Z, et al. Molecular identification of parasites causing cutaneous leishmaniasis in Panama. Am J Trop Med Hyg. 2021;104:1326–34.
48. Maloney JG, Molokin A, Santin M. Next generation amplicon sequencing improves detection of *Blastocystis* mixed subtype infections. Infect Genet Evol. 2019;73:119–25.
49. Montalvo AM, Fraga J, Tirado D, Blandon G, Alba A, Van der Auwera G, et al. Detection and identification of *Leishmania* spp.: application of two hsp70-based PCR-RFLP protocols to clinical samples from the New World. Parasitol Res. 2017;116:1843–8.
50. Montalvo AM, Fraga J, Maes I, Dujardin JC, Van der Auwera G. Three new sensitive and specific heat-shock protein 70 PCRs for global *Leishmania* species identification. Eur J Clin Microbiol Infect Dis. 2012;31:1453–61.
51. 51. Leon CM, Munoz M, Hernandez C, Ayala MS, Florez C, Teheran A, et al. Analytical performance of four polymerase chain reaction (PCR) and real time PCR (qPCR) assays for the detection of six *Leishmania* species DNA in Colombia. Front Microbiol. 2017;8:1907.
52. Filgueira CPB, Moreira OC, Cantanhede LM, de Farias HMT, Porrozzi R, Britto C, et al. Comparison and clinical validation of qPCR assays targeting *Leishmania* 18S rDNA and HSP70 genes in patients with American tegumentary leishmaniasis. PLoS Negl Trop Dis. 2020;14:e0008750.
53. Folgueira C, Canavate C, Chicharro C, Requena JM. Genomic organization and expression of the HSP70 locus in New and Old World *Leishmania* species. Parasitology. 2007;134:369–77.
54. Ramirez CA, Requena JM, Puerta CJ. Identification of the HSP70-II gene in *Leishmania braziliensis* HSP70 locus: genomic organization and UTRs characterization. Parasit Vectors. 2011;4:166.
55. Trueb I, Portela RD, Franke CR, Carneiro IO, Ribeiro GJ Jr, Soares RP, et al. *Trypanosoma cruzi* and *Leishmania* sp. infection in wildlife from urban rainforest fragments in Northeast Brazil. J Wildl Dis. 2018;54:76–84.
56. Orduna-Mayares D, Hernandez-Camacho N, Escobedo-Ortegon FJ, Canche-Pool EB, Sosa-Gallegos SL, SalvadorZamora L, et al. *Trypanosoma cruzi* and *Leishmania* spp in rodents in a peri-urban area of Central Mexico. Vet Parasitol Reg Stud Rep. 2022;35:100779.
57. Bastrenta B, Mita N, Buitrago R, Vargas F, Flores M, Machane M, et al. Human mixed infections of *Leishmania* spp. and *Leishmania-Trypanosoma cruzi*. in a sub Andean Bolivian area: identification by polymerase chain reaction/hybridization and isoenzyme. Mem Inst Oswaldo Cruz. 2003;98:255–64.
58. Mendes DG, Lauria-Pires L, Nitz N, Lozzi SP, Nascimento RJ, Monteiro PS, et al. Exposure to mixed asymptomatic infections with *Trypanosoma cruzi*, *Leishmania braziliensis* and *Leishmania chagasi* in the human population of the greater Amazon. Trop Med Int Health. 2007;12:629–36.

59. Castillo-Castaneda AC, Patino LH, Zuniga MF, Cantillo-Barraza O, Ayala MS, Segura M, et al. An overview of the trypanosomatid (Kinetoplastida: Trypanosomatidae) parasites infecting several mammal species in Colombia. Parasit Vectors. 2022;15:471.
60. Patino LH, Mendez C, Rodriguez O, Romero Y, Velandia D, Alvarado M, et al. Spatial distribution, *Leishmania* species and clinical traits of cutaneous leishmaniasis cases in the Colombian army. PLoS Negl Trop Dis. 2017;11:e0005876.
61. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS ONE. 2012;7:e35671.
62. Delgado-Noguera LA, Hernandez-Pereira CE, Castillo-Castaneda AC, Patino LH, Castaneda S, Herrera G, et al. Diversity and geographical distribution of *Leishmania* species and the emergence of *Leishmania* (*Leishmania*) *infantum* and *L.* (*Viannia*) *panamensis* in Central-Western Venezuela. Acta Trop. 2023;242:106901.
63. Gonzalez C, Leon C, Paz A, Lopez M, Molina G, Toro D, et al. Diversity patterns, *Leishmania* DNA detection, and bloodmeal identification of Phlebotominae sand flies in villages in northern Colombia. PLoS ONE. 2018;13:e0190686.
64. Ardila MM, Carrillo-Bonilla L, Pabon A, Robledo SM. Surveillance of phlebotomine fauna and *Didelphis marsupialis* (Didelphimorphia: Didelphidae) infection in an area highly endemic for visceral leishmaniasis in Colombia. Biomedica. 2019;39:252–64.
65. Rugani JN, Quaresma PF, Gontijo CF, Soares RP, Monte-Neto RL. Intraspecies susceptibility of *Leishmania* (*Viannia*) *braziliensis* to antileishmanial drugs: Antimony resistance in human isolates from atypical lesions. Biomed Pharmacother. 2018;108:1170–80.
66. de Oliveira GM, Madeira Mde F, Oliveira FS, Pires MQ, Pacheco Rda S. Canine cutaneous leishmaniasis: Dissemination and tissue tropism of genetically distinct *Leishmania* (*Viannia*) *braziliensis* populations. Vet Med Int. 2013;2013:982183.
67. Velez ID, Carrillo LM, Lopez L, Rodriguez E, Robledo SM. An epidemic outbreak of canine cutaneous leishmaniasis in Colombia caused by *Leishmania braziliensis* and *Leishmania panamensis*. Am J Trop Med Hyg. 2012;86:807–11.
68. Silva TF, Tomiotto-Pellissier F, Pasquali AKS, Pinto-Ferreira F, Pavanelli WR,Conchon-Costa I, et al. Phenotypical and genotypical differences among *Leishmania* (*Leishmania*) *amazonensis* isolates that caused different clinical frames in humans and dogs: A systematic review. Acta Trop. 2021;221:106018.
69. Cardoso T, Bezerra C, Medina LS, Ramasawmy R, Scheriefer A, Bacellar O, et al. *Leishmania braziliensis* isolated from disseminated leishmaniasis patients downmodulate neutrophil function. Parasite Immunol. 2019;41:e12620.
70. Rugani JN, Gontijo CMF, Frezard F, Soares RP, Monte-Neto RLD. Antimony resistance in *Leishmania* (*Viannia*) *braziliensis* clinical isolates from atypical lesions associates with increased ARM56/ARM58 transcripts and reduced drug uptake. Mem Inst Oswaldo Cruz. 2019;114:e190111.
71. Trainor KE, Porter BF, Logan KS, Hoffman RJ, Snowden KF. Eight cases of feline cutaneous leishmaniasis in Texas. Vet Pathol. 2010;47:1076–81.
72. Hopke K, Meyers A, Auckland L, Hamer S, Florin D, Diesel A, et al. *Leishmania mexicana* in a central Texas cat: clinical presentation, molecular identification, sandfly vector collection and novel management. JFMS Open Rep. 2021;7:2055116921999595.
73. Rivas AK, Alcover M, Martinez-Orellana P, Montserrat-Sangra S, NachumBiala Y, Bardagi M, et al. Clinical and diagnostic aspects of feline cutaneous leishmaniosis in Venezuela. Parasit Vectors. 2018;11:141.
74. Mendoza Yeimar CA, Hernández-Pereira Carlos E, Shaban Maryia V. Cutaneous leishmaniosis due to *Leishmania mexicana* in a cat treated with cryotherapy. Vet Dermatol. 2021;33:450–3.
75. Paniz Mondolfi AE, Colmenares Garmendia A, Mendoza Perez Y, Hernandez-Pereira CE, Medina C, Vargas F, et al. Autochthonous cutaneous leishmaniasis in urban domestic animals (*Felis catus*/*Canis lupus familiaris*) from central-western Venezuela. Acta Trop. 2019;191:252–60.
76. Dalvi APR, Carvalho TDG, Werneck GL. Is there an association between exposure to cats and occurrence of visceral leishmaniasis in humans and dogs? Vector Borne Zoonotic Dis. 2018;18:335–42.
77. Asfaram S, Fakhar M, Teshnizi SH. Is the cat an important reservoir host for visceral leishmaniasis? A systematic review with meta-analysis. J Venom Anim Toxins Incl Trop Dis. 2019;25:e20190012.
78. Akhtardanesh B, Moeini E, Sharifi I, Saberi M, Sadeghi B, Ebrahimi M, et al. *Leishmania* infection in cats positive for immunodeficiency virus and feline leukemia virus in an endemic region of Iran. Vet Parasitol Reg Stud Rep. 2020;20:100387.
79. Nascimento LFJ, Cirilo TM, Gomes DS, Gomes ACA, Lima VFS, Scher R, et al. Epidemiological and diagnostic aspects of feline leishmaniasis with emphasis on Brazil: a narrative review. Parasitol Res. 2022;121:21–34.
80. Gosch CS, Resende BS, Amorim CB, Marques CP, Pereira LIA, Pinto SA, et al. Case report: atypical cutaneous leishmaniasis in a patient with mixed *Leishmania guyanensis* and *Leishmania amazonensis* infection. Am J Trop Med Hyg. 2018;99:1165–9.
81. Martinez E, Mollinedo S, Torrez M, Munoz M, Banuls AL, Le Pont F. Coinfection by *Leishmania amazonensis* and *L. infantum/L chagasi* in a case of diffuse cutaneous leishmaniasis in Bolivia. Trans R Soc Trop Med Hyg. 2002;96:529–32.
82. Bartolome C, Buendia M, Benito M, De la Rua P, Ornosa C, Martin-Hernandez R, et al. A new multiplex PCR protocol to detect mixed trypanosomatid infections in species of *Apis* and *Bombus*. J Invertebr Pathol. 2018;154:37–41.
83. Derghal M, Tebai A, Balti G, Souguir-Omrani H, Chemkhi J, Rhim A, et al. High-resolution melting analysis identifies reservoir hosts of zoonotic *Leishmania* parasites in Tunisia. Parasit Vectors. 2022;15:12.
84. Ferreira Ede C, Cruz I, Canavate C, de Melo LA, Pereira AA, Madeira FA, et al. Mixed infection of *Leishmania infantum* and *Leishmania braziliensis* in rodents from endemic urban area of the New World. BMC Vet Res. 2015;11:71.
85. Herrera L, Morocoima A, Lozano-Arias D, Garcia-Alzate R, Viettri M, Lares M, et al. Infections and coinfections by trypanosomatid parasites in a rural community of Venezuela. Acta Parasitol. 2022;67:1015–23.
86. Ma L, Jakobiec FA, Dryja TP. A review of next-generation sequencing (NGS): applications to the diagnosis of ocular infectious diseases. Semin Ophthalmol. 2019;34:223–31.
87. Jain M, Olsen HE, Paten B, Akeson M. The Oxford Nanopore MinION: delivery of nanopore sequencing to the genomics community. Genome Biol. 2016;17:239.
88. Su J, Lui WW, Lee Y, Zheng Z, Siu GK, Ng TT, et al. Evaluation of *Mycobacterium tuberculosis* enrichment in metagenomic samples using ONT adaptive sequencing and amplicon sequencing for identification and variant calling. Sci Rep. 2023;13:5237.

## Publisher's Note

## 3.3. CAPÍTULO 3

**Título:** Diversidad genética de aislamientos autóctonos de *Leishmania infantum* en Colombia y sus relaciones filogenéticas intra-especie a nivel global

Teniendo presente que el estudio genómico a través de plataformas NGS de los agentes infecciosos son de gran importancia ya que tienen un impacto crucial para entender el agente etiológico de las enfermedades, en nuestro caso *L. infantum,* su divergencia y los patrones que modulan el curso de la enfermedad a nivel nacional, regional y mundial propusimos esta investigación. Puntualmente quisimos evaluar la arquitectura genómica de aislamientos de *L. infantum* originarias de Colombia desde un enfoque comparativo con aislamientos de diferentes ubicaciones geográficas a nivel mundial; así como, la diversidad genómica de la especie con un análisis de relación entre los resultados obtenidos en términos de sensibilidad diagnóstica de antígenos. Para esto, se cultivaron y secuenciaron por primera vez 4 genomas de aislamientos de *L. infantum* procedentes de Colombia por medio de HiSeq X-Ten system; a la vez que, se descargaron 183 genomas curados disponibles en bases de datos de acceso público como DDBJ/ENA/GenBank.

Dentro de los análisis realizados, se caracterizó la somía, se identificaron CNV´s, SNP´s e INDEL´s en los genomas agrupados en 5 grupos Colombia, América, África, Europa y Asia; por otra parte, la estructura poblacional se analizó a partir de la frecuencia alélica, diversidad intra-grupo e inter-grupo y los análisis de hetero y homocigosidad; adicionalmente, se analizó la diversidad genética y las relaciones filogenéticas de los antígenos usados para el diagnóstico inmunocromatográfico de la LV: *rK39* y *rK28*. En el global de los genomas, se identificó una alta homogeneidad genética y una extensiva aneuploidía, la circulación de cuatro poblaciones de *L. infantum* alrededor del mundo. Para el caso puntual de los genomas colombianos, se observó una mayor relación filogenética con los genomas de la cuenca del Mediterráneo y África del norte que con los genomas de América y Brasil. Adicionalmente se demostró la alta variabilidad genética del antígeno *rK39* a nivel mundial.

Este estudio permitió la publicación de un artículo científico:

Patino LH, Castillo-Castañeda A, Muñoz M, Muskus C, Rivero-Rodríguez M, Pérez-Doria A, Bejarano EE, Ramírez JD. Revisiting the heterogeneous global genomic population structure of *Leishmania infantum*. Microb Genom. 2021 Sep;7(9):000640. doi: 10.1099/mgen.0.000640. PMID: 34491157; PMCID: PMC8715437.

MICROBIAL GENOMICS

RESEARCH ARTICLE
Patino *et al.*, *Microbial Genomics* 2021;7:000640
DOI 10.1099/mgen.0.000640

MICROBIOLOGY SOCIETY
OPEN DATA OPEN ACCESS

# Revisiting the heterogeneous global genomic population structure of *Leishmania infantum*

Luz H. Patino[1], Adriana Castillo-Castañeda[1], Marina Muñoz[1], Carlos Muskus[2], Matilde Rivero-Rodríguez[3,4], Alveiro Pérez-Doria[3], Eduar E. Bejarano[3] and Juan David Ramírez[1,*]

## Abstract

*Leishmania infantum* is the main causative agent responsible for visceral leishmaniasis (VL), a disease with global distribution. The genomic structure and genetic variation of this species have been widely studied in different parts of the world. However, in some countries, this information is still yet unknown, as is the genomic behaviour of the main antigens used in VL diagnosis (rK39 and rK28), which have demonstrated variable sensitivity and specificity in a manner dependent on the geographic region analysed. The objective of this study was to explore the genomic architecture and diversity of four Colombian *L. infantum* isolates obtained in this study and to compare these results with the genetic analysis of 183 *L. infantum* isolates from across the world (obtained from public databases), as well as to analyse the whole rK39 and rK28 antigen sequences in our dataset. The results showed that, at the global level, *L. infantum* has high genetic homogeneity and extensive aneuploidy. Furthermore, we demonstrated that there are distinct populations of *L. infantum* circulating in various countries throughout the globe and that populations of distant countries have close genomic relationships. Additionally, this study demonstrated the high genetic variability of the rK28 antigen worldwide. In conclusion, our study allowed us to (i) expand our knowledge of the genomic structure of global *L. infantum*; (ii) describe the intra-specific genomic variability of this species; and (iii) understand the genomic characteristics of the main antigens used in the diagnosis of VL. Additionally, this is the first study to report whole-genome sequences of Colombian *L. infantum* isolates.

## DATA SUMMARY

The four genomes sequenced for this study can be found in the EBI European Nucleotide Archive (ENA) under project number PRJEB42749. The 183 sequences used in our comparative analyses were download from ENA under accession numbers PRJEB2473, PRJNA494801, PRJEB15063, PRJEB2600, PRJNA589999, PRJNA607007, PRJNA658892 and PRJEB3283. All sequences are summarized along with accession numbers in Table S1 (available in the online version of this article).

## INTRODUCTION

Visceral leishmaniasis (VL) is considered the most severe form of leishmaniasis in the world [1, 2]. This disease is endemic on the Indian subcontinent, and in East Africa and the Americas (mainly in Brazil) [1, 3]. VL pathology is caused mainly by parasites of the *Leishmania donovani* complex (*L. infantum/chagasi* and *L. donovani*) [4], and *L. infantum* is the species responsible for VL in the Americas [5–7].

Since 2007, when the whole genome of *L. infantum* was sequenced and published for the first time, various studies using DNA- sequencing technology revealed important findings for this species. Those research efforts facilitated an understanding of its genetic structure [8]; the analysis of the genomic variations associated with the clinical features, hosts and treatment responses [9, 10]; the identification of markers of drug resistance [11]; the determination of the genomic diversity in geographically restricted regions [9, 10, 12, 13] and globally [14]; as well as the low plasticity and intra- specific genomic variability that characterize this *Leishmania* species [10, 14, 15]. To date, these studies have focused mainly in specific geographical areas, such as Brazil, leaving the genomic diversity of this species in other regions of the world unclear. Although the incidence of VL is low in other regions, there has been an increasing number of cases in recent years [3], such as in Colombia, which has reported 244 cases of VL in the last 10 years [16].

Because of the clinical and epidemiological importance of VL worldwide, different serological and molecular tools currently exist that allow fast and effective diagnosis [17, 18]. One of the serological techniques used is the immunochromatographic rapid diagnostic test, which is based on two antigens: the rK39 recombinant antigen, derived from part of an *L. infantum* kinesin-related gene [19], and the rK28 recombinant antigen, developed by fusing multiple tandem repeat sequences of *L. donovani* haspb1 and k39 kinesin genes [20]. Although these techniques are rapid and easy to perform, their sensitivity is variable, and the diagnostic accuracy has been shown to depend on the geographic region. Although the rK39 antigen test has been reported to have high sensitivity in Indian populations (95 –97.0 %), in other parts of the world, the reported sensitivities are variable or generally lower, as is the case in populations in East Africa, where a sensitivity of 75–85.3 % has been reported [17, 20–22]; France, where the test has a sensitivity of 88.5 % [23]; Brazil, where it has a sensitivity of 84.7–93 % [22, 24]; and finally Colombia, which has reported a sensitivity of 82.9–91.5 % [18]. Regarding the recombinant antigen (rK28), reports have described a high sensitivity for the diagnosis of VL in countries such as Sudan, Ethiopia, India and Brazil, in which sensitivities between 94 and 100 % were observed [25–27]. The reason for the different test performances in these areas is unclear, but one reason may be the ability of different *Leishmania* strains to induce the production of specific host antibodies [23, 24]. Another possibility is the molecular diversity of the rK39/rK28 sequences among global strains, which could induce a differential antibody response in the host, antibodies that could have a low affinity by the recombinant K39/K28 antigen commonly used in the immunochromatographic tests, making this test decrease its diagnostic performance. Based on this hypothesis, we believe it is necessary to evaluate the currently unknown rK39/rK28 sequences of strains from different parts of the world.

To date, no studies have analysed the whole genomes of *L. infantum* strains in Colombia or analysed and compared the genomic sequences of the main antigens used in VL

Received 24 March 2021; Accepted 22 June 2021; Published 07 September 2021

Author affiliations: [1]Centro de Investigaciones en Microbiología y Biotecnología-U R (CIMBIUR), Facultad de Ciencias Naturales, Universidad del Rosario, Bogotá, Colombia; [2]Programa de Estudios y Control de Enfermedades Tropicales (PECET), Facultad de Medicina, Universidad de Antioquia, Medellín, Colombia; [3]Grupo de Investigaciones Biomédicas, Universidad de Sucre, Sincelejo, Colombia; [4]Candidata a doctor en Medicina Tropical, Universidad de Cartagena- SUE Caribe, Colombia.

*Correspondence: Juan David Ramírez, juand. ramirez@ urosario. edu. co

Keywords: Aneuploidy; genomic diversity; *Leishmania*; whole genome sequencing.

The four samples sequenced for this study were deposited in the EBI European Nucleotide Archive (ENA) under project number PRJEB42749. Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Five supplementary tables and four supplementary figures are available with the online version of this article.

diagnosis (rK39/rK28) throughout the world. Therefore, the focus of this study was to analyse the whole genome of four different *L. infantum* isolates collected from humans and dogs in two regions of Colombia, evaluate the genomic variation among the isolates and diversity across the globe (ploidy changes, structural variations and single- point mutations), and analyse the phylogenomic relationships (based on the nuclear/mitochondrial genomes and the recombinants antigens rK39/rK28) within 183 publicly available *L. infantum* genomes from the five continents corresponding to the known broad geographic distribution of the parasite.

**Impact Statement**

*Leishmania infantum* is a protozoan parasite responsible for one of the most severe forms of leishmaniasis in the world, the visceral leishmaniasis (VL). In this work we used DNA-sequencing to analyse the genetic structure of this parasite and to describe the genomic behaviour of the main antigens used in VL diagnosis (rK39 and rK28 antigen). The results obtained allow us to expand the knowledge about the intraspecific genomic variability of this parasite to global level and to understand the possible reasons why the tests used in the VL diagnosis present a variable sensitivity and specificity according to the geographical area evaluated.

## METHODS

### Ethics statement

The genomes were obtained from isolates collected from human patients and dogs (*Canis lupus familiaris*). To collect and use these samples, this study was approved by the Ethics Committee of the Universidad de Antioquia (Number VRI3445/2010) and the Universidad de Sucre in accordance with resolution numbers 36 836 and 2–2012, respectively. Written informed consent was obtained from the patients from whom the strains were isolated.

### Study population

A total of 187 whole genomes, which were retrieved from ENA databases, were analysed. These included four new Colombian genomes sequenced in this investigation, two of which were isolated from humans and two from dogs with clinical signs of VL. The other 183 were downloaded from public databases: 36 isolates from Asia (Turkey *N*=13, Israel *N*=8, China *N*=6, Cyprus *N*=5, Uzbekistan *N*=2 and Palestine *N*=2) [12, 14], 17 from Europe (France *N*=5, Italy *N*=5, Portugal *N*=2 and Spain *N*=5) [14, 28], 9 from North Africa (Morocco *N*=2, Zaghouan *N*=1, Nabeul *N*=1, Kairouan *N*=5) [13, 14, 29], and 121 from Central and South America (Brazil *N*=118, Honduras *N*=2 and Panama *N*=1) [9, 14, 15, 28, 30]. All metadata on the 187 genomes, including ENA accession numbers for individual samples, are summarized in Table S1. The public dataset was downloaded using the tools described in https:// github. com/ EnzoAndree/ getENA.

### *Leishmania infantum* culture conditions, DNA extraction and species identification

The promastigotes from four Colombian clinical isolates were grown in Schneider medium supplemented with 10 % (v/v) foetal bovine serum and cultured at 26 °C with 5 % $CO_2$. Approximately $1\times10^6$ promastigotes in the late logarithmic growth phase were cultured and harvested by centrifugation for subsequent DNA extraction, which was conducted using the High Pure PCR Template Preparation Kit (Roche Life Science, Mannheim, Germany) in accordance with the manufacturer's instructions. The DNA concentrations were determined with the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the DNA quality and integrity were determined by 1 % agarose gel electrophoresis. Once the quality of the DNA was verified, each sample was divided into two aliquots: one for species identification and the other for whole-g enome sequencing. Species identification was performed using direct Sanger sequencing of genes encoding cytochrome b molecules and heat shock protein 70, as described previously [31, 32]. The amplification products were purified with EXOSAP (Affymetrix, Santa Clara, CA, USA) and sequenced using the dideoxy-t erminal method on an automated capillary sequencer (AB3730; Applied Biosystems, Foster City, CA, USA). Subsequently, the sequences were subjected to blastn [33] for a similarity search with the *Leishmania* sequences deposited in GenBank [31]. The HSP-70 sequences obtained from the four Colombian clinical isolates were aligned with HSP70 sequences from other *Leishmania* species using clustal W. The analysis was done with sequences of *L. peruviana* (HF586368.1), *L. braziliensis* (GU071173.1), *L. guyanensis* (EU599093.1), *L. panamensis* (FN395055.1), *L. mexicana* (HF586413.1), *L. amazonensis* (HF586354.1) and *L. infantum* (JN676923.1). Later, maximum- likelihood (ML) trees were inferred using IQ-T REE 2 [34]. The robustness of the nodes was evaluated using the bootstrap method (BT, with 1000 replicates). Finally, the obtained tree was visualized and edited using the Interactive Tree Of Life V4 (http:// itol. embl. de) [35].

### Whole-genome sequencing of clinical isolates and DNA mapping

The extracted whole- genome DNA was sequenced on a HiSeq X- Ten system (Illumina; Novogene Bioinformatics Technology, Beijing, PR China). Briefly, mate- paired libraries were constructed by end repair (350 bp insert size) and subjected to paired- end sequencing (2×150 bp read length). Paired reads were discarded when reads with adapter contamination, >10 % uncertain nucleotides, or >50 % low-q uality nucleotides (base quality <5) were identified [36]. The paired- end Illumina reads of four Colombian clinical isolates and of the 183 global genomes (data downloaded from DDBJ/ENA/GenBank (http://www. ebi. ac. uk/ ena) were mapped to the reference *L. infantum* JPCM5 [37] and assembled with the smalt program (version 0.7.4) (http://www. sanger. ac. uk/ science/ tools/ smalt- 0). The JPCM5 genome was used as a reference in this study because so far it is the most comprehensive reference genome available, with a high quality, a robust assembly and without discontinuities and undetermined sequence. The mapping involved the following parameters: exhaustive search option ($-x$ and $-y$ of 0.8), a reference hash index of 13 bases, and a sliding step of 3. An identity threshold of $y$=0.8 was used to prevent the mapping of non- *Leishmania* reads to the reference sequences. The reads file merging, sorting, and elimination of PCR duplicates were implemented with SAMtools (version 0.1.18) and Picard (version 1.85: http:// broadinstitute. github. io/ picard/) [38].

### Phylogenomic inferences

The SNP alignments from whole nuclear and mitochondrial genomes for the complete dataset ($N$=187), as well as the alignment obtained for each antigen gene, rK39 (GenBank accession number: L07879), rK28 (GenBank accession number: HM594686) and K9/K26 (GenBank accession numbers: AF131227/AF131228, respectively), were used to evaluate the phylogenomic relationships among each *L. infantum* isolate. Maximum-l ikelihood (ML) trees were inferred using IQ- TREE 2 [34]. The robustness of the nodes was evaluated using the bootstrap method (BT, with 1000 replicates). The obtained tree was visualized and edited using the Interactive Tree Of Life V4 (http://i tol. embl. de) [35]. To detect recombination signatures in the 187 genomes analysed, phylogenetic networks were built in SplitsTree5 [39] using the neighbor-n et method. For all inferences, we included *L. infantum* JPCM5 as the reference genome [37] and, for the outgroup, we used the *L. donovani* (Ld ERR013297) genome assembly from DDBJ/ ENA/GenBank database (http://www. ebi. ac. uk/ ena) under Run accession ERR013297.

### Evaluation of chromosome and gene copy number variations (CNVs)

For the chromosomal somy estimation, the median read depth of each chromosome was initially calculated (di). All positions with a read depth of >1 standard deviations away from this initial median were then removed, and the di was recalculated from high- quality reads. Subsequently, the median depth of the 36 chromosomes (dm) of *L. infantum* was calculated, and the somy (S-v alue) of each chromosome was obtained with the following formula: $S$=2×di/dm [40]. The somy values calculated from sequencing data were averaged across the potentially variable somy of these cells. For this reason, somy values may be non- integers, representing the mean value of a mixed population. The ranges of monosomy, disomy, trisomy, tetrasomy and pentasomy were then used to define the full-c ell normalized chromosome depth or somy ($S$) as $S<1.5$, $1.5\leq S<2.5$, $2.5\leq S<3.5$, $3.5\leq S<4.5$ and $4.5\leq S<5.5$, respectively, as previously described [41].

To evaluate the CNVs at the gene level, we defined average haploid depth per gene without somy effect as dHG and the full cell depth with somy effect as dFG. Their relationship was defined as dFG=$S$× dHG. We evaluated the gene and chromosome copy numbers by considering their statistical significance. Significance was set at a $z$- score cutoff of >2 and an adjusted $P$- value (Student's $t$-t est) of <0.05. The heatmaps were created using the Heatmap3 package in R [42]. Finally, the genes that presented CNVs were subjected to Gene Ontology enrichment analyses using TriTrypDB tools (http:// tritrypdb. org), and Fisher's exact test was used to maintain the FDR below 0.05. The GO terms were submitted to REVIGO [43].

### Variant prediction calling and SNP filtering

With the purpose to detect SNPs in the 187 samples analysed, the reads of each genome were aligned to the reference *L. infantum* JPCM5 using the smalt program (version 0.7.4) (http://www. sanger. ac. uk/ science/ tools/ smalt-0 ). The Picard program (version 1.85) (http:// broadinstitute. github. io/ picard/) was used for merging and sorting bam files and marking duplicated reads, as previously described [41]. SNP calling was completed in GATK v4.0.2 https:// gatk.b roadinstitute. org/ hc/e n- us. More specifically, we used GATK's HaplotypeCaller to produce genotype VCF files for every isolate, combine GVCFs to merge the genotype VCF files of all isolates, genotype GVCFs to perform joint genotyping, and finally, select variants to filter the SNPs. We realigned around indels to remove these and retrieved only the SNPs. Low-quality SNPs were excluded using VariantFiltration when QUAL<500, DP <5, QD <2.0, FS >60.0, MQ <40.0, MQRankSum < −12.5, or ReadPosRankSum < −8.0. All candidate SNPs were visually inspected in the Integrative Genomic Viewer (IGV_2_3_47) [41] and SAMtools to avoid false positives. Considering the total number of SNPs and using the snp-d istans programme (https://g ithub. com/ tseemann/ snp- dists), we generated a pairwise distance matrix, and the results obtained were graphically represented. The SnpEff programme (version v4.1) [41] was used to classify all SNPs based on their functional impact, and from this selection, we identified the unique/shared SNPs between/within the five geographical groups evaluated; the data were included in an Excel matrix, which was used to perform the comparative analysis. Finally, from the allele frequency estimation data, we determined the homozygous and heterozygous variants. Allele shifts of <0.2 or >0.80 were considered homozygous variants, while allele shifts between 0.2 and 0.8 were heterozygous variants [44]. Once the variants were identified, they were counted using an Excel matrix.

### Genetic diversity and differentiation analysis

The genetic population structure of *L. infantum* was inferred from whole- genome SNP alignments using FastStructure v 1.0 [45]. The appropriate number of model components that explained the structure in the dataset was determined by the application of the algorithm for multiple choices of *K*, ranging from *K* = 1 to *K* = 10, considering nine independent simulations, which were conducted to estimate the cross-v alidation error values [46] (http:// dalexander. github. io/ admixture/ admixture- manual. pdf). The expected admixture proportions inferred by FastStructure were graphically represented by Distruct plots using the mean of the variational posterior distribution. Later, we grouped the 187 genomes included in this study according to their geographical distribution (Colombia, the Americas, Asia, North Africa and Europe). We evaluated and compared the genetic diversity between/within each of the groups using DnaSP software v.5.0. Finally, to evaluate the degree of genetic differentiation and gene flow among the five populations, we calculated the pairwise $F_{\mathrm{ST}}$ value using Arlequin3.5 software [47]. On the basis of the data obtained, we generated a pairwise distance matrix, which was graphically represented.

### rK39 and rK28 analysis

The Short Read Sequencing Typing 2 tool [48] was used to extract the rK39 and rK28 gene sequences from the whole genomes of the 187 isolates. Because rK28 was developed by fusing multiple tandem repeat sequences of three proteins, K26, LdK39 and K9, we also extracted and analysed these independently. The consensus sequences of each gene were aligned using the multiple sequence alignment programme mafft v7 (Suita, Osaka, Japan) [49], additionally the rK39 gene sequence was translated to amino acids (aa) using UGENE software [50], the DNA and amino acid sequences were grouped according to geographical origin (Colombia, the Americas, Asia, North Africa and Europe). The genomic diversity of each group of sequences was calculated. Diversity indices, such as number of haplotypes (h), haplotype diversity (Hd), and nucleotide diversity (π), were estimated using DnaSP software v.5.0. Neutrality indices, Tajima's D and Fu's Fs, were calculated using Arlequin v. 3.5.2.2 [47]. Additionally, to determine the potential evolutionary selection that occurred in these genes, the d*N*/d*S* rates were assessed using the Datamonkey programme (http://www. datamonkey. org) [51]. Finally, the nucleotide sequences were used to perform phylogenetic analysis following the parameters previously described in the Phylogenomic inferences section.

## RESULTS

### Identification of *Leishmania infantum*

The phylogenetic analysis based on HSP-70 sequences showed that the four Colombian Clinical isolates were closely related with *L. infantum*, while species of subgenera *Viannia* formed an independent cluster (Fig. S1). Once the Colombian samples were typed as *L. infantum*, we submitted the isolates to whole-g enome sequencing. The Colombian genomes were sequenced to a mean depth of coverage of 127X (Table S2).

### Nuclear and mitochondrial phylogenomic inferences

Two alignments were used to conduct phylogenomic analyses for 187 genome sequences (including four new genomes obtained in this study). The first corresponded to SNPs from nuclear genomes and the second to SNPs from mitochondrial genomes (Fig. 1). For both cases, *L. donovani* (Ld ERR013297) genomes were used as the outgroup. The phylogenetic reconstruction based on nuclear genome SNP alignments (Fig. 1a) showed *L. infantum* to be separated into various groups, some of which coincided with geographic origin. Tree topology revealed the existence of two clusters with well- supported nodes (with bootstrap ≥90.0). Cluster 1 (represented by a grey line outside the circle) was divided into three subgroups (1a to 1 c): subgroups 1a and 1b (highlighted in dark and medium beige, respectively) represented the five sequences from Cyprus included in this study, and subgroup 1c (highlighted in light beige) included 12 of the 13 sequences from Turkey, including the divergent sequence LI_ERR328068. Cluster 2 (represented by a black line outside the circle), like cluster 1, was subdivided in three subgroups (2a to 2c): subgroup 2a (highlighted in strong magenta) included all sequences from Uzbekistan and China used in this study; subgroup 2b (highlighted in violet) included five sequences from North Africa (Kairouan), sequences from Palestine and Israel, including one divergent sequence from Israel (LI_ERR205797); and the most heterogeneous subgroup, subgroup 2c (highlighted in light violet), included sequences from Europe, South and Central America, North Africa, one sequence from Israel (LI_ERR230194), and the four sequences from Colombia. We observed that Colombian genomes were closely related to sequences from Morocco and Italy. Finally, we highlighted the results obtained from genome LI_ERR205774 (Brazil), which showed high divergence compared with other sequences from the same or closer regions and genomes LI_SRR1109864, LI_SRR11098648 (North Africa), LI_ERR205735 (France), LI_ERR205787 (Panama) and LI_ERR205752 (Turkey), which were identified as outliers (Fig. 1a).

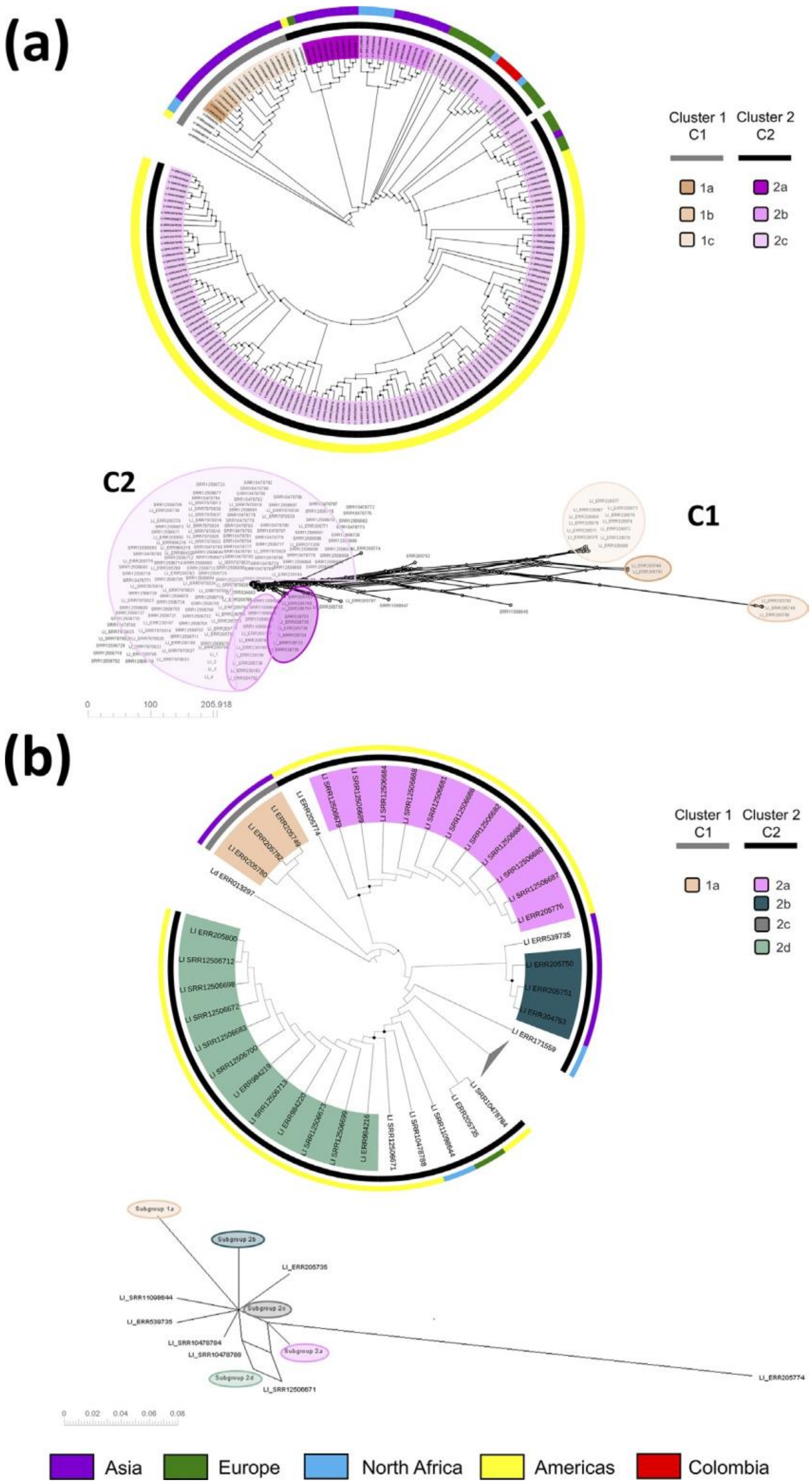


**Fig. 1.** Nuclear and mitochondrial phylogenies of 187 genomes of *L. infantum.* The trees located at the top of the figure represent the phylogenomic analysis based on SNP alignments for nuclear (a) and mitochondrial (maxicircle) (b) genomes of 187 genomic sequences analysed in this study. We used *L. infantum* JPCM5 as the reference genome (REF) and *L. donovani* (Ld ERR013297) as the outgroup. The first lines (grey and black) located on the outside of the circle represent the identified clusters, and the external lines (different colours) represent the geographical regions. Black dots represent well-s upported nodes (bootstrap ≥90). The figures located at the bottom of each tree represent the phylogenetic network (neighbor- net) constructed in SplitsTree 5 based on nuclear and mitochondrial (maxicircle) genome alignments. The grey triangle in the mitochondrial tree represents genomes with identical sequences (total of 151 genomes).

In the tree topology based on mitochondrial genome SNP alignment (Fig. 1b), we observed clustering events that showed little concordance with those observed in the topology of the nuclear genome. In general, we observed two clusters with well- supported nodes (with bootstrap ≥90.0). Cluster 1 (represented by a grey line outside the circle) was represented by the single subgroup 1a (highlighted in dark beige), which included three sequences from Cyprus. Cluster 2 (represented by a black line outside the circle) was divided into four subgroups (2a to 2d): subgroup 2a (highlighted in light purple) included sequences from Brazil, subgroup 2b (highlighted in teal) included sequences from Asia (Uzbekistan and Israel), subgroup 2c (denoted by a grey triangle) was represented by 151 genomes with identical sequences and distributed in different geographical regions, and subgroup 2d (highlighted in mint green) was represented by sequences from Brazil (Fig. 1b). Finally, we identified seven genomes that were considered outliers, including the highly divergent genome LI_ERR205774 (Brazil) (Fig. 1b). All findings were supported by phylogenetic tree topologies obtained in SplitsTree5 [38] (using the neighbor-net method), in which the members of these groups were consistently clustered together (bottom panel Fig. 1a, b).

## Chromosome/gene copy number variation within Colombian genomes and between worldwide genomes

### Chromosome copy number variation

Initially we analysed the somy values for the four Colombian clinical isolates obtained in this study. The results showed moderate aneuploidy in some genomes. In the LI_2 genome, we observed an unchanged (disomic) karyotype in 35/36 chromosomes, contrasting with what was observed in the three remaining genomes, which showed some degree of aneuploidy (Fig. S2). Analysis of the LI_1 genome provided evidence of an increase in the somy in 8/36 chromosomes (Chrs 1, 5, 13, 15, 20, 22, 26 and 31); the LI_3 genome showed aneuploidy in 5/36 chromosomes (Chrs 5, 8, 20, 23, and 31), and the LI_4 genome showed changes in nine chromosomes: Chrs 6, 7, 9, 22, 23 26 and 29 (trisomic); Chr 20 (tetrasomic); and Chr 31 (pentasomic) (Fig. S2). The allele frequency counts for each predicted heterozygous SNP did not exhibit discordance between read depths and allele frequencies, confirming the accuracy of the previously described somy profiles.

Later, the somy values for the 183 currently available genomes were estimated, and the results were compared to values obtained for the Colombian isolates. In general, the results evidenced moderate aneuploidy in the *L. infantum* genomes, and some chromosomes showed similar behaviour in all geographical regions, namely chromosomes 31 (tetrasomic) and 23 (trisomic) (Fig. 2a). Additionally, we found that some chromosomes presented behaviours particular to some geographical regions; for example, chromosome 5 was trisomic in all geographical regions except Europe; chromosome 20 remained trisomic in all groups except the American genomes; and finally, chromosomes 33 and 35 were trisomic only in the American and European genomes, respectively (Fig. 2b). Interestingly, the American *L. infantum* genomes (mainly from Brazil) showed different behaviour in the ploidy; 25 % of genomes evaluated (30/121) evidenced a low number of copies per chromosome compared with the remaining 75 %, which presented moderate ploidy (Fig. 2a, b).

### Gene copy number variations

After analysing the variations at the chromosomal level, we evaluated the CNVs for each gene ($z$- score cutoff >2 and adjusted $P$- value <0.05) and compared the occurrence in the four Colombian genomes. The results obtained did not reveal significant differences among them: the genome LI_4 showed the highest number of genes with CNVs (187 genes), followed by genome LI_3 (183 genes), genome LI_1 (177 genes) and finally genome LI_2 (174 genes). Later, with the purpose of analysing the genes with CNVs shared among the four Colombian genomes, we averaged the values obtained among the genomes, calculated standard deviations $\sigma$ and the corresponding $z$- score, the results revealed 188 genes shared among them; 143 (76 %) were annotated with known functions and 45 genes (24 %) as hypothetical proteins (Fig. 3a). Their functional annotations included mainly proteophosphoglycan, amastin surface glycoprotein, GP63-leishmanolysin, tb-292 membrane associated protein-like protein, elongation factor 1- alpha, beta tubulin, and heat shock protein 83–17 (Table S3). The next step was to evaluate and compare gene CNVs between the 187 genomes analysed, including the Colombian genomes. Initially, we averaged the values of the isolates for each geographical region and made a comparison between them. The initial results evidenced a moderate increase in the number of genes with CNVs in the Colombian genomes (188 genes) compared with genomes from other regions (the Americas: 124 genes, Asia: 131 genes, North Africa: 122 genes, and Europe: 137 genes) (Fig. 3a). Later, we studied in detail the genes with CNV in each geographical region, and the results demonstrated that the gene encoding proteophosphoglycan (LINF_350010200) presented significant CNVs in the 187 genomes. Additionally, we identified other genes with CNVs in some regions; for example, CNVs were observed in genes encoding rRNA, tb-292 membrane-associated protein- like protein, GP63-leishmanolysin, and putative amastin surface glycoprotein in Colombia and the Americas; genes encoding rRNA, tb-292 membrane associated protein-like protein, and putative amastin surface glycoprotein (LI_310009800) in Asia and North Africa;

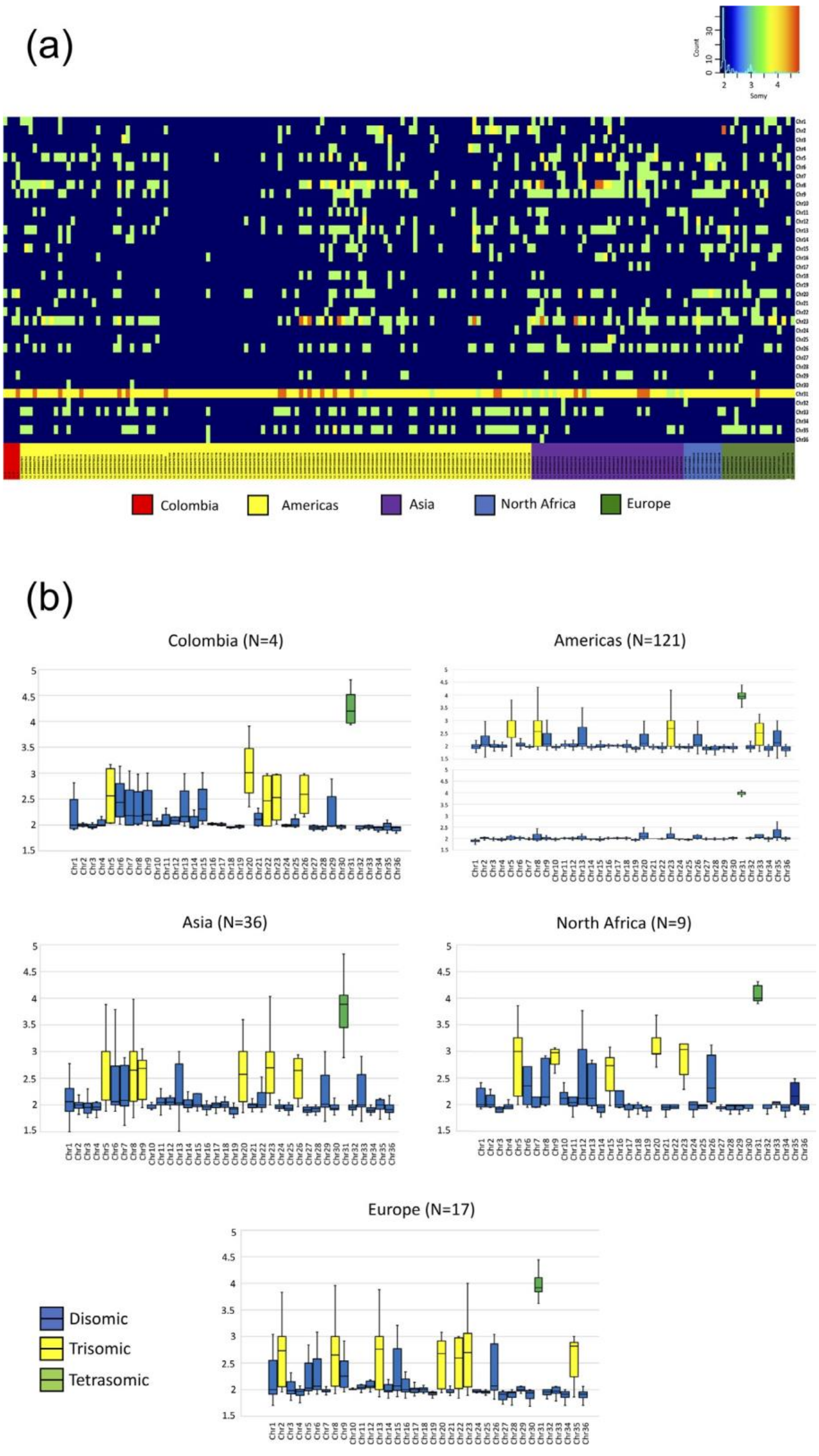
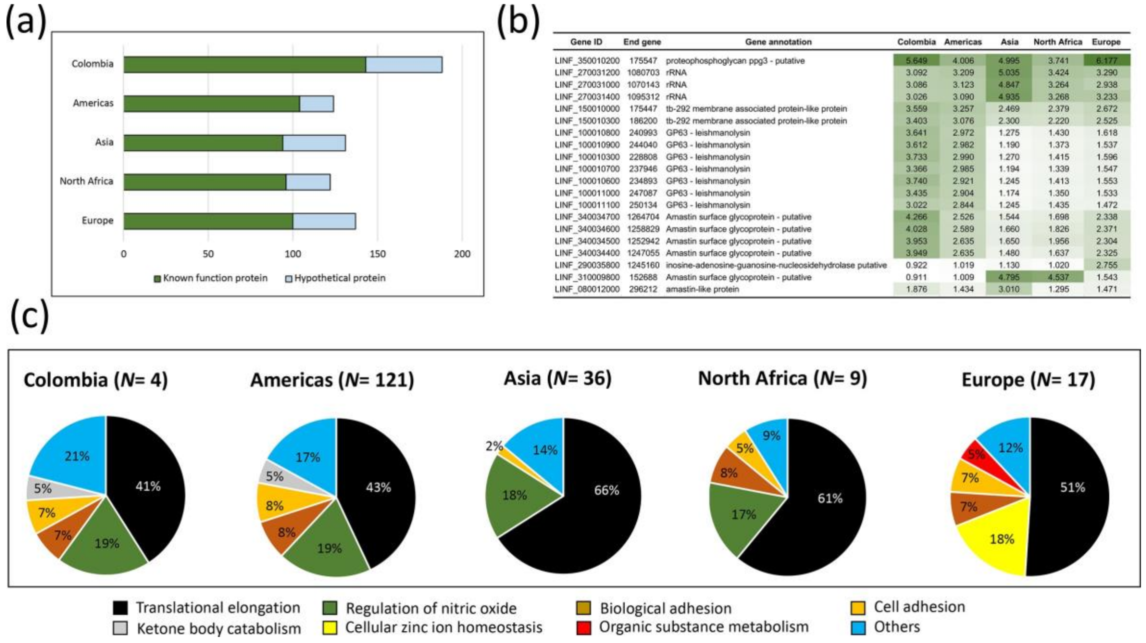


**Fig. 2.** Evaluation of chromosomal copy number in 187 *L. infantum* genomes. (a) Heatmap shows the estimated copy numbers of the 36 chromosomes (*y*-a xis) in the 187 genomes (*x*- axis). Disomic (blue), trisomic (green), tetrasomic (yellow), and pentasomic (orange). (b) Average copy number by chromosome in the five geographical regions evaluated. In the American panel, the top graphic represents the genomes with moderate ploidy (91 genomes), and in the bottom panel, it represents the genomes that presented low ploidy (30 genomes).



| Gene ID | End gene | Gene annotation | Colombia | Americas | Asia | North Africa | Europe |
|---|---|---|---|---|---|---|---|
| LINF_350010200 | 175547 | proteophosphoglycan ppg3 - putative | 5.649 | 4.006 | 4.995 | 3.741 | 6.177 |
| LINF_270031200 | 1080703 | rRNA | 3.092 | 3.209 | 5.035 | 3.424 | 3.290 |
| LINF_270031000 | 1070143 | rRNA | 3.086 | 3.123 | 4.847 | 3.264 | 2.938 |
| LINF_270031400 | 1095312 | rRNA | 3.026 | 3.090 | 4.935 | 3.268 | 3.233 |
| LINF_150010000 | 175447 | tb-292 membrane associated protein-like protein | 3.559 | 3.257 | 2.469 | 2.379 | 2.672 |
| LINF_150010300 | 186200 | tb-292 membrane associated protein-like protein | 3.403 | 3.076 | 2.300 | 2.220 | 2.525 |
| LINF_100010800 | 240993 | GP63 - leishmanolysin | 3.641 | 2.972 | 1.275 | 1.430 | 1.618 |
| LINF_100010900 | 244040 | GP63 - leishmanolysin | 3.612 | 2.982 | 1.190 | 1.373 | 1.537 |
| LINF_100010300 | 228808 | GP63 - leishmanolysin | 3.733 | 2.990 | 1.270 | 1.415 | 1.596 |
| LINF_100010700 | 237946 | GP63 - leishmanolysin | 3.366 | 2.985 | 1.194 | 1.339 | 1.547 |
| LINF_100010600 | 234893 | GP63 - leishmanolysin | 3.740 | 2.921 | 1.245 | 1.413 | 1.553 |
| LINF_100011000 | 247087 | GP63 - leishmanolysin | 3.435 | 2.904 | 1.174 | 1.350 | 1.533 |
| LINF_100011100 | 250134 | GP63 - leishmanolysin | 3.022 | 2.844 | 1.245 | 1.435 | 1.472 |
| LINF_340034700 | 1264704 | Amastin surface glycoprotein - putative | 4.266 | 2.526 | 1.544 | 1.698 | 2.338 |
| LINF_340034600 | 1258829 | Amastin surface glycoprotein - putative | 4.028 | 2.589 | 1.660 | 1.826 | 2.371 |
| LINF_340034500 | 1252942 | Amastin surface glycoprotein - putative | 3.953 | 2.635 | 1.650 | 1.956 | 2.304 |
| LINF_340034400 | 1247055 | Amastin surface glycoprotein - putative | 3.949 | 2.635 | 1.480 | 1.637 | 2.325 |
| LINF_290035800 | 1245160 | inosine-adenosine-guanosine-nucleosidehydrolase putative | 0.922 | 1.019 | 1.130 | 1.020 | 2.755 |
| LINF_310009800 | 152688 | Amastin surface glycoprotein - putative | 0.911 | 1.009 | 4.795 | 4.537 | 1.543 |
| LINF_080012000 | 296212 | amastin-like protein | 1.876 | 1.434 | 3.010 | 1.295 | 1.471 |

**Fig. 3.** Evaluation of gene copy number variation (CNV) and gene ontology enrichment analyses in 187 *L. infantum* genomes distributed in five geographical regions. (a) Number of genes that presented CNVs by geographical region. (b) Name and ID of genes that presented the highest CNVs. (c) Gene Ontology enrichment analyses for biological processes found in the five geographical regions.

and those encoding rRNA, tb-292 membrane associated protein- like protein, putative amastin surface glycoprotein, and putative inosine- adenosine- guanosine- nucleoside hydrolase in Europe (Fig. 3b). Finally, Gene Ontology enrichment analysis of the genes that presented CNVs indicated that between 41 and 66 % of the enriched terms in isolates from the five geographical regions were associated with translational elongation (with the highest percentages in Asia and North Africa [66 and 61 %, respectively]), and 19 % of genes were associated with the regulation of nitric oxide (except the isolates from Europe). Curiously, only the European isolates presented genes with CNVs that were involved in cellular zinc ion homeostasis (Fig. 3c).

## Variant prediction calling and SNP filtering

Initially, we analysed the four new Colombian genomes and compared them with the reference genome. The results revealed the total number of SNPs in the four isolates ranged from 1147 (LI_3 isolate) to 1182 (LI_4 isolate) without significant differences among them. The four genomes were characterized by low heterozygosity (~80 % of SNP loci were homozygous) (Fig. S3a). Of the total number of SNPs identified, 25 % had potential effects on gene function (high and moderate impact), and of these, ~98 % were synonymous variants. However, in the four Colombian genomes, we identified some variants with high functional impact (stop_gained and stop_lost), the SNPs associated with stop_gained were detected in genes encoding hypothetical proteins and, in a gene, encoding to known function protein as putative ubiquitin- conjugating enzyme. Interestingly, the four Colombian genomes shared the same SNPs associated with stop_lost, which were identified in the gene encoding kinetoplast- associated protein- like protein (LINF_270007500) (Table 1). When we evaluated the number of SNPs with functional impact for each chromosome per isolate; we observed that the distribution of SNPs among the chromosomes was homogeneous, with chromosomes 12 and 36 having the highest number of SNPs (Fig. S3b). Finally, we analysed the shared/unique SNPs among the four Colombian genomes, and 215 SNPs with high and moderate functional impact were found to be shared. Of these, 56 % (121 SNPs) were found in genes encoding proteins of known function (Table S4). When we evaluated and compared the unique SNPs in all isolates, we observed that the LI_2 and LI_4 genomes had the greatest numbers of unique SNPs (25 and 28, respectively) not found in the other analysed isolates (Fig. S3c).

Later, we focused our analysis on the 183 genomes from across the world and compared those from the different geographical regions. Initially, we considered the SNP density (including in Colombian genomes) and observed a variable density ranging from 2.7 SNP/Kb to 0.013 SNPs/Kb. The genomes with greatest variability were from Cyprus (2.7 to 2.2 SNPs/Kb), followed by genomes from Turkey (1.8 to 1.4 SNPs/Kb), one genome from Brazil: LI_ERR205774 (1.5 SNPs/Kb), and the genomes from Zaghouan and Nabeul (1.43 and 1.40 SNPs/Kb, respectively). The genomes with the lowest variability were Brazilian (Figs 4a and S4).

**Table 1.** List of genes that showed SNPs with high functional impact, in the four Colombian genomes analysed (a) SNP position on the gene. (b) Change of nucleotide respect to reference (Ref/Alt).

| **LI_1** | | | | |
|---|---|---|---|---|
| **Gene ID** | | **a** | **b** | **Gene annotation** |
| LINF_270007500 | stop_Lost | 62 087 | A/C | kinetoplast- associated_protein- like_protein |
| **LI_2** | | | **b** | |
| **Gene ID** | | **a** | | **Gene annotation** |
| LINF_200019900 | stop_gained | 648 231 | G/A | hypothetical_protein_-_conserved |
| LINF_270007500 | stop_gained | 61 967 | C/A | kinetoplast- associated_protein- like_protein |
| LINF_270007500 | stop_lost | 62 087 | A/C | kinetoplast- associated_protein- like_protein |
| LINF_330039100 | stop_gained | 1 344 679 | C/G | hypothetical_protein_-_conserved |
| **LI_3** | | | **b** | |
| **Gene ID** | | **a** | | **Gene annotation** |
| LINF_270007500 | stop_lost | 62 087 | A/C | kinetoplast- associated_protein- like_protein |
| LINF_330039100 | stop_gained | 1 344 679 | C/G | hypothetical_protein_-_conserved |
| **LI_4** | | | **b** | |
| **Gene ID** | | **a** | | **Gene annotation** |
| LINF_130021300 | stop_gained | 598 485 | C/T | ubiquitin- conjugating_enzyme_-_putative |
| LINF_270007500 | stop_lost | 62 087 | A/C | kinetoplast- associated_protein- like_protein |
| LINF_360069500 | stop_gained | 2 335 305 | C/A | glycerophosphoryl_diester_phosphodiesterase_-_putative |

The results showed the largest number of variants in chromosomes 12, 22 and 27 and less variability in chromosomes 2, 5 and 7 (Fig. 4b). Finally, we analysed the number of shared SNPs within and between each group evaluated. The results showed the greatest number of shared SNPs in the Colombian genomes (862 shared SNPs; *N*=4), and there were moderate numbers of shared SNPs in the other geographical regions: the Americas (205 SNPs; *N*=121), Asia (187 SNPs; *N*=36), North Africa (209 SNPs; *N*=9) and Europe (121 SNPs; *N*=17). During this analysis, we discovered that all genomes belonging to certain countries shared SNPs (high functional impact) that were not found in other regions (e.g. the 121 genomes from Brazil shared a mutation in the gene LINF 080015600, histone deacetylase [putative], which was not found in other countries), and the same occurred with genomes from Honduras, China and Cyprus (Table 2). We evaluated the shared SNPs between the different geographical regions and found the regions sharing the most SNPs were Colombia–North Africa and Colombia–Europe (202 and 193 shared SNPs, respectively), and the group with the least shared SNPs was North Africa– America (85 SNPs). Additionally, we identified 49 shared SNPs between the 187 genomes analysed, 38 % of which (19 SNPs) were located in genes with known functions. We highlighted those SNPs located in genes involved in resistance to pentamidine (LINF_230007800), in pteridine/ glucose transport (LINF_100009100 LINF_330008300), and in glycolysis and gluconeogenesis, that is, glucose 6 phosphate isomerase (LINF_120010600), as well as genes associated with the intracellular signal pathway, such as phosphatidylinositol 3 kinase (LINF_270015200) (Table of Fig. 4c)

## Genetic diversity and differentiation analysis

The population organization of the 187 isolates was analysed with FastStructure software, which indicated the existence of four different populations (*K*=4). The largest population—POP1 (highlighted in blue)—comprised 100 % of Colombian, Honduran and Brazilian genomes (except LI_ERR205774) and genomes from Europe (France, Italy, Spain and Portugal), North Africa (Morocco and Kairouan) and Asia (Israel and Palestine). POP2 (highlighted in green) represented 100 % of the genomes from China and Uzbekistan, one genome from France, and curiously, the unique Panamanian genome. POP3 (highlighted in orange) included all genomes from Turkey (except LI_ERR205752) and some genomes from Cyprus, while POP4 (highlighted in light purple) included the remaining genomes from Cyprus. Additionally, we identified four highly heterogeneous genomes: LI_ SRR11098647 (Nabeul), LI_ SRR11098648 (Zaghouan), LI_ERR205752 (Turkey) and LI_ERR205774 (Brazil) (Fig. 5). Later, the 187 genomes were grouped according to their geographical location three sequences from Asia; subgroup 2c (highlighted in light beige), which was the most heterogeneous, included sequences from three of the five geographical regions analysed (Asia, Europe, Americas [Brazil and Panama]); and subgroup 2d (highlighted in light green) was represented mainly by sequences from Brazil, the two sequences from Honduras, and some from Asia and Europe (Fig. 6a). The analysis performed on the amino acid sequence of rK39 protein, specifically to the region used as target in the diagnostic tests (39 amino acid repeated 6.5 times), revealed a non- synonymous substitution in the amino acid 27, we observed a change of a methionine (M) or serine (S) by a threonine (T). This substitution was identified in four different sites of the protein (residues 720, 759, 836 and 876) (Fig. 7).

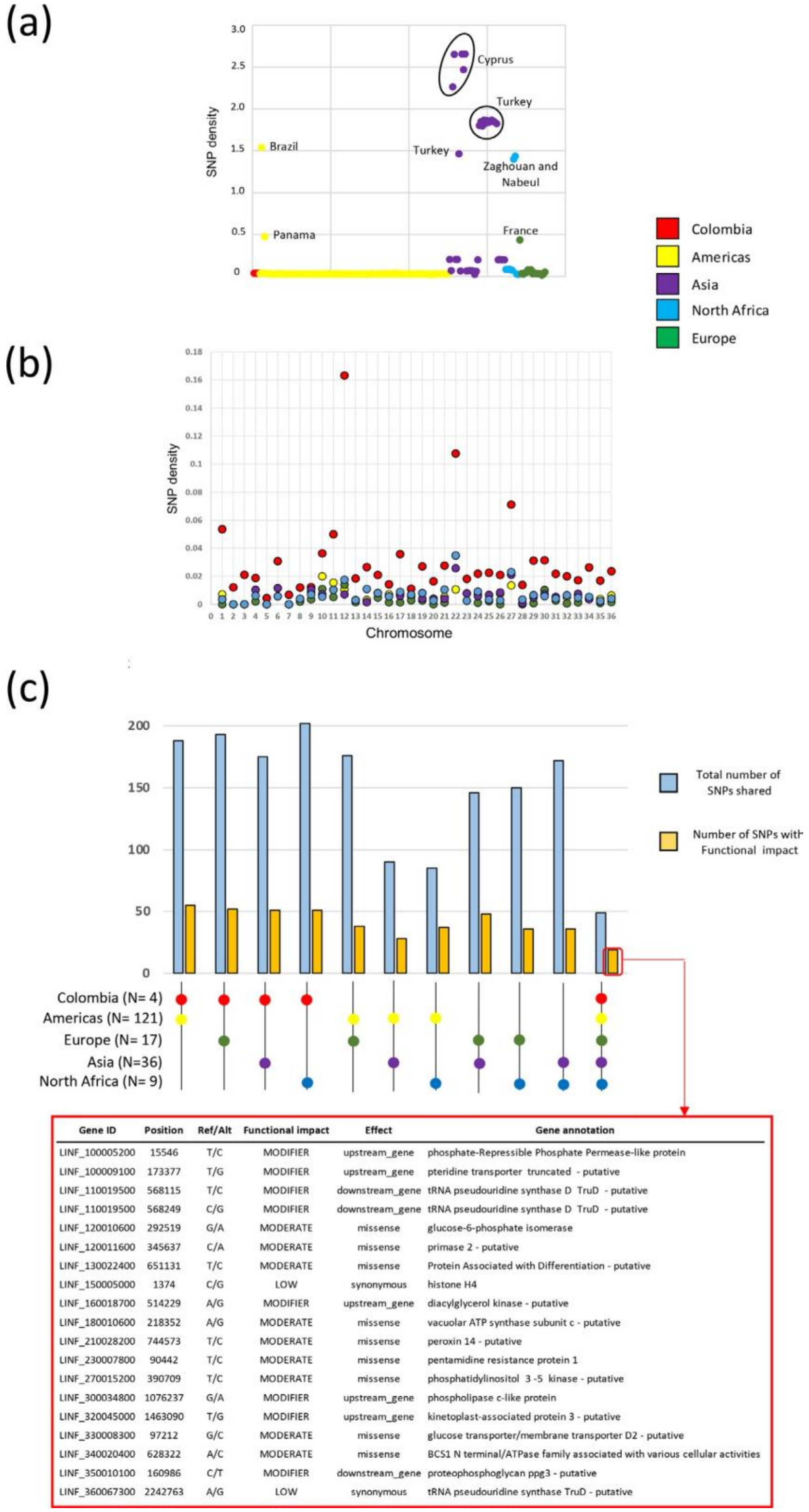
Considering that rK28 is a chimeric antigen composed of three amino acid sequences from different proteins, we analysed not only the complete chimaera (rK28fus) but also each component protein separately (K9, K26 and LdK39). Analysis of the rK28fus protein revealed the formation of two well-s upported clusters. Cluster 1 (represented by a grey line outside the circle) was represented by 17/121 (14 %) sequences from Brazil and four samples from Asia. Cluster 2 (represented by a black line outside the circle), which showed high heterogeneity, was divided into eight groups: subgroup 2a (highlighted in violet) included sequences from Brazil; subgroup 2b (highlighted in yellow) included sequences from Brazil, one sequence from Colombia, and some sequences from Europe and Asia; subgroup 2 c (highlighted in light beige) was represented mainly by sequences from Brazil, three sequences from Colombia, two sequences from North Africa, and one sequence from Asia and Europe; subgroups 2d–2g were the most heterogeneous subgroups and included sequences from various geographical regions; and subgroup 2h (highlighted in turquoise) was represented mainly by sequences from Brazil and one sequence from each of North Africa and Europe (Fig. 6b). As observed for the two previous genes, K9 was grouped into two clusters. Cluster 1 was divided into two groups (subgroups 1a and 1b) and cluster 2 was divided into three groups (subgroups 2a–2c), with each one revealing highly heterogeneous grouping regarding the geographical distribution (Fig. 6c). Finally, the analysis of K26 and LdK39 proteins revealed great differences between each of them and the sequences analysed, and the variation generated gaps that did not allow us to make an adequate phylogenetic analysis.

Additionally, we evaluated and compared the genomic sequence diversity of rK39, rK28fus and K9 while considering geographical origin. Of the three genes, rK28 presented the highest number of haplotypes in all geographical regions (Table 3). On the basis of the haplotype (Hd) and nucleotide diversity indexes ($\pi$) the genes rK39 and rK28fus, the North African sequences presented the greatest genetic (Hd=1) and nucleotide diversities ($\pi$=0.014 and $\pi$=0.015, respectively), contrary to what was observed for gene K9, which contained the greatest genetic (Hd=1) and nucleotide diversities ($\pi$=0.015) in Colombia. Tajima's D index was then used to determine the extent of neutral selection. Although the values were not statistically significant ($P > 0.05$), the Tajima tests for rK39 showed negative values for all geographical regions, except Asia.

For rK28, negative Tajima values were observed for the Americas, North Africa and Europe, and positive values were observed for Colombia and Asia. Finally, the results for K9 were negative for genomes from Colombia and Asia and positive for those from Europe, America and North Africa. Regarding Fu's Fs index, the values for genes rK39 and rK28fus were negative and significant ($P < 0.05$) in sequences from the Americas, Asia, North Africa and Europe; and for gene K9, negative and significant values were observed in sequences from the Americas, Asia and Europe (Table 3). Finally, a test for positive selection was carried out by analysing the $dN/dS$ ratio. Interestingly, the results revealed a ratio of >1.0 for the rK28fus gene in all geographical regions (except Colombia) and ratios of <1.0 for rK39 and K9 (Table 3).

## DISCUSSION

The objective of this study was to understand the phylogenomic relationships, genetic structure and intraspecific variability of *L. infantum* worldwide. A phylogenetic analysis of the nuclear genomes allowed us to identify the presence of different *L. infantum* phylogroups circulating in continents around the world (Fig. 1a). These findings were consistent with the population structures defined by STRUCTURE and the *F* statistics ($F_{ST}$) (Fig. 5, Table S5), which revealed the existence of genetically distinct



| Gene ID | Position | Ref/Alt | Functional impact | Effect | Gene annotation |
|---|---|---|---|---|---|
| LINF_100005200 | 15546 | T/C | MODIFIER | upstream_gene | phosphate-Repressible Phosphate Permease-like protein |
| LINF_100009100 | 173377 | T/G | MODIFIER | upstream_gene | pteridine transporter truncated - putative |
| LINF_110019500 | 568115 | T/C | MODIFIER | downstream_gene | tRNA pseudouridine synthase D TruD - putative |
| LINF_110019500 | 568249 | C/G | MODIFIER | downstream_gene | tRNA pseudouridine synthase D TruD - putative |
| LINF_120010600 | 292519 | G/A | MODERATE | missense | glucose-6-phosphate isomerase |
| LINF_120011600 | 345637 | C/A | MODERATE | missense | primase 2 - putative |
| LINF_130022400 | 651131 | T/C | MODERATE | missense | Protein Associated with Differentiation - putative |
| LINF_150005000 | 1374 | C/G | LOW | synonymous | histone H4 |
| LINF_160018700 | 514229 | A/G | MODIFIER | upstream_gene | diacylglycerol kinase - putative |
| LINF_180010600 | 218352 | A/G | MODERATE | missense | vacuolar ATP synthase subunit c - putative |
| LINF_210028200 | 744573 | T/C | MODERATE | missense | peroxin 14 - putative |
| LINF_230007800 | 90442 | T/C | MODERATE | missense | pentamidine resistance protein 1 |
| LINF_270015200 | 390709 | T/C | MODERATE | missense | phosphatidylinositol 3 -5 kinase - putative |
| LINF_300034800 | 1076237 | G/A | MODIFIER | upstream_gene | phospholipase c-like protein |
| LINF_320045000 | 1463090 | T/G | MODIFIER | upstream_gene | kinetoplast-associated protein 3 - putative |
| LINF_330008300 | 97212 | G/C | MODERATE | missense | glucose transporter/membrane transporter D2 - putative |
| LINF_340020400 | 628322 | A/C | MODERATE | missense | BCS1 N terminal/ATPase family associated with various cellular activities |
| LINF_350010100 | 160986 | C/T | MODIFIER | downstream_gene | proteophosphoglycan ppg3 - putative |
| LINF_360067300 | 2242763 | A/G | LOW | synonymous | tRNA pseudouridine synthase TruD - putative |

**Fig. 4.** Overview of SNPs identified in 187 *L. infantum* genomes. (a) Density of SNPs in each of the 187 genomes. (b) Relationship between SNP density and chromosomes in each of the five geographical regions. (c) Number of shared SNPs (total number represented by blue bars, and number of SNPs with functional impact represented by orange bars) between the five geographical regions. The coloured dots located in the

**Table 2.** List of genes with unique SNPs representing high functional impact in each region analysed. (a) SNP position on the gene. (b) Change of nucleotide respect to reference (Ref/Alt).

| **Brazil** | | | | |
|---|---|---|---|---|
| **Gene ID** | | **a** | **b** | **Gene annotation** |
| LINF 080015600 | stop gained | 437 460 | C/A | histone deacetylase - putative |
| **Honduras** | | | | |
| **Gene ID** | | **a** | **b** | **Gene annotation** |
| LINF 080011500 | stop gained | 266 177 | C/T | hypothetical protein - conserved |
| LINF 270033100 | stop gained | 1 130 864 | T/G | tubulin- tyrosine ligase- like protein |
| LINF 330041100 | stop gained | 1 482 887 | G/A | Holliday- junction resolvase- like of SPT6/SH2 domain |
| **China** | | | | |
| **Gene ID** | | **a** | **b** | **Gene annotation** |
| LINF 170016300 | stop gained | 445 751 | G/T | ATP-d ependent RNA helicase - putative |
| LINF 360033600 | stop gained | 1 067 054 | G/A | related to multifunctional cyclin- dependent kinase pho85- like protein |
| **Cyprus** | | | | |
| **Gene ID** | | **a** | **b** | **Gene annotation** |
| LINF 260014500 | stop gained | 276 029 | G/T | hypothetical protein - conserved |
| LINF 340005800 | stop gained | 30 935 | C/T | hypothetical protein - conserved |

**Fig. 5.** Estimated population structure of 187 *L. infantum* genomes. Bar graph generated by FastStructure software inferring the genetic structure of 187 genomes of *L. infantum*. Each genome is represented by a single vertical line in the barplots; each colour represents one population (*K*=4), and the lengths of the colour segments show the genomes' estimated proportion in that population. The bars at the bottom of the graph represent the geographical origin.

*L. infantum* populations, with the first being represented by genomes from the Americas, Europe, and some from Asia and Africa; the second and third populations represented by Chinese and Turkish genomes; and a fourth population represented by genomes from Cyprus; although the clustering of Turkish genomes was probably related to the organism from which it was isolated (sandfly vector. Overall, however, *L. infantum* demonstrated low genetic variability (Fig. 4a), the results obtained in this study

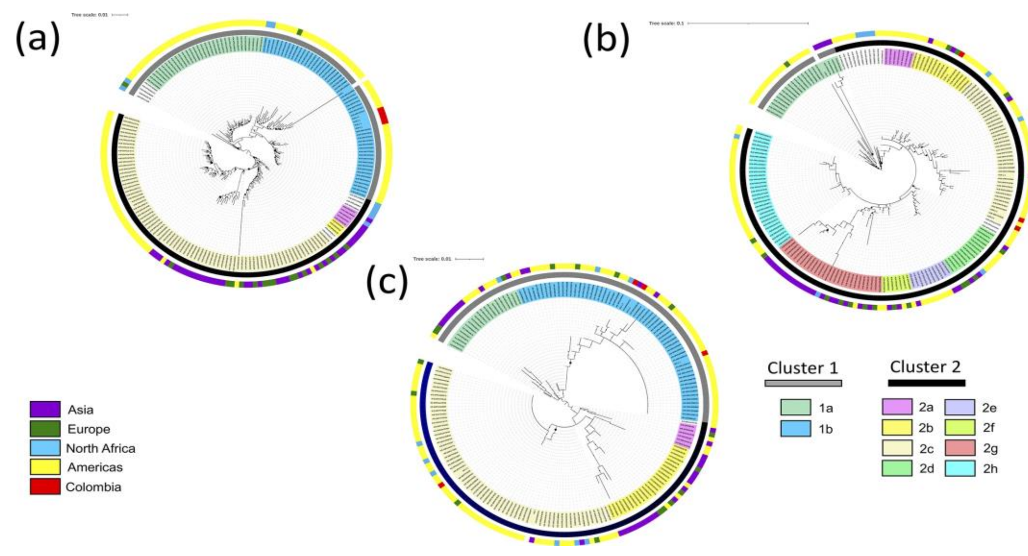


**Fig. 6.** Phylogenetic analysis of rK39, rK28 and K9 genes obtained from 187 *L. infantum* genomes. The trees represent the phylogenetic analysis based on rK39 (a), rK28fus (b) and K9 (c) genes. We used the sequences of rK39 (L07879), rK28 (HM594686) and K9 (AF131227) genes from GenBank as a reference (Ref). The colours inside the circle represent the subgroups, the first lines (grey and black) on the outside represent the identified clusters, and the external lines (different colours) represent the geographical regions. The black dots represent well-supported nodes (bootstrap nodes (bootstrap ≥90)

suggest there is genetic diversification at a global level in this species, diversification that could be generated by the immense human migrations between countries and/or by the adaptation of the parasite to the distinct genetic structures of the sandfly vector/reservoirs, which have been demonstrated to play an important role in the genetic structure of *L. infantum* [52, 53]. Additionally, we found evidence of the close phylogenomic relationships between genomes from different geographical regions (e.g. between genomes from China, Panama and France; between genomes from Italy, Portugal and Israel; and between genomes from Colombia, Europe and North Africa) (Figs 1a and 5), which is relevant, considering that the appearance of *L. infantum* strains in different regions could generate fitness with potential implications in the spread of virulent strains, the exploitation of different and new vectors and hosts, resistance to anti-leishmanial agents, and adaptation to new ecological niches.

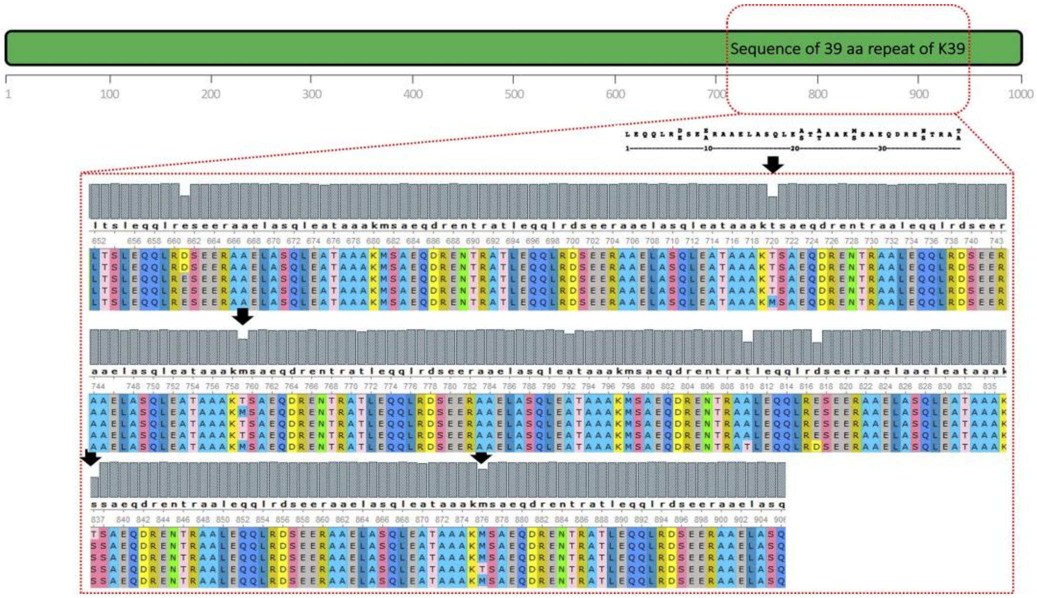


**Fig. 7.** Multiple alignment of rK39 protein between the 187 *L. infantum* genomes. The top bar represents the sequence of K39 protein (amino acid 1–950). In this sequence, the dotted square shows the region used as target in the immunochromatographic diagnostic test (39 amino acid repeated 6.5 times). The bottom shows the multiple alignment of the target region, among the 187 *L. infantum* genomes. The non- synonymous substitutions found between the genomes are represented with black arrows.

**Table 3.** Diversity indexes calculated for the rK39, rK28fus and K9 genes in the different geographical regions evaluated

| | rK39 gene | | | | | rK28fus gene | | | | | rK9 gene | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Colombia** | **Americas** | **North Africa** | **Asia** | **Europe** | **Colombia** | **Americas** | **North Africa** | **Asia** | **Europe** | **Colombia** | **Americas** | **North Africa** | **Asia** | **Europe** |
| Sample size | 4 | 121 | 9 | 36 | 17 | 4 | 121 | 9 | 36 | 17 | 4 | 121 | 9 | 36 | 17 |
| Haplotypes | 3 | 66 | 9 | 32 | 16 | 3 | 82 | 9 | 34 | 16 | 4 | 33 | 5 | 25 | 12 |
| No. of sites with substitutions (S) | 4 | 57 | 10 | 29 | 41 | 6 | 36 | 23 | 47 | 19 | 10 | 14 | 12 | 25 | 13 |
| Haplotype diversity | 0.833 | 0.974 | 1.000 | 0.992 | 0.993 | 0.833 | 0.985 | 1.000 | 0.993 | 0.992 | 1.000 | 0,863 | 0,833 | 0,971 | 0,949 |
| Nucleotide diversity (π) | 0.002 | 0.008 | 0.014 | 0.011 | 0.011 | 0.004 | 0.011 | 0.015 | 0.014 | 0.006 | 0.015 | 0,014 | 0,013 | 0,014 | 0,013 |
| Tajima's D | −0.781 | −1.222 | −0.437 | 0.778 | −1.147 | 0.179 | −0.554 | −0.498 | 0.031 | −0.651 | −0.528 | 2.427 | 0,269 | −0,251 | 0,645 |
| Fs | 0.133 | −24.98 * | −6.462 * | −22.722* | −7.431* | 0.888 | −24.758* | `−3.539* | −17.949* | −11.825* | −0.480 | −11.816* | 1.043 | −15.078* | −4.210* |
| dN/dS index | 0.321 | 0.409 | 0.247 | 0.180 | 0.453 | 0.382 | 1.940 | 4.01 | 1.053 | 1.42 | 0.838 | 0,991 | 0,924 | 0,876 | 0,993 |

*$P<0.05$.

The close relationships between the Colombian genomes and the European/North African genomes (Fig. 1a), the low genetic divergence among genomes from these regions

($F_{ST}$ = 0.10230 and 0.0608, respectively) (Table S5), the high number of shared SNPs (Fig. 4c), and the population structure obtained (POP1) (Fig. 5) support the hypothesis that *L. infantum* was introduced several times and at several points along the American continent from Europe, possibly during Spanish and Portuguese colonizations [53–56]. From the evidence, we propose that North African strains were introduced into Colombia possibly during the arrival of African immigrants/slaves [57], as occurred with *Plasmodium falciparum*, which came to the Americas via the trans-Atlantic slave trade [58]. We theorize that the establishment of North African genotypes in Colombia but not in regions geographically close to Colombia (e.g. Brazil), as well as the distant genomic relationship between the Colombian and Brazilian genomes (Fig. 1a, Table S5), could be associated with the geographical distribution of vectors and reservoirs species found in both countries (*Pintomyia evansi* and *Didelphis marsupialis* in Colombia [59, 60] and *Lutzomyia cruzi* and *D. albiventris* [61, 62] in Brazil). The geographical distributions of some of these vectors are restricted and limited to specific biomes (sylvatic, domestic or peridomestic), which could affect not only the dynamics of *L. infantum* transmission but also the epidemiology of the disease. Together, these findings led us to elucidate the possible origin of *L. infantum* strains in Colombia and to understand the mode of dispersion of this parasite in the American territory. However, we believe that genotyping more strains from South America (mainly from Colombia) would help to test this hypothesis. Additionally, the phylogenetic analysis of mitochondrial (maxicircle) genomes allowed us to identify the high level of intra-specific conservation of *L. infantum* worldwide (Fig. 1b) and once again demonstrated the utility of this region as suitable phylogenetic markers for *Leishmania* typing and for exploring evolutionary relationships within the trypanosomatids [63, 64].

We subsequently analysed the structural changes at the chromosome/gene level. Previously, various studies have described aneuploidy as an important mechanism of adaptation used by Old and New World *Leishmania* species [36, 40, 41, 65, 66]; however, few studies have focused on analysing whether the structural changes observed in species are maintained across different geographical regions. The results obtained confirm not only the widely described [10, 12–15, 41, 65, 67] extensive aneuploidy of *L. infantum* but also the conservation of these structural changes at the global level (Figs 2 and S2), which suggests that, regardless of the genetic background of the host and the eco-epidemiological niche in which the parasite exists, the chromosomal architecture of *L. infantum* needed to undergo extensive changes during possible adaptation mechanisms. On the other hand, and despite observing a low number of genes with CNVs in our dataset (Fig. 3a), the ontology enrichment analysis revealed an increase, at the global level, in genes directly involved with the virulence and infectivity of the parasite (e.g. genes associated with translational elongation). There was also an increase (except in European genomes) in genes involved in defenses against oxidative stress (regulation of nitric oxide) [68] (Fig. 3c). These findings highlighted and confirmed the importance of these genes in *L. infantum* as possible mechanisms of survival and adaptation in both the vector and the mammal host [69]. Finally, we identified an increase in genes involved in the transport and homeostasis of zinc (a component crucial in the cellular functions of this parasite) [70]. Despite these genes having been previously reported in *L. infantum* [70] and other *Leishmania* species [71, 72], it is interesting that the increase was observed only in the European genomes. Considering these findings, we believe that further studies are necessary to determine if this variation depends on the geographical area analysed or if it is the result of the culture conditions.

To evaluate the intraspecific genomic variability of *L. infantum* worldwide, we analysed the presence of nucleotide- level variations (SNPs) in the 187 genomes. The results demonstrated that, in general, *L. infantum* presents low genetic diversity compared with other *Leishmania* species [41, 66, 73]. When we analysed and compared this diversity among the five geographical regions, we found *L. infantum* from South America had the lowest genetic heterogeneity (Figs 4a and S4), a property previously ascribed to Brazilian genomes [9, 10, 14, 74]. We consider that this low diversity could be associated with (i) the relatively recent arrival of *L. infantum* to the Americas [53, 55]; (ii) the number of vectors and/or animal reservoirs involved in the transmission cycle [75, 76] where *L. infantum* could have easily adapted, thus favouring certain genotypes and promoting the stochastic loss of others (as has been described in *Trypanosoma brucei* [77] and suggested for other *Leishmania* species, such as *L. panamensis* [72]); or (iii) the low number of samples included in the study (Colombia *N*=4), which could influence the accuracy of the results. Therefore, additional studies involving representative sampling from Colombia are necessary to confirm these findings. Additionally, the SNP analysis allowed us to identify, at the global level, great variations in the chromosomes (Fig. 4b) containing genes involved in parasite virulence or defence against harmful oxidative effects, such as the A2 family genes found on chromosome 22 required for visceralization [78] and recently assembled for *L. infantum* [37], and heat shock protein 70 and trypanothione synthetase genes found on chromosome 27, which are associated with resistance and metabolism antimony. Another interesting finding was the identification of 29 SNPs shared between the 187 genomes that were located in genes associated with drug resistance, metabolism and signal pathways (Fig. 4c). These results agree with the results of previous studies [14, 79] and suggest that some of these changes could be the result of positive/purifying selection events that bestowed certain advantages upon the parasite in its adaption to ecological niches or promoted the transmission cycle.

Finally, the data for the 187 genomes included in the study were used to analyse the genetic diversity of the antigens used in VL diagnosis (rK28 and rK39). The results revealed there is high molecular divergence at the global level among the genomes (Fig. 6), which agrees with studies conducted on *L. donovani*, demonstrating the extensive genetic diversity among genotypes and geographical origins [21, 80, 81]. The rK39 nucleotide sequence revealed a moderate genetic heterogeneity of this protein mainly in samples from Europe and Africa (Fig. 6a); genetic variability that could generate a differential antibody response in the host, impacting directly the immunochromatographic tests performance. The results obtained in this study and the hypothesis proposed is in line with the low sensitivity of these diagnostic tests, reported in East Africa, France and Spain, that have showed sensitivity percentages of 75–85.3 %, 88.5 and 78 %, respectively [17, 20–23, 82]. Contrasting the results observed in Colombia and some regions of Brazil where the sensitivity percentage ranked between 85–93 % [18, 24], findings that could agree with the low genomic variability (Fig. 6a) described in this study.

Despite the three markers (rK28/K9 and rK39) showed genetic variability in many of the geographical regions evaluated, we found rK28fus had the highest variability, which was evidenced in the highest number of clustering events (Fig. 6b), the highest haplotypic diversity and the number of substitution sites (*S*), as well as the highest neutrality test (Tajima's D and Fu's Fs) and d*N*/d*S* index scores (Table 3). Despite some studies describe that the rK28- based tests present a high sensitivity (95–100 %) in countries such as Sudan, Ethiopia and India suggesting a drastic genetic drift in the sequences evaluated. Herein, the results demonstrate that rK28 is a protein highly diverse worldwide, mainly in countries of North Africa and Asia. This variability could impact indirectly in the sensitivity of diagnostic tests. Likewise, to analyse target sequence of rK39 protein used in the diagnostic tests, an interesting non- synonymous substitution in the amino acid 27 of the protein was observed (change of a methionine [M] or serine [S] by a threonine [T]) (Fig. 7). Considering the biochemical characteristics of the amino acid and the fundamental role they play in both the structure and the functionality of the protein, we consider that additional studies should be performed to evaluate the possible consequences of the change observed.

The high polymorphism found in the rK28 marker at the global level, the low phylogenomic relationships observed among sequences from geographically close countries (e.g. Brazil and Colombia) (Fig. 6b), the limited specificity of this marker in some geographical regions [26, 83], and the discrepancies in the specificity found among kits based on this or other antigens (e.g. rK39) [18] used in different geographical regions [20, 26] highlight the need for diagnostic tests designed that contain as base specific antigens from each geographical region. More specific tests will contribute considerably to the rapid and effective global diagnosis of canine and human VL, as well as decrease the frequency of false-negative reports (~10.5 %) [84] and the degree of cross-reactivity with other diseases (e.g. malaria, intestinal schistosomiasis, and tuberculosis) observed [26, 85].

In summary our whole-genome sequence data represents, to date, the biggest global distribution of *L. infantum* analysis. The results obtained for this species demonstrate the low variation at the gene level, the extensive aneuploidy, the low levels of allelic diversity, and the presence of different phylogroups of *L. infantum* circulating in the world. Furthermore, we found a close relationship between the Colombian *L. infantum* genomes and the North African and European genomes. Finally, this was the first study to use whole-genome sequencing that demonstrated the high genetic variability of the main antigens used in the diagnosis of VL at the global level. These findings are relevant, as they facilitate an understanding of the genetic behaviour of these antigens according to geographical area, and the data indicate there is a need to develop for new diagnostic targets, which would contribute to improving the rapid diagnostic techniques for VL.

Funding information

This research was funded by Dirección de Investigación e Innovación from Universidad del Rosario.

Acknowledgements

We would like to thank the Programa de Control y Estudio de Enfermedades Tropicales (PECET) and Universidad Sucre (Colombia) for the assistance with the sampling. We also thank Dr. Hideo Imamura for assistance in the analyses. This work was funded by DIRECCIÓN DE INVESTIGACIÓN E INNOVACIÓN from Universidad del Rosario. Juan David Ramírez González, Ph.D. is a Latin American fellow in the Biomedical Sciences supported by The Pew Charitable Trusts. We thank Suzanne Leech, Ph.D., from Edanz Group (https:// en- author- services. edanz. com/ ac) for editing a draft of this manuscript.

### Author contributions

Conceptualization: L.H.P and J.D.R. Methodology: L.H.P, M.M and A.C. Formal analysis: L.H.P and M.M. Investigation: L.H.P and J.D.R. Supervision: J.D.R. Writing – original draft: L.H.P and J.D.R. Writing – review and editing: L.H.P, J.D.R. A.C, M.M, C.M, M.R. and E.B.

### Conflicts of interest

The authors declare that there are no conflicts of interest.

### Ethical statement

This study was approved by the Ethics Committee of the Universidad de Antioquia (Number VRI3445/2010) and the Universidad de Sucre in accordance with the resolution numbers 36836 and 2–2012, respectively. Written informed consent was obtained from the patients from which the strains were isolated.

### References

1. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P. Leishmaniasis worldwide and global estimates of its incidence. *PloS one* 2012;7:e35671.
2. Burza S, Croft SL, Boelaert M. Leishmaniasis. *Lancet* 2018;392:951–970.
3. Organization. PAH. Epidemiological report of the Americas. Report N° 7 Dec-2019. 2019.
4. Chappuis F, Sundar S, Hailu A, Ghalib H, Rijal S. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nat Rev Microbiol* 2007;5:873–882.
5. Pennisi MG, Persichetti MF. Feline leishmaniosis: Is the cat a small dog? *Vet Parasitol* 2018;251:131–137.
6. Dantas- Torres F. *Canine leishmaniosis* in South America. *Parasites Vectors* 2009;2:S1.
7. Kaszak I, Planellas M, Dworecka- Kaszak B. *Canine leishmaniosis* - an emerging disease. *Ann Parasitol* 2015;61:69–76.
8. Peacock CS, Seeger K, Harris D, Murphy L, Ruiz JC. Comparative genomic analysis of three *Leishmania* species that cause diverse human disease. *Nat Genet* 2007;39:839–847.
9. Carnielli JBT, Crouch K, Forrester S, Silva VC, Carvalho SFG. A Leishmania infantum genetic marker associated with miltefosine treatment failure for visceral leishmaniasis. *EBioMedicine* 2018;36:83–91.
10. Teixeira DG, Monteiro GRG, Martins DRA, Fernandes MZ, Macedo- Silva V. Comparative analyses of whole genome sequences of *Leishmania infantum* isolates from humans and dogs in northeastern Brazil. *Int J Parasitol* 2017;47:655–665.
11. Yasur- Landau D, Jaffe CL, David L, Doron- Faigenboim A, Baneth G. Resistance of *Leishmania infantum* to allopurinol is associated with chromosome and gene copy number variations including decrease in the S- adenosylmethionine synthetase (METK) gene copy number. *Int J Parasitol Drugs Drug Resist* 2018;8:403–410.
12. Rogers MB, Downing T, Smith BA, Imamura H, Sanders M. Genomic confirmation of hybridisation and recent inbreeding in a vector- isolated Leishmania population. *PLoS Genet* 2014;10:e1004092.
13. Bussotti G, Benkahla A, Jeddi F, Souiai O, Aoun K. Nuclear and mitochondrial genome sequencing of North- African Leishmania infantum isolates from cured and relapsed visceral leishmaniasis patients reveals variations correlating with geography and phenotype. *Microb Genom* 2020;6.
14. Franssen SU, Durrant C, Stark O, Moser B, Downing T. Global genome diversity of the *Leishmania donovani* complex. *eLife* 2020;9.
15. Carvalho KSS, da Silva Júnior WJ, da Silveira Regueira Neto M, Silva VC, de Sá Leitão Paiva Júnior S, *et al*. Application of Next Generation sequencing (NGS) for descriptive analysis of 30 genomes of Leishmania infantum isolates in middle-n orth Brazil. *Sci Rep* 2020;10:12321.
16. SIVIGILA. Boletin epidemiologico. *Sistema Nacional de Vigilancia en Salud Publica* 2019.
17. Kuhne V, Rezaei Z, Pitzinger P, Buscher P. Systematic review on antigens for serodiagnosis of visceral leishmaniasis, with a focus on East Africa. *PLoS Negl Trop Dis* 2019;13:e0007658.
18. Herrera G, Castillo A, Ayala MS, Florez C, Cantillo- Barraza O. Evaluation of four rapid diagnostic tests for canine and human visceral *Leishmaniasis* in Colombia. *BMC Infect Dis* 2019;19:747.
19. Burns JM, Shreffler WG, Benson DR, Ghalib HW, Badaro R. Molecular characterization of a kinesin-r elated antigen of *Leishmania chagasi* that detects specific antibody in African and American visceral leishmaniasis. *Proc Natl Acad Sci USA* 1993;90:775–779.
20. Pattabhi S, Whittle J, Mohamath R, El- Safi S, Moulton GG. Design, development and evaluation of rK28- based point-o f-care tests for improving rapid diagnosis of visceral leishmaniasis. *PLoS Negl Trop Dis* 2010;4.
21. Bhattacharyya T, Boelaert M, Miles MA. Comparison of visceral leishmaniasis diagnostic antigens in African and Asian *Leishmania donovani* reveals extensive diversity and region-s pecific polymorphisms. *PLoS Negl Trop Dis* 2013;7:e2057.
22. Cunningham J, Hasker E, Das P, El Safi S, Goto H. A global comparative evaluation of commercial immunochromatographic rapid diagnostic tests for visceral leishmaniasis. *Clin Infect Dis* 2012;55:1312–1319.
23. Abass E, Kang C, Martinkovic F, Semiao- Santos SJ, Sundar S. Heterogeneity of *Leishmania donovani* parasites complicates diagnosis of visceral leishmaniasis: comparison of different serological tests in three endemic regions. *PloS one* 2015;10:e0116408.
24. da Silva MRB, Brandao NAA, Colovati M, de Sousa MMP, de Lima LC. Performance of two immunochromatographic tests for diagnosis of visceral leishmaniasis in patients coinfected with HIV. *Parasitol Res* 2018;117:419–427.
25. Mukhtar M, Abdoun A, Ahmed AE, Ghalib H, Reed SG. Diagnostic accuracy of rK28- based immunochromatographic rapid diagnostic tests for visceral leishmaniasis: a prospective clinical cohort study in Sudan. *Trans R Soc Trop Med Hyg* 2015;109:594–600.
26. Bezuneh A, Mukhtar M, Abdoun A, Teferi T, Takele Y. Comparison of point- of- care tests for the rapid diagnosis of visceral leishmaniasis in East African patients. *Am J Trop Med Hyg* 2014;91:1109–1115.
27. Venturin GL, Bragato JP, Silva KL, de Lima VM. Recombinant K28 antigen in ELISA in the diagnosis of canine visceral leishmaniosis. *Parasite Immunol* 2015;37:670–673.
28. Schwabl P, Boite MC, Bussotti G, Jacobs A, Andersson B. Colonization and genetic diversification processes of *Leishmania infantum* in the Americas. *Commun Biol* 2021;4:139.
29. Brotherton MC, Bourassa S, Leprohon P, Legare D, Poirier GG. Proteomic and genomic analyses of antimony resistant *Leishmania infantum* mutant. *PloS one* 2013;8:e81899.

30. Valdivia HO, Almeida LV, Roatt BM, Reis- Cunha JL, Pereira AA. Comparative genomics of canine-i solated *Leishmania* (Leishmania) amazonensis from an endemic focus of visceral leishmaniasis in Governador Valadares, southeastern Brazil. *Sci Rep* 2017;7:40804.
31. Ramirez JD, Hernandez C, Leon CM, Ayala MS, Florez C. Taxonomy, diversity, temporal and geographical distribution of Cutaneous Leishmaniasis in Colombia: A retrospective study. *Sci Rep* 2016;6:28266.
32. Patino LH, Mendez C, Rodriguez O, Romero Y, Velandia D. Spatial distribution, *Leishmania* species and clinical traits of Cutaneous Leishmaniasis cases in the Colombian army. *PLoS Negl Trop Dis* 2017;11:e0005876.
33. Mount DW. Using the Basic Local Alignment Search Tool (BLAST). *CSH Protoc* 2007;2007:pdb
34. Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD. IQ- TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. *Mol Biol Evol* 2020;37:1530–1534.
35. Letunic I, Bork P. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Res* 2019;47:W256–W259.
36. Patino LH, Imamura H, Cruz- Saavedra L, Pavia P, Muskus C. Major changes in chromosomal somy, gene expression and gene dosage driven by Sb(III) in Leishmania braziliensis and *Leishmania panamensis*. *Sci Rep* 2019;9:9485.
37. Gonzalez- de la Fuente S, Peiro- Pastor R, Rastrojo A, Moreno J, Carrasco- Ramiro F. Resequencing of the *Leishmania infantum* (strain JPCM5) genome and de novo assembly into 36 contigs. *Sci Rep* 2017;7:18050.
38. Ilg T. Proteophosphoglycans of *Leishmania*. *Parasitol Today* 2000;16:489–497.
39. Huson DH, Bryant D. Application of phylogenetic networks in evolutionary studies. *Mol Biol Evol* 2006;23:254–267.
40. Cuypers B, Berg M, Imamura H, Dumetz F, De Muylder G. Integrated genomic and metabolomic profiling of ISC1, an emerging *Leishmania donovani* population in the Indian subcontinent. *Infect Genet Evol* 2018;62:170–178.
41. Dumetz F, Imamura H, Sanders M, Seblova V, Myskova J. Modulation of aneuploidy in *Leishmania donovani* during adaptation to different *in vitro* and *in vivo* environments and its impact on gene expression. *mBio* 2017;8.
42. Zhao S, Guo Y, Sheng Q, Shyr Y. Advanced heat map and clustering analysis using heatmap3. *Biomed Res Int* 2014;2014:986048.
43. Supek F, Bosnjak M, Skunca N, Smuc T. REVIGO summarizes and visualizes long lists of gene ontology terms. *PloS one* 2011;6:e21800.
44. Tihon E, Imamura H, Van den Broeck F, Vermeiren L, Dujardin JC. Genomic analysis of Isometamidium Chloride resistance in Trypanosoma congolense. *Int J Parasitol Drugs Drug Resist* 2017;7:350–361.
45. Raj A, Stephens M, Pritchard JK. fastSTRUCTURE: variational inference of population structure in large SNP data sets. *Genetics* 2014;197:573–589.
46. Evanno G, Regnaut S, Goudet J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol Ecol* 2005;14:2611–2620.
47. Excoffier L, Lischer HE. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Mol Ecol Resour* 2010;10:564–567.
48. Inouye M, Conway TC, Zobel J, Holt KE. Short read sequence typing (SRST): multi- locus sequence types from short reads. *BMC genomics* 2012;13:338.
49. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 2013;30:772–780.
50. Okonechnikov K, Golosova O, Fursov M, team U. Unipro UGENE: a unified bioinformatics toolkit. *Bioinformatics* 2012;28:1166–1167.
51. Poon AF, Frost SD, Pond SL. Detecting signatures of selection from DNA sequences using Datamonkey. *Methods Mol Biol* 2009;537:163–183.
52. Ribolla PE, Gushi LT, Pires E, Costa CH, Costa DL. *Leishmania infantum* genetic diversity and lutzomyia longipalpis mitochondrial haplotypes in Brazil. *Biomed Res Int* 2016;2016:9249217.
53. Boite MC, Spath GF, Bussotti G, Porrozzi R, Morgado FN. Trans-atlantic spill over: deconstructing the ecological adaptation of *Leishmania infantum* in the Americas. *Genes (Basel*) 2019;11.
54. Leblois R, Kuhls K, Francois O, Schonian G, Wirth T. Guns, germs and dogs: On the origin of *Leishmania chagasi*. *Infect Genet Evol* 2011;11:1091–1095.
55. Lukes J, Mauricio IL, Schonian G, Dujardin JC, Soteriadou K. Evolutionary and geographical history of the *Leishmania donovani* complex with a revision of current taxonomy. *Proc Natl Acad Sci USA* 2007;104:9375–9380.
56. Kuhls K, Alam MZ, Cupolillo E, Ferreira GE, Mauricio IL. Comparative microsatellite typing of new world leishmania infantum reveals low heterogeneity among populations and its recent old world origin. *PLoS Negl Trop Dis* 2011;5:e1155.
57. Nozais JP. [Hypotheses on the origin of certain parasites on the Latin American continent]. *Bull Soc Pathol Exot Filiales* 1985;78:401–412.
58. Tagliamonte MS, Yowell CA, Elbadry MA, Boncy J, Raccurt CP. Genetic markers of adaptation of plasmodium falciparum to transmission by american vectors identified in the genomes of parasites from Haiti and South America. *mSphere* 2020;5.
59. Gonzalez C, Cabrera OL, Munstermann LE, Ferro C. [Distribution of *Leishmania infantum* vector species in Colombia]. *Biomedica* 2006;26:64–72.
60. Ardila MM, Carrillo- Bonilla L, Pabon A, Robledo SM. Surveillance of phlebotomine fauna and *Didelphis marsupialis* (Didelphimorphia: Didelphidae) infection in an area highly endemic for visceral leishmaniasis in Colombia. *Biomedica* 2019;39:252–264.
61. Falcao de Oliveira E, Galati EAB, Oliveira AG, Rangel EF, Carvalho BM. Ecological niche modelling and predicted geographic distribution of *Lutzomyia cruzi*, vector of *Leishmania infantum* in South America. *PLoS Negl Trop Dis* 2018;12:e0006684.
62. Silva EM, Alves LC, Guerra NR, Farias MP, Oliveira EL. *Leishmania* spp. in *Didelphis* spp. from Northeastern Brazil. *J Zoo Wildl Med* 2016;47:942–944.
63. Kaufer A, Barratt J, Stark D, Ellis J. The complete coding region of the maxicircle as a superior phylogenetic marker for exploring evolutionary relationships between members of the *Leishmaniinae*. *Infect Genet Evol* 2019;70:90–100.
64. Camacho E, Rastrojo A, Sanchiz A, Gonzalez- de la Fuente S, Aguado B. Leishmania mitochondrial genomes: maxicircle structure and heterogeneity of minicircles. *Genes (Basel*) 2019;10.
65. Patino LH, Muskus C, Munoz M, Ramirez JD. Genomic analyses reveal moderate levels of ploidy, high heterozygosity and structural variations in a Colombian isolate of *Leishmania* (Leishmania) amazonensis. *Acta Trop* 2019;203:105296.
66. Patino Luz HM, Carlos M, David RJ. Genomic diversification, structural plasticity, and hybridization in *Leishmania* (Viannia) braziliensis. *Front Cell Infect Microbiol* 2020;10.

67. Bussotti G, Gouzelou E, Cortes Boite M, Kherachi I, Harrat Z. Leishmania genome dynamics during environmental adaptation reveal strain-s pecific differences in gene copy number variation, karyotype instability, and telomeric amplification. *mBio* 2018;9.

68. Olekhnovitch R, Ryffel B, Muller AJ, Bousso P. Collective nitric oxide production provides tissue- wide immunity during *Leishmania* infection. *J Clin Invest* 2014;124:1711–1722.

69. Timm T, Annoscia G, Klein J, Lochnit G. The Eukaryotic Elongation Factor 1 Alpha (eEF1alpha) from the Parasite *Leishmania infantum* is modified with the immunomodulatory substituent phosphorylcholine (PC). *Molecules* 2017;22.

70. Carvalho S, Barreira da Silva R, Shawki A, Castro H, Lamy M. LiZIP3 is a cellular zinc transporter that mediates the tightly regulated import of zinc in *Leishmania infantum* parasites. *Mol Microbiol* 2015;96:581–595.

71. Saini S, Bharati K, Shaha C, Mukhopadhyay CK. Zinc depletion promotes apoptosis- like death in drug- sensitive and antimony- resistance *Leishmania donovani*. *Sci Rep* 2017;7:10488.

72. Patino LH, Munoz M, Muskus C, Mendez C, Ramirez JD. Intraspecific genomic divergence and minor structural variations in *Leishmania* (Viannia) panamensis. *Genes (Basel*) 2020;11.

73. Iantorno SA, Durrant C, Khan A, Sanders MJ, Beverley SM. Gene expression in leishmania is regulated predominantly by gene dosage. *mBio* 2017;8.

74. Segatto M, Ribeiro LS, Costa DL, Costa CH, Oliveira MR. Genetic diversity of Leishmania infantum field populations from Brazil. *Mem Inst Oswaldo Cruz* 2012;107:39–47.

75. Diniz SA, Silva FL, Carvalho Neta AC, Bueno R, Guerra RM. Animal reservoirs for visceral leishmaniasis in densely populated urban areas. *J Infect Dev Ctries* 2008;2:24–33.

76. Gonzalez C, Paz A, Ferro C. Predicted altitudinal shifts and reduced spatial distribution of Leishmania infantum vector species under climate change scenarios in Colombia. *Acta Trop* 2014;129:83–90.

77. Oberle M, Balmer O, Brun R, Roditi I. Bottlenecks and the maintenance of minor genotypes during the life cycle of *Trypanosoma brucei*. *PLoS Pathog* 2010;6:e1001023.

78. McCall LI, Zhang WW, Matlashewski G. Determinants for the development of visceral leishmaniasis disease. *PLoS Pathog* 2013;9:e1003053.

79. Cordeiro AT, Michels PA, Delboni LF, Thiemann OH. The crystal structure of glucose-6-p hosphate isomerase from *Leishmania mexicana* reveals novel active site features. *Eur J Biochem* 2004;271:2765–2772.

80. Kuhls K, Keilonat L, Ochsenreither S, Schaar M, Schweynoch C. Multilocus microsatellite typing (MLMT) reveals genetically isolated populations between and within the main endemic regions of visceral leishmaniasis. *Microbes Infect* 2007;9:334–343.

81. Alam MZ, Kuhls K, Schweynoch C, Sundar S, Rijal S. Multilocus microsatellite typing (MLMT) reveals genetic homogeneity of *Leishmania donovani* strains in the Indian subcontinent. *Infect Genet Evol* 2009;9:24–31.

82. Bangert M, Flores- Chavez MD, Llanes- Acevedo IP, Arcones C, Chicharro C. Validation of rK39 immunochromatographic test and direct agglutination test for the diagnosis of Mediterranean visceral leishmaniasis in Spain. *PLoS Negl Trop Dis* 2018;12:e0006277.

83. WHO DEST. Visceral leishmaniasis rapid diagnostic test performance. 2010.

84. Oliveira Dos Santos Maciel M, Soares MF, Costa SF, Bragato JP, Rebech GT, *et al*. Plasmonic rK28 ELISA improves the diagnosis of canine Leishmania infection. *Parasite Immunol* 2020;42:e12684.

85. Vaish M, Bhatia A, Reed SG, Chakravarty J, Sundar S. Evaluation of rK28 antigen for serodiagnosis of visceral Leishmaniasis in India. *Clin Microbiol Infect* 2012;18:81–85.

## 3.4. CAPÍTULO 4

**Título:** Caracterización cualitativa y cuantitativa de la proteómica en una cepa *Leishmania infantum* resistente al estibogluconato de sodio trivalente.

Los antimoniales son el tratamiento antiparasitario de primera línea más importantes contra las leishmaniasis; sin embargo, cada vez es más frecuente el reporte de fallas terapéuticas asociadas a la resistencia a estos medicamentos, lo que ha limitado la disponibilidad de las alternativas terapéuticas en algunas regiones del mundo. En este estudio, se determinó el IC50 de formas promastigotes de *L. infantum* de la región de las américas (cepa de referencia aislada en Brasil), frente al estibogluconato de sodio trivalente (SbIII), posteriormente se indujo a resistencia antiparasitaria *in vitro* por medio de aumento escalonado del SbIII durante aproximadamente 4 meses; posterior a la confirmación del fenotipo resistente al SbIII, Se compararon la tasa de crecimiento de la línea resistente SbR con su contraparte silvestre Wt, además de realizar un análisis proteómico cualitativo y cuantitativo mediante LC-MS/MS label free para ambos grupos de parásitos.

El análisis proteómico reveló concentraciones de proteínas similares por célula pero con diferentes números de copias de moléculas. Identificamos 165 proteínas con concentración aumentada y 56 con concentración disminuida en la línea resistente. El aumento de las proteínas se asoció con actividades como la transcripción, traducción, metabolismo de lípidos, metabolismo energético y biogénesis de peroxisomas, mientras que la disminución se relacionó con la adquisición y metabolismo de metales, especialmente hierro. Estos hallazgos sugieren una nueva perspectiva sobre la resistencia a los antimonios, destacando la importancia de la regulación postranscripcional y postraduccional, junto con la compensación del gasto energético y las alteraciones en la composición lipídica de la membrana de los orgánulos en los parásitos resistentes a los antimonios. En resumen, nuestro estudio proporciona información crucial sobre el perfil proteómico de las cepas resistentes al estibogluconato, enriqueciendo nuestra comprensión del complejo panorama de la resistencia a los antiparasitarios en *L. infantum*.

Este estudio permitió el sometimiento para la publicación de un artículo científico:

Castillo-Castañeda A, Patiño LH, Muro A, Manzano R, López-Abán J, Ramírez JD. Characterizing *Leishmania infantum*-induced resistance to trivalent stibogluconate (SbIII) through deep proteomics. International Journal of Parasitology. (sometido)

# International Journal for Parasitology

# Characterizing Leishmania infantum-induced resistance to trivalent stibogluconate (SbIII) through deep proteomics

--Manuscript Draft--

| | |
|---|---|
| **Manuscript Number:** | |
| **Article Type:** | Full Length Article |
| **Keywords:** | L. infantum, stibogluconate-resistance, quantitative proteomic, RNA metabolism, energetic compensation, lipid biosynthesis, peroxisome biogenesis |
| **Corresponding Author:** | Juan David Ramírez, Ph.D<br>Icahn School of Medicine at Mount Sinai<br>Bogota, COLOMBIA |
| **First Author:** | Adriana Castillo-Castañeda |
| **Order of Authors:** | Adriana Castillo-Castañeda |
| | Luz Helena Patiño |
| | Antonio Muro |
| | Julio Lopez |
| | Raul Manzano |
| | Juan David Ramírez, Ph.D |
| **Abstract:** | Across the world, various species within the Leishmania donovani complex are implicated in cases of visceral leishmaniasis. Notably, Leishmania infantum predominates in regions spanning the Mediterranean Basin, the Middle East, and Central Asia, as well as countries in South and Central America. Traditionally, antimonial compounds have served as the primary antiparasitic treatment for leishmaniasis. However, the global spread of resistance to these compounds has posed a significant challenge. Unfortunately, the emergence of resistance has outpaced the development of new therapeutic options. In this study, we aimed to investigate resistance development to trivalent sodium stibogluconate in vitro using promastigotes from a wild strain of L. infantum. We compared the growth rates between the resistant and wild strain groups and conducted label-free quantitative mass spectrometry-based proteomic analyses to assess proteomic expression changes. Statistical and bioinformatics analyses were employed to evaluate the significance of protein concentration changes, protein identity annotation, GO term analysis, biosynthetic pathways, and protein-protein interactions. Our findings revealed that despite both groups of parasites exhibiting a logarithmic growth phase, the resistant line displayed a notable reduction in growth rate. Proteomic analysis unveiled similar protein concentrations per cell but with differing copy molecule numbers. We identified 165 proteins with increased concentration and 56 with decreased concentration in the resistant line. Increased protein groups were associated with transcription and translation activities, lipid metabolism, energy metabolism, and peroxisome biogenesis, while decreased protein groups were linked to metal acquisition and metabolism, particularly iron. These results suggest a novel perspective on antimonial resistance, highlighting the importance of post-transcriptional and post-translational regulation, alongside energy expenditure compensation and alterations in organelle membrane lipid composition in antimonial-resistant parasites. Overall, our study provides insights into the proteomic profile of stibogluconate-resistant strains, contributing to our understanding of the complex landscape of antiparasitic resistance in L. infantum. |
| **Suggested Reviewers:** | Carlos Muskus<br>carmusk@yahoo.com<br>Expert in molecular biology of Leishmania |
| | Patricia Cuervo<br>patricia.cuervo@fiocruz.br<br>Expert in proteomics of Leishmania |
| | |
| | Antonio Marcilla<br>antonio.marcilla@uv.es<br>Expert in Parasite proteomics |

*Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation*

# Characterizing *Leishmania infantum*-induced resistance to trivalent stibogluconate (SbIII) through deep proteomics

Castillo-Castañeda, Adriana[1]; Patiño, Luz H[1]; Muro Antonio[2]; López Julio[2]; Manzano, Raúl[2]; Ramírez, Juan David[1,3]

[1] Centro de Investigaciones en Microbiología y Biotecnología-UR (CIMBIUR), Facultad de Ciencias Naturales, Universidad del Rosario, Bogotá, Colombia.

[2] Infectious and Tropical Diseases Research Group (e-INTRO), Biomedical Research Institute of Salamanca-Research Centre for Tropical Diseases at the University of Salamanca (IBSAL-CIETUS), Faculty of Pharmacy, University of Salamanca, 37008, Salamanca, Spain

[3] Department of Pathology, Molecular and Cell-Based Medicine, Molecular Microbiology Laboratory, Icahn School of Medicine at Mount Sinai, New York, NY, USA.

**Corresponding author:** juand.ramirez@urosario.edu.co

**E-mail addresses:**

ACC: adrianac.castillo@urosario.edu.co

LHP: luzh.patino@urosario.edu.co

RM: rmanzano@usal.es

JL: jlaban@usal.es

AM: ama@usal.es

## ABSTRACT

Across the world, various species within the *Leishmania donovani* complex are implicated in cases of visceral leishmaniasis. Notably, *Leishmania infantum* predominates in regions spanning the Mediterranean Basin, the Middle East, and Central Asia, as well as countries in South and Central America. Traditionally, antimonial compounds have served as the primary antiparasitic treatment for leishmaniasis. However, the global spread of resistance to these compounds has posed a significant challenge. Unfortunately, the emergence of resistance has outpaced the development of new therapeutic options. In this study, we aimed to investigate resistance development to trivalent sodium stibogluconate *in vitro* using promastigotes from a wild strain of *L. infantum*. We compared the growth rates between the resistant and wild strain groups and conducted label-free quantitative mass spectrometry-based proteomic analyses to assess proteomic expression changes. Statistical and bioinformatics analyses were employed to evaluate the significance of protein concentration changes, protein identity annotation, GO term analysis, biosynthetic pathways, and protein-protein interactions. Our findings revealed that despite both groups of parasites exhibiting a logarithmic growth phase, the resistant line displayed a notable reduction in growth rate. Proteomic analysis unveiled similar protein concentrations per cell but with differing copy molecule numbers. We identified 165 proteins with increased concentration and 56 with decreased concentration in the resistant line. Increased protein groups were associated with transcription and translation activities, lipid metabolism, energy metabolism, and peroxisome biogenesis, while decreased protein groups were linked to metal acquisition and metabolism, particularly iron. These results suggest a novel perspective on antimonial resistance, highlighting the importance of post-transcriptional and post-translational regulation, alongside energy expenditure compensation and alterations in organelle membrane lipid composition in antimonial-resistant parasites. Overall, our study provides insights into the proteomic profile of

stibogluconate-resistant strains, contributing to our understanding of the complex landscape of antiparasitic resistance in *L. infantum*.

**Keywords***:* *L. infantum*, stibogluconate-resistance, quantitative proteomic, RNA metabolism, energetic compensation, lipid biosynthesis, peroxisome biogenesis.

## Introduction

Visceral leishmaniasis (VL) is a severe form of leishmaniasis, a parasitic disease transmitted by vectors, often affecting neglected communities. VL presents a grave risk to life as the parasite invades crucial tissues and organs like the bone marrow, liver, and spleen. This invasion frequently leads to severe complications, including pancytopenia, recurrent infections, anemia, fever, and weight loss (van Griensven and Diro, 2012). Visceral leishmaniasis (VL) disproportionately affects vulnerable populations, especially children (Castillo-Castañeda et al., 2021) and individuals with weakened immune systems (Castillo-Castañeda et al., 2022; Lindoso et al., 2018). This disease primarily affects tropical regions, however, due to globalization and migratory patterns, there has been an increase in reported cases in subtropical regions in recent years (Wamai et al., 2020). In 2022, the World Health Organization (WHO) reported 13,012 new cases of VL in endemic countries, along with 69 imported cases (https://apps.who.int/neglected_diseases/ntddata/leishmaniasis/leishmaniasis.htmL). These data highlight the importance of considering underreporting, as the WHO estimates reporting rates to be only between 25% and 45% (WHO, 2020). VL is primarily caused by two main species: *Leishmania donovani* and *L. infantum.* The latter species is associated with the disease in regions spanning the Mediterranean Basin, Central Asia, the Middle East, and the Americas (Franssen et al., 2020).

In cases of leishmaniasis, therapeutic failure is mainly associated with treatment resistance, particularly with antimonial compounds showing the lowest therapeutic response (Ponte-Sucre et al., 2017). Since the 1920s, pentavalent antimonials such as sodium stibogluconate (Sb) and meglumine antimoniate (Ma) have been developed as the first drugs effective against leishmaniasis, demonstrating significant therapeutic success (Madusanka et al., 2024), however, resistance to antimonials emerged in the 1950s in the Indian subcontinent, subsequently expanding to various countries in the region and beyond (Ponte-Sucre et al., 2017). This emergence of resistance to antimonials led to a 60% rate of therapeutic failure, prompting the suspension of Sb as a first-line treatment for leishmaniasis in India (Croft et al., 2006). Inappropriate therapeutic regimens and the use of heavy metal-containing medications have been associated with the development of resistance to Sb (Cuypers et al., 2018; Decuypere et al., 2012; Dumetz et al., 2018). Currently, reports of resistance to this drug in the Americas are limited, making it a viable treatment option in affected populations where miltefosine and amphotericin have seen limited use due to ineffective or high costs, respectively (PAHO, 2022). For instance, in countries like Colombia and Brazil, some cases of VL have been attributed to therapeutic failure due to natural resistance to miltefosine in *L. infantum* (Carnielli et al., 2018; Zambrano Hernandez et al., 2015).

Stibogluconate (Sb) is a pentavalent antimony (SbV) derivative synthesized from the reaction between stibonic and gluconic acids (Roberts and Rainey, 1993). After administration, the drug reaches optimal concentrations within the body, effectively penetrating macrophages (Croft et al., 2006), within these cells, the compound undergoes metabolism, being reduced to its trivalent form (SbIII). In the parasite, this affects the trypanothione, beta-oxidation, and glycolysis pathways, along with inducing DNA fragmentation (Berman et al., 1985; Sereno et al., 2001). In response, *Leishmania* spp. have developed several resistance mechanisms against the Sb, this phenomenon has been extensively studied involving the characterization of parasites from clinical isolates and those induced to resist the drug *in vitro* (Downing et al., 2011; Patino et al., 2019a, 2019b; Silva et al., 2018). Descriptions and hypotheses concerning Sb resistance mechanisms primarily stem from specific investigations into DNA and RNA, and to a lesser extent with protein analysis, conducted mainly through techniques such as tandem mass spectrometry (MS-MS) (Andrade and Murta, 2014; Brotherton et al., 2013; Douanne et al., 2020; Leprohon et al., 2009; Matrangolo et al., 2013). A wide array of genomic changes in antimonial response, including copy number variations (CNVs) across chromosomes and genes, as well Single Nucleotide Polymorphisms (SNPs), have been identified (Kamran et al., 2023; Patino et al., 2019a). Studies utilizing qPCR and RNAseq have observed a decrease in the expression of genes encoding aquaporins (AQP1) and kinetoplastid membrane protein (KMP-11), along with overexpression of genes related to superoxide dismutase (SOC), ATP-binding cassette (ABC), and multidrug resistance protein 1 (MDR1) (El Fadili et al., 2009; Ponte-Sucre et al., 2017; Tessarollo et al., 2015).

Investigations into clinical isolates of *L. donovani* exhibiting cross-resistance to MIL and AMB by Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) revealed two proteins: heat shock protein HSP83 and small kinetoplastid calpain-related protein (SKCRP14.1). These proteins were found to be closely associated with the drug-induced programmed cell death (PCD) phenotype (Vergnes et al., 2007). Additionally, analyses of *L. donovani* isolates resistant to Sb using MALDI-TOF/TOF identified major proteins in the membrane-enriched fraction. These included ABC transporter, HSP-83, GPI protein transamidase, cysteine–leucine-rich protein, and 60S ribosomal protein L23a (Kumar et al., 2010). Proteomics studies employing Two-dimensional Difference Gel Electrophoresis (2D-DIGE) in *L. amazonensis, L. braziliensis,* and *L. infantum* have unveiled proteins linked to vesicle-mediated transport, tryparedoxin, oxidative stress response, and protein-protein interactions, such as heat shock proteins (Lima et al., 2020).

In addition, comparative proteomic analysis in antimony-susceptible and resistant lines of *L. braziliensis* and *L. infantum chagasi* was conducted using two-dimensional gel electrophoresis (2-DE) followed by liquid chromatography/mass spectrometry (LC/MS/MS), this analysis revealed overexpression of tryparedoxin peroxidase, alpha-tubulin, HSP70, HSP83, and HSP60 in both resistant lines, while cyclophilin-A showed lower expression in both (Matrangolo et al., 2013). Recent in-depth quantitative proteomics analysis of a MIL-resistant strain of *L. infantum* has revealed notable changes, there was a significant increase observed in oxidative phosphorylation complexes, particularly complex IV, along with heightened activity in β-oxidation of fatty acids and ABC transporters (Saboia-Vahia et al., 2022). Surprisingly, proteins traditionally associated with reactive oxygen species (ROS) detoxification did not appear to contribute to the resistance process (Saboia-Vahia et al., 2022). Despite our understanding of some mechanisms underlying Sb resistance, certain aspects of its action remain incompletely understood. Therefore, conducting an integrative proteomic analysis is necessary to achieve a comprehensive understanding.

Given the ongoing gaps in our comprehension of antiparasitic resistance, particularly evident in the discrepancies between transcriptomic and proteomic data in strains of *Leishmania* spp. (Gutierrez Guarnizo et al., 2023), resistant to antimonials, and recognizing the wealth of data produced by current proteomic methodologies with their heightened resolution and precision, it becomes imperative to undertake comprehensive quantitative proteomic investigations. These efforts would not only enhance our understanding of the proteomic changes underlying antimonial resistance but also facilitate the identification of new therapeutic targets or the optimization of current medications. This proactive approach is crucial given the anticipated increase in leishmanicidal resistance globally, affecting *L. infantum* and other species. Our focus on *L. infantum* is driven by its substantial impact on morbidity and mortality, especially in regions where it is endemic and among vulnerable populations.

## 1. Materials and methods

### 1.1. *Parasite Culture, Growth Curve, and Susceptibility Test*

### 1.1.1. *Parasite culture*

In this study, we utilized the reference strain MHOM/BR/1974/PP75 of *L. infantum* was utilized. Initially, promastigotes were cultured in a biphasic medium consisting of NNN and Schneider’s medium (Sigma-Aldrich, St. Louis, MO, USA). The medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), L-glutamine (Sigma-Aldrich, St. Louis, MO, USA), and penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and maintained at 26°C. After three passages in the biphasic medium, the parasites were transferred to a liquid phase of Schneider's medium supplemented as aforementioned, for an additional three passages.

### 1.1.2. *Growth curve*

To analyze the growth curve, promastigotes (1x$10^5$ parasites) were inoculated in Schneider’s medium supplemented as described above, and parasite density was measured every 24 hours for eleven days using a Neubauer's chamber and light microscopy at 40X magnification. For all experiments, promastigotes in the logarithmic phase were utilized.

### 1.1.3. *Susceptibility test*

To determine the Inhibitory Concentration (IC) of trivalent sodium stibogluconate (SbIII, Sigma Chemical Co., St. Louis, MO, USA), 1x$10^6$ parasites were cultured in a 96-well tissue culture plate with varying concentrations of SbIII, spanning from 500 µg/mL to 0.98 µg/mL. A control medium without the drug was also included. Additionally, amphotericin B at a concentration of 10 µg/mL served as a control for mortality. After a 72-hour incubation period at 26°C, the cytotoxicity of each well was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay (Invitrogen, Carlsbad, CA, USA). The optical density of the parasites in each well was measured using a spectrophotometer (SpectraMax—Molecular Devices, San Jose, CA, USA) at wavelengths of 540 nm and

630 nm. The effect of drug treatment on cell viability was quantified as the IC50, representing the concentration resulting in 50% lysis of the parasites within 72 hours. Each experiment was independently conducted three times in triplicate.

### 1.2. *Resistance induction*

For the *in vitro* resistance induction, promastigotes were cultivated with increasing concentrations of SbIII, beginning with 10 µg/mL and added in a stepwise manner (20, 40, 80, and 130 µg/mL), following previously described protocols (Liarte and Murta, 2010). This process was conducted over approximately 4 months. The *L. infantum* wild-type (Wt) parasites were maintained under the same incubation conditions and passage numbers as the resistant line (SbR) but without drug pressure. After the drug pressure period, the SbR line was cultured in a medium without SbIII for four weeks. Subsequently, the parasites were exposed again to SbIII at the last concentration to confirm the resistant phenotype. The growth profile and IC50 of the SbR line and Wt strain were determined again using the previously described methods.

### 1.3. *Sample preparation for deep proteomics*

Promastigotes of wild type and SbR line were cultured in a drug-free medium with an initial inoculum of 1x10$^6$ parasites/mL, they were then incubated at 26°C for five days to ensure that both lines reached the logarithmic phase of growth. Following this incubation period, the promastigotes were collected by centrifugation to 3000 rpm for 10 minutes, washed three times with PBS, and resuspended to determine the parasite concentration per milliliter. A total of five independent biological replicates of *L. infantum* SbIII resistant line (SbR) and four *L. infantum* parental wild-type strains (Wt) were utilized.

### *1.4. Protein extraction*

Proteomic sample preparation followed the SDS lysis protocol described by Kukal et al 2014 (Kulak et al., 2014). For each lysis reaction, approximately 2x$10^8$ logarithmic phase promastigotes were used, along with a hot buffer containing Tris-HCL 100mM (pH 8.5) (Sigma, Darmstadt, Germany, Cat. T6066) and SDS 2% (w/v) (Merck, Hohenbrunn, Germany, Cat. 8.22050.0100). The samples were then boiled for 10 minutes in a water bath. After a brief incubation at room temperature, the lysates were clarified by centrifugation at 10,000 rpm for 5 minutes. The resulting supernatants were transferred to new tubes and sonicated for 3 cycles of 30 seconds each, with a 1/1-second pulse and an amplitude of 30 for each cycle. The total protein concentration per sample was measured using the PierceTM BCA Protein Assay kit following the manufacturer's instructions (ThermoScientific, Cat. 23227) and spectrophotometer readings were taken at 562nm using a Biotek Synergy 4 (Vermont, United States). All protein samples (50 µg total protein) were initially digested with Tris (2-carboxyethyl)phosphine (TCEP) 100mM and cloracetamyde (CAA) 40mM to 95°C for 10 minutes, Subsequently, the solution was diluted to 1µg/µL with water and incubated to 37°C overnight with Trypsin/Lys-C protease (ThermoScientific, Japan, ref. 90307) in a 1:50 enzyme-to-protein ratio. Peptides were collected, concentrated, and desalted using Empore$^{TM}$ styrene divinyl benzene (SDB-RPS) disks (Merk, Darmstadt, Germany). The columns underwent conditioning with isopropanol, followed by several solutions: solution A (5% ammonium hydroxide (NH4OH) and 80% acetonitrile (ACN)), solution B (0.2% trifluoroacetic acid (TFA)), and solution C (1% TFA and isopropanol). Sample purification was achieved using solution C, while a combination of solution C and solution D (0.2% TFA and 5% ACN), and then solution A again to elute the peptides (Hernandez-Valladares et al., 2016; Rappsilber et al., 2007). The samples were then vacuum-dried until completely dry.

### *1.5. LC-MS/MS processing*

The samples underwent analysis via reverse-phase liquid chromatography (LC) using a nanoUPLC NanoElute (Bruker) system connected with the mass spectrophotometer TIMSTOF Pro (Bruker). Peptide elution was achieved through a gradient from 2 to 17% of ACN / 0,1% FA over 60 minutes, followed by 17 to 25% over 30 minutes, and finally 25 to 37% over 10 minutes. This process utilized a C18 5um 300ID 5mm (Thermo) and an Aurora column C18 1.9um 75ID 25cms (IonOpticks). Acquisition was conducted in positive mode using a data-dependent acquisition (DDA) method employing Parallel Accumulation Serial Fragmentation (PASEF) (Meier et al., 2018). To ensure a 100% duty cycle, a ramp and accumulation time of 100ms for the electric field gradient were set. The acquisition method employed topN acquisition cycles, incorporating full m/z frame acquisition – ionic mobility, and 10 PASEF MS/MS. Each PASEF cycle consisted of 12 spectra MS/MS, resulting in a total time of 1.1 seconds. The MS spectra were acquired for ions with charges ranging from 0 to 5, and both MS and MS/MS spectra were collected in the range of 100 to 1700 *m/z*. Precursors for PASEF were selected by the PASEF algorithm, and a polygon filter was applied to the m/z - ion mobility area to exclude precursors with a single charge and low m/z from the PASEF selection. The precursors were analyzed within a range of ion mobility (1/K0) of 0.6–1.6 Versus/cm$^2$. A minimum precursor signal of 2,500 was established for consideration, and a target value of 20,000 was set to trigger the reprogramming of MS precursors for PASEF MS/MS.

### *1.6. Data analysis*

Proteomic data were analyzed using the Andromeda search engine included in the MSFragger (version 3.8) (Kong et al., 2017). *L. infantum* sequences available at UniProtKB/Swiss-Prot (downloaded in May 2023, proteome:UP000008153) were used for searching the mass spectra obtained. Reversed proteins were included as decoys, and the "matching between runs" option was used for searching, along with common human sequences as contaminants. For all cases, the fragment ion mass tolerance was set at 0.5 Da, and parent ion tolerance was set at 20 ppm. Protein discovery rate and the maximum false peptide were set at 1% FDR. Variable modifications included oxidation on methionine and N-terminal acetylation of proteins, while the fixed modification was carbamidomethylation of cysteine. The MaxLFQ algorithm was employed to compute the relative abundance of each protein (Cox and Mann, 2008). Subsequently, spectral protein intensity (raw intensities) was utilized to calculate protein absolute abundances using the total protein approach (TPA). The 'proteomic ruler' approach (Wiśniewski, 2017) was then applied to estimate the absolute protein copy numbers per cell (Wiśniewski, 2017). These calculations regarding total protein, protein concentration, and copy number were conducted in Microsoft Excel utilizing data from the proteome available in UniprotKB.

Data validation and statistical analysis of significant differences in protein abundances were conducted using Perseus software (version 2.0.7.0) (Tyanova et al., 2016) and Amica (version 3.0.1, accessed on March 2024) (Didusch et al., 2022). The minimal number of valid values was set to 3 per protein in at least one group, and missing values were imputed from a normal distribution. The significance was determined using the Student's t-test with a false discovery rate (FDR) threshold of 3%, and the data are presented as the mean ± standard deviation (SD). All mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD###.

### *1.7. Enrichment analysis based on gene ontology and metabolic pathway annotations*

These analyses aimed to identify specific groups of proteins present in the SbR line that may contribute to the development of the resistant phenotype. To conduct enrichment analysis of gene ontology and metabolic pathway annotations, only proteins exhibiting statistically significant differences in concentration between the Wt and SbR parasites were considered. The enrichment analysis was performed using the tool available at Tritrypdb (http://tritrypdb.org, v64, accessed on March 2024) (Alvarez-Jarreta et al., 2024). The parameters were configured as follows: organism - *L. infantum* JPCM5, evidence computed and curated, with a p-value cutoff of 0.05. The search encompassed gene ontology categories including Biological Process, Cellular Component, and Molecular Function, using OmicsBox (version 3.1) ("OmicsBox – Bioinformatics Made Easy," 2019), following the functional annotation pipeline, Blast2Go was used (Gotz et al., 2008). Additionally, differential proteins were mapped to *L. infantum* JPCM5 (T number: T01112) in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. (Kanehisa, 2000) Furthermore, the proteins were analyzed in STRING (version 12.0, accessed on March 2024)(Szklarczyk et al., 2019) to identify protein-protein interactions. Graphics were generated using R software. (version 4.3) (R Core Team, 2021)

### *1.8. Statistical Analysis*

Growth curves and IC50 analyses were conducted using GraphPad Prism (version 8.0) for Windows (GraphPad Software, San Diego, CA, USA). Asterisks denote significant differences, with a significance threshold set at $p < 0.05$. Statistical significance between the Wt strain and SbR line was analyzed using Student's t-test and two-way ANOVA.

## **2. Results**

### *2.1. In vitro-selected L. infantum SbIII-Resistant line (SbR) exhibits distinct growth kinetics compared to Wild Type strain (Wt)*

Based on the 50% inhibitory concentration identified in the *L. infantum* wild-type strain (IC50 13.8± 0.8 µg/mL), we initiated the *in vitro* resistance induction protocol with 10µg/mL SbIII, gradually increasing the concentration until reaching 130µg/mL. The promastigotes underwent approximately four months of consecutive passages under drug pressure by SbIII, equivalent to approximately 30 passages. Subsequently, the SbR line was cultured without SbIII for five passages (four weeks) to confirm the stability of the resistant phenotype. IC50 assays were then performed again for both the wild-type strain and the SbR line. The promastigotes of the SbR line exhibited an IC50 of 125.27±1.40µg/mL, while the IC50 of SbIII for the wild-type strain was 13.2±1.1µg/mL, remained stable under the same culture conditions and time frame as the SbR line. This confirms that the IC50 increased by a factor of 9.5 in the SbR line compared to its Wt parent.

When analyzing the *in vitro* growth pattern of promastigotes from both the Wt strain and SbR lines, both displayed typical logarithmic and stationary phases. However, notably, the growth rate of the SbR line was markedly lower compared to the Wt strain (see Fig. 1a). The late logarithmic phase occurred on day 3 for the Wt strain and on day 4 for the SbR line, respectively. Interestingly, no morphological changes were observed in the resistant promastigotes.

### *2.2. Total proteins per cell were similar in both the Wild-type strain (Wt) and SbIII-Resistant line (SbR), but the protein composition varied*

To delve deeper into the molecular mechanisms driving the development of the SbIII-resistant phenotype, we conducted a comprehensive quantitative comparison of the proteomes between the *L. infantum* SbIII-resistant line and its parental wild-type strain. Lysates from promastigotes in the early logarithmic phase were prepared from five independent biological replicates for the SbIII-resistant line and four replicates for the wild-type strain. All samples underwent analysis by LC-MS/MS. We identified approximately ~57,000 peptides corresponding to ~5,577 protein groups, covering approximately ~70% of the predicted proteome of *L. infantum* (~8,045 protein-coding genes predicted), using the JPCM5 reference *L. infantum* strain (González-de la Fuente et al., 2017). We considered that each gene codes for only one protein (González-de la Fuente et al., 2017; Saboia-Vahia et al., 2022). We observed significant differences in the average counts, with 4,813 for Wt and 4,373 for SbR, $p < 0.01$ (Supplementary Table S1). When analyzing the protein sequences of at least five peptides, we were able to identify nearly 3,707 protein groups (~65%). Pearson's correlation analysis conducted on the proteomics data among the different biological replicates in each experimental group revealed high reproducibility, with a coefficient of approximately ±93% for Wt and ±95% for SbR (Fig. 1b). Principal Component Analysis (PCA) revealed very compact clusters for each set of biological replicates of the Wt strain and SbR line. Additionally, it demonstrated a clear separation between the two, confirming the existence of significant differences in protein abundance and composition between the study groups (Fig. 1c).

Both the total protein approach (TPA) and the histone ruler methods were employed to determine the total protein contents, protein concentrations, and protein copy numbers (Pinho et al., 2020; Wiśniewski, 2017). These calculations were based on the reported genome content (32.8Mb) of the *L. infantum* reference strain JPCM5 (González-de la Fuente et al., 2017) with a DNA content of 0.036 pg per haploid genome (0.072 pg per diploid genome) (Saboia-Vahia et al., 2022). We assumed a diploid state for both the Wt strain and SbR line, considering that several *L. donovani* and *L. infantum* isolates have shown a disomic genome overall, with occasional trisomy for chromosomes 8, 9, and 23, and tetraploidy for chromosome 31 (Franssen et al., 2020). The "protein ruler" analysis data indicated a similar protein content per cell in both groups (see Fig. 1d), with a value of 4.86 ± 0.425 pg in the Wt parental strain and 4.96 ± 0.724 pg in the SbR line (Supplementary Table S2). To calculate the copy number of each protein and the total number of protein molecules per parasite, we utilized the total protein values. This estimation yielded a total of $7.46 \pm 0.63 \times 10^7$ molecules per parasite for Wt and $8.18 \pm 1.11 \times 10^7$ molecules for SbR. Notably, we did not observe significant differences between the two groups. This result aligns with the content of protein per cell and the number of copies of the proteins (Supplementary Table S2).

### *2.3. The general protein profile of the SbIII-Resistant line (SbR) exhibited a trend of increase compared to the parental strain*

Protein identification underwent statistical validation using Perseus and Amica software, revealing 5,577 and 5,638 proteins, respectively. Notably, both tools identified proteins with significant concentration changes in a similar manner. Initially, we conducted coefficient variation analysis using the LFQ intensities (%) (see Supplementary Fig. S1a) and their imputed intensities for both groups (see Supplementary Fig. S1b), demonstrating the reproducibility of data obtained between replicates. When normalizing the intensity values for each protein in terms of Log2, the change in the production pattern of proteins between the Wt strain and SbR line became evident (see Fig. 2a). There was a noticeable tendency for increased protein production in the resistant phenotype, which was expected considering the metabolic and physiological adaptations of the parasite against SbIII.

### *2.4. Significant differences in protein abundance were observed between the Wild-type strain and the SbIII-resistant line*

We conducted a Student's t-test with an FDR of 3% to pinpoint significant differences between the Wt strain and SbR line. Specifically, we considered only proteins with a Log2FC threshold of 1.5 (SbR vs. Wt). This analysis revealed alterations in the abundance of 350 proteins (see Fig. 2b, Supplementary Table S3). Out of these, 254 proteins showed higher abundance in the SbR line compared to the Wt strain, while 96 proteins had lower concentrations (see Fig. 2b). Furthermore, among the up-regulated proteins in the SbR line, 91 were classified as uncharacterized or uncharacterized fragments, while 40 fell into the same category among the down-regulated protein (see Supplementary Table S3). Consequently, our analysis focused exclusively on characterized differential proteins, comprising 163 enriched and 56 reduced proteins in the SbR line. We utilized Tritrypdb enrichment tool and Omicsbox to identify protein groups enriched in gene ontology categories: Molecular Function (MF), Biological Process (BP), and Cellular Component (CC). To aid visual comprehension, we chose specific GO terms for bounding and visualization (Fig. 3).

In the Molecular Function (MF) GO terms of the SbR line, we observed an over-representation of functions associated with replication, transcription, and translation. Specifically, this included ribonucleoside triphosphate phosphatase activity, ribonucleoside binding, purine nucleoside activity, nucleoside activity, and modified amino acid binding. Additionally, metabolic functions such as catalytic activity, binding, oxidoreductase activity, and carbohydrate derivative activity were enriched. However, functions related to the use or acquisition of cofactors like metals were underrepresented. Notably, this group lacked terms such as ferroxidase activity, ferrous iron binding, metal ion binding, iron chaperone activity, and response to oxidative stress, including superoxide dismutase activity (see Fig. 3a).

In the Biological Process (BP) GO terms of the SbR line, the up-concentrated proteins were linked to processes such as ribonucleoprotein biogenesis, nucleotide metabolic processes, reproductive processes, response to stimuli, and regulation of biosynthetic, metabolic, cellular, and biological processes, including lipid biosynthetic processes. Additionally, the iron homeostasis also corresponded to the previous MF panorama observed (Fig. 3b).

Finally, an interesting pattern of Cellular Component (CC) GO terms was identified in the SbR line. There was a significant enrichment observed in terms related to the mitochondrial membrane, mitochondrial respiratory chain, nucleus, endoplasmic reticulum lumen, and Golgi apparatus membrane (see Fig. 3c).

### *2.5. Annotations of metabolic pathways and protein-protein interaction networks support the high regulation of proteins involved in RNA metabolism, energy production, and lipid metabolism.*

We utilized the Assign KO tool of KEGG Mapper to assign KO numbers to the ID proteins of the SbR line, both for up-regulated (163) and down-regulated (56) proteins. We focused on the Trypanosomatidae family (code 5654) to search our protein-coding genes and employed the Reconstruct Pathway tool. For the up-regulated proteins, we identified seven pathways. These pathways encompass genes involved in lipid, carbohydrate, energy, and nucleotide metabolisms (see Supplementary Fig. S2a). Within the lipid metabolism pathway, several key genes associated with fatty acid metabolism, fatty acid elongation, glycerolipid metabolism, sphingolipid metabolism, and biosynthesis of unsaturated fatty acids were found. In carbohydrate metabolism, the genes were associated to pivotal pathways such as the pentose phosphate pathway, pentose and glucuronate interconversions, amino sugar and nucleotide sugar metabolism, pyruvate metabolism, N-Glycan biosynthesis, and inositol phosphate metabolism. Regarding energy metabolism, genes participating in crucial pathways such as oxidative phosphorylation, carbon fixation, and sulfur metabolism were observed. Lastly, concerning nucleotides, we identified pathways encompassing purine and pyrimidine metabolism. Conversely, for the down-regulated proteins in the SbR line, only three pathways were implicated: amino acid metabolism, metabolism of cofactors, and carbohydrate metabolism (see Supplementary Fig. S2b).

STRING software was used to construct an interaction network for the up-regulated proteins, employing the k-means clustering option with a specified value of k=8 (see Fig. 4). The resulting clusters comprised varying numbers of genes, with the first four clusters containing 25, 9, 8, and 3 proteins, respectively, while the remaining clusters each contained two proteins. Notably, the largest cluster primarily consisted of proteins related to transcriptional and translational activity (see Fig. 5a and Table 1), the second cluster encompassed proteins involved in carbohydrate and glycan metabolism (see Fig. 5b and Table 1), the third cluster included proteins associated with lipid metabolism (see Fig. 5c and Table 1), lastly the fourth cluster contained proteins associated with peroxisome biogenesis (see Fig. 5d and Table 1).

## 3. DISCUSSION

When analyzing the *in vitro* growth profiles of promastigotes from the Wt strain and SbR line, both exhibited logarithmic and stationary phases. However, notably, the SbR line displayed a significantly lower growth rate compared to the Wt strain (see Fig. 1a). These characteristics mirror findings observed in *L. infantum* strains with miltefosine-induced resistance, but our SbIII-resistant promastigotes did not exhibit any morphological changes, which contrasts with the morphological changes reported in this miltefosine-resistant *L. infantum* line by Saboia-Vahia et al (Saboia-Vahia et al., 2022). This observation could suggest that the mechanisms of action of SbIII do not involve the disruption of actin and tubulin proteins (Frézard et al., 2009). Further indicating that structural changes in *L. infantum* may not be necessary to develop the SbIII-resistant phenotype. However, specific studies such as immunofluorescence and knock-out assays are necessary to corroborate this hypothesis.

Several reports have linked morphological changes in microorganisms to a loss of fitness. For instance, in *L. donovani*, such changes have been associated with increased difficulty in completing metacyclogenesis in *in vitro* cultures (Vergnes et al., 2007). Furthermore, miltefosine-resistant strains of *L. infantum* have shown negative changes in proliferation, infectivity, and dissemination capacity. These changes have been linked to an insufficient stress response across *in vivo* assays (Hendrickx et al., 2016). In contrast, paromomycin (PP) and antimonial-resistant phenotypes of some species of *Leishmania* have demonstrated advantages in amastigotes. These include enhanced growth potential, improved intracellular stress response, and increased virulence (Lira et al., 1999; Mukherjee et al., 2021; Ouakad et al., 2011). This enhanced fitness could be one of the factors contributing to *Leishmania* spp. resistance to Sb, potentially explaining its more widespread dissemination within a region compared to miltefosine-resistant strains (Sienkiewicz et al., 2018).

Further investigations into proteomic changes associated with fitness are crucial for a comprehensive understanding of the implications of Sb resistance. Both *in vitro* and *in vivo* assays, focusing on how SbR lines modulate infection in vectors and mammals should be conducted to thoroughly evaluate this hypothesis. This is especially important considering the enzootic and zoonotic transmission cycles of *L. donovani* and *L. infantum*, respectively. Considering the parasite's life cycle and the possibility of promastigotes transferring genes linked to Sb resistance and virulence factors via exovesicles within the vector (Dong et al., 2019; Inbar et al., 2019), as well as the potential for more efficient and aggressive infection in mammals due to heightened fitness, it's imperative to verify the stability of the resistant phenotype across all stages of the parasite's life cycle.

Principal component analysis (PCA) revealed high reproducibility among biological replicates with clear differentiation between the two groups (Fig. 1c). This confirms distinct compositions for each proteome, consistent with calculations using the proteomic ruler and total protein approach (TPA) calculations of the identified protein groups and total protein molecules per cell (Supplementary Table

S2). We observed a low correlation among the protein groups identified, protein concentration per cell, and the copy number in each group. This observation is consistent with previous findings in *Leishmania (Viannia), Saccharomyces cerevisiae*, and *Escherichia coli* protein abundance, where moderate or no correlation between protein size and abundance was found (Pinho et al., 2020; Warringer and Blomberg, 2006; Wiśniewski and Rakus, 2014). In our dataset, the SbR line exhibited a higher quantity of protein molecules per cell (copies), compensating for the fewer identified protein groups compared to the Wt strain. This finding elucidates why a similar protein concentration per cell was calculated for both parasite groups.

*Leishmania* spp. can display a variable correlation between gene and protein expression levels due to the absence of promoter-mediated regulation of transcription initiation for nuclear genes. Consequently, the expression of individual genes is primarily regulated post-transcriptionally (Clayton, 2019; Clayton and Shapira, 2007; Iantorno et al., 2017). In contrast, genomic and transcriptional comparisons of drug-resistant lines of several species of *Leishmania* have revealed more correlation. For example, in Sb-resistant lines of *L. amazonensis*, *L. braziliensis*, and *L. panamensis*, most of the gene expression changes were concordant with the chromosomal changes, with up-regulated genes primarily located on chromosomes 4, 8, 11, 13, and 31 (Patino et al., 2019a, 2019b). Additionally, mainly SNPs found on chromosome 32 were related to aquaporin-like protein, mitogen-activated protein kinase, and ABC transporter-like protein (Patino et al., 2019a, 2019b). A similar finding regarding somy and gene expression was reported for *L. donovani* lines resistant to SbIII, AMB, MIL, and PP (Rastrojo et al., 2018). Given these considerations, it's crucial to understand the translation process of this parasite genus to prevent biases in conclusions, because the abundance of mRNA transcripts does not always correspond to the final protein product (Brotherton et al., 2013; Chawla et al., 2011; Pinho et al., 2020). Furthermore, a multiomics approach is necessary to unveil the concept of resistance in *Leishmania* parasites.

Through Gene Ontology, KEGG pathway, and STRING analysis, we revealed a consistent proteomic pattern featuring proteins related to DNA stability and RNA metabolism (Fig. 4). In our SbR line, we identified numerous enriched proteins associated with key regulators of the parasite life cycle. Particularly noteworthy were proteins involved in DNA replication such as putative DNA replication factors, thymidine kinase, nucleolar GTP-binding protein, putative RAS-like small GTPases, and histone H4. These proteins are essential for maintaining the structural integrity of chromosomes. For instance, RAS-like proteins are involved in various cellular processes such as cell cycle progression, vesicle trafficking, and cytoskeletal dynamics during differentiation, cell-cell adhesion, growth, and apoptosis (Van Dam et al., 2011). Thymidine kinase plays a pivotal role by catalyzing the transfer of a phosphate group from ATP to thymidine, converting it into thymidine monophosphate (TMP). This enzymatic process is indispensable for generating thymidine nucleotides, which serve as crucial building blocks for DNA replication and repair and contribute to adaptation to stress and cellular differentiation in trypanosomatid parasitic protozoa (Cayla et al., 2022; Silva et al., 2021). Enriched changes in histone proteins, such as Histone 4, can significantly impact gene expression patterns, DNA repair mechanisms, and overall genomic stability (De Lima et al., 2020). These processes are crucial for cell function and survival (Sereno et al., 2001). Further characterization of these proteins and their specific roles in the resistance mechanism would be valuable for understanding the molecular mechanisms underlying drug resistance in the parasite. Additionally, it could provide insights into novel therapeutic targets for combating drug-resistant *Leishmania* infections. Investigating the specific role of histone H4 in antimonial resistance may also provide insights into the epigenetic regulation of drug resistance mechanisms in *Leishmania* parasites, potentially opening new avenues for therapeutic interventions targeting epigenetic regulators.

In terms of transcription, our analysis identified several significant proteins, including RNA polymerase subunit beta, ATP-dependent RNA helicase, putative RNA-binding protein, and putative

Pumilio protein 8. Interestingly, our findings align with those reported by Andrade et al., who investigated antimony-resistant *L. infantum* lines using RNAseq (Andrade et al., 2020). Of these proteins, we highlight Pumilio proteins which play critical roles in mRNA regulation by binding to specific sequences in 3′-UTRs. This binding recruits proteins that facilitate mRNA degradation, co-transcriptional repression, and translational repression (Kramer et al., 2023; Kramer and Carrington, 2011). Moreover, the conservation of Pumilio proteins across species underscores their importance in fundamental cellular processes(Alves et al., 2010). Exploring the role of Pumilio proteins in *Leishmania* spp. could, therefore, offer new avenues for understanding the mechanisms of resistance in *Leishmania* parasites.

Considering the translation process, our analysis revealed the presence of proteins such as putative ribosome biogenesis protein, SRP54 putative, prolyl-tRNA synthetase, methyltransferase superfamily protein, putative rRNA methyltransferase, and nucleolar protein related to the methyltransferase superfamily. Interestingly, Andrade et al. reported a downregulation of processes related to translation in *L. infantum* Sb-resistant strains, including rRNA processing, ribosome biogenesis, ribonucleoprotein complex, nucleosome assembly, and overall translation (Andrade et al., 2020). Our results are consistent with recent studies on *L. tropica* antimony-resistant strains, which also revealed a notable increase in proteins linked to translation (Gutierrez Guarnizo et al., 2023). This implies that changes in ribosomal proteins and translation initiation factors could be pivotal in the observed translational alterations, possibly serving as preemptive adaptations to drug exposure. Additionally, in Archaea and Eukarya, the conserved ribonucleoproteic core comprises two proteins: SRP19 and the essential GTPase SRP54, along with an SPR RNA. This complex facilitates the GTP-dependent interaction between SRP and its receptor SR, enabling the transfer of ribosomes carrying nascent polypeptide chains to the protein translocation apparatus at the membrane (Egea et al., 2008).

Indeed, our findings underscore the significance of comprehensive proteomic analyses. In trypanosomatids, where transcription is polycistronic and mRNA maturation requires splicing and polyadenylation, intricate control over mRNA processing occurs during the protein translation and degradation (Bunnik et al., 2013; Clayton, 2019; Clayton and Shapira, 2007). This understanding of translation and its regulatory mechanisms holds immense potential for advancing the development of new or improved antileishmanial treatments.

The notable increase in lipid-related proteins detected in our SbR line implies a potential link between the resistance phenotype and lipid composition and metabolism (see Fig. 4 and Table 1). Lipids serve vital functions in numerous cellular processes, spanning energy metabolism and storage, cell membrane structure, transport mechanisms, signaling pathways, proliferation, and autophagy (Biagiotti et al., 2017). In *Leishmania*, specific lipids have been suggested as potential biomarkers for leishmaniasis disease and resistance to antimonials (Andrade et al., 2020; De Azevedo et al., 2014) and amphotericin B (Pountain and Barrett, 2019). For instance, research employing gas chromatography and mass spectrometry (GC-MS) in *L. donovani* has shown that antimony exposure results in elevated levels of long fatty acids. (Haimeur et al., 1999). The presence of Delta-4 fatty acid desaturase, Delta-12 fatty acid desaturase, and fatty acid desaturase in *L. infantum* SbR suggests that these enzymes may contribute to protective mechanisms against antimony-induced stress (Biagiotti et al., 2017; Mathur et al., 2015).

The lipid membrane composition in clinical isolates of *L. donovani*, both antimonial-resistant and sensitive, has shown significant changes, including increased levels of unsaturated diacyl-PC and diacyl-PE in the drug-resistant isolates (t'Kindt et al., 2010). Similarly, in *Leishmania* strains with induced AMB resistance, lipid composition analysis revealed predominance of fatty acids, with stearic acid being the major fatty acid. Additionally, the major sterol identified was an ergosterol precursor, cholesta-5,7,24-trien-3beta-ol, instead of ergosterol, as observed in the AMB-sensitive strain (Mbongo et al., 1998). Other lipid-related proteins identified in the SbR line included members of the DEGS subfamily, putative 1-acyl-sn-glycerol-3-phosphate acyltransferase-like protein, putative serine palmitoyltransferase, and

SDR family. Notably, lipidomic assays have underscored the importance of these macromolecules as potential novel therapeutic targets (Gutierrez Guarnizo et al., 2021b). Our findings underscore the need for further lipidomic studies in promastigotes and amastigotes of this SbR line. Such investigations would serve to confirm the stability of this lipid pattern among changes in parasite morphology and to elucidate its role in resistance maintenance.

The elevated concentrations of mannosyltransferases, ribokinase, and lipophosphoglycan biosynthetic proteins such as Lpg2 in the SbR line compared to the Wt strain are intriguing findings. Additionally, the reduction in genes encoding proteins involved in carbohydrate transport adds another layer of interest. These observations are noteworthy, especially considering that most trypanosomatid species primarily rely on glycolysis, amino acid metabolism, and the fatty acid pathway for energy generation, particularly in promastigotes maintained in culture (Bringaud et al., 2006; Maugeri et al., 2011; Van Hellemond et al., 2005). Furthermore, the heightened glucose metabolism can lead to an increase in the formation of reactive oxygen species (ROS) and exacerbate oxidative stress through glucose auto-oxidation and protein glycosylation(Yang et al., 2021). It's plausible that the *L. infantum* SbR line undergoes metabolic compensation, favoring the utilization of energy through fatty long-chain substrates for energy acquisition (Leroux et al., 2023).  This adaptive shift could be accompanied by a reduction in glucose uptake, thereby mitigating the formation of reactive oxygen species (ROS) and enhancing the parasite's survival in the oxidative environment induced by the drug (Machuca et al., 2006).

Indeed, carbohydrate metabolism plays a crucial role in driving the life cycle and optimal growth of *Leishmania* spp. (Subramanian et al., 2015). Pathways such as glucose uptake, succinate fermentation, glutamate biosynthesis, and an active tricarboxylic acid cycle are indispensable for the parasite 's life cycle. The reduction of proteins associated with these pathways in the SbR line could account for the observed slow and reduced growth rate. Metabolomic assays have identified several genes and proteins related to carbohydrate metabolism as potential therapeutic targets in other trypanosomatids (Fall et al., 2022).

For years, transporter proteins of the ABC family and certain antioxidant responses have been known to play crucial roles in drug resistance mechanisms across various infectious agents (Abou Ammar et al., 2013; Manzano et al., 2013; Perea et al., 2016; Ren et al., 2014). However, recent studies have shown that these responses come with a high energetic cost for Leishmania parasites. The observed optimization of energy metabolism in resistant parasites during basal changes supports the idea of preemptive adaptation (Gutierrez Guarnizo et al., 2023). The proponents of this theory suggest that once the resistant profile is established, the parasite no longer needs to continuously respond to antimonial because the compound is no longer toxic, and rendering the high energy expenditure is unnecessary. Therefore, our data align with the current findings of Gutiérrez-Guarnizo in *L. major* antimonial-resistant strain, proposing possible changes in the lipidome and metabolome in resistant parasites (Gutierrez Guarnizo et al., 2021a, 2021b), as a way to optimize energy metabolism, supplying the energy required to fuel the antioxidant response via thiol metabolism and the subsequent thiol-metal complex conformation, as well iron related-antioxidant defense, offers an energy-saving alternative to efficiently respond to the drugs (Goto et al., 2024; Gutierrez Guarnizo et al., 2023). Integrative omics studies are necessary to study this phenomenon since an integrative comprehension.

When analyzing the GO terms related to Cellular Components (see Fig. 3c) and the protein-protein interaction data (see Fig. 5d), we noted proteins linked to endoplasmic reticulum and peroxisome biogenesis. Notably, ATP-binding cassette protein subfamily D, members 1 and 3 (ABCD), which belong to the ALD subfamily, play crucial roles in peroxisomal import of fatty acids, fatty acyl-CoAs, and maintenance of lipid homeostasis within the organelle (Oliveira et al., 2020). These proteins, located on the peroxisomal membrane, likely play crucial roles in peroxisome biogenesis and detoxification

(Sauvage et al., 2009). Specifically, in terms of detoxification, the ABCD proteins are significant as they facilitate the ATP-dependent transport of various chemotherapeutic drugs away from their targets within the parasite (Pramanik et al., 2019). The pressence of ABC transporters in protozoan parasites like *Toxoplasma gondii*, *Trypanosoma brucei, Leishmania* spp., and P*lasmodium* spp. underscores their importance as resistance mechanisms and potential therapeutic targets  (Klokouzas, 2003).

Additionally, we noted the presence of the peroxin Pex19, which along with Pex3, playing an indispensable role in peroxisomal membrane protein (PMP) biogenesis (de novo) from the endoplasmic reticulum (ER), as well as in the division of pre-existing peroxisomes and their growth (Agrawal et al., 2017). Both Pex3 and Pex19 are essentia l for these processes, as their absence prevents the formation of mature peroxisomes (Schrader and Fahimi, 2006; Tabak et al., 2013). Peroxisomes are vital organelles involved in maintaining cellular metabolic homeostasis. Depending on the metabolic needs of the cell, peroxisomes can alter their enzyme content and modulate their number and size, requiring both Pex3 and Pex19 for these. These trypanosomatid proteins have been targeted for study due to their low amino acid sequence conservation with human proteins. Altered interaction between Pex proteins in *T. brucei* disrupts glycosome biogenesis, leading to the mislocalization of glycosomal enzymes to the cytosol and causing lethality in the parasite (Banerjee et al., 2021). These findings provide starting points for further optimization and development of novel therapies against trypanosomatid parasite infections (Li et al., 2021).

Lastly, it's important to acknowledge some limitations in our study. Firstly, we found 91 and 40 differential proteins categorized as uncharacterized or uncharacterized fragments in the enriched and reduced proteins in the SbR line, respectively. Unfortunately, the reference proteomic database lacks complete information on all proteins, which may have resulted in missed identifications or misannotations, leading to the loss of valuable information. Secondly, although our proteomic in-depth study utilized a reference strain, we acknowledge the importance and necessity of conducting studies with strains isolated from patients. Thirdly, our analysis concentrated solely on intracellular protein contents, overlooking the potential contribution of exosome contents. Exosomes play crucial roles in intercellular communication and can contain biologically significant proteins that were not captured in our study. Fourthly, to derive more robust conclusions and gain deeper insights into our research objectives, integration with other omics data is essential. However, this integration requires a complex and sophisticated computational approach, which was beyond the scope of this study. Addressing these limitations in future research endeavors will be critical for advancing our understanding of antimonial resistance mechanisms in Leishmania parasites.

## 4. Conclusion

The comprehensive proteomic analysis in our study illuminates the intricate biological mechanisms driving antimonial resistance in *L. infantum*. We discovered that changes in growth rate, protein abundance, post-transcriptional and post-translational modifications, energy source utilization, and lipid composition within intracellular membranes could all play roles in the development of antimonial resistance. Furthermore, mechanisms such as energetic compensation and preemptive adaptation may reduce antioxidative stress response, modify iron metabolism, form metal-thiol complexes, and decrease efflux pump production. To gain a comprehensive understanding of the stability of this phenotype over time and under various conditions, similar studies should be conducted in the *L. infantum* amastigote-SbR line. Additionally, rigorous experimental designs, encompassing *in vitro* and *in vivo* assays in vectors and mammalian cells, are essential to minimize biases in the findings. Moreover, complementary studies such as translatome, lipidomic, and metabolomic analyses are crucial for both promastigotes and amastigotes of the L. infantum SbR line. These additional approaches can provide valuable insights and help evaluate the hypotheses proposed in this paper. Lastly, considering the unique transcriptional and translational regulation observed in *L. infantum*, there is a pressing need for studies on natural and induced resistant parasites. These studies should focus on targeting master regulators of drug-induced selective translation, which could lead to the discovery of novel therapeutic targets and facilitate the design and development of new treatments.

## Acknowledgments

This work was funded by Dirección de Investigación e Innovación from Universidad del Rosario. Juan David Ramírez González is a Latin American fellow in the biomedical sciences, supported by The Pew Charitable Trusts. Gratitude to María Nieves Ibarrola and Rosa María Degano, my mentors in proteomics, for their warm welcome during my stay in Salamanca and their unwavering support throughout this process.

## References

Abou Ammar, G., Tryono, R., Döll, K., Karlovsky, P., Deising, H.B., Wirsel, S.G.R., 2013. Identification of ABC Transporter Genes of Fusarium graminearum with Roles in Azole Tolerance and/or Virulence. PLoS ONE 8, e79042. https://doi.org/10.1371/journal.pone.0079042

Agrawal, G., Shang, H.H., Xia, Z.-J., Subramani, S., 2017. Functional regions of the peroxin Pex19 necessary for peroxisome biogenesis. J. Biol. Chem. 292, 11547–11560. https://doi.org/10.1074/jbc.M116.774067

Alvarez-Jarreta, J., Amos, B., Aurrecoechea, C., Bah, S., Barba, M., Barreto, A., Basenko, E.Y., Belnap, R., Blevins, A., Böhme, U., Brestelli, J., Brown, S., Callan, D., Campbell, L.I., Christophides, G.K., Crouch, K., Davison, H.R., DeBarry, J.D., Demko, R., Doherty, R., Duan, Y., Dundore, W., Dyer, S., Falke, D., Fischer, S., Gajria, B., Galdi, D., Giraldo-Calderón, G.I., Harb, O.S., Harper, E., Helb, D., Howington, C., Hu, S., Humphrey, J., Iodice, J., Jones, A., Judkins, J., Kelly, S.A., Kissinger, J.C., Kittur, N., Kwon, D.K., Lamoureux, K., Li, W., Lodha, D., MacCallum, R.M., Maslen, G., McDowell, M.A., Myers, J., Nural, M.V., Roos, D.S., Rund, S.S.C., Shanmugasundram, A., Sitnik, V., Spruill, D., Starns, D., Tomko, S.S., Wang, H., Warrenfeltz, S., Wieck, R., Wilkinson, P.A., Zheng, J., 2024. VEuPathDB: the eukaryotic pathogen, vector and host bioinformatics resource center in 2023. Nucleic Acids Res. 52, D808–D816. https://doi.org/10.1093/nar/gkad1003

Alves, L.R., Ávila, A.R., Correa, A., Holetz, F.B., Mansur, F.C.B., Manque, P.A., De Menezes, J.P.B., Buck, G.A., Krieger, M.A., Goldenberg, S., 2010. Proteomic analysis reveals the dynamic association of proteins with translated mRNAs in Trypanosoma cruzi. Gene 452, 72–78. https://doi.org/10.1016/j.gene.2009.12.009

Andrade, J.M., Gonçalves, L.O., Liarte, D.B., Lima, D.A., Guimarães, F.G., De Melo Resende, D., Santi, A.M.M., De Oliveira, L.M., Velloso, J.P.L., Delfino, R.G., Pescher, P., Späth, G.F., Ruiz, J.C., Murta, S.M.F., 2020. Comparative transcriptomic analysis of antimony resistant and susceptible Leishmania infantum lines. Parasit. Vectors 13, 600. https://doi.org/10.1186/s13071-020-04486-4

Andrade, J.M., Murta, S.M.F., 2014. Functional analysis of cytosolic tryparedoxin peroxidase in antimony-resistant and –susceptible Leishmania braziliensis and Leishmania infantum lines. Parasit. Vectors 7, 406. https://doi.org/10.1186/1756-3305-7-406

Banerjee, H., LaPointe, P., Eitzen, G., Rachubinski, R.A., 2021. A Small Molecule Inhibitor of Pex3–Pex19 Interaction Disrupts Glycosome Biogenesis and Causes Lethality in Trypanosoma brucei. Front. Cell Dev. Biol. 9, 703603. https://doi.org/10.3389/fcell.2021.703603

Berman, J.D., Waddell, D., Hanson, B.D., 1985. Biochemical mechanisms of the antileishmanial activity of sodium stibogluconate. Antimicrob. Agents Chemother. 27, 916–920. https://doi.org/10.1128/AAC.27.6.916

Biagiotti, M., Dominguez, S., Yamout, N., Zufferey, R., 2017. Lipidomics and anti-trypanosomatid chemotherapy. Clin. Transl. Med. 6, e27. https://doi.org/10.1186/s40169-017-0160-7

Bringaud, F., Rivière, L., Coustou, V., 2006. Energy metabolism of trypanosomatids: Adaptation to available carbon sources. Mol. Biochem. Parasitol. 149, 1–9. https://doi.org/10.1016/j.molbiopara.2006.03.017

Brotherton, M.-C., Bourassa, S., Leprohon, P., Légaré, D., Poirier, G.G., Droit, A., Ouellette, M., 2013. Proteomic and Genomic Analyses of Antimony Resistant Leishmania infantum Mutant. PLoS ONE 8, e81899. https://doi.org/10.1371/journal.pone.0081899

Bunnik, E.M., Chung, D.-W., Hamilton, M., Ponts, N., Saraf, A., Prudhomme, J., Florens, L., Le Roch, K.G., 2013. Polysome profiling reveals translational control of gene expression in the human malaria parasite Plasmodium falciparum. Genome Biol. 14, R128. https://doi.org/10.1186/gb-2013-14-11-r128

Carnielli, J.B.T., Crouch, K., Forrester, S., Silva, V.C., Carvalho, S.F.G., Damasceno, J.D., Brown, E., Dickens, N.J., Costa, D.L., Costa, C.H.N., Dietze, R., Jeffares, D.C., Mottram, J.C., 2018. A Leishmania infantum genetic marker associated with miltefosine treatment failure for visceral leishmaniasis. EBioMedicine 36, 83–91. https://doi.org/10.1016/j.ebiom.2018.09.029

Castillo-Castañeda, A., Herrera, G., Ayala, M.S., Fuya, P., Ramírez, J.D., 2021. Spatial and Temporal Variability of Visceral Leishmaniasis in Colombia, 2007 to 2018. Am. J. Trop. Med. Hyg. 105, 144–155. https://doi.org/10.4269/ajtmh.21-0103

Castillo-Castañeda, A., Patiño, L.H., Muñoz, M., Ayala, M.S., Segura, M., Bautista, J., Shaban, M.V., Paniz-Mondolfi, A., Ramírez, J.D., 2022. Amplicon-based next-generation sequencing reveals the co-existence of multiple Leishmania species in patients with visceral leishmaniasis. Int. J. Infect. Dis. 115, 35–38. https://doi.org/10.1016/j.ijid.2021.11.029

Cayla, M., Nievas, Y.R., Matthews, K.R., Mottram, J.C., 2022. Distinguishing functions of trypanosomatid protein kinases. Trends Parasitol. 38, 950–961. https://doi.org/10.1016/j.pt.2022.08.009

Chawla, B., Jhingran, A., Panigrahi, A., Stuart, K.D., Madhubala, R., 2011. Paromomycin Affects Translation and Vesicle-Mediated Trafficking as Revealed by Proteomics of Paromomycin –Susceptible –Resistant Leishmania donovani. PLoS ONE 6, e26660. https://doi.org/10.1371/journal.pone.0026660

Clayton, C., 2019. Regulation of gene expression in trypanosomatids: living with polycistronic transcription. Open Biol 9, 190072. https://doi.org/10.1098/rsob.190072

Clayton, C., Shapira, M., 2007. Post-transcriptional regulation of gene expression in trypanosomes and leishmanias. Mol. Biochem. Parasitol. 156, 93–101. https://doi.org/10.1016/j.molbiopara.2007.07.007

Cox, J., Mann, M., 2008. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. 26, 1367–1372. https://doi.org/10.1038/nbt.1511

Croft, S.L., Sundar, S., Fairlamb, A.H., 2006. Drug Resistance in Leishmaniasis. CMR 19, 111–126. https://doi.org/10.1128/CMR.19.1.111-126.2006

Cuypers, B., Berg, M., Imamura, H., Dumetz, F., De Muylder, G., Domagalska, M.A., Rijal, S., Bhattarai, N.R., Maes, I., Sanders, M., Cotton, J.A., Meysman, P., Laukens, K., Dujardin, J.-C., 2018. Integrated genomic and metabolomic profiling of ISC1, an emerging Leishmania donovani population in the Indian subcontinent. Infect. Genet. Evol. 62, 170–178. https://doi.org/10.1016/j.meegid.2018.04.021

De Azevedo, A.F., De Lisboa Dutra, J.L., Barbosa Santos, M.L., De Alexandria Santos, D., Alves, P.B., De Moura, T.R., De Almeida, R.P., Fernandes, M.F., Scher, R., Fernandes, R.P.M., 2014. Fatty acid profiles in Leishmania spp. isolates with natural resistance to nitric oxide and trivalent antimony. Parasitol. Res. 113, 19–27. https://doi.org/10.1007/s00436-013-3621-y

De Lima, L.P., Poubel, S.B., Yuan, Z.-F., Rosón, J.N., Vitorino, F.N.D.L., Holetz, F.B., Garcia, B.A., Da Cunha, J.P.C., 2020. Improvements on the quantitative analysis of Trypanosoma cruzi histone post translational modifications: Study of changes in epigenetic marks through the parasite's metacyclogenesis and life cycle. J. Proteomics 225, 103847. https://doi.org/10.1016/j.jprot.2020.103847

Decuypere, S., Vanaerschot, M., Brunker, K., Imamura, H., Müller, S., Khanal, B., Rijal, S., Dujardin, J.-C., Coombs, G.H., 2012. Molecular Mechanisms of Drug Resistance in Natural Leishmania Populations Vary with Genetic Background. PLoS Negl Trop Dis 6, e1514. https://doi.org/10.1371/journal.pntd.0001514

Didusch, S., Madern, M., Hartl, M., Baccarini, M., 2022. amica: an interactive and user-friendly web-platform for the analysis of proteomics data. BMC Genomics 23, 817. https://doi.org/10.1186/s12864-022-09058-7

Dong, G., Filho, A.L., Olivier, M., 2019. Modulation of Host-Pathogen Communication by Extracellular Vesicles (EVs) of the Protozoan Parasite Leishmania. Front Cell Infect Microbiol 9, 100. https://doi.org/10.3389/fcimb.2019.00100

Douanne, N., Wagner, V., Roy, G., Leprohon, P., Ouellette, M., Fernandez-Prada, C., 2020. MRPA-independent mechanisms of antimony resistance in Leishmania infantum. Int. J. Parasitol. Drugs Drug Resist. 13, 28–37. https://doi.org/10.1016/j.ijpddr.2020.03.003

Downing, T., Imamura, H., Decuypere, S., Clark, T.G., Coombs, G.H., Cotton, J.A., Hilley, J.D., de Doncker, S., Maes, I., Mottram, J.C., Quail, M.A., Rijal, S., Sanders, M., Schönian, G., Stark, O., Sundar, S., Vanaerschot, M., Hertz-Fowler, C., Dujardin, J.-C., Berriman, M., 2011. Whole genome sequencing of multiple Leishmania donovani clinical isolates provides insights into population structure and mechanisms of drug resistance. Genome Res 21, 2143–2156. https://doi.org/10.1101/gr.123430.111

Dumetz, F., Cuypers, B., Imamura, H., Zander, D., D'Haenens, E., Maes, I., Domagalska, M.A., Clos, J., Dujardin, J.-C., De Muylder, G., 2018. Molecular Preadaptation to Antimony Resistance in Leishmania donovani on the Indian Subcontinent. mSphere 3. https://doi.org/10.1128/mSphere.00548-17

Egea, P.F., Napetschnig, J., Walter, P., Stroud, R.M., 2008. Structures of SRP54 and SRP19, the Two Proteins that Organize the Ribonucleic Core of the Signal Recognition Particle from Pyrococcus furiosus. PLoS ONE 3, e3528. https://doi.org/10.1371/journal.pone.0003528

El Fadili, K., Drummelsmith, J., Roy, G., Jardim, A., Ouellette, M., 2009. Down regulation of KMP-11 in Leishmania infantum axenic antimony resistant amastigotes as revealed by a proteomic screen. Exp. Parasitol. 123, 51–57. https://doi.org/10.1016/j.exppara.2009.05.013

Fall, F., Mamede, L., Schioppa, L., Ledoux, A., De Tullio, P., Michels, P., Frédérich, M., Quetin-Leclercq, J., 2022. Trypanosoma brucei: Metabolomics for analysis of cellular metabolism and drug discovery. Metabolomics 18, 20. https://doi.org/10.1007/s11306-022-01880-0

Franssen, S.U., Durrant, C., Stark, O., Moser, B., Downing, T., Imamura, H., Dujardin, J.-C., Sanders, M.J., Mauricio, I., Miles, M.A., Schnur, L.F., Jaffe, C.L., Nasereddin, A., Schallig, H., Yeo, M., Bhattacharyya, T., Alam, M.Z., Berriman, M., Wirth, T., Schönian, G., Cotton, J.A., 2020. Global genome diversity of the Leishmania donovani complex. eLife 9, e51243. https://doi.org/10.7554/eLife.51243

Frézard, F., Demicheli, C., Ribeiro, R., 2009. Pentavalent Antimonials: New Perspectives for Old Drugs. Molecules 14, 2317–2336. https://doi.org/10.3390/molecules14072317

González-de la Fuente, S., Peiró-Pastor, R., Rastrojo, A., Moreno, J., Carrasco-Ramiro, F., Requena, J.M., Aguado, B., 2017. Resequencing of the Leishmania infantum (strain JPCM5) genome and de novo assembly into 36 contigs. Sci Rep 7, 18050. https://doi.org/10.1038/s41598-017-18374-y

Goto, Y., Ito, T., Ghosh, S., Mukherjee, B., 2024. Access and utilization of host-derived iron by *Leishmania* parasites. J. Biochem. (Tokyo) 175, 17–24. https://doi.org/10.1093/jb/mvad082

Gotz, S., Garcia-Gomez, J.M., Terol, J., Williams, T.D., Nagaraj, S.H., Nueda, M.J., Robles, M., Talon, M., Dopazo, J., Conesa, A., 2008. High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Res. 36, 3420–3435. https://doi.org/10.1093/nar/gkn176

Gutierrez Guarnizo, S.A., Karamysheva, Z.N., Galeano, E., Muskus, C.E., 2021a. Metabolite Biomarkers of Leishmania Antimony Resistance. Cells 10, 1063. https://doi.org/10.3390/cells10051063

Gutierrez Guarnizo, S.A., Tikhonova, E.B., Karamyshev, A.L., Muskus, C.E., Karamysheva, Z.N., 2023. Translational reprogramming as a driver of antimony-drug resistance in Leishmania. Nat. Commun. 14, 2605. https://doi.org/10.1038/s41467-023-38221-1

Gutierrez Guarnizo, S.A., Tikhonova, E.B., Zabet-Moghaddam, M., Zhang, K., Muskus, C., Karamyshev, A.L., Karamysheva, Z.N., 2021b. Drug-Induced Lipid Remodeling in Leishmania Parasites. Microorganisms 9, 790. https://doi.org/10.3390/microorganisms9040790

Haimeur, A., Guimond, C., Pilote, S., Mukhopadhyay, R., Rosen, B.P., Poulin, R., Ouellette, M., 1999. Elevated levels of polyamines and trypanothione resulting from overexpression of the ornithine decarboxylase gene in arsenite-resistant Leishmania. Mol Microbiol 34, 726–735. https://doi.org/10.1046/j.1365-2958.1999.01634.x

Hendrickx, S., Beyers, J., Mondelaers, A., Eberhardt, E., Lachaud, L., Delputte, P., Cos, P., Maes, L., 2016. Evidence of a drug-specific impact of experimentally selected paromomycin and miltefosine resistance on parasite fitness in *Leishmania infantum*. J. Antimicrob. Chemother. 71, 1914–1921. https://doi.org/10.1093/jac/dkw096

Hernandez-Valladares, M., Aasebø, E., Mjaavatten, O., Vaudel, M., Bruserud, Ø., Berven, F., Selheim, F., 2016. Reliable FASP-based procedures for optimal quantitative proteomic and phosphoproteomic analysis on samples from acute myeloid leukemia patients. Biol. Proced. Online 18, 13. https://doi.org/10.1186/s12575-016-0043-0

Iantorno, S.A., Durrant, C., Khan, A., Sanders, M.J., Beverley, S.M., Warren, W.C., Berriman, M., Sacks, D.L., Cotton, J.A., Grigg, M.E., 2017. Gene Expression in *Leishmania* Is Regulated Predominantly by Gene Dosage. mBio 8. https://doi.org/10.1128/mBio.01393-17

Inbar, E., Shaik, J., Iantorno, S.A., Romano, A., Nzelu, C.O., Owens, K., Sanders, M.J., Dobson, D., Cotton, J.A., Grigg, M.E., Beverley, S.M., Sacks, D., 2019. Whole genome sequencing of experimental hybrids supports meiosis-like sexual recombination in Leishmania. PLOS Genet. 15, e1008042. https://doi.org/10.1371/journal.pgen.1008042

Kamran, Mohd., Bhattacharjee, R., Das, S., Mukherjee, S., Ali, N., 2023. The paradigm of intracellular parasite survival and drug resistance in leishmanial parasite through genome plasticity and epigenetics: Perception and future perspective. Front. Cell. Infect. Microbiol. 13, 1001973. https://doi.org/10.3389/fcimb.2023.1001973

Kanehisa, M., 2000. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res. 28, 27–30. https://doi.org/10.1093/nar/28.1.27

Klokouzas, A., 2003. ABC transporters and drug resistance in parasitic protozoa. Int. J. Antimicrob. Agents 22, 301–317. https://doi.org/10.1016/S0924-8579(03)00210-3

Kong, A.T., Leprevost, F.V., Avtonomov, D.M., Mellacheruvu, D., Nesvizhskii, A.I., 2017. MSFragger: ultrafast and comprehensive peptide identification in mass spectrometry–based proteomics. Nat. Methods 14, 513–520. https://doi.org/10.1038/nmeth.4256

Kramer, S., Carrington, M., 2011. Trans-acting proteins regulating mRNA maturation, stability and translation in trypanosomatids. Trends Parasitol. 27, 23–30. https://doi.org/10.1016/j.pt.2010.06.011

Kramer, S., Karolak, N.K., Odenwald, J., Gabiatti, B., Castañeda Londoño, P.A., Zavřelová, A., Freire, E.R., Almeida, K.S., Braune, S., Moreira, C., Eder, A., Goos, C., Field, M., Carrington, M., Holetz, F., Górna, M.W., Zoltner, M., 2023. A unique mRNA decapping complex in trypanosomes. Nucleic Acids Res. 51, 7520–7540. https://doi.org/10.1093/nar/gkad497

Kulak, N.A., Pichler, G., Paron, I., Nagaraj, N., Mann, M., 2014. Minimal, encapsulated proteomic-sample processing applied to copy-number estimation in eukaryotic cells. Nat. Methods 11, 319–324. https://doi.org/10.1038/nmeth.2834

Kumar, A., Sisodia, B., Misra, P., Sundar, S., Shasany, A.K., Dube, A., 2010. Proteome mapping of overexpressed membrane-enriched and cytosolic proteins in sodium antimony gluconate (SAG) resistant clinical isolate of Leishmania donovani: Overexpressed proteins in SAG resistant L. donovani. Br. J. Clin. Pharmacol. 70, 609–617. https://doi.org/10.1111/j.1365-2125.2010.03716.x

Leprohon, P., Legare, D., Raymond, F., Madore, E., Hardiman, G., Corbeil, J., Ouellette, M., 2009. Gene expression modulation is associated with gene amplification, supernumerary chromosomes and chromosome loss in antimony-resistant Leishmania infantum. Nucleic Acids Res. 37, 1387–1399. https://doi.org/10.1093/nar/gkn1069

Leroux, M., Luquain-Costaz, C., Lawton, P., Azzouz-Maache, S., Delton, I., 2023. Fatty Acid Composition and Metabolism in Leishmania Parasite Species: Potential Biomarkers or Drug Targets for Leishmaniasis? Int. J. Mol. Sci. 24, 4702. https://doi.org/10.3390/ijms24054702

Li, M., Gaussmann, S., Tippler, B., Ott, J., Popowicz, G.M., Schliebs, W., Sattler, M., Erdmann, R., Kalel, V.C., 2021. Novel Trypanocidal Inhibitors that Block Glycosome Biogenesis by Targeting PEX3–PEX19 Interaction. Front. Cell Dev. Biol. 9, 737159. https://doi.org/10.3389/fcell.2021.737159

Liarte, D.B., Murta, S.M.F., 2010. Selection and phenotype characterization of potassium antimony tartrate-resistant populations of four New World Leishmania species. Parasitol. Res. 107, 205–212. https://doi.org/10.1007/s00436-010-1852-8

Lima, B.S. de S., Esteves, B.B., Fialho-Júnior, L.C., Mendes, T.A. de O., Pires, S. da F., Chapeourouge, A., Perales, J., de Andrade, H.M., 2020. Study of the differentially abundant proteins among Leishmania amazonensis, L. braziliensis, and L. infantum. PLoS ONE 15, e0240612. https://doi.org/10.1371/journal.pone.0240612

Lindoso, J., Moreira, C., Cunha, M., Queiroz, I.T., 2018. Visceral leishmaniasis and HIV coinfection: current perspectives. HIV Volume 10, 193–201. https://doi.org/10.2147/HIV.S143929

Lira, R., Sundar, S., Makharia, A., Kenney, R., Gam, A., Saraiva, E., Sacks, D., 1999. Evidence that the High Incidence of Treatment Failures in Indian Kala-Azar Is Due to the Emergence of Antimony-Resistant Strains of *Leishmania donovani*. J. Infect. Dis. 180, 564–567. https://doi.org/10.1086/314896

Machuca, C., Rodríguez, A., Herrera, M., Silva, S., Ponte-Sucre, A., 2006. Leishmania amazonensis: Metabolic adaptations induced by resistance to an ABC transporter blocker. Exp. Parasitol. 114, 1–9. https://doi.org/10.1016/j.exppara.2006.02.008

Madusanka, R.K., Karunaweera, N.D., Silva, H., Selvapandiyan, A., 2024. Antimony resistance and gene expression in *Leishmania* : spotlight on molecular and proteomic aspects. Parasitology 151, 1–14. https://doi.org/10.1017/S0031182023001129

Manzano, J.I., García-Hernández, R., Castanys, S., Gamarro, F., 2013. A New ABC Half-Transporter in Leishmania major Is Involved in Resistance to Antimony. Antimicrob. Agents Chemother. 57, 3719–3730. https://doi.org/10.1128/AAC.00211-13

Mathur, R., Das, R.P., Ranjan, A., Shaha, C., 2015. Elevated ergosterol protects *Leishmania* parasites against antimony-generated stress. FASEB J. 29, 4201–4213. https://doi.org/10.1096/fj.15-272757

Matrangolo, F.S.V., Liarte, D.B., Andrade, L.C., de Melo, M.F., Andrade, J.M., Ferreira, R.F., Santiago, A.S., Pirovani, C.P., Silva-Pereira, R.A., Murta, S.M.F., 2013. Comparative proteomic analysis of antimony-resistant and -susceptible Leishmania braziliensis and Leishmania infantum chagasi lines. Mol. Biochem. Parasitol. 190, 63–75. https://doi.org/10.1016/j.molbiopara.2013.06.006

Maugeri, D.A., Cannata, J.J.B., Cazzulo, J.-J., 2011. Glucose metabolism in *Trypanosoma cruzi*. Essays Biochem. 51, 15–30. https://doi.org/10.1042/bse0510015

Mbongo, N., Loiseau, P.M., Billion, M.A., Robert-Gero, M., 1998. Mechanism of amphotericin B resistance in Leishmania donovani promastigotes. Antimicrob Agents Chemother 42, 352–357.

Meier, F., Brunner, A.-D., Koch, S., Koch, H., Lubeck, M., Krause, M., Goedecke, N., Decker, J., Kosinski, T., Park, M.A., Bache, N., Hoerning, O., Cox, J., Räther, O., Mann, M., 2018. Online Parallel Accumulation–Serial Fragmentation (PASEF) with a Novel Trapped Ion Mobility Mass Spectrometer. Mol. Cell. Proteomics 17, 2534–2545. https://doi.org/10.1074/mcp.TIR118.000900

Mukherjee, B., Mukherjee, K., Nanda, P., Mukhopadhayay, R., Ravichandiran, V., Bhattacharyya, S.N., Roy, S., 2021. Probing the molecular mechanism of aggressive infection by antimony resistant Leishmania donovani. Cytokine 145, 155245. https://doi.org/10.1016/j.cyto.2020.155245

Oliveira, I.H.R., Figueiredo, H.C.P., Rezende, C.P., Verano-Braga, T., Melo-Braga, M.N., Reis Cunha, J.L., De Andrade, H.M., 2020. Assessing the composition of the plasma membrane of Leishmania (Leishmania) infantum and L. (L.) amazonensis using label-free proteomics. Exp. Parasitol. 218, 107964. https://doi.org/10.1016/j.exppara.2020.107964

OmicsBox – Bioinformatics Made Easy, 2019.

Ouakad, M., Vanaerschot, M., Rijal, S., Sundar, S., Speybroeck, N., Kestens, L., Boel, L., De Doncker, S., Maes, I., Decuypere, S., Dujardin, J.-C., 2011. Increased metacyclogenesis of antimony-resistant *Leishmania donovani* clinical lines. Parasitology 138, 1392–1399. https://doi.org/10.1017/S0031182011001120

PAHO, W., 2022. Guideline for the Treatment of Leishmaniasis in the Americas. Second Edition, Second. ed. Pan American Health Organization. https://doi.org/10.37774/9789275125038

Patino, L.H., Imamura, H., Cruz-Saavedra, L., Pavia, P., Muskus, C., Méndez, C., Dujardin, J.C., Ramírez, J.D., 2019a. Major changes in chromosomal somy, gene expression and gene dosage driven by SbIII in Leishmania braziliensis and Leishmania panamensis. Sci. Rep. 9. https://doi.org/10.1038/s41598-019-45538-9

Patino, L.H., Muskus, C., Ramírez, J.D., 2019b. Transcriptional responses of Leishmania (Leishmania) amazonensis in the presence of trivalent sodium stibogluconate. Parasit. Vectors 12. https://doi.org/10.1186/s13071-019-3603-8

Perea, A., Manzano, J.I., Castanys, S., Gamarro, F., 2016. The LABCG2 Transporter from the Protozoan Parasite Leishmania Is Involved in Antimony Resistance. Antimicrob. Agents Chemother. 60, 3489–3496. https://doi.org/10.1128/AAC.02813-15

Pinho, N., Wiśniewski, J.R., Dias-Lopes, G., Saboia-Vahia, L., Bombaça, A.C.S., Mesquita-Rodrigues, C., Menna-Barreto, R., Cupolillo, E., De Jesus, J.B., Padrón, G., Cuervo, P., 2020. In-depth quantitative proteomics uncovers specie-specific metabolic programs in Leishmania (Viannia) species. PLoS Negl. Trop. Dis. 14, e0008509. https://doi.org/10.1371/journal.pntd.0008509

Ponte-Sucre, A., Gamarro, F., Dujardin, J.-C., Barrett, M.P., López-Vélez, R., García-Hernández, R., Pountain, A.W., Mwenechanya, R., Papadopoulou, B., 2017. Drug resistance and treatment failure in leishmaniasis: A 21st century challenge. PLoS Negl. Trop. Dis. 11, e0006052. https://doi.org/10.1371/journal.pntd.0006052

Pountain, A.W., Barrett, M.P., 2019. Untargeted metabolomics to understand the basis of phenotypic differences in amphotericin B-resistant Leishmania parasites. Wellcome Open Res. 4, 176. https://doi.org/10.12688/wellcomeopenres.15452.1

Pramanik, P.K., Alam, M.N., Roy Chowdhury, D., Chakraborti, T., 2019. Drug Resistance in Protozoan Parasites: An Incessant Wrestle for Survival. J. Glob. Antimicrob. Resist. 18, 1–11. https://doi.org/10.1016/j.jgar.2019.01.023

R Core Team, 2021. R: A Language and Environment for Statistical Computing.

Rappsilber, J., Mann, M., Ishihama, Y., 2007. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat. Protoc. 2, 1896–1906. https://doi.org/10.1038/nprot.2007.261

Rastrojo, A., García-Hernández, R., Vargas, P., Camacho, E., Corvo, L., Imamura, H., Dujardin, J.-C., Castanys, S., Aguado, B., Gamarro, F., Requena, J.M., 2018. Genomic and transcriptomic alterations in Leishmania donovani lines experimentally resistant to antileishmanial drugs. Int. J. Parasitol. Drugs Drug Resist. 8, 246–264. https://doi.org/10.1016/j.ijpddr.2018.04.002

Ren, B., Dai, H.-Q., Pei, G., Tong, Y.-J., Zhuo, Y., Yang, N., Su, M.-Y., Huang, P., Yang, Y.-Z., Zhang, L.-X., 2014. ABC transporters coupled with the elevated ergosterol contents contribute to the azole resistance and amphotericin B susceptibility. Appl. Microbiol. Biotechnol. 98, 2609–2616. https://doi.org/10.1007/s00253-013-5425-5

Roberts, W.L., Rainey, P.M., 1993. Antileishmanial activity of sodium stibogluconate fractions. Antimicrob. Agents Chemother. 37, 1842–1846. https://doi.org/10.1128/AAC.37.9.1842

Saboia-Vahia, L., Cuervo, P., Wiśniewski, J.R., Dias-Lopes, G., Pinho, N., Padrón, G., De Pilla Varotti, F., Murta, S.M.F., 2022. In-Depth Quantitative Proteomics Characterization of In Vitro Selected Miltefosine Resistance in Leishmania infantum. Proteomes 10, 10. https://doi.org/10.3390/proteomes10020010

Sauvage, V., Aubert, D., Escotte-Binet, S., Villena, I., 2009. The role of ATP-binding cassette (ABC) proteins in protozoan parasites. Mol. Biochem. Parasitol. 167, 81–94. https://doi.org/10.1016/j.molbiopara.2009.05.005

Schrader, M., Fahimi, H.D., 2006. Growth and Division of Peroxisomes, in: International Review of Cytology. Elsevier, pp. 237–290. https://doi.org/10.1016/S0074-7696(06)55005-3

Sereno, D., Holzmuller, P., Mangot, I., Cuny, G., Ouaissi, A., Lemesre, J.-L., 2001. Antimonial-Mediated DNA Fragmentation in Leishmania infantum Amastigotes. Antimicrob. Agents Chemother. 45, 2064–2069. https://doi.org/10.1128/AAC.45.7.2064-2069.2001

Sienkiewicz, N., Ong, H.B., Fairlamb, A.H., 2018. Characterisation of a putative glutamate 5-kinase from *Leishmania donovani*. FEBS J. 285, 2662–2678. https://doi.org/10.1111/febs.14511

Silva, G.L.A., Tosi, L.R.O., McCulloch, R., Black, J.A., 2021. Unpicking the Roles of DNA Damage Protein Kinases in Trypanosomatids. Front. Cell Dev. Biol. 9, 636615. https://doi.org/10.3389/fcell.2021.636615

Silva, S.C., Guimarães, L.H., Silva, J.A., Magalhães, V., Medina, L., Queiroz, A., Machado, P.R.L., Schriefer, A., 2018. Molecular epidemiology and in vitro evidence suggest that Leishmania braziliensis strain helps determine antimony response among American tegumenary leishmaniasis patients. Acta Trop 178, 34–39. https://doi.org/10.1016/j.actatropica.2017.10.010

Subramanian, A., Jhawar, J., Sarkar, R.R., 2015. Dissecting Leishmania infantum Energy Metabolism - A Systems Perspective. PLOS ONE 10, e0137976. https://doi.org/10.1371/journal.pone.0137976

Szklarczyk, D., Gable, A.L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., Simonovic, M., Doncheva, N.T., Morris, J.H., Bork, P., Jensen, L.J., Mering, C. von, 2019. STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res. 47, D607–D613. https://doi.org/10.1093/nar/gky1131

t'Kindt, R., Scheltema, R.A., Jankevics, A., Brunker, K., Rijal, S., Dujardin, J.-C., Breitling, R., Watson, D.G., Coombs, G.H., Decuypere, S., 2010. Metabolomics to Unveil and Understand Phenotypic Diversity between Pathogen Populations. PLoS Negl. Trop. Dis. 4, e904. https://doi.org/10.1371/journal.pntd.0000904

Tabak, H.F., Braakman, I., Zand, A.V.D., 2013. Peroxisome Formation and Maintenance Are Dependent on the Endoplasmic Reticulum. Annu. Rev. Biochem. 82, 723–744. https://doi.org/10.1146/annurev-biochem-081111-125123

Tessarollo, N.G., Andrade, J.M., Moreira, D.S., Murta, S.M.F., 2015. Functional analysis of iron superoxide dismutase-A in wild-type and antimony-resistant Leishmania braziliensis and Leishmania infantum lines. Parasitol. Int. 64, 125–129. https://doi.org/10.1016/j.parint.2014.11.001

Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M.Y., Geiger, T., Mann, M., Cox, J., 2016. The Perseus computational platform for comprehensive analysis of (prote)omics data. Nat. Methods 13, 731–740. https://doi.org/10.1038/nmeth.3901

Van Dam, T.J.P., Bos, J., Snel, B., 2011. Evolution of the Ras-like small GTPases and their regulators. Small GTPases 2, 4–16. https://doi.org/10.4161/sgtp.2.1.15113

van Griensven, J., Diro, E., 2012. Visceral Leishmaniasis. Infect. Dis. Clin. North Am. 26, 309–322. https://doi.org/10.1016/j.idc.2012.03.005

Van Hellemond, J.J., Bakker, B.M., Tielens, A.G.M., 2005. Energy Metabolism and Its Compartmentation in Trypanosoma brucei, in: Advances in Microbial Physiology. Elsevier, pp. 199–226. https://doi.org/10.1016/S0065-2911(05)50005-5

Vergnes, B., Gourbal, B., Girard, I., Sundar, S., Drummelsmith, J., Ouellette, M., 2007. A Proteomics Screen Implicates HSP83 and a Small Kinetoplastid Calpain-related Protein in Drug Resistance in Leishmania donovani Clinical Field Isolates by Modulating Drug-induced Programmed Cell Death. Mol. Cell. Proteomics 6, 88–101. https://doi.org/10.1074/mcp.M600319-MCP200

Wamai, R.G., Kahn, J., McGloin, J., Ziaggi, G., 2020. Visceral leishmaniasis: a global overview. J. Glob. Health Sci. 2, e3. https://doi.org/10.35500/jghs.2020.2.e3

Warringer, J., Blomberg, A., 2006. Evolutionary constraints on yeast protein size. BMC Evol. Biol. 6, 61. https://doi.org/10.1186/1471-2148-6-61

WHO, 2020. Global leishmaniasis surveillance, 2017–2018, and first report on 5 additional indicators (No. 25). World Orgaization Health.

Wiśniewski, J.R., 2017. Label-Free and Standard-Free Absolute Quantitative Proteomics Using the “Total Protein” and “Proteomic Ruler” Approaches, in: Methods in Enzymology. Elsevier, pp. 49–60. https://doi.org/10.1016/bs.mie.2016.10.002

Wiśniewski, J.R., Rakus, D., 2014. Multi-enzyme digestion FASP and the ‘Total Protein Approach’-based absolute quantification of the Escherichia coli proteome. J. Proteomics 109, 322–331. https://doi.org/10.1016/j.jprot.2014.07.012

Yang, Y., Wu, Y., Sun, X.-D., Zhang, Y., 2021. Reactive Oxygen Species, Glucose Metabolism, and Lipid Metabolism, in: Huang, C., Zhang, Y. (Eds.), Oxidative Stress. Springer Singapore, Singapore, pp. 213–235. https://doi.org/10.1007/978-981-16-0522-2_9

Zambrano Hernandez, C. del P., Ayala Sotelo, M.S., Fuya Oviedo, O.P., Montenegro Puentes, C.A., Aya Vanegas, N.M., Rodriguez Toro, J.G., Becerra Osorio, S.L., Aguilera Jaramillo, G., Lozano Polanco, C.A., Rojas Garcia, M.C., Blásquez, O., 2015. Brote urbano de leishmaniasis visceral en Neiva (Huila), 2012. Rev Salud Pública 17, 514–527. https://doi.org/10.15446/rsap.v17n4.44663

## Figures and Legends

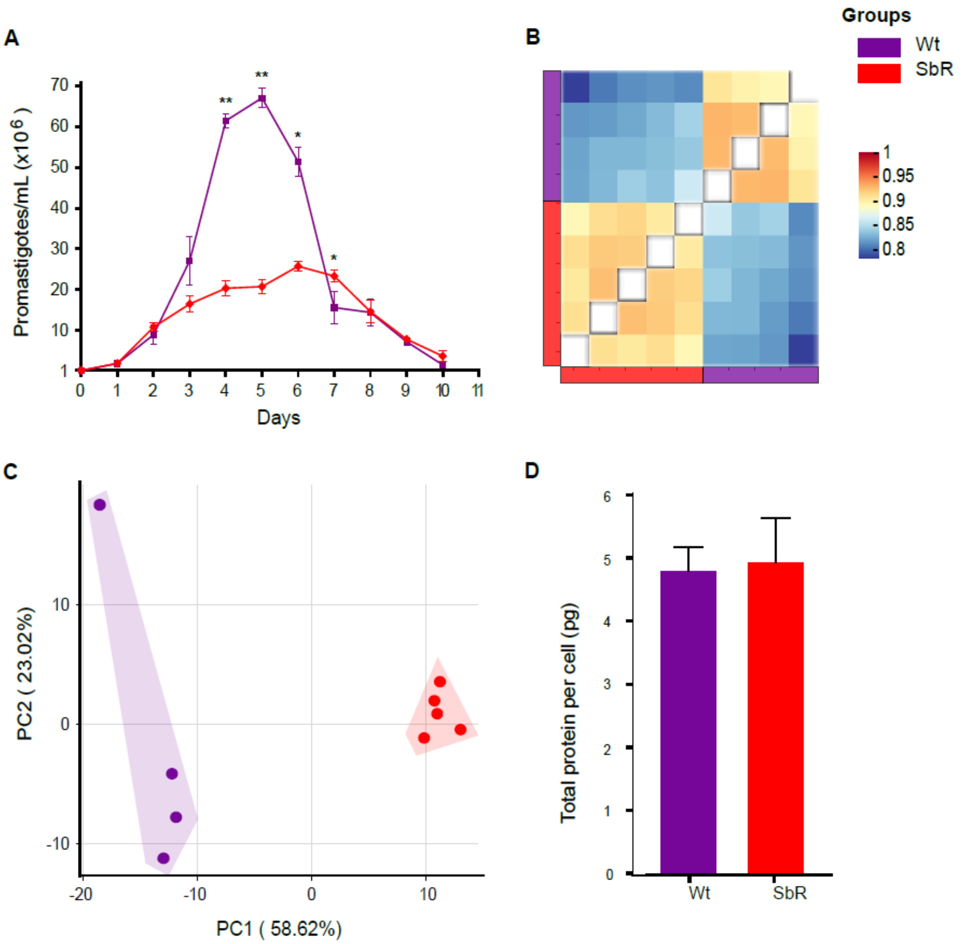


**Fig. 1.** Biological comparisons between *L. infantum* wild-type strain (purple) and sodium stibogluconate resistant (SbR) line (red). (a) *In vitro* promastigote growth curves expressed as mean ± SD by day of three biological and technical replicates, significant differences were determined by two-way ANOVA and Student's t-test * $p < 0.05$ and ** $p < 0.01$. (b) Pearson's correlation analysis conducted on the proteomics

data among the different biological replicates in each experimental group, $p < 0.05$, scale color shows the correlation between replicates and groups, wild-type strain n = 4; resistant line n = 5. (c) Principal component analysis (PCA) of protein concentration values of validated and quantified proteins. (d) The total protein value per cell (pg) was determined by the histone ruler method, bar graphs represent the mean ± SD of all independent experiments. significant difference was determined by unpaired Student's t-test, * $p < 0.05$.

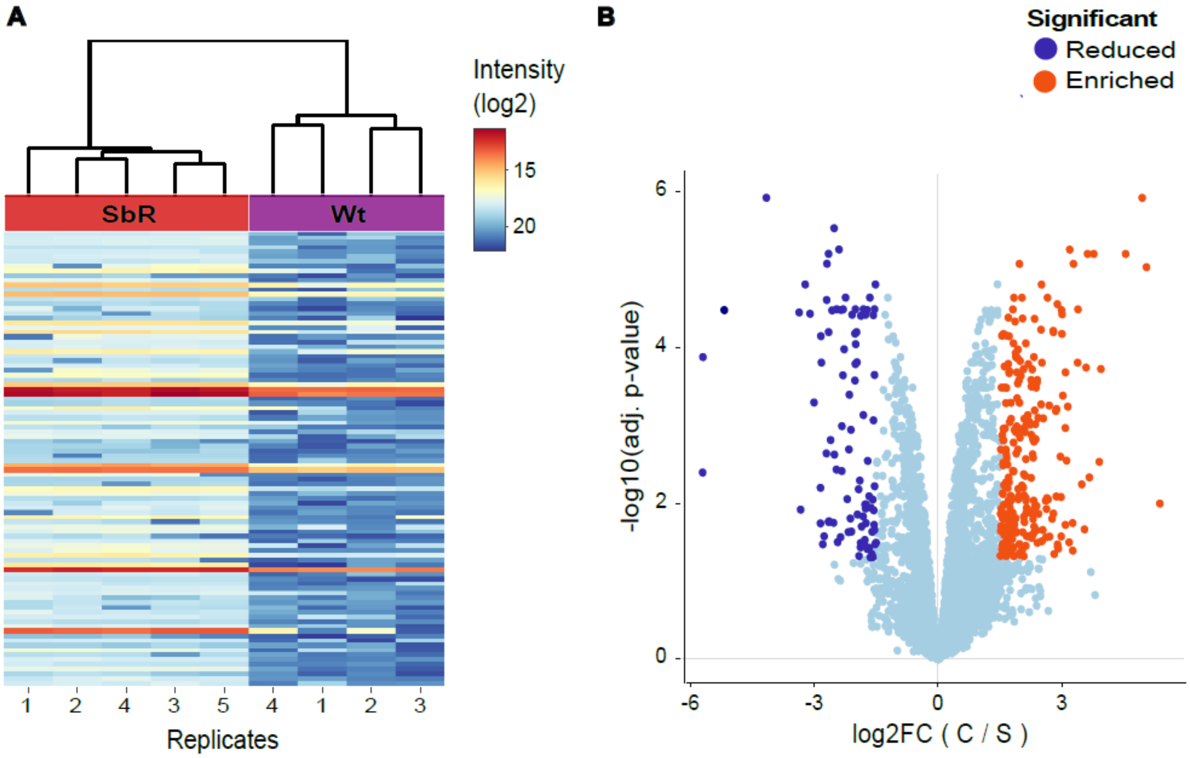


**Fig. 2.** Representation of the significant differences in protein concentration values between the wild-type strain *L. infantum* (purple) and sodium stibogluconate resistant (SbR) line (red) normalized in Log2. (a) Heatmap: values are colored according to the color scale, and (b) Volcano diagram: plotted values represent the average concentration of replicates from each strain, proteins with reduced concentration are blue, while proteins with enriched concentration are orange, significance was determined by t-test with 3% FDR.


A
ubiquitin binding
translation release factor activity
transferase activity
thioredoxin-disulfide reductase (NADP) activity
superoxide dismutase activity
sulfur compound binding
structural molecule activity
structural constituent of ribosome
sphingosine N-acyltransferase activity
small molecule binding
small GTPase binding
S-methyltransferase activity
ribonucleoside triphosphate phosphatase activity
ribonucleoside binding
pyrophosphatase activity
pyridoxal phosphate binding
purine nucleoside binding
protein tag activity
protein serine/threonine phosphatase activity
protein-folding chaperone binding
peptide and protein bindig
peptidase activity
oxidoreductase activity
organic cyclic compound binding
oligopeptide binding
nucleoside binding
nuclear export signal receptor activity
molecular function
modified amino acid binding
hydrolase activity
hexosyltransferase activity
heterocyclic compound binding
GTPase activity
GTP binding
glycosyltransferase activity
glutamate-5-semialdehyde dehydrogenase activity
ferric iron-related
enzyme regulator activity
dephospho-CoA kinase activity
deaminase activity
catalytic activity
carbon-oxygen lyase activity
carbohydrate derivative binding
binding
ATP binding-activity
arylformamidase activity
anion binding
amide binding
alpha-1,3-mannosyltransferase activity
aldehyde-lyase activity
agmatinase activity
adenosine deaminase activity
acyltransferase activity
acetate:proton symporter activity
2 sulfur cluster binding
1
2
3
4
5
6
7
Molecular function
B
vesicle-mediated transp
tryptophan catp to kynurenine
transp
translational termination
translation
superoxide mep
succinate transm transp
small molecule mep
signal transduction
S-methylmethionine cycle
ribosomal small subunit export from nucleus
ribosomal large subunit export from nucleus
ribonucleoprot complex biogenesis
response to stimulus
reproductive process
reg of mep
reg of cp
reg of bp
putrescine biop from arginine using agmatinase
proteolysis
prot transp to vacuole involved in ubiquitin-dependent prot
prot sumoylation
prot phosphorylation
prot localization
prot export from nucleus
proline biop
primary mep
phosphorus mep
phosphatidylinositol dephosphorylation
organophosphate mep
organonitrogen com mep
organic substance mep
organic substance catp
organic substance biop
organic cyclic com mep
nucleotide mep
nucleoside triphosphate mep
nucleoside phosphate mep
nucleobase-containing com mep
mRNA splicing
microtubule cytoskeleton orgnn
methionine biop
mep
localization
iron incorporation into metallo-sulfur cluster
iron-sulfur cluster assembly
intracell transp
intracell prot transp
intracell iron ion homeostasis
inosine biop
endosome transp via multivesicular body sorting pathway
endoplasmic reticulum to Golgi vesicle-mediated transp
endoplasmic reticulum orgnn
cp
COPII-coated vesicle cargo loading
coenzyme A biop
cell mep
cell component orgnn
cell catp
cell biop
cell adhesion
carbohydrate mep
bbp
adenosine catp
acetate transm transp
[2Fe-2S] cluster assembly
5
10
15
20
Biological Process
C
Groups
Reduced
Enriched
U2-type catalytic step 2 spliceosome
transmembrane transporter complex
transferase complex
respiratory chain complex
respirasome
subcomplex of 90S preribosome
Prp19 complex
protein-containing complex
preribosome
post-mRNA release spliceosomal complex
plastid
plasma membrane
oxidoreductase complex
organelle membrane or lumen
oligosaccharyltransferase complex
nucleus-nucleolus
non-membrane-bounded organelle
neuromuscular junction
NADH dehydrogenase complex
MKS complex
mitocondrial membrane and matriz
membrane and protein complex
late endosome membrane
large ribosomal subunit
intraciliary transport particle B
intracel. membrane and organelle
Golgi apparatus
external compound of cell
endomembrane system
end. reticulum
cytosol
cytoskeleton
cytoplasm
COPII vesicle coat
COP9 signalosome
cis-Golgi
ciliary related
catalytic complex
BBSome
axoneme
90S preribosome
5
10
15
20
Cellular Component

**Fig. 3.** Gene Ontology terms of characterized proteins with significant concentration changes in the sodium stibogluconate-resistant line. (a) Molecular Function, (b) Biological Process, and (c) Cellular Component. On the x-axis are the reduced (blue) and enriched (orange) proteins, and on the y-axis are the respective GO terms. The plot size corresponds to the protein number in each GO term

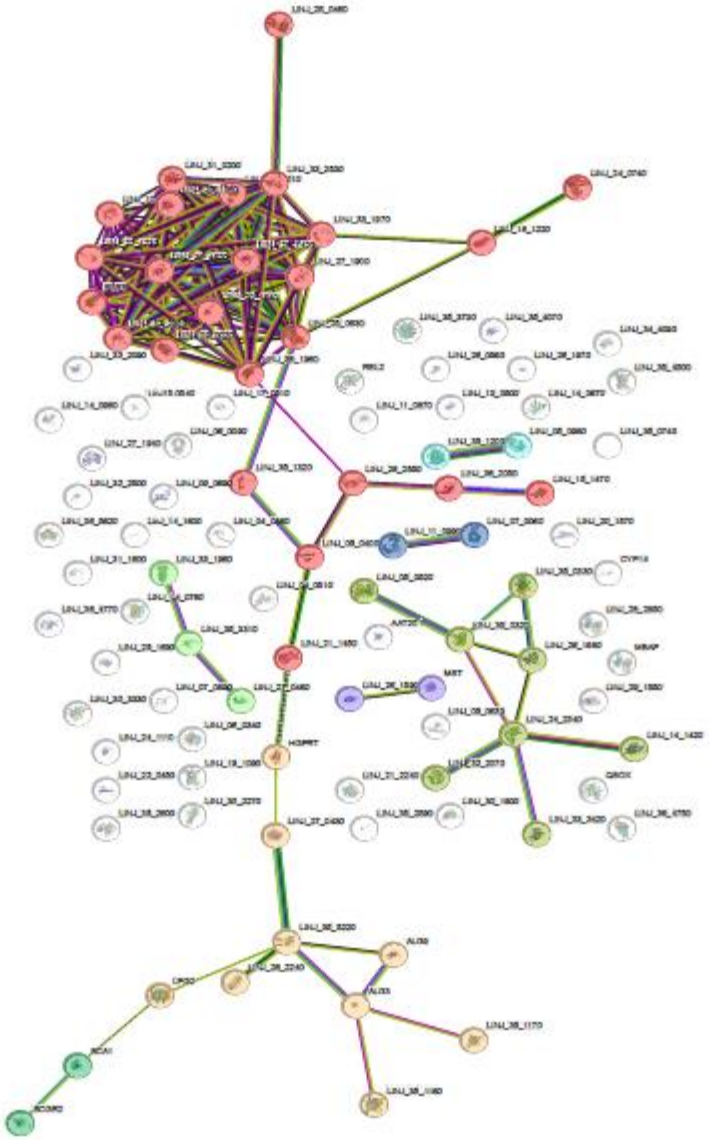


**Fig. 4.** Protein-protein interaction network of enriched proteins in the sodium stibogluconate-resistant line, utilizing the k-means clustering option with a specified value of k=8. The distance among nodes and clusters is not scaled.

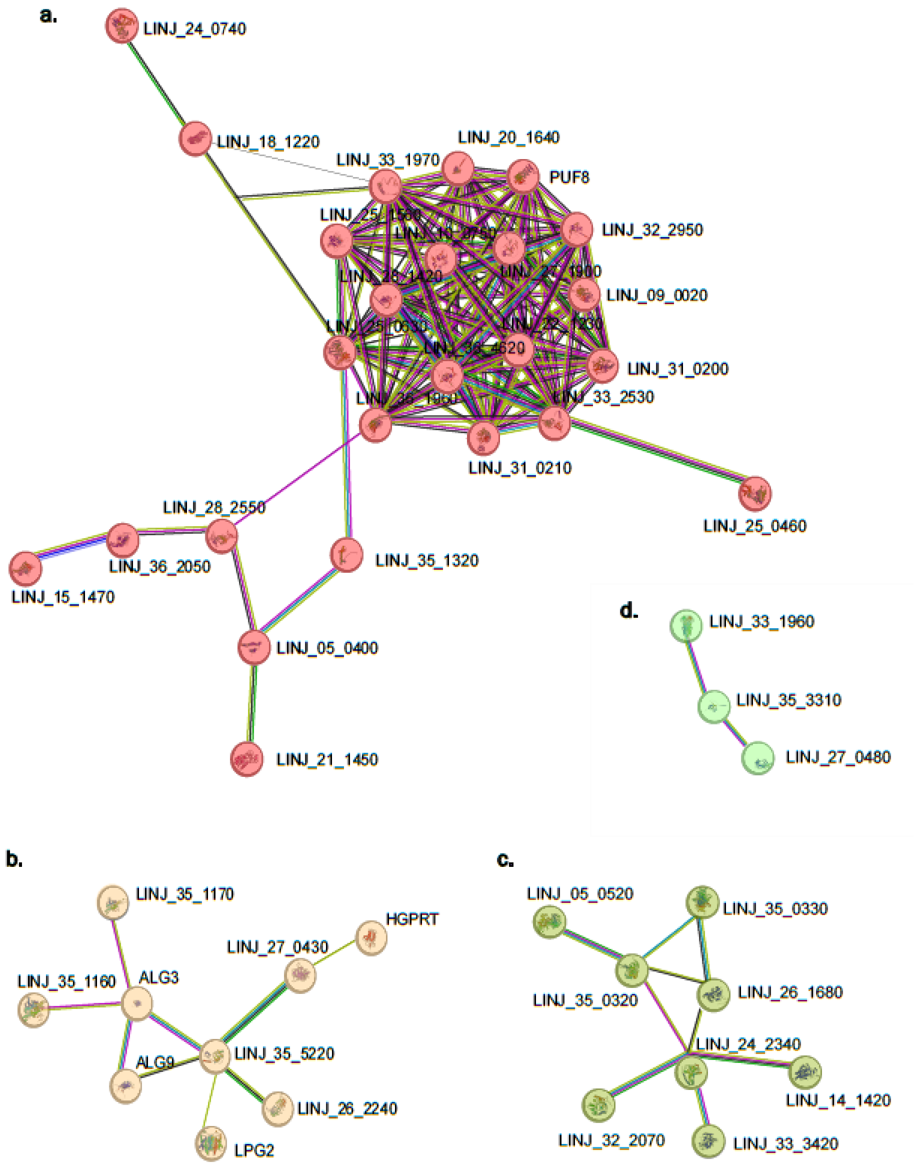
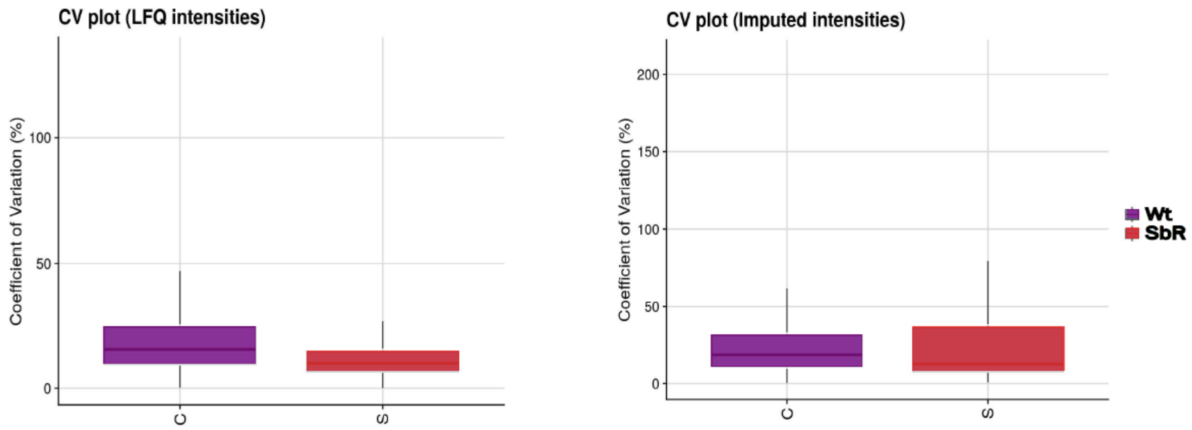
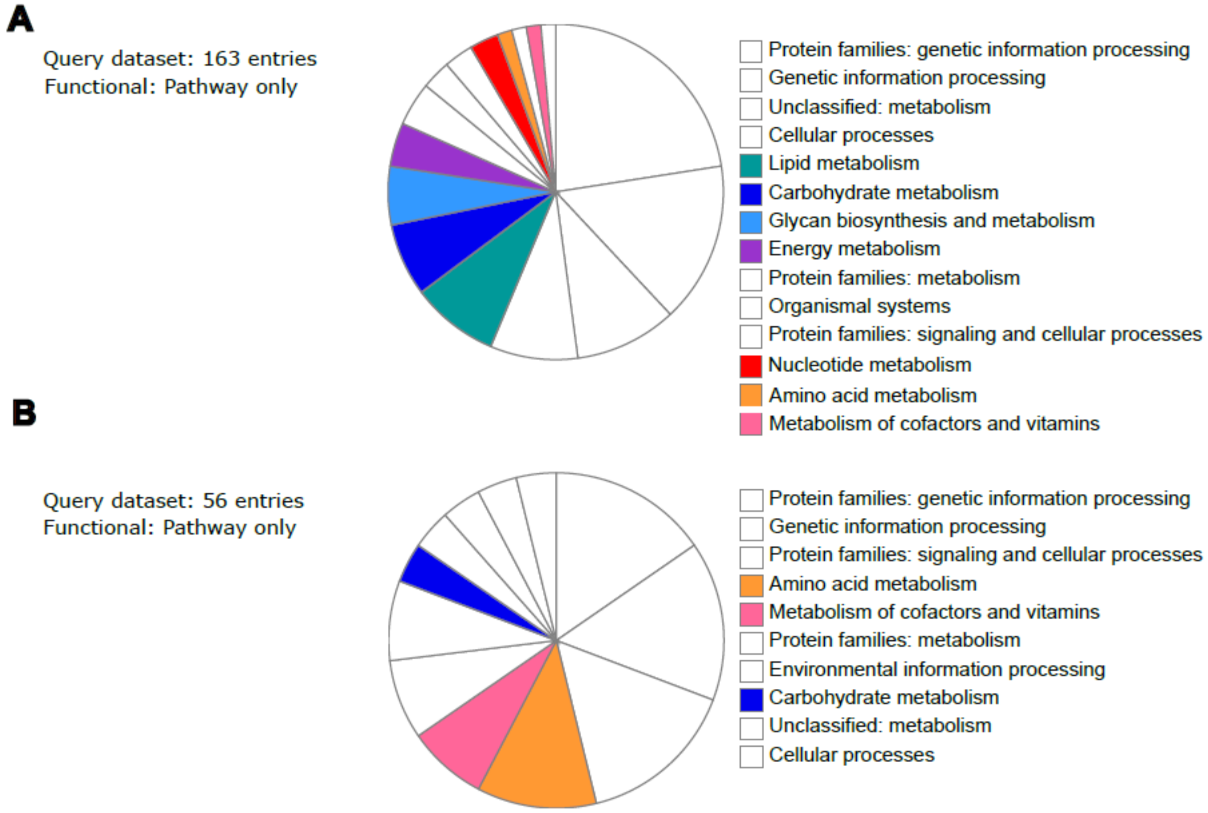


**Fig. 5.** Main clusters of the protein-protein interaction network of enriched proteins in the sodium stibogluconate-resistant line. (a) Proteins related to transcriptional and translational activity (red plots), (b) Proteins involved in carbohydrate and glycan metabolism (brown), (c) proteins associated with lipid metabolism (olive), and (d) proteins associated with peroxisome biogenesis (green). The distance among nodes and clusters is not scaled.



**Supplementary Fig. S1.** Coefficient variation analysis made from intensities measured for the wild-type strain *L. infantum* (purple) and sodium stibogluconate resistant (SbR) line (red). (a) Coefficient variation plot with the LFQ intensity percentages and (b) Coefficient variation plot with imputed intensities.



**Supplementary Fig. S2.** Kegg pathway analysis made from significantly different proteins in sodium stibogluconate resistant (SbR) line. (a) enriched proteins and (b) reduced proteins.

## Tables and Legends

**Table 1.** The main clusters identified through STRING of enriched protein in *L. infantum* SbII-resistant and protein constituents of each cluster

| CLUSTER NUMBER | PROTEIN NAME | PROTEIN IDENTIFIER | PROTEIN DESCRIPTION |
|---|---|---|---|
| **a**<br>**color: red**<br>**gene count: 25** | LINJ_05_0400 | 5671.A4HSG2 | Structural maintenance of chromosomes protein. |
| | LINJ_09_0020 | 5671.A4HTR8 | Uncharacterized protein. |
| | LINJ_10_0750 | 5671.A4HUI2 | Uncharacterized protein. |
| | LINJ_15_1470 | 5671.E9AGN0 | Putative mismatch repair protein MSH3. |
| | LINJ_18_1220 | 5671.A4HXZ5 | Putative prolyl-tRNA synthetase. |
| | LINJ_20_1640 | 5671.A4HYZ6 | Putative ribosome biogenesis protein. |
| | LINJ_21_1450 | 5671.A4HZ97 | Thymidine kinase. |
| | LINJ_22_1230 | 5671.E9AH06 | Protein MAK16 homolog; Belongs to the MAK16 family. |
| | LINJ_24_0740 | 5671.A4I0R1 | Signal recognition particle 54 kDa protein; Binds to the signal sequence of presecretory protein when they emerge from the ribosomes and transfers them to TRAM (translocating chain-associating membrane protein). Belongs to the GTP-binding SRP family. SRP54 subfamily. |
| | LINJ_25_0460 | 5671.A4I188 | G domain-containing protein. |
| | LINJ_25_0630 | 5671.A4I1A3 | DNA-directed RNA polymerase subunit beta; DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates. |
| | LINJ_25_1560 | 5671.A4I1J6 | Uncharacterized protein; Belongs to the class I-like SAM-binding methyltransferase superfamily. RsmB/NOP family. |
| | LINJ_27_1900 | 5671.A4I305 | Putative rRNA methyltransferase; Probable methyltransferase involved in the maturation of rRNA and in the biogenesis of ribosomal subunits. |
| | LINJ_28_1420 | 5671.A4I3L0 | Putative ATP-dependent RNA helicase; Belongs to the DEAD box helicase family. |
| | LINJ_28_2550 | 5671.A4I3W9 | Putative DNA replication licensing factor; Belongs to the MCM family. |
| | LINJ_31_0200 | 5671.A4I6B1 | Putative nucleolar protein; Belongs to the class I-like SAM-binding methyltransferase superfamily. RsmB/NOP family. |
| | LINJ_31_0210 | 5671.A4I6B2 | WD_REPEATS_REGION domain-containing protein. |
| | LINJ_32_2950 | 5671.A4I8A2 | Putative RNA-binding protein. |

| | | | |
|---|---|---|---|
| | LINJ_33_1970 | 5671.A4I949 | Nucleolar GTP-binding protein 1; Involved in the biogenesis of the 60S ribosomal subunit. Belongs to the TRAFAC class OBG-HflX-like GTPase superfamily. OBG GTPase family. NOG subfamily. |
| | LINJ_33_2530 | 5671.E9AHQ8 | Putative ras-like small GTPases. |
| | LINJ_35_1320 | 5671.A4IB25 | Histone H4; Core component of nucleosome. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones |
| | LINJ_36_1960 | 5671.A4IDF9 | Uncharacterized protein. |
| | LINJ_36_2050 | 5671.A4IDG7 | Putative mismatch repair protein MSH8. |
| | LINJ_36_4620 | 5671.A4IDL9 | ATP-dependent RNA helicase-like protein. |
| | PUF8 | 5671.A4I1T6 | Putative pumillio protein 8. |
| **b**<br>**color: brown**<br>**gene count: 9** | ALG3 | 5671.A4IDH7 | Putative dolichyl-P-Man:GDP-Man5GlcNAc2-PP-dolichyl alpha-1,3-mannosyltransferase. |
| | ALG9 | 5671.E9AGA5 | Mannosyltransferase. |
| | HGPRT | 5671.E9AGU6 | Hypoxanthine phosphoribosyltransferase; Belongs to the purine/pyrimidine phosphoribosyltransferase family. |
| | LINJ_26_2240 | 5671.A4I2F0 | Putative GDP-mannose 4,6 dehydratase. |
| | LINJ_27_0430 | 5671.A4I2M8 | Ribokinase; Catalyzes the phosphorylation of ribose at O-5 in a reaction requiring ATP and magnesium. The resulting D-ribose-5-phosphate can then be used either for sythesis of nucleotides, histidine, and tryptophan, or as a component of the pentose phosphate pathway. |
| | LINJ_35_1160 | 5671.A4IB10 | Oligosaccharyl transferase-like protein. |
| | LINJ_35_1170 | 5671.E9AHU4 | Putative oligosaccharyl transferase subunit. |
| | LINJ_35_5220 | 5671.A4IC43 | Putative alpha-1,2-mannosyltransferase. |
| | LPG2 | 5671.A4IA88 | Lipophosphoglycan biosynthetic protein (Lpg2). |
| **c**<br>**color: olive**<br>**gene count: 8** | LINJ_05_0520 | 5671.A4HSH5 | Nuclear receptor binding factor-like protein. |
| | LINJ_14_1420 | 5671.A4HW80 | Delta-4 fatty acid desaturase,putativewith. |
| | LINJ_24_2340 | 5671.A4I134 | Putative fatty-acid desaturase. |
| | LINJ_26_1680 | 5671.A4I2A2 | Putative fatty acid desaturase; Belongs to the fatty acid desaturase type 1 family. DEGS subfamily. |
| | LINJ_32_2070 | 5671.A4I816 | Putative 1-acyl-sn-glycerol-3-phosphateacyltransferase-like protein. |

| | | | |
|---|---|---|---|
| | LINJ_33_3420 | 5671.A4I9G8 | Delta-12 fatty acid desaturase. |
| | LINJ_35_0320 | 5671.A4IAT3 | Putative serine palmitoyltransferase. |
| | LINJ_35_0330 | 5671.A4IAT4 | Putative short chain dehydrogenase; Belongs to the short-chain dehydrogenases/reductases (SDR) family. |
| **d**<br>**color: green**<br>**gene count: 3** | LINJ_27_0480 | 5671.A4I2N3 | ATP-binding cassette protein subfamily D, member 1. putative ABCD1 |
| | LINJ_33_1960 | 5671.A4I948 | ATP-binding cassette protein subfamily D, member 3. putative ABCD3 |
| | LINJ_35_3310 | 5671.A4IBL7 | Peroxin 19. |

**Supplementary Table S1.** Protein groups identified in each group and their replicates, and histone ruler calculations.

**Supplementary Table S2.** Comparison of general information about the proteomes of *L. infantum* wild-type strain and SbIII-resistant line.

**Supplementary Table S3.** Proteins of *L. infantum* SbIII-resistant line with Log fold change in abundance compared to the control group, along with associated statistical significance and functional annotations.

**Author contributions:**

**Adriana Castillo-Castañeda:** Conceptualization, Methodology, Software, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization. **Luz Helena Patiño:** Conceptualization, Writing - Review & Editing. **Raúl Manzano:** Validation, Writing - Review & Editing, Supervision. **Julio López:** Resources, Writing - Review & Editing, Supervision. **Antonio Muro:** Resources, Writing - Review & Editing. **Juan David Ramírez:** Conceptualization, Resources, Writing - Review & Editing, Supervision, Funding acquisition.

# 4. CONCLUSIONES

## CAPÍTULO 1

- Se corrobora que la mayor incidencia de casos de LV se da en niños entre los 0 – 7 años en situación de vulnerabilidad asociada a la pobreza.
- No se evidencian diferencias significativas de la ocurrencia de casos por sexo.
- En términos generales, aunque la enfermedad no tiene una alta tasa de mortalidad, es importante considerar el subregistro y la calidad del dato de notificación en el SIVIGILA.
- Se evidencian cambios en el reporte de casos de LV a través del tiempo; así como, la incidencia en los departamentos endémicos.
- La presencia de vectores de *L. infantum* y la alta tasa de migración humana en las zonas fronterizas puede influir en la aparición de nuevos focos de transmisión para esta y otras ETV.

## CAPÍTULO 2

- Los hallazgos obtenidos a partir del amplicon-based sequencing refutan el paradigma tradicional de la leishmaniasis como una infección impulsada por una sola especie y replantean la dinámica de la interacción huésped-patógeno en el contexto del multiparasitismo.
- La metodología propuesta de amplicon-based sequensing tiene una alta capacidad para detectar e identificar especies de tripanosomátidos en reservorios y vectores flebotomineos; lo que puede ser útil en términos de análisis de muestras y tratamiento, sobre todo en zonas endémicas para LC, LV, y/o Enfermedad de Chagas.
- La técnica permitió corroborar la circulación de especies de *Leishmania* en departamentos recientemente reportados; así como la circulación de nuevas especies en departamentos con reciente reporte de casos de LV.
- Al analizar muestras de pacientes con LV, vectores y caninos del departamento de Sucre se puede evidenciar la trazabilidad de la coinfección en estos.
- El muestreo en diferentes reservorios mamíferos silvestres permitió describir por primera vez la presencia de *Leishmania* spp. en chigüiros en Colombia.
- Teniendo en cuenta la ocurrencia de casos de LC y LV, se evidencian escenarios de estudio interesantes en los departamentos de Antioquía, Casanare, La Guajira y Sucre.
- Las metodologías adapatadas para amplicon-based sequencing de fragmento HSP70-largo, presentaró una destacada especificidad y eficacia para la identificación de *Leishmania* spp. y los eventos de coinfección.
- Particularmente la plataforma usada de secuenciación MinIon$^{TM}$ tiene características considerables para su uso en campo, menor costo y tiempo de procesamiento, y la generación de lecturas largas.

## CAPÍTULO 3

- En general, los genomas de *L. infantum* analizados presentaron una alta homogeneidad y una extensiva aneuploidía.
- Existe una baja diversidad en los genomas de los aislamientos de *L. infantum* autóctonos de Colombia.
- Los genomas de Colombia tuvieron una mayor relación filogenética con los genomas de *L. infantum* provenientes de la cuenca del Mediterráneo y el Norte de África que con los genomas de las Américas e incluso Brasil.
- Demostramos que existen poblaciones distintas de *L. infantum* circulando en varios países alrededor del mundo y que las poblaciones de países distantes tienen relaciones genómicas cercanas.
- En cuanto a los antígenos rK28 y rK39, nuestro estudio demostró una alta variabilidad genética del rK28 a nivel mundial; mientras que, la secuencia de nucleótidos rK39 reveló una heterogeneidad genética moderada principalmente en muestras de Europa y África.

## CAPÍTULO 4

- Se evidenciaron cambios importantes en la cinética del crecimiento, cantidad de grupos proteicos y el número de copias de moléculas de proteínas entre la línea de *L. infantum* con resistencia inducida al SbIII en comparación con su parental silvestre.
- El aumento de las proteínas se asoció con actividades como la transcripción, traducción, metabolismo de lípidos, metabolismo energético y biogénesis de peroxisomas.
- La reducción en proteínas de interés biológico se relacionó con la adquisición y metabolismo de metales, especialmente hierro.
- Se sugiere una nueva perspectiva sobre la resistencia a los antimonios, destacando la importancia de la regulación postranscripcional y postraduccional.
- Los resultados obtenidos apuntan a una importante compensación del gasto energético, asociado con alteraciones en la composición lipídica de la membrana de los orgánulos, metabolización de los carbohidratos y una adaptación preventiva que evita el uso de mecanismos de resistencia que conllevan a un alto gasto energético en los parásitos resistentes a los antimonios.

# 5. PROSPECTIVAS

- Realizar estudios de seroprevalencia y seroconversión en la población infantil del área periurbana afectada por la leishmaniasis visceral (LV) en la ciudad de Neiva. Esto cobra particular relevancia dado que este municipio ha sido seleccionado como piloto para el programa de eliminación de la LV por parte de la Organización Panamericana de la Salud, el Ministerio de Salud y Protección Social, y el Instituto Nacional de Salud. Estos estudios serían de gran utilidad para evaluar la efectividad de las estrategias implementadas en la zona.

- Conducir de estudios de seroprevalencia y seroconversión en la población infantil del área rural del municipio de Ovejas, uno de los municipios con mayor incidencia sostenida de leishmaniasis visceral en Colombia. En este contexto, se ha observado la presencia de coinfección en vectores, reservorios y pacientes, así como la circulación de especies de Leishmania previamente no identificadas en la zona

- El municipio de Hatonuevo en la Guajira es un escenario interesante para la implementación de estudios One Health, considerando que es un nuevo foco de transmisión para leishmaniasis visceral, la población afectada presenta la singularidad de pertenecer a comunidad indígena y los análisis moleculares han identificado material genétco de *L. amazonensis* en los diferentes actores del ciclo de vida del parásito del municipio: vectores, pacientes con LV y perros.

- Implementar la vigilancia genómica activa en el municipio de Hatonuevo en la Guajira con apoyo de la comunidad indígena y los actores de la salud pública del departamento puede ayudar a comprender las características particulares de la enfermedad en esta población; así como, servir de herramienta preventiva y predictiva para la ocurrencia de nuevos casos.

- Continuar con el análisis molecular de reservorios y vectores de forma extensiva en el tiempo y espacio, priorizando las zonas endémicas de leishmaniasis en Colombia. Con el fin de caracterizar las especies de *Leishmania* spp. y otros tripanosomátidos circulantes en los territorios usando una metodología costo-efectiva, sensible y de alta resolución; como lo es el amplicon-based sequencing del *HSP70* desarrollada e implementada en la actual tesis.

- Hacer estudios tipo WGS (Whole Genomic Sequensing) en muestras de pacientes con LV generarían una fuente de información genómica valiosa para realizar estudios de mayor robustes en la comparación de la diversidad genética y las relaciones filogenéticas de la especie a nivel global y regional. Por ejemplo, sería de gran utilidad para el diseño de pruebas inmunocromatográficas (Pruebas rápidas de caset o tiras) para la identificación de antígenos recombinantes que tengan una alta sensibilidad, especificidad y robustez frente a la zona geográfica de la ocurrencia de casos.

- A partir del uso de la línea *L. infantum* resistente al SbIII generada en la actual tesis doctoral, se pueden desarrollar estudios de lipidómica para caracterizar la composición de lípidos a nivel de la membrana celular y de organelos intracelulares de gran interés como el retículo endoplasmático y los peroxisomas, y así establecer la significancia y el impacto de los posibles cambios y su relación con el fenotipo resistente presentado.

- Sería interesante llevar a cabo un estudio dual del translatoma y el metaboloma de la línea resistente a SbIII, y poder realizar la trazabilidad y cuantificación de los productos metabólicos finales en la línea resistente. Por ejemplo, sería de gran utilidad para el diseño de pruebas inmunocromatográficas (Pruebas rápidas de caset o tiras) que permitan la identificación de marcadores de resistencia, y así poder guiar de manera asertiva el tratamiento a administrar a los pacientes con LV.

- Adicionalmente, el uso de la línea *L. infantum* resistente al SbIII para medir y modelar la ingesta, producción y gasto energético de esta línea celular en comparación con su parental silvestre.

- Considerando que el ciclo de vida de *Leishmania* spp. comprende dos estadios morfológica y metabólicamente diferenciados para cada actor del ciclo de vida; promastigote en el vector y amastigote en el mamífero. En los cuales, el parásito debe enfrentar se a procesos de adaptación y respuesta a las condiciones del ambiente y el sistema inmunológico del individuo; se propone evaluar la estabilidad del fenotipo resistente inducido *in vitro* a lo largo del ciclo de vida del parásito *in vivo*.

- Alineado a la prospectiva anterior, y considerando que los promastigotes de los tripanosomátidos (en nuestro caso *Leishmania*) se pueden comunicar a través de exovesículas; transfiendosen una gran variedad de materiales biológicos como: ADN, ARN, lípidos, factores de virulencia, factore de resistencia y leishmaniavirus. Adicionalmente que, en un mismo individuo pueden co-existir diferentes especies de tripanosomátidos sería interesante evaluar el secretoma del parásito en modelos de la enfermedad *in vivo*, ya que pausiblemente los amastigotes pueden también realizar esta actividad.

- A partir de la integración de los datos ómicos de la actual tesis, datos de acceso público y nuevos estudios en líneas de *L. infantum* resistentes a los antimoniales, proponer nuevos blancos terapéuticos, mejoras o combinaciones terapéuticas de los medicamentos existentes

# 6. ANEXOS

## 6.1. Productos de la tesis

A continuación, se listan los productos generados durante el desarrollo de la presente tesis. Estos anexos los puede encontrar en el siguiente link: 6. ANEXOS TESIS ADRIANA CASTILLO CASTAÑEDA

:

### 6.1.1. Lista de publicaciones aportadas e índices de calidad

**Artículo 1:**

Castillo-Castañeda A, Herrera G, Ayala MS, Fuya P, Ramírez JD. Spatial and Temporal Variability of Visceral Leishmaniasis in Colombia, 2007 to 2018. Am J Trop Med Hyg. 2021 Jul 7;105(1):144-155. doi: 10.4269/ajtmh.21-0103. PMID: 34232908; PMCID: PMC8274786

<table>
<tr><td rowspan="6">Indicadores de calidad de la revista</td><td colspan="6"><b>WoS: Journal Citation Reports (JCR–SCIE edition)</b></td></tr>
<tr><td colspan="6">JIF 2021: 3.707</td></tr>
<tr><td colspan="3">Categoría:<br>Tropical Medicine, Public, Environmental & Occupational Health</td><td colspan="3">Ranking - Cuartil - Percentil:<br>20/137 Q1 85.77</td></tr>
<tr><td colspan="6"><b>SCOPUS y SCImago Journal Rank (SJR)</b></td></tr>
<tr><td colspan="2">CiteScore: 3.2</td><td colspan="2">SJR 2021: 1.253</td><td colspan="2">SNIP 2021: 1.368</td></tr>
<tr><td colspan="3">Categoría:<br>Tropical medicine</td><td colspan="3">Ranking - Cuartil - Percentil:<br>20/137 Q1 85.77</td></tr>
</table>

**Artículo 2:**

Patiño LH, Castillo-Castañeda AC, Muñoz M, Jaimes JE, Luna-Niño N, Hernández C, Ayala MS, Fuya P, Mendez C, Hernández-Pereira CE, Delgado L, Sandoval-Ramírez CM, Urbano P, Paniz-Mondolfi A, Ramírez JD. Development of an Amplicon-Based Next-Generation Sequencing Protocol to Identify *Leishmania* Species and Other Trypanosomatids in Leishmaniasis Endemic Areas. Microbiol Spectr. 2021 Oct 31;9(2):e0065221. doi: 10.1128/Spectrum.00652-21. Epub 2021 Oct 13. PMID: 34643453; PMCID: PMC8515931.

<table>
<tr><td rowspan="6">Indicadores de calidad de la revista</td><td colspan="2"><b>WoS: Journal Citation Reports (JCR–SCIE edition)</b></td></tr>
<tr><td colspan="2">JIF 2021: 9.043</td></tr>
<tr><td>Categoría:<br>Microbiology</td><td>Ranking - Cuartil - Percentil:<br>5/28 Q1 83.93</td></tr>
<tr><td colspan="2"><b>SCOPUS y SCImago Journal Rank (SJR)</b></td></tr>
<tr><td>CiteScore: 7.97</td><td>SJR 2021: 2.148</td></tr>
<tr><td>Categoría:<br>Microbiology</td><td>Ranking - Cuartil - Percentil:<br>5/28 Q1 83.93</td></tr>
</table>

### Artículo 3:

Castillo-Castañeda A, Patiño LH, Muñoz M, Ayala MS, Segura M, Bautista J, Shaban MV, Paniz-Mondolfi A, Ramírez JD. Amplicon-based next-generation sequencing reveals the co-existence of multiple *Leishmania* species in patients with visceral leishmaniasis. Int J Infect Dis. 2022 Feb;115:35-38. doi: 10.1016/j.ijid.2021.11.029. Epub 2021 Dec 1. PMID: 34863923.

| | | |
|---|---|---|
| Indicadores de calidad de la revista | **WoS: Journal Citation Reports (JCR–SCIE edition)** | |
| | JIF 2022: 8.4 | |
| | Categoría:<br>Infectious diseases | Ranking - Cuartil - Percentil:<br>13/96 Q1 87 |
| | **SCOPUS y SCImago Journal Rank (SJR)** | |
| | CiteScore: 11.203 | SJR 2022: 2.433 |
| | Categoría:<br>Infectious diseases | Ranking - Cuartil - Percentil:<br>13/96 Q1 87 |

### Artículo 4:

Castillo-Castañeda AC, Patiño LH, Zuñiga MF, Cantillo-Barraza O, Ayala MS, Segura M, Bautista J, Urbano P, Jaimes-Dueñez J, Ramírez JD. An overview of the trypanosomatid (Kinetoplastida: Trypanosomatidae) parasites infecting several mammal species in Colombia. Parasit Vectors. 2022 Dec 16;15(1):471. doi: 10.1186/s13071-022-05595-y. PMID: 36522757; PMCID: PMC9756507.

| | | |
|---|---|---|
| Indicadores de calidad de la revista | WoS: Journal Citation Reports (JCR–SCIE edition) | |
| | JIF 2022: 3.2 | |
| | Categoría:<br>Parasitology, Tropical Medicine | Ranking - Cuartil - Percentil:<br>6/24 Q1 77.1 |
| | SCOPUS y SCImago Journal Rank (SJR) | |
| | CiteScore: 3.064 | SJR 2022: 0.961 |
| | Categoría:<br>Medicine, infectious diseases | Ranking - Cuartil - Percentil:<br>6/24 Q1 77.1 |

### Artículo 5:

Patiño LH, Ballesteros N, Muñoz M, Jaimes J, Castillo-Castañeda AC, Madigan R, Paniz-Mondolfi A, Ramírez JD. Validation of Oxford nanopore sequencing for improved New World *Leishmania* species identification via analysis of 70-kDA heat shock protein. Parasit Vectors. 2023 Dec 18;16(1):458. doi: 10.1186/s13071-023-06073-9. PMID: 38111024; PMCID: PMC10726620.

| | | |
|---|---|---|
| Indicadores de calidad de la revista | WoS: Journal Citation Reports (JCR–SCIE edition) | |
| | JIF 2023: 3.2 | |
| | Categoría:<br>Parasitology, Tropical Medicine | Ranking - Cuartil - Percentil:<br>6/24 Q1 77.1 |
| | SCOPUS y SCImago Journal Rank (SJR) | |

|  |  |  |
|---|---|---|
|  | CiteScore: 3.064 | SJR 2023: 0.967 |
|  | Categoría:<br>Parasitology, infectious diseases | Ranking - Cuartil - Percentil:<br>6/24 Q1 77.1 |

**Artículo 6:**

Patino LH, Castillo-Castañeda A, Muñoz M, Muskus C, Rivero-Rodríguez M, Pérez-Doria A, Bejarano EE, Ramírez JD. Revisiting the heterogeneous global genomic population structure of *Leishmania infantum*. Microb Genom. 2021 Sep;7(9):000640. doi: 10.1099/mgen.0.000640. PMID: 34491157; PMCID: PMC8715437

<table>
<tr><td rowspan="6">Indicadores de calidad de la revista</td><td colspan="3"><b>WoS: Journal Citation Reports (JCR–SCIE edition)</b></td></tr>
<tr><td colspan="3">JIF 2021: 4.868</td></tr>
<tr><td colspan="2">Categoría:<br>Genethics and heredity, microbiology</td><td>Ranking - Cuartil - Percentil:<br>39/175 Q1 78</td></tr>
<tr><td colspan="3"><b>SCOPUS y SCImago Journal Rank (SJR)</b></td></tr>
<tr><td>CiteScore: 4.08</td><td>SJR 2021: 1.575</td><td>SNIP 2021: 1.368</td></tr>
<tr><td colspan="2">Categoría:<br>Genethics and heredity, microbiology</td><td>Ranking - Cuartil - Percentil:<br>39/175 Q1 78</td></tr>
</table>

**Artículo 7:**

Castillo-Castañeda A, Patiño LH, Muro A, Manzano R, López-Abán J, Ramírez JD. Characterizing *Leishmania infantum*-induced resistance to trivalent stibogluconate (SbIII) through deep proteomics. International Journal for Parasitology. (sometido)

### 6.1.2. Presentaciones en eventos

- Nombre del evento: X curso de biología molecular de tripanosomátidos y X simposio de biología celular y molecular de la enfermedad de Chagas
  Tipo de evento: curso/simposio
  Nombre de la ponencia: Leishmaniasis in Colombia, molecular epidemiology
  Tipo de producto: presentación oral
  Fecha del evento: 24 a 29 de julio de 2023

- Nombre del evento: Seminarios "CIETUS"
  Tipo de evento: Conferencia
  Ámbito: Internacional
  Nombre ponencia: Análisis epidemiológico, genómico y proteómico de *Leishmania infantum* en Colombia

Tipo de producto: Presentación oral
Fecha: mayo de 2023

- Nombre del evento: XVIII Congreso Colombiano de Parasitología y Medicina Tropical
  Tipo de evento: Congreso
  Nombre de la ponencia: Identificación de tripanosomatidos a partir de mamíferos silvestres y domésticos en diferentes departamentos de Colombia
  Tipo de producto: presentación oral
  Fecha: noviembre de 2022

- Nombre del evento: WorldLeish 2022, Cartagena, Colombia
  Tipo de evento: Congreso
  Nombre de la ponencia: Retrospective epidemiological analysis of visceral leishmaniasis in Colombia
  Tipo de producto: presentación oral
  Fecha: agosto de 2022

- ICOPA 2022, Copenhague, Dinamarca
  Tipo de evento: Congreso
  Nombre de la ponencia: The approach of amplicon-based next-generation sequencing shows the simultaneous presence of several Leishmania species in visceral leishmaniasis cases
  Tipo de producto: presentación poster
  Fecha: agosto de 2022

### 6.1.3. Pasantías

**Lugar:** Centro de Investigación de Enfermedades Tropicales de la Universidad de Salamanca (CIETUS)

**Duración:** 6 meses

## 6.2. Becas y reconocimientos

- Beca comité doctoral, Universidad del Rosario. 3er puesto entre aspirantes al doctorado
- Beca pasantía estudios doctorales, Universidad del Rosario. Asistente graduado
- Beca para la participación al X curso de tripanosomáticos y el simposio de la Enfermedad de Chagas – CONICET – UNOBIOLAC
- Beca para la participación al curso: International Spring School in Global Health-2023

### 6.3. Publicaciones en índice de impacto sobre epidemiología molecular leishmaniasis

- Ramírez JD, Cao L, Castillo-Castañeda AC, Patino LH, Ayala MS, Cordon-Cardo C, Sordillo EM, Paniz-Mondolfi A. Clinical performance of a quantitative pan-genus *Leishmania* Real-time PCR assay for diagnosis of cutaneous and visceral leishmaniasis. Pract Lab Med. 2023 Oct 5;37:e00341. doi: 10.1016/j.plabm.2023.e00341. PMID: 37842331; PMCID: PMC10570565.

- Jaimes-Dueñez J, Castillo-Castañeda A, Jiménez-Leaño Á, Duque JE, Cantillo-Barraza O, Cáceres-Rivera DI, Granada Y, Triana-Chávez O, Ramírez JD. Epidemiological features of *Leishmania infantum* in dogs (Canis lupus familiaris) suggest a latent risk of visceral leishmaniasis in the metropolitan area of Bucaramanga, Santander, Eastern Colombia. Prev Vet Med. 2023 Oct;219:106021. doi: 10.1016/j.prevetmed.2023.106021. Epub 2023 Sep 9. PMID: 37738753.

- Posada-López L, Velez-Mira A, Cantillo O, Castillo-Castañeda A, Ramírez JD, Galati EAB, Galvis-Ovallos F. Ecological interactions of sand flies, hosts, and *Leishmania panamensis* in an endemic area of cutaneous leishmaniasis in Colombia. PLoS Negl Trop Dis. 2023 May 11;17(5):e0011316. doi: 10.1371/journal.pntd.0011316. PMID: 37167348; PMCID: PMC10204979.

- Delgado-Noguera LA, Hernández-Pereira CE, Castillo-Castañeda AC, Patiño LH, Castañeda S, Herrera G, Mogollón E, Muñoz M, Duran A, Loyo D, Pacheco M, Arena L, Isquiel G, Yepez L, Colmenarez B, Caviedes M, Mendez Y, Herrera S, Ramírez JD, Paniz-Mondolfi AE. Diversity and geographical distribution of Leishmania species and the emergence of *Leishmania (Leishmania) infantum* and *L. (Viannia) panamensis* in Central-Western Venezuela. Acta Trop. 2023 Jun;242:106901. doi: 10.1016/j.actatropica.2023.106901. Epub 2023 Mar 20. PMID: 36940857.

### 6.4. Publicaciones en índice de impacto en otras enfermedades infecciosas

- Ramírez JD, Florez C, Muñoz M, Hernández C, Castillo A, Gomez S, Rico A, Pardo L, Barros EC, Castañeda S, Ballesteros N, Martínez D, Vega L, Jaimes JE, Cruz-Saavedra L, Herrera G, Patiño LH, Teherán AA, Gonzalez-Reiche AS, Hernandez MM, Sordillo EM, Simon V, van Bakel H, Paniz-Mondolfi A. The arrival and spread of SARS-CoV-2 in Colombia. J Med Virol. 2021 Feb;93(2):1158-1163. doi: 10.1002/jmv.26393. Epub 2020 Aug 13. PMID: 32761908; PMCID: PMC7436700.

## 7. BIBLIOGRAFÍA

1. WHO. (2020). *Leishmaniasis: Newsroom*. https://www.who.int/news-room/fact-sheets/detail/leishmaniasis
2. PAHO, W. (2019). *Epidemiological Report of the Americas. Leishmaniases* (7; p. 8). http://iris.paho.org/xmlui/handle/123456789/50505
3. Sanchez, J. P., Cañola, J., Molina, J. P., Bejarano, N., Vélez-Mira, A., Vélez, I. D., & Robledo, S. M. (2020). Ecoepidemiología de la leishmaniasis visceral en Colombia (1943-2019): Revisión sistemática. *Hechos Microbiológicos*, *11*(1 y 2), 22-60. https://doi.org/10.17533/udea.hm.v11n1a03
4. WHO. (2020). *Global leishmaniasis surveillance, 2017–2018, and first report on 5 additional indicators* (25; WER No. 25, 2020, 95, 265–280, pp. 265-280). World Orgaization Health. https://www.who.int/publications/i/item/who-wer9525
5. Braz, B. M. de A., Silva, R. B. S., Lins, S. C., Silva, D. R. X., Ramalho, W. M., & Melo, M. A. de. (2021). Demographic and spatial study of visceral leishmaniasis in the state of Alagoas, Brazil, during 2007-2018. *Revista da Sociedade Brasileira de Medicina Tropical*, *54*, e06102020. https://doi.org/10.1590/0037-8682-0610-2020
6. Jiang, D., Ma, T., Hao, M., Qian, Y., Chen, S., Meng, Z., Wang, L., Zheng, C., Qi, X., Wang, Q., & Ding, F. (2021). Spatiotemporal patterns and spatial risk factors for visceral leishmaniasis from 2007 to 2017 in Western and Central China: A modelling analysis. *Science of The Total Environment*, *764*, 144275. https://doi.org/10.1016/j.scitotenv.2020.144275
7. Cloots, K., Burza, S., Malaviya, P., Hasker, E., Kansal, S., Mollett, G., Chakravarty, J., Roy, N., Lal, B. K., Rijal, S., Sundar, S., & Boelaert, M. (2020). Male predominance in reported Visceral Leishmaniasis cases: Nature or nurture? A comparison of population-based with health facility-reported data. *PLOS Neglected Tropical Diseases*, *14*(1), e0007995. https://doi.org/10.1371/journal.pntd.0007995
8. Tovar A, C., & Yasnot A, M. (2017). Leishmaniasis visceral en América Latina y perspectivas terapéuticas. *Revista MVZ Córdoba*, 6075-6088. https://doi.org/10.21897/rmvz.1077
9. Zambrano Hernandez, C. del P., Ayala Sotelo, M. S., Fuya Oviedo, O. P., Montenegro Puentes, C. A., Aya Vanegas, N. M., Rodriguez Toro, J. G., Becerra Osorio, S. L., Aguilera Jaramillo, G., Lozano Polanco, C. A., Rojas Garcia, M. C., & Blásquez, O. (2015). Brote urbano de leishmaniasis visceral en Neiva (Huila), 2012. *Revista de Salud Pública*, *17*(4), 514-527. https://doi.org/10.15446/rsap.v17n4.44663
10. Manotas-Berdugo, H., Toro Maldonado, J. F., Rodríguez-Rodríguez, J., & Salgado-García, D. (2018). Brote urbano de leishmaniasis en Colombia. *Revista de Salud Pública*, *20*(1), 89-93. https://doi.org/10.15446/rsap.v20n1.47135
11. Zambrano, P., Ayala, M. S., Fuya, P., Barraza, O., & Rodríguez Toro, G. (2016). *Cartagena: New Urban Focus of Visceral Leishmaniasis in Colombia.* *7*((1)), 83-91. http://www.scielo.org.co/scielo.php?script=sci_arttext&pid=S0121-74882016000100011
12. Cortés, L. A. (2012). Foco de leishmaniasis en El Hobo, municipio de El Carmen de Bolívar, Bolívar, Colombia. *Biomédica*, *26*(1), 236. https://doi.org/10.7705/biomedica.v26i1.1518
13. Hotez, P., Ottesen, E., Fenwick, A., & Molyneux, D. (2006). The Neglected Tropical Diseases: The Ancient Afflictions of Stigma and Poverty and the Prospects for their Control and Elimination. En A. J. Pollard & A. Finn (Eds.), *Hot Topics in Infection and Immunity in Children III* (Vol. 582, pp. 23-33). Springer US. https://doi.org/10.1007/0-387-33026-7_3

14. Romero, M. H., López, M. C., & Sanchez, J. A. (2009). Búsqueda activa de casos de leishmaniasis visceral zoonótica en población infantil indígena y canina colombiana. *Revista de Salud Pública*, *11*(6), 944-951. https://doi.org/10.1590/S0124-00642009000600010
15. Romero, M. H., & Sánchez, J. A. (2009). Evaluación De Factores De Riesgo De La Leishmaniasis Visceral En Seis Municipios Endémicos Colombianos. *Revista Hacia la Promoción de la Salud*, *14*, 66-74. https://www.redalyc.org/articulo.oa?id=309126692003
16. Grillet, M. E., Hernández-Villena, J. V., Llewellyn, M. S., Paniz-Mondolfi, A. E., Tami, A., Vincenti-Gonzalez, M. F., Marquez, M., Mogollon-Mendoza, A. C., Hernandez-Pereira, C. E., Plaza-Morr, J. D., Blohm, G., Grijalva, M. J., Costales, J. A., Ferguson, H. M., Schwabl, P., Hernandez-Castro, L. E., Lamberton, P. H. L., Streicker, D. G., Haydon, D. T., … Alarcón-de Noya, B. (2019). Venezuela's humanitarian crisis, resurgence of vector-borne diseases, and implications for spillover in the region. *The Lancet Infectious Diseases*, *19*(5), e149-e161. https://doi.org/10.1016/S1473-3099(18)30757-6
17. Rodríguez-Morales, A. J., Suárez, J. A., Risquez, A., Villamil-Gómez, W. E., & Paniz-Mondolfi, A. (2019). Consequences of Venezuela's massive migration crisis on imported malaria in Colombia, 2016–2018. *Travel Medicine and Infectious Disease*, *28*, 98-99. https://doi.org/10.1016/j.tmaid.2019.02.004
18. Patino, L. H., Mendez, C., Rodriguez, O., Romero, Y., Velandia, D., Alvarado, M., Pérez, J., Duque, M. C., & Ramírez, J. D. (2017). Spatial distribution, Leishmania species and clinical traits of Cutaneous Leishmaniasis cases in the Colombian army. *PLOS Neglected Tropical Diseases*, *11*(8), e0005876. https://doi.org/10.1371/journal.pntd.0005876
19. Berry, I., & Berrang-Ford, L. (2016). Leishmaniasis, conflict, and political terror: A spatio-temporal analysis. *Social Science & Medicine*, *167*, 140-149. https://doi.org/10.1016/j.socscimed.2016.04.038
20. Vannier-Santos, M., Martiny, A., & Souza, W. (2002). Cell Biology of Leishmania spp.: Invading and Evading. *Current Pharmaceutical Design*, *8*(4), 297-318. https://doi.org/10.2174/1381612023396230
21. Espinosa, O. A., Serrano, M. G., Camargo, E. P., Teixeira, M. M. G., & Shaw, J. J. (2018). An appraisal of the taxonomy and nomenclature of trypanosomatids presently classified as *Leishmania* and *Endotrypanum*. *Parasitology*, *145*(4), 430-442. https://doi.org/10.1017/S0031182016002092
22. Alvar, J., Vélez, I. D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., Boer, M. den, & the WHO Leishmaniasis Control Team. (2012). Leishmaniasis Worldwide and Global Estimates of Its Incidence. *PLoS ONE*, *7*(5), e35671. https://doi.org/10.1371/journal.pone.0035671
23. Gramiccia, M., & Gradoni, L. (2005). The current status of zoonotic leishmaniases and approaches to disease control. *International Journal for Parasitology*, *35*(11-12), 1169-1180. https://doi.org/10.1016/j.ijpara.2005.07.001
24. Franssen, S. U., Durrant, C., Stark, O., Moser, B., Downing, T., Imamura, H., Dujardin, J.-C., Sanders, M. J., Mauricio, I., Miles, M. A., Schnur, L. F., Jaffe, C. L., Nasereddin, A., Schallig, H., Yeo, M., Bhattacharyya, T., Alam, M. Z., Berriman, M., Wirth, T., … Cotton, J. A. (2020). Global genome diversity of the Leishmania donovani complex. *eLife*, *9*, e51243. https://doi.org/10.7554/eLife.51243

25. Paranaiba, L. F., Pinheiro, L. J., Torrecilhas, A. C., Macedo, D. H., Menezes-Neto, A., Tafuri, W. L., & Soares, R. P. (2017). Leishmania enriettii (Muniz & Medina, 1948): A highly diverse parasite is here to stay. *PLOS Pathogens*, *13*(5), e1006303. https://doi.org/10.1371/journal.ppat.1006303
26. Rodriguez-Bonfante, C., Bonfante-Garrido, R., Grimaldi, G., Momen, H., & Cupolillo, E. (2003). Genotypically distinct Leishmania colombiensis isolates from Venezuela cause both cutaneous and visceral leishmaniasis in humans. *Infection, Genetics and Evolution*, *3*(2), 119-124. https://doi.org/10.1016/S1567-1348(03)00012-1
27. Aleixo, J. A., Nascimento, E. T., Monteiro, G. R., Fernandes, M. Z., Ramos, A. M. O., Wilson, M. E., Pearson, R. D., & Jeronimo, S. M. B. (2006). Atypical American visceral leishmaniasis caused by disseminated Leishmania amazonensis infection presenting with hepatitis and adenopathy. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *100*(1), 79-82. https://doi.org/10.1016/j.trstmh.2005.06.025
28. Carvalho, E. M., Momem, H., Grimaldi, G., Barral-Netto, M., Badaró, R., & Barral, A. (1986). Isolation of Leishmania Mexicana Amazonensis from the Bone Marrow in a Case of American Visceral Leishmaniasis. *The American Journal of Tropical Medicine and Hygiene*, *35*(4), 732-734. https://doi.org/10.4269/ajtmh.1986.35.732
29. Fakhar, M., Pouladfar, G. R., Alborzi, A., Kadivar, M. R., Hatam, G. R., & Motazedian, M. H. (2008). Isolation of Leishmania tropica from a Patient with Visceral Leishmaniasis and Disseminated Cutaneous Leishmaniasis, Southern Iran. *The American Journal of Tropical Medicine and Hygiene*, *79*(3), 435-437. https://doi.org/10.4269/ajtmh.2008.79.435
30. Herrera, G., Castillo, A., Ayala, M. S., Flórez, C., Cantillo-Barraza, O., & Ramirez, J. D. (2019). Evaluation of four rapid diagnostic tests for canine and human visceral Leishmaniasis in Colombia. *BMC Infectious Diseases*, *19*(1), 747. https://doi.org/10.1186/s12879-019-4353-0
31. Muskus, C. E., & Marín Villa, M. (2002). Metaciclogénesis: Un proceso fundamental en la biología de Leishmania. *Biomédica*, *22*(2), 167. https://doi.org/10.7705/biomedica.v22i2.1156
32. Torres-Guerrero, E., Quintanilla-Cedillo, M. R., Ruiz-Esmenjaud, J., & Arenas, R. (2017). Leishmaniasis: A review. *F1000Research*, *6*. https://doi.org/10.12688/f1000research.11120.1
33. Dostálová, A., & Volf, P. (2012). Leishmania development in sand flies: Parasite-vector interactions overview. *Parasites & Vectors*, *5*(1), 276. https://doi.org/10.1186/1756-3305-5-276
34. Killick-Kendrick, R. (1990). The life-cycle of *Leishmania* in the sandfly with special reference to the form infective to the vertebrate host. *Annales de Parasitologie Humaine et Comparée*, *65*, 37-42. https://doi.org/10.1051/parasite/1990651037
35. Gossage, S. M., Rogers, M. E., & Bates, P. A. (2003). Two separate growth phases during the development of Leishmania in sand flies: Implications for understanding the life cycle. *International Journal for Parasitology*, *33*(10), 1027-1034. https://doi.org/10.1016/S0020-7519(03)00142-5
36. Dvorak, V., Shaw, J., & Volf, P. (2018). Parasite Biology: The Vectors. En F. Bruschi & L. Gradoni (Eds.), *The Leishmaniases: Old Neglected Tropical Diseases* (pp. 31-77). Springer International Publishing. https://doi.org/10.1007/978-3-319-72386-0_3
37. Secundino, N. F. C., Eger-Mangrich, I., Braga, E. M., Santoro, M. M., & Pimenta, P. F. P. (2005). *Lutzomyia longipalpis* Peritrophic Matrix: Formation, Structure, and Chemical Composition. *Journal of Medical Entomology*, *42*(6), 928-938. https://doi.org/10.1093/jmedent/42.6.928

38. Boulanger, N., Lowenberger, C., Volf, P., Ursic, R., Sigutova, L., Sabatier, L., Svobodova, M., Beverley, S. M., Spath, G., Brun, R., Pesson, B., & Bulet, P. (2004). Characterization of a Defensin from the Sand Fly Phlebotomus duboscqi Induced by Challenge with Bacteria or the Protozoan Parasite Leishmania major. *Infection and Immunity*, *72*(12), 7140-7146. https://doi.org/10.1128/IAI.72.12.7140-7146.2004

39. Diaz-Albiter, H., Sant'Anna, M. R. V., Genta, F. A., & Dillon, R. J. (2012). Reactive Oxygen Species-mediated Immunity against *Leishmania mexicana* and *Serratia marcescens* in the Phlebotomine Sand Fly *Lutzomyia longipalpis*. *Journal of Biological Chemistry*, *287*(28), 23995-24003. https://doi.org/10.1074/jbc.M112.376095

40. Volf, P., Hajmova, M., Sadlova, J., & Votypka, J. (2004). Blocked stomodeal valve of the insect vector: Similar mechanism of transmission in two trypanosomatid models. *International Journal for Parasitology*, *34*(11), 1221-1227. https://doi.org/10.1016/j.ijpara.2004.07.010

41. Alemayehu, B., & Alemayehu, M. (2017). Leishmaniasis: A Review on Parasite, Vector and Reservoir Host. *Health Science Journal*, *11*(4). https://doi.org/10.21767/1791-809X.1000519

42. Ready, P. (2014). Epidemiology of visceral leishmaniasis. *Clinical Epidemiology*, 147. https://doi.org/10.2147/CLEP.S44267

43. Maroli, M., Feliciangeli, M. D., Bichaud, L., Charrel, R. N., & Gradoni, L. (2013). Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health concern. *Medical and Veterinary Entomology*, *27*(2), 123-147. https://doi.org/10.1111/j.1365-2915.2012.01034.x

44. Torres-Guerrero, E., Quintanilla-Cedillo, M. R., Ruiz-Esmenjaud, J., & Arenas, R. (2017). Leishmaniasis: A review. *F1000Research*, *6*, 750. https://doi.org/10.12688/f1000research.11120.1

45. Bejarano, E. E., Sierra, D., & Vélez, I. D. (2003). Novedades en la distribución geográfica del grupo verrucarum (Diptera: Psychodidae) en Colombia. *Biomédica*, *23*(3), 341. https://doi.org/10.7705/biomedica.v23i3.1228

46. González, C., Cabrera, O. L., Munstermann, L. E., & Ferro, C. (2012). Distribución de los vectores de Leishmania infantum (Kinetoplastida: Trypanosomatidae) en Colombia. *Biomédica*, *26*(1), 64. https://doi.org/10.7705/biomedica.v26i1.1501

47. Ferro, C., López, M., Fuya, P., Lugo, L., Cordovez, J. M., & González, C. (2015). Spatial Distribution of Sand Fly Vectors and Eco-Epidemiology of Cutaneous Leishmaniasis Transmission in Colombia. *PLOS ONE*, *10*(10), e0139391. https://doi.org/10.1371/journal.pone.0139391

48. Paternina, L. E., Verbel-Vergara, D., Romero-Ricardo, L., Pérez-Doria, A., Paternina-Gómez, M., Martínez, L., & Bejarano, E. E. (2016). Evidence for anthropophily in five species of phlebotomine sand flies (Diptera: Psychodidae) from northern Colombia, revealed by molecular identification of bloodmeals. *Acta Tropica*, *153*, 86-92. https://doi.org/10.1016/j.actatropica.2015.10.005

49. INS. (2018). *Informe de vigilancia entomológica de leishmaniasis, Colombia 2018*. Instituto Nacional de Salud.

50. González, C., León, C., Paz, A., López, M., Molina, G., Toro, D., Ortiz, M., Cordovez, J. M., Atencia, M. C., Aguilera, G., & Tovar, C. (2018). Diversity patterns, Leishmania DNA detection, and bloodmeal identification of Phlebotominae sand flies in villages in northern Colombia. *PLOS ONE*, *13*(1), e0190686. https://doi.org/10.1371/journal.pone.0190686

51. Sandoval-Ramírez, C. M., Hernández, C., Teherán, A. A., Gutierrez-Marin, R., Martínez-Vega, R. A., Morales, D., Hoyos-Lopez, R., Araque-Mogollón, A., & Ramírez, J. D. (2020). Complex ecological

interactions across a focus of cutaneous leishmaniasis in Eastern Colombia: Novel description of *Leishmania* species, hosts and phlebotomine fauna. *Royal Society Open Science*, *7*(7), 200266. https://doi.org/10.1098/rsos.200266

52. López, M., Erazo, D., Hoyos, J., León, C., Fuya, P., Lugo, L., Cordovez, J. M., & González, C. (2021). Measuring spatial co-occurrences of species potentially involved in Leishmania transmission cycles through a predictive and fieldwork approach. *Scientific Reports*, *11*(1), 6789. https://doi.org/10.1038/s41598-021-85763-9
53. Thies, S. F., Bronzoni, R. V. de M., Espinosa, M. M., Souza, C. de O., Ribeiro, A. L. M., Santos, E. S. dos, Dias, E. S., & Damazo, A. S. (2016). Frequency and diversity of phlebotomine sand flies (Diptera: Psychodidae) in Sinop, State of Mato Grosso, Brazil. *Revista da Sociedade Brasileira de Medicina Tropical*, *49*(5), 544-552. https://doi.org/10.1590/0037-8682-0251-2016
54. de Santana Martins Rodgers, M., Bavia, M. E., Fonseca, E. O. L., Cova, B. O., Silva, M. M. N., Carneiro, D. D. M. T., Cardim, L. L., & Malone, J. B. (2019). Ecological niche models for sand fly species and predicted distribution of Lutzomyia longipalpis (Diptera: Psychodidae) and visceral leishmaniasis in Bahia state, Brazil. *Environmental Monitoring and Assessment*, *191*(S2), 331. https://doi.org/10.1007/s10661-019-7431-2
55. Silva Neto, A. B. da, Oliveira, E. F. de, Encina, C. C. C., Figueiredo, H. R. de, Paranhos Filho, A. C., & Oliveira, A. G. de. (2020). Effects of El Niño-Southern Oscillation on human visceral leishmaniasis in the Brazilian State of Mato Grosso do Sul. *Memórias do Instituto Oswaldo Cruz*, *115*, e190298. https://doi.org/10.1590/0074-02760190298
56. Sadlova, J., Yeo, M., Seblova, V., Lewis, M. D., Mauricio, I., Volf, P., & Miles, M. A. (2011). Visualisation of Leishmania donovani Fluorescent Hybrids during Early Stage Development in the Sand Fly Vector. *PLoS ONE*, *6*(5), e19851. https://doi.org/10.1371/journal.pone.0019851
57. Coelho, A. C., Leprohon, P., & Ouellette, M. (2012). Generation of Leishmania Hybrids by Whole Genomic DNA Transformation. *PLoS Neglected Tropical Diseases*, *6*(9), e1817. https://doi.org/10.1371/journal.pntd.0001817
58. Calvo-Álvarez, E., Álvarez-Velilla, R., Jiménez, M., Molina, R., Pérez-Pertejo, Y., Balaña-Fouce, R., & Reguera, R. M. (2014). First Evidence of Intraclonal Genetic Exchange in Trypanosomatids Using Two Leishmania infantum Fluorescent Transgenic Clones. *PLoS Neglected Tropical Diseases*, *8*(9), e3075. https://doi.org/10.1371/journal.pntd.0003075
59. Romano, A., Inbar, E., Debrabant, A., Charmoy, M., Lawyer, P., Ribeiro-Gomes, F., Barhoumi, M., Grigg, M., Shaik, J., Dobson, D., Beverley, S. M., & Sacks, D. L. (2014). Cross-species genetic exchange between visceral and cutaneous strains of *Leishmania* in the sand fly vector. *Proceedings of the National Academy of Sciences*, *111*(47), 16808-16813. https://doi.org/10.1073/pnas.1415109111
60. Seblova, V., Myskova, J., Hlavacova, J., Votypka, J., Antoniou, M., & Volf, P. (2015). Natural hybrid of Leishmania infantum/L. donovani: Development in Phlebotomus tobbi, P. perniciosus and Lutzomyia longipalpis and comparison with non-hybrid strains differing in tissue tropism. *Parasites & Vectors*, *8*(1), 605. https://doi.org/10.1186/s13071-015-1217-3
61. Rogers, M. B., Downing, T., Smith, B. A., Imamura, H., Sanders, M., Svobodova, M., Volf, P., Berriman, M., Cotton, J. A., & Smith, D. F. (2014). Genomic Confirmation of Hybridisation and Recent Inbreeding in a Vector-Isolated Leishmania Population. *PLoS Genetics*, *10*(1), e1004092. https://doi.org/10.1371/journal.pgen.1004092

62. Cardoso, M. S., Bento, G. A., de Almeida, L. V., de Castro, J. C., Reis-Cunha, J. L., Barbosa, V. de A., de Souza, C. F., Brazil, R. P., Valdivia, H. O., & Bartholomeu, D. C. (2019). Detection of multiple circulating Leishmania species in Lutzomyia longipalpis in the city of Governador Valadares, southeastern Brazil. *PLOS ONE*, *14*(2), e0211831. https://doi.org/10.1371/journal.pone.0211831

63. Van Wynsberghe, N. R., Canto-Lara, S. B., Sosa-Bibiano, E. I., Rivero-Cárdenas, N. A., & Andrade-Narváez, F. J. (2009). Comparison of small mammal prevalence of Leishmania (Leishmania) mexicana in five foci of cutaneous leishmaniasis in the State of Campeche, Mexico. *Revista do Instituto de Medicina Tropical de São Paulo*, *51*(2), 87-94. https://doi.org/10.1590/S0036-46652009000200006

64. Alemayehu, T., van Griensven, A., Woldegiorgis, B. T., & Bauwens, W. (2017). An improved SWAT vegetation growth module and its evaluation for four tropical ecosystems. *Hydrology and Earth System Sciences*, *21*(9), 4449-4467. https://doi.org/10.5194/hess-21-4449-2017

65. Akuffo, H., Costa, C., van Griensven, J., Burza, S., Moreno, J., & Herrero, M. (2018). New insights into leishmaniasis in the immunosuppressed. *PLOS Neglected Tropical Diseases*, *12*(5), e0006375. https://doi.org/10.1371/journal.pntd.0006375

66. Kassahun, A., Sadlova, J., Dvorak, V., Kostalova, T., Rohousova, I., Frynta, D., Aghova, T., Yasur-Landau, D., Lemma, W., Hailu, A., Baneth, G., Warburg, A., Volf, P., & Votypka, J. (2015). Detection of Leishmania donovani and L. tropica in Ethiopian wild rodents. *Acta Tropica*, *145*, 39-44. https://doi.org/10.1016/j.actatropica.2015.02.006

67. Rohousova, I., Talmi-Frank, D., Kostalova, T., Polanska, N., Lestinova, T., Kassahun, A., Yasur-Landau, D., Maia, C., King, R., Votypka, J., Jaffe, C. L., Warburg, A., Hailu, A., Volf, P., & Baneth, G. (2015). Exposure to Leishmania spp. And sand flies in domestic animals in northwestern Ethiopia. *Parasites & Vectors*, *8*(1), 360. https://doi.org/10.1186/s13071-015-0976-1

68. Roque, A. L. R., & Jansen, A. M. (2014). Wild and synanthropic reservoirs of Leishmania species in the Americas. *International Journal for Parasitology: Parasites and Wildlife*, *3*(3), 251-262. https://doi.org/10.1016/j.ijppaw.2014.08.004

69. Medkour, H., Davoust, B., Dulieu, F., Maurizi, L., Lamour, T., Marié, J.-L., & Mediannikov, O. (2019). Potential animal reservoirs (dogs and bats) of human visceral leishmaniasis due to Leishmania infantum in French Guiana. *PLOS Neglected Tropical Diseases*, *13*(6), e0007456. https://doi.org/10.1371/journal.pntd.0007456

70. Zhao, S., Kuang, Y., Wu, C.-H., Ben-Arieh, D., Ramalho-Ortigao, M., & Bi, K. (2016). Zoonotic visceral leishmaniasis transmission: Modeling, backward bifurcation, and optimal control. *Journal of Mathematical Biology*, *73*(6-7), 1525-1560. https://doi.org/10.1007/s00285-016-0999-z

71. Picón, Y., Almario, G., Rodríguez, V., & Garcia, N. V. (2020). Seroprevalence, clinical, and pathological characteristics of canine leishmaniasis in a central region of Colombia. *Journal of Veterinary Research*, *0*(0). https://doi.org/10.2478/jvetres-2020-0011

72. Dantas-Torres, F., Solano-Gallego, L., Baneth, G., Ribeiro, V. M., de Paiva-Cavalcanti, M., & Otranto, D. (2012). Canine leishmaniosis in the Old and New Worlds: Unveiled similarities and differences. *Trends in Parasitology*, *28*(12), 531-538. https://doi.org/10.1016/j.pt.2012.08.007

73. Otranto, D., & Dantas-Torres, F. (2013). The prevention of canine leishmaniasis and its impact on public health. *Trends in Parasitology*, *29*(7), 339-345. https://doi.org/10.1016/j.pt.2013.05.003

74. Le Rutte, E. A., van Straten, R., & Overgaauw, P. A. M. (2018). Awareness and control of canine leishmaniosis: A survey among Spanish and French veterinarians. *Veterinary Parasitology*, *253*, 87-93. https://doi.org/10.1016/j.vetpar.2018.01.013
75. Dantas-Torres, F., Miró, G., Baneth, G., Bourdeau, P., Breitschwerdt, E., Capelli, G., Cardoso, L., Day, M. J., Dobler, G., Ferrer, L., Irwin, P., Jongejan, F., Kempf, V. A. J., Kohn, B., Lappin, M., Little, S., Madder, M., Maggi, R., Maia, C., … Otranto, D. (2019). Canine Leishmaniasis Control in the Context of One Health. *Emerging Infectious Diseases*, *25*(12), 1-4. https://doi.org/10.3201/eid2512.190164
76. Sevá, A. P., Ovallos, F. G., Amaku, M., Carrillo, E., Moreno, J., Galati, E. A. B., Lopes, E. G., Soares, R. M., & Ferreira, F. (2016). Canine-Based Strategies for Prevention and Control of Visceral Leishmaniasis in Brazil. *PLOS ONE*, *11*(7), e0160058. https://doi.org/10.1371/journal.pone.0160058
77. Fernández Manrique, J., C, T., Bello, F., Escovar, J., LOZANO, C., Ayala, M., NICHOLLS, R., VARGAS, J., Moncada, L., A, A., & López, M. (2002). Prevalence of canine visceral leishmaniasis in municipalities of Huila, Colombia. *Revista de Salud Pública*, *4*, 278-285.
78. Paternina-Gómez, M., Díaz-Olmos, Y., Paternina, L. E., & Bejarano, E. E. (2013). Alta prevalencia de infección por Leishmania (Kinetoplastidae: Trypanosomatidae) en caninos del norte de Colombia. *Biomédica*, *33*(3). https://doi.org/10.7705/biomedica.v33i3.780
79. Paternina Tuirán, L. E., Díaz-Olmos, Y. A., Paternina-Gómez, M., Carrillo-Bonilla, L. M., Vélez, I. D., & Bejarano Martínez, E. E. (2015). DETECCIÓN DE ANTICUERPOS ANTI-Leishmania (Trypanosomatidae) EN POBLACIONES CANINAS DEL DEPARTAMENTO DE SUCRE, COLOMBIA. *Acta Biológica Colombiana*, *21*(1). https://doi.org/10.15446/abc.v21n1.48845
80. Arbeláez, N., Moreno, J., Murillo, J., Montoya, A., Robledo, S. M., Vélez, A., & Vélez, I. D. (2020). First Report of an Urban Case of Canine Visceral Leishmaniasis in the Municipality of Cali, Colombia. *The American Journal of Tropical Medicine and Hygiene*, *102*(2), 289-293. https://doi.org/10.4269/ajtmh.19-0515
81. González-Colonia, L., Bonilla-Aldana, D., Álvarez-Serrano, M., Granados-Hincapie, L., Pinilla-Ticora, L., & Rodriguez-Morales, A. (2021). An Urban Case of Canine Visceral Leishmaniasis in the Municipality of Pereira. *World s Veterinary Journal*, *11*, 115-118.
82. Soares, I. R., Silva, S. O., Moreira, F. M., Prado, L. G., Fantini, P., Maranhão, R. de P. A., da Silva Filho, J. M., Melo, M. N., & Palhares, M. S. (2013). First evidence of autochthonous cases of Leishmania (Leishmania) infantum in horse (Equus caballus) in the Americas and mixed infection of Leishmania infantum and Leishmania (Viannia) braziliensis. *Veterinary Parasitology*, *197*(3), 665-669. https://doi.org/10.1016/j.vetpar.2013.06.014
83. Viettri, M., Herrera, L., Aguilar, C. M., Morocoima, A., Reyes, J., Lares, M., Lozano-Arias, D., García-Alzate, R., Chacón, T., Feliciangeli, M. D., & Ferrer, E. (2018). Molecular diagnosis of Trypanosoma cruzi/Leishmania spp. Coinfection in domestic, peridomestic and wild mammals of Venezuelan co-endemic areas. *Veterinary Parasitology: Regional Studies and Reports*, *14*, 123-130. https://doi.org/10.1016/j.vprsr.2018.10.002
84. Cotton, J., Durrant, C., Franssen, S., Gelanew, T., Hailu, A., Mateus, D., Sanders, M., Berriman, M., Volf, P., Miles, M., & Yeo, M. (2019). Genomic analysis of natural intra-specific hybrids among Ethiopian isolates of Leishmania donovani. *bioRxiv*. https://doi.org/10.1101/516211
85. Gelanew, T., Kuhls, K., Hurissa, Z., Weldegebreal, T., Hailu, W., Kassahun, A., Abebe, T., Hailu, A., & Schönian, G. (2010). Inference of Population Structure of Leishmania donovani Strains Isolated from

Different Ethiopian Visceral Leishmaniasis Endemic Areas. *PLoS Neglected Tropical Diseases*, *4*(11), e889. https://doi.org/10.1371/journal.pntd.0000889

86. Gelanew, T., Hailu, A., Schőnian, G., Lewis, M. D., Miles, M. A., & Yeo, M. (2014). Multilocus sequence and microsatellite identification of intra-specific hybrids and ancestor-like donors among natural Ethiopian isolates of Leishmania donovani. *International Journal for Parasitology*, *44*(10), 751-757. https://doi.org/10.1016/j.ijpara.2014.05.008
87. Kato, H., Gomez, E. A., Seki, C., Furumoto, H., Martini-Robles, L., Muzzio, J., Calvopiña, M., Velez, L., Kubo, M., Tabbabi, A., Yamamoto, D. S., & Hashiguchi, Y. (2019). PCR-RFLP analyses of Leishmania species causing cutaneous and mucocutaneous leishmaniasis revealed distribution of genetically complex strains with hybrid and mito-nuclear discordance in Ecuador. *PLOS Neglected Tropical Diseases*, *13*(5), e0007403. https://doi.org/10.1371/journal.pntd.0007403
88. Veland, N., Adaui, V., Llanos-Cuentas, A., Arevalo, J., Boggild, A. K., Valencia, B. M., & Alba, M. (2013). Simultaneous Infection with Leishmania (Viannia) braziliensis and L. (V.) lainsoni in a Peruvian Patient with Cutaneous Leishmaniasis. *The American Journal of Tropical Medicine and Hygiene*, *88*(4), 774-777. https://doi.org/10.4269/ajtmh.12-0594
89. Nolder, D., Miles, M. A., Llanos-Cuentas, A., Davies, C. R., & Roncal, N. (2007). MULTIPLE HYBRID GENOTYPES OF LEISHMANIA (VIANNIA) IN A FOCUS OF MUCOCUTANEOUS LEISHMANIASIS. *The American Journal of Tropical Medicine and Hygiene*, *76*(3), 573-578. https://doi.org/10.4269/ajtmh.2007.76.573
90. Inbar, E., Shaik, J., Iantorno, S. A., Romano, A., Nzelu, C. O., Owens, K., Sanders, M. J., Dobson, D., Cotton, J. A., Grigg, M. E., Beverley, S. M., & Sacks, D. (2019). Whole genome sequencing of experimental hybrids supports meiosis-like sexual recombination in Leishmania. *PLOS Genetics*, *15*(5), e1008042. https://doi.org/10.1371/journal.pgen.1008042
91. Lukeš, J., Lys Guilbride, D., Votýpka, J., Zíková, A., Benne, R., & Englund, P. T. (2002). Kinetoplast DNA Network: Evolution of an Improbable Structure. *Eukaryotic Cell*, *1*(4), 495-502. https://doi.org/10.1128/EC.1.4.495-502.2002
92. Telleria, J., Lafay, B., Virreira, M., Barnabé, C., Tibayrenc, M., & Svoboda, M. (2006). Trypanosoma cruzi: Sequence analysis of the variable region of kinetoplast minicircles. *Experimental Parasitology*, *114*(4), 279-288. https://doi.org/10.1016/j.exppara.2006.04.005
93. Yatawara, L., Le, T. H., Wickramasinghe, S., & Agatsuma, T. (2008). Maxicircle (mitochondrial) genome sequence (partial) of Leishmania major: Gene content, arrangement and composition compared with Leishmania tarentolae. *Gene*, *424*(1-2), 80-86. https://doi.org/10.1016/j.gene.2008.07.026
94. Kaufer, A., Barratt, J., Stark, D., & Ellis, J. (2019). The complete coding region of the maxicircle as a superior phylogenetic marker for exploring evolutionary relationships between members of the Leishmaniinae. *Infection, Genetics and Evolution*, *70*, 90-100. https://doi.org/10.1016/j.meegid.2019.02.002
95. Coutinho-Abreu, I. V., Serafim, T. D., Meneses, C., Kamhawi, S., Oliveira, F., & Valenzuela, J. G. (2019). *Distinct gene expression patterns in vector-residing* Leishmania infantum *identify parasite stage-enriched markers* [Preprint]. Microbiology. https://doi.org/10.1101/679712
96. Schwabl, P., Boité, M. C., Bussotti, G., Jacobs, A., Andersson, B., Moreira, O., Freitas-Mesquita, A. L., Meyer-Fernandes, J. R., Telleria, E. L., Traub-Csekö, Y., Vaselek, S., Leštinová, T., Volf, P., Morgado, F. N., Porrozzi, R., Llewellyn, M., Späth, G. F., & Cupolillo, E. (2021). Colonization and genetic

diversification processes of Leishmania infantum in the Americas. *Communications Biology*, *4*(1), 139. https://doi.org/10.1038/s42003-021-01658-5

97. Patino, L. H., Imamura, H., Cruz-Saavedra, L., Pavia, P., Muskus, C., Méndez, C., Dujardin, J. C., & Ramírez, J. D. (2019). Major changes in chromosomal somy, gene expression and gene dosage driven by SbIII in Leishmania braziliensis and Leishmania panamensis. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-45538-9
98. Ballesteros, N., Vásquez, N., Patino, L., Cruz-Saavedra, L., & Ramírez, J. (2019). Short-term temperature stress does not affect chromosomal ploidy but cause transcriptomic changes in Leishmania braziliensis promastigotes in vitro. *Manuscrito en proceso*.
99. González-de la Fuente, S., Peiró-Pastor, R., Rastrojo, A., Moreno, J., Carrasco-Ramiro, F., Requena, J. M., & Aguado, B. (2017). Resequencing of the Leishmania infantum (strain JPCM5) genome and de novo assembly into 36 contigs. *Scientific Reports*, *7*(1), 18050. https://doi.org/10.1038/s41598-017-18374-y
100. Ivens, A. C. (2005). The Genome of the Kinetoplastid Parasite, Leishmania major. *Science*, *309*(5733), 436-442. https://doi.org/10.1126/science.1112680
101. Peacock, C. S., Seeger, K., Harris, D., Murphy, L., Ruiz, J. C., Quail, M. A., Peters, N., Adlem, E., Tivey, A., Aslett, M., Kerhornou, A., Ivens, A., Fraser, A., Rajandream, M.-A., Carver, T., Norbertczak, H., Chillingworth, T., Hance, Z., Jagels, K., … Berriman, M. (2007). Comparative genomic analysis of three Leishmania species that cause diverse human disease. *Nature Genetics*, *39*(7), 839-847. https://doi.org/10.1038/ng2053
102. Rogers, M. B., Hilley, J. D., Dickens, N. J., Wilkes, J., Bates, P. A., Depledge, D. P., Harris, D., Her, Y., Herzyk, P., Imamura, H., Otto, T. D., Sanders, M., Seeger, K., Dujardin, J.-C., Berriman, M., Smith, D. F., Hertz-Fowler, C., & Mottram, J. C. (2011). Chromosome and gene copy number variation allow major structural change between species and strains of Leishmania. *Genome Research*, *21*(12), 2129-2142. https://doi.org/10.1101/gr.122945.111
103. Downing, T., Imamura, H., Decuypere, S., Clark, T. G., Coombs, G. H., Cotton, J. A., Hilley, J. D., de Doncker, S., Maes, I., Mottram, J. C., Quail, M. A., Rijal, S., Sanders, M., Schönian, G., Stark, O., Sundar, S., Vanaerschot, M., Hertz-Fowler, C., Dujardin, J.-C., & Berriman, M. (2011). Whole genome sequencing of multiple Leishmania donovani clinical isolates provides insights into population structure and mechanisms of drug resistance. *Genome Research*, *21*(12), 2143-2156. https://doi.org/10.1101/gr.123430.111
104. Coughlan, S., Taylor, A. S., Feane, E., Sanders, M., Schonian, G., Cotton, J. A., & Downing, T. (2018). *Leishmania naiffi* and *Leishmania guyanensis* reference genomes highlight genome structure and gene evolution in the *Viannia* subgenus. *Royal Society Open Science*, *5*(4), 172212. https://doi.org/10.1098/rsos.172212
105. Valdivia, H. O., Reis-Cunha, J. L., Rodrigues-Luiz, G. F., Baptista, R. P., Baldeviano, G. C., Gerbasi, R. V., Dobson, D. E., Pratlong, F., Bastien, P., Lescano, A. G., Beverley, S. M., & Bartholomeu, D. C. (2015). Comparative genomic analysis of Leishmania (Viannia) peruviana and Leishmania (Viannia) braziliensis. *BMC Genomics*, *16*(1). https://doi.org/10.1186/s12864-015-1928-z
106. Lin, W., Batra, D., Narayanan, V., Rowe, L. A., Sheth, M., Zheng, Y., Juieng, P., Loparev, V., & de Almeida, M. (2019). First Draft Genome Sequence of Leishmania (Viannia) lainsoni Strain 216-34,

Isolated from a Peruvian Clinical Case. *Microbiology Resource Announcements*, *8*(6), MRA.01524-18, e01524-18. https://doi.org/10.1128/MRA.01524-18

107. Raymond, F., Boisvert, S., Roy, G., Ritt, J.-F., Légaré, D., Isnard, A., Stanke, M., Olivier, M., Tremblay, M. J., Papadopoulou, B., Ouellette, M., & Corbeil, J. (2012). Genome sequencing of the lizard parasite Leishmania tarentolae reveals loss of genes associated to the intracellular stage of human pathogenic species. *Nucleic Acids Research*, *40*(3), 1131-1147. https://doi.org/10.1093/nar/gkr834
108. Coughlan, S., Mulhair, P., Sanders, M., Schonian, G., Cotton, J. A., & Downing, T. (2017). The genome of Leishmania adleri from a mammalian host highlights chromosome fission in Sauroleishmania. *Scientific Reports*, *7*(1), 43747. https://doi.org/10.1038/srep43747
109. Butenko, A., Kostygov, A. Y., Sádlová, J., Kleschenko, Y., Bečvář, T., Podešvová, L., Macedo, D. H., Žihala, D., Lukeš, J., Bates, P. A., Volf, P., Opperdoes, F. R., & Yurchenko, V. (2019). Comparative genomics of Leishmania (Mundinia). *BMC Genomics*, *20*(1), 726. https://doi.org/10.1186/s12864-019-6126-y
110. Padrón-Nieves, M., & Ponte-Sucre, A. (Eds.). (2018). *Drug Resistance in Leishmania Parasites: Consequences, Molecular Mechanisms and Possible Treatments* (2nd ed. 2018). Springer International Publishing : Imprint: Springer. https://doi.org/10.1007/978-3-319-74186-4
111. Holzer, T. R., McMaster, W. R., & Forney, J. D. (2006). Expression profiling by whole-genome interspecies microarray hybridization reveals differential gene expression in procyclic promastigotes, lesion-derived amastigotes, and axenic amastigotes in Leishmania mexicana. *Molecular and Biochemical Parasitology*, *146*(2), 198-218. https://doi.org/10.1016/j.molbiopara.2005.12.009
112. El-Sayed, N. M. (2005). Comparative Genomics of Trypanosomatid Parasitic Protozoa. *Science*, *309*(5733), 404-409. https://doi.org/10.1126/science.1112181
113. Cohen-Freue, G., Holzer, T. R., Forney, J. D., & McMaster, W. R. (2007). Global gene expression in Leishmania. *International Journal for Parasitology*, *37*(10), 1077-1086. https://doi.org/10.1016/j.ijpara.2007.04.011
114. Rougeron, V., De Meeus, T., Hide, M., Waleckx, E., Bermudez, H., Arevalo, J., Llanos-Cuentas, A., Dujardin, J.-C., De Doncker, S., Le Ray, D., Ayala, F. J., & Banuls, A.-L. (2009). Extreme inbreeding in Leishmania braziliensis. *Proceedings of the National Academy of Sciences*, *106*(25), 10224-10229. https://doi.org/10.1073/pnas.0904420106
115. Rogers, M. B., Hilley, J. D., Dickens, N. J., Wilkes, J., Bates, P. A., Depledge, D. P., Harris, D., Her, Y., Herzyk, P., Imamura, H., Otto, T. D., Sanders, M., Seeger, K., Dujardin, J.-C., Berriman, M., Smith, D. F., Hertz-Fowler, C., & Mottram, J. C. (2011). Chromosome and gene copy number variation allow major structural change between species and strains of Leishmania. *Genome Research*, *21*(12), 2129-2142. https://doi.org/10.1101/gr.122945.111
116. Teixeira, D. G., Monteiro, G. R. G., Martins, D. R. A., Fernandes, M. Z., Macedo-Silva, V., Ansaldi, M., Nascimento, P. R. P., Kurtz, M. A., Streit, J. A., Ximenes, M. F. F. M., Pearson, R. D., Miles, A., Blackwell, J. M., Wilson, M. E., Kitchen, A., Donelson, J. E., Lima, J. P. M. S., & Jeronimo, S. M. B. (2017). Comparative analyses of whole genome sequences of Leishmania infantum isolates from humans and dogs in northeastern Brazil. *International Journal for Parasitology*, *47*(10-11), 655-665. https://doi.org/10.1016/j.ijpara.2017.04.004

117. Sterkers, Y., Lachaud, L., Bourgeois, N., Crobu, L., Bastien, P., & Pagès, M. (2012). Novel insights into genome plasticity in Eukaryotes: Mosaic aneuploidy in *Leishmania*: Mosaic aneuploidy in *Leishmania*. *Molecular Microbiology*, *86*(1), 15-23. https://doi.org/10.1111/j.1365-2958.2012.08185.x
118. Samarasinghe, S. R., Samaranayake, N., Kariyawasam, U. L., Siriwardana, Y. D., Imamura, H., & Karunaweera, N. D. (2018). Genomic insights into virulence mechanisms of Leishmania donovani: Evidence from an atypical strain. *BMC Genomics*, *19*(1), 843. https://doi.org/10.1186/s12864-018-5271-z
119. Lukes, J., Mauricio, I. L., Schonian, G., Dujardin, J.-C., Soteriadou, K., Dedet, J.-P., Kuhls, K., Tintaya, K. W. Q., Jirku, M., Chocholova, E., Haralambous, C., Pratlong, F., Obornik, M., Horak, A., Ayala, F. J., & Miles, M. A. (2007). Evolutionary and geographical history of the Leishmania donovani complex with a revision of current taxonomy. *Proceedings of the National Academy of Sciences*, *104*(22), 9375-9380. https://doi.org/10.1073/pnas.0703678104
120. Bussotti, G., Gouzelou, E., Côrtes Boité, M., Kherachi, I., Harrat, Z., Eddaikra, N., Mottram, J. C., Antoniou, M., Christodoulou, V., Bali, A., Guerfali, F. Z., Laouini, D., Mukhtar, M., Dumetz, F., Dujardin, J.-C., Smirlis, D., Lechat, P., Pescher, P., El Hamouchi, A., … Späth, G. F. (2018). *Leishmania* Genome Dynamics during Environmental Adaptation Reveal Strain-Specific Differences in Gene Copy Number Variation, Karyotype Instability, and Telomeric Amplification. *mBio*, *9*(6), e01399-18, /mbio/9/6/mBio.01399-18.atom. https://doi.org/10.1128/mBio.01399-18
121. Kuhls, K., Alam, M. Z., Cupolillo, E., Ferreira, G. E. M., Mauricio, I. L., Oddone, R., Feliciangeli, M. D., Wirth, T., Miles, M. A., & Schönian, G. (2011). Comparative Microsatellite Typing of New World Leishmania infantum Reveals Low Heterogeneity among Populations and Its Recent Old World Origin. *PLoS Neglected Tropical Diseases*, *5*(6), e1155. https://doi.org/10.1371/journal.pntd.0001155
122. Leblois, R., Kuhls, K., François, O., Schönian, G., & Wirth, T. (2011). Guns, germs and dogs: On the origin of Leishmania chagasi. *Infection, Genetics and Evolution*, *11*(5), 1091-1095. https://doi.org/10.1016/j.meegid.2011.04.004
123. Carvalho, K. S. S., da Silva Júnior, W. J., da Silveira Regueira Neto, M., Silva, V. C., de Sá Leitão Paiva Júnior, S., Balbino, V. Q., Costa, D. L., & Costa, C. H. N. (2020). Application of next generation sequencing (NGS) for descriptive analysis of 30 genomes of Leishmania infantum isolates in Middle-North Brazil. *Scientific Reports*, *10*(1), 12321. https://doi.org/10.1038/s41598-020-68953-9
124. S L Figueiredo de Sá, B., Rezende, A. M., Melo Neto, O. P. de, Brito, M. E. F. de, & Brandão Filho, S. P. (2019). Identification of divergent Leishmania (Viannia) braziliensis ecotypes derived from a geographically restricted area through whole genome analysis. *PLoS Neglected Tropical Diseases*, *13*(6), e0007382. https://doi.org/10.1371/journal.pntd.0007382
125. Zhang, W., & Matlashewski, G. (2001). Characterization of the A2-A2rel gene cluster in Leishmania donovani: Involvement of A2 in visceralization during infection. *Molecular microbiology*, *39*, 935-948. https://doi.org/10.1046/j.1365-2958.2001.02286.x
126. Franssen, S. U., Takele, Y., Adem, E., Sanders, M. J., Müller, I., Kropf, P., & Cotton, J. A. (2021). *Diversity and within-host evolution of parasites from VL and VL/HIV patients in Northern Ethiopia* [Preprint]. Infectious Diseases (except HIV/AIDS). https://doi.org/10.1101/2021.04.01.21254750

127. Callahan, H. L., & Beverley, S. M. (1991). Heavy metal resistance: A new role for P-glycoproteins in Leishmania. *The Journal of Biological Chemistry*, *266*(28), 18427-18430.
128. Ashutosh, Garg, M., Sundar, S., Duncan, R., Nakhasi, H. L., & Goyal, N. (2012). Downregulation of Mitogen-Activated Protein Kinase 1 of Leishmania donovani Field Isolates Is Associated with Antimony Resistance. *Antimicrob. Agents Chemother.*, *56*(1), 518-525. https://doi.org/10.1128/AAC.00736-11
129. Pérez-Victoria, F. J., Sánchez-Cañete, M. P., Castanys, S., & Gamarro, F. (2006). Phospholipid Translocation and Miltefosine Potency Require Both L. donovani Miltefosine Transporter and the New Protein LdRos3 in Leishmania Parasites. *Journal of Biological Chemistry*, *281*(33), 23766-23775. https://doi.org/10.1074/jbc.M605214200
130. Carnielli, J. B. T., Crouch, K., Forrester, S., Silva, V. C., Carvalho, S. F. G., Damasceno, J. D., Brown, E., Dickens, N. J., Costa, D. L., Costa, C. H. N., Dietze, R., Jeffares, D. C., & Mottram, J. C. (2018). A Leishmania infantum genetic marker associated with miltefosine treatment failure for visceral leishmaniasis. *EBioMedicine*, *36*, 83-91. https://doi.org/10.1016/j.ebiom.2018.09.029
131. Akhoundi, M., Downing, T., Votýpka, J., Kuhls, K., Lukeš, J., Cannet, A., Ravel, C., Marty, P., Delaunay, P., Kasbari, M., Granouillac, B., Gradoni, L., & Sereno, D. (2017). Leishmania infections: Molecular targets and diagnosis. *Molecular Aspects of Medicine*, *57*, 1-29. https://doi.org/10.1016/j.mam.2016.11.012
132. Kuhls, K., Keilonat, L., Ochsenreither, S., Schaar, M., Schweynoch, C., Presber, W., & Schönian, G. (2007). Multilocus microsatellite typing (MLMT) reveals genetically isolated populations between and within the main endemic regions of visceral leishmaniasis. *Microbes and Infection*, *9*(3), 334-343. https://doi.org/10.1016/j.micinf.2006.12.009
133. Zemanová, E., Jirků, M., Mauricio, I. L., Horák, A., Miles, M. A., & Lukeš, J. (2007). The Leishmania donovani complex: Genotypes of five metabolic enzymes (ICD, ME, MPI, G6PDH, and FH), new targets for multilocus sequence typing. *International Journal for Parasitology*, *37*(2), 149-160. https://doi.org/10.1016/j.ijpara.2006.08.008
134. Mauricio, I. L., Yeo, M., Baghaei, M., Doto, D., Pratlong, F., Zemanova, E., Dedet, J.-P., Lukes, J., & Miles, M. A. (2006). Towards multilocus sequence typing of the Leishmania donovani complex: Resolving genotypes and haplotypes for five polymorphic metabolic enzymes (ASAT, GPI, NH1, NH2, PGD). *International Journal for Parasitology*, *36*(7), 757-769. https://doi.org/10.1016/j.ijpara.2006.03.006
135. Boité, M. C., Mauricio, I. L., Miles, M. A., & Cupolillo, E. (2012). New Insights on Taxonomy, Phylogeny and Population Genetics of Leishmania (Viannia) Parasites Based on Multilocus Sequence Analysis. *PLoS Neglected Tropical Diseases*, *6*(11), e1888. https://doi.org/10.1371/journal.pntd.0001888
136. Herrera, G., Hernández, C., Ayala, M. S., Flórez, C., Teherán, A. A., & Ramírez, J. D. (2017). Evaluation of a Multilocus Sequence Typing (MLST) scheme for Leishmania (Viannia) braziliensis and Leishmania (Viannia) panamensis in Colombia. *Parasites & Vectors*, *10*(1), 236. https://doi.org/10.1186/s13071-017-2175-8
137. Downing, T., Stark, O., Vanaerschot, M., Imamura, H., Sanders, M., Decuypere, S., de Doncker, S., Maes, I., Rijal, S., Sundar, S., Dujardin, J.-C., Berriman, M., & Schönian, G. (2012). Genome-wide SNP and microsatellite variation illuminate population-level epidemiology in the Leishmania donovani

species complex. *Infection, Genetics and Evolution*, *12*(1), 149-159. https://doi.org/10.1016/j.meegid.2011.11.005

138. El Baidouri, F., Diancourt, L., Berry, V., Chevenet, F., Pratlong, F., Marty, P., & Ravel, C. (2013). Genetic Structure and Evolution of the Leishmania Genus in Africa and Eurasia: What Does MLSA Tell Us. *PLoS Neglected Tropical Diseases*, *7*(6), e2255. https://doi.org/10.1371/journal.pntd.0002255
139. Schwenkenbecher, J. M., Wirth, T., Schnur, L. F., Jaffe, C. L., Schallig, H., Al-Jawabreh, A., Hamarsheh, O., Azmi, K., Pratlong, F., & Schönian, G. (2006). Microsatellite analysis reveals genetic structure of Leishmania tropica. *International Journal for Parasitology*, *36*(2), 237-246. https://doi.org/10.1016/j.ijpara.2005.09.010
140. Mahnaz, T., Al-Jawabreh, A., Kuhls, K., & Schönian, G. (2011). Multilocus microsatellite typing shows three different genetic clusters of Leishmania major in Iran. *Microbes and Infection*, *13*(11), 937-942. https://doi.org/10.1016/j.micinf.2011.05.005
141. Kuhls, K., Cupolillo, E., Silva, S. O., Schweynoch, C., Côrtes Boité, M., Mello, M. N., Mauricio, I., Miles, M., Wirth, T., & Schönian, G. (2013). Population Structure and Evidence for Both Clonality and Recombination among Brazilian Strains of the Subgenus Leishmania (Viannia). *PLoS Neglected Tropical Diseases*, *7*(10), e2490. https://doi.org/10.1371/journal.pntd.0002490
142. Gouzelou, E., Haralambous, C., Amro, A., Mentis, A., Pratlong, F., Dedet, J.-P., Votypka, J., Volf, P., Ozensoy Toz, S., Kuhls, K., Schönian, G., & Soteriadou, K. (2012). Multilocus Microsatellite Typing (MLMT) of Strains from Turkey and Cyprus Reveals a Novel Monophyletic L. donovani Sensu Lato Group. *PLoS Neglected Tropical Diseases*, *6*(2), e1507. https://doi.org/10.1371/journal.pntd.0001507
143. Hide, M., Marion, E., Pomares, C., Fisa, R., Marty, P., & Bañuls, A. L. (2013). Parasitic genotypes appear to differ in leishmaniasis patients compared with asymptomatic related carriers. *International Journal for Parasitology*, *43*(5), 389-397. https://doi.org/10.1016/j.ijpara.2012.12.008
144. Segatto, M., Ribeiro, L. S., Costa, D. L., Costa, C. H. N., Oliveira, M. R. de, Carvalho, S. F. G., Macedo, A. M., Valadares, H. M. S., Dietze, R., Brito, C. F. A. de, & Lemos, E. M. (2012). Genetic diversity of Leishmania infantum field populations from Brazil. *Memórias do Instituto Oswaldo Cruz*, *107*(1), 39-47. https://doi.org/10.1590/S0074-02762012000100006
145. Ferreira, G. E. M., dos Santos, B. N., Dorval, M. E. C., Ramos, T. P. B., Porrozzi, R., Peixoto, A. A., & Cupolillo, E. (2012). The Genetic Structure of Leishmania infantum Populations in Brazil and Its Possible Association with the Transmission Cycle of Visceral Leishmaniasis. *PLoS ONE*, *7*(5), e36242. https://doi.org/10.1371/journal.pone.0036242
146. Boité, M. C., Späth, G. F., Bussotti, G., Porrozzi, R., Morgado, F. N., Llewellyn, M., Schwabl, P., & Cupolillo, E. (2019). Trans-Atlantic Spillover: Deconstructing the Ecological Adaptation of Leishmania infantum in the Americas. *Genes*, *11*(1), 4. https://doi.org/10.3390/genes11010004
147. Samorodnitsky, E., Jewell, B. M., Hagopian, R., Miya, J., Wing, M. R., Lyon, E., Damodaran, S., Bhatt, D., Reeser, J. W., Datta, J., & Roychowdhury, S. (2015). Evaluation of Hybridization Capture Versus Amplicon-Based Methods for Whole-Exome Sequencing. *Human Mutation*, *36*(9), 903-914. https://doi.org/10.1002/humu.22825
148. Gnirke, A., Melnikov, A., Maguire, J., Rogov, P., LeProust, E. M., Brockman, W., Fennell, T., Giannoukos, G., Fisher, S., Russ, C., Gabriel, S., Jaffe, D. B., Lander, E. S., & Nusbaum, C. (2009).

Solution hybrid selection with ultra-long oligonucleotides for massively parallel targeted sequencing. *Nature Biotechnology*, *27*(2), 182-189. https://doi.org/10.1038/nbt.1523

149. Sulonen, A.-M., Ellonen, P., Almusa, H., Lepistö, M., Eldfors, S., Hannula, S., Miettinen, T., Tyynismaa, H., Salo, P., Heckman, C., Joensuu, H., Raivio, T., Suomalainen, A., & Saarela, J. (2011). Comparison of solution-based exome capture methods for next generation sequencing. *Genome Biology*, *12*(9), R94. https://doi.org/10.1186/gb-2011-12-9-r94
150. Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K., & Schloss, P. D. (2013). Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform. *Applied and Environmental Microbiology*, *79*(17), 5112-5120. https://doi.org/10.1128/AEM.01043-13
151. Lundberg, D. S., Yourstone, S., Mieczkowski, P., Jones, C. D., & Dangl, J. L. (2013). Practical innovations for high-throughput amplicon sequencing. *Nature Methods*, *10*(10), 999-1002. https://doi.org/10.1038/nmeth.2634
152. Clark, M. J., Chen, R., Lam, H. Y. K., Karczewski, K. J., Chen, R., Euskirchen, G., Butte, A. J., & Snyder, M. (2011). Performance comparison of exome DNA sequencing technologies. *Nature Biotechnology*, *29*(10), 908-914. https://doi.org/10.1038/nbt.1975
153. Chilamakuri, C. S., Lorenz, S., Madoui, M.-A., Vodák, D., Sun, J., Hovig, E., Myklebost, O., & Meza-Zepeda, L. A. (2014). Performance comparison of four exome capture systems for deep sequencing. *BMC Genomics*, *15*(1), 449. https://doi.org/10.1186/1471-2164-15-449
154. Chang, F., & Li, M. M. (2013). Clinical application of amplicon-based next-generation sequencing in cancer. *Cancer Genetics*, *206*(12), 413-419. https://doi.org/10.1016/j.cancergen.2013.10.003
155. Wen, C., Wu, L., Qin, Y., Van Nostrand, J. D., Ning, D., Sun, B., Xue, K., Liu, F., Deng, Y., Liang, Y., & Zhou, J. (2017). Evaluation of the reproducibility of amplicon sequencing with Illumina MiSeq platform. *PLOS ONE*, *12*(4), e0176716. https://doi.org/10.1371/journal.pone.0176716
156. Grubaugh, N. D., Gangavarapu, K., Quick, J., Matteson, N. L., De Jesus, J. G., Main, B. J., Tan, A. L., Paul, L. M., Brackney, D. E., Grewal, S., Gurfield, N., Van Rompay, K. K. A., Isern, S., Michael, S. F., Coffey, L. L., Loman, N. J., & Andersen, K. G. (2019). An amplicon-based sequencing framework for accurately measuring intrahost virus diversity using PrimalSeq and iVar. *Genome Biology*, *20*(1), 8. https://doi.org/10.1186/s13059-018-1618-7
157. Uyaguari-Diaz, M. I., Chan, M., Chaban, B. L., Croxen, M. A., Finke, J. F., Hill, J. E., Peabody, M. A., Van Rossum, T., Suttle, C. A., Brinkman, F. S. L., Isaac-Renton, J., Prystajecky, N. A., & Tang, P. (2016). A comprehensive method for amplicon-based and metagenomic characterization of viruses, bacteria, and eukaryotes in freshwater samples. *Microbiome*, *4*(1), 20. https://doi.org/10.1186/s40168-016-0166-1
158. Moreno, Y., Moreno-Mesonero, L., Amorós, I., Pérez, R., Morillo, J. A., & Alonso, J. L. (2018). Multiple identification of most important waterborne protozoa in surface water used for irrigation purposes by 18S rRNA amplicon-based metagenomics. *International Journal of Hygiene and Environmental Health*, *221*(1), 102-111. https://doi.org/10.1016/j.ijheh.2017.10.008
159. Tanaka, R., Hino, A., Tsai, I. J., Palomares-Rius, J. E., Yoshida, A., Ogura, Y., Hayashi, T., Maruyama, H., & Kikuchi, T. (2014). Assessment of Helminth Biodiversity in Wild Rats Using 18S rDNA Based Metagenomics. *PLoS ONE*, *9*(10), e110769. https://doi.org/10.1371/journal.pone.0110769

160. Holmgaard, D. B., Barnadas, C., Mirbarati, S. H., O'Brien Andersen, L., Nielsen, H. V., & Stensvold, C. R. (2020). Detection and Identification of *Acanthamoeba* and Other Nonviral Causes of Infectious Keratitis in Corneal Scrapings by Real-Time PCR and Next-Generation Sequencing-Based 16S-18S Gene Analysis. *Journal of Clinical Microbiology*, *59*(2), e02224-20, /jcm/59/2/JCM.02224-20.atom. https://doi.org/10.1128/JCM.02224-20

161. Llewellyn, M. S., Messenger, L. A., Luquetti, A. O., Garcia, L., Torrico, F., Tavares, S. B. N., Cheaib, B., Derome, N., Delepine, M., Baulard, C., Deleuze, J.-F., Sauer, S., & Miles, M. A. (2015). Deep sequencing of the Trypanosoma cruzi GP63 surface proteases reveals diversity and diversifying selection among chronic and congenital Chagas disease patients. *PLoS Neglected Tropical Diseases*, *9*(4), e0003458. https://doi.org/10.1371/journal.pntd.0003458

162. Abbasi, I., Nasereddin, A., & Warburg, A. (2019). Development of a next generation DNA sequencing-based multi detection assay for detecting and identifying Leishmania parasites, blood sources, plant meals and intestinal microbiome in phlebotomine sand flies. *Acta Tropica*, *199*, 105101. https://doi.org/10.1016/j.actatropica.2019.105101

163. Kocher, A., Valière, S., Bañuls, A.-L., & Murienne, J. (2018). High-throughput sequencing of kDNA amplicons for the analysis of *Leishmania* minicircles and identification of Neotropical species. *Parasitology*, *145*(5), 585-594. https://doi.org/10.1017/S0031182017002013

164. Cooper, C., Keatley, S., Northover, A., Gofton, A. W., Brigg, F., Lymbery, A. J., Pallant, L., Clode, P. L., & Thompson, R. C. A. (2018). Next generation sequencing reveals widespread trypanosome diversity and polyparasitism in marsupials from Western Australia. *International Journal for Parasitology: Parasites and Wildlife*, *7*(1), 58-67. https://doi.org/10.1016/j.ijppaw.2018.01.005

165. Barbosa, A. D., Gofton, A. W., Paparini, A., Codello, A., Greay, T., Gillett, A., Warren, K., Irwin, P., & Ryan, U. (2017). Increased genetic diversity and prevalence of co-infection with Trypanosoma spp. In koalas (Phascolarctos cinereus) and their ticks identified using next-generation sequencing (NGS). *PLOS ONE*, *12*(7), e0181279. https://doi.org/10.1371/journal.pone.0181279

166. Dario, M. A., Moratelli, R., Schwabl, P., Jansen, A. M., & Llewellyn, M. S. (2017). Small subunit ribosomal metabarcoding reveals extraordinary trypanosomatid diversity in Brazilian bats. *PLoS Neglected Tropical Diseases*, *11*(7), e0005790. https://doi.org/10.1371/journal.pntd.0005790

167. Maiguashca Sánchez, J., Sueto, S. O. B., Schwabl, P., Grijalva, M. J., Llewellyn, M. S., & Costales, J. A. (2020). Remarkable genetic diversity of Trypanosoma cruzi and Trypanosoma rangeli in two localities of southern Ecuador identified via deep sequencing of mini-exon gene amplicons. *Parasites & Vectors*, *13*(1), 252. https://doi.org/10.1186/s13071-020-04079-1

168. Rijal, S., Ostyn, B., Uranw, S., Rai, K., Bhattarai, N. R., Dorlo, T. P. C., Beijnen, J. H., Vanaerschot, M., Decuypere, S., Dhakal, S. S., Das, M. L., Karki, P., Singh, R., Boelaert, M., & Dujardin, J.-C. (2013). Increasing Failure of Miltefosine in the Treatment of Kala-azar in Nepal and the Potential Role of Parasite Drug Resistance, Reinfection, or Noncompliance. *Clinical Infectious Diseases*, *56*(11), 1530-1538. https://doi.org/10.1093/cid/cit102

169. Mueller, M., Ritmeijer, K., Balasegaram, M., Koummuki, Y., Santana, M. R., & Davidson, R. (2007). Unresponsiveness to AmBisome in some Sudanese patients with kala-azar. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *101*(1), 19-24. https://doi.org/10.1016/j.trstmh.2006.02.005

170. Salih, N. A., van Griensven, J., Chappuis, F., Antierens, A., Mumina, A., Hammam, O., Boulle, P., Alirol, E., Alnour, M., Elhag, M. S., Manzi, M., Kizito, W., & Zachariah, R. (2014). Liposomal amphotericin B for complicated visceral leishmaniasis (kala-azar) in eastern Sudan: How effective is treatment for this neglected disease? *Tropical Medicine & International Health*, *19*(2), 146-152. https://doi.org/10.1111/tmi.12238
171. Sundar, S., More, D. K., Singh, M. K., Singh, V. P., Sharma, S., Makharia, A., Kumar, P. C. K., & Murray, H. W. (2000). Failure of Pentavalent Antimony in Visceral Leishmaniasis in India: Report from the Center of the Indian Epidemic. *Clinical Infectious Diseases*, *31*(4), 1104-1107. https://doi.org/10.1086/318121
172. Uliana, S. R. B., Trinconi, C. T., & Coelho, A. C. (2018). Chemotherapy of leishmaniasis: Present challenges. *Parasitology*, *145*(4), 464-480. https://doi.org/10.1017/S0031182016002523
173. Lindoso, J. A., Cota, G. F., da Cruz, A. M., Goto, H., Maia-Elkhoury, A. N. S., Romero, G. A. S., de Sousa-Gomes, M. L., Santos-Oliveira, J. R., & Rabello, A. (2014). Visceral Leishmaniasis and HIV Coinfection in Latin America. *PLoS Neglected Tropical Diseases*, *8*(9), e3136. https://doi.org/10.1371/journal.pntd.0003136
174. Singh, B., Fakiola, M., Sudarshan, M., Oommen, J., Singh, S. S., Sundar, S., & Blackwell, J. M. (2019). HLA-DR Class II expression on myeloid and lymphoid cells in relation to HLA-DRB1 as a genetic risk factor for visceral leishmaniasis. *Immunology*, *156*(2), 174-186. https://doi.org/10.1111/imm.13018
175. Oliveira-Sena, I. V., & Werneck, G. L. (2019). Risk factors for in-hospital mortality from visceral leishmaniasis: A case-control study. *Journal of Infection and Public Health*, S1876034119303181. https://doi.org/10.1016/j.jiph.2019.10.003
176. Asín-Prieto, E., Rodríguez-Gascón, A., & Isla, A. (2015). Applications of the pharmacokinetic/pharmacodynamic (PK/PD) analysis of antimicrobial agents. *Journal of Infection and Chemotherapy*, *21*(5), 319-329. https://doi.org/10.1016/j.jiac.2015.02.001
177. Dorlo, T. P. C., Eggelte, T. A., Schoone, G. J., de Vries, P. J., & Beijnen, J. H. (2012). A Poor-Quality Generic Drug for the Treatment of Visceral Leishmaniasis: A Case Report and Appeal. *PLoS Neglected Tropical Diseases*, *6*(5), e1544. https://doi.org/10.1371/journal.pntd.0001544
178. Voak, A. A., Harris, A., Qaiser, Z., Croft, S. L., & Seifert, K. (2017). Pharmacodynamics and Biodistribution of Single-Dose Liposomal Amphotericin B at Different Stages of Experimental Visceral Leishmaniasis. *Antimicrobial Agents and Chemotherapy*, *61*(9), e00497-17, e00497-17. https://doi.org/10.1128/AAC.00497-17
179. Van Bocxlaer, K., Yardley, V., Murdan, S., & Croft, S. L. (2016). Drug permeation and barrier damage in *Leishmania* -infected mouse skin. *Journal of Antimicrobial Chemotherapy*, *71*(6), 1578-1585. https://doi.org/10.1093/jac/dkw012
180. Vanaerschot, M., Dumetz, F., Roy, S., Ponte-Sucre, A., Arevalo, J., & Dujardin, J.-C. (2014). Treatment failure in leishmaniasis: Drug-resistance or another (epi-) phenotype? *Expert Review of Anti-Infective Therapy*, *12*(8), 937-946. https://doi.org/10.1586/14787210.2014.916614
181. Ouakad, M., Vanaerschot, M., Rijal, S., Sundar, S., Speybroeck, N., Kestens, L., Boel, L., De Doncker, S., Maes, I., Decuypere, S., & Dujardin, J.-C. (2011). Increased metacyclogenesis of antimony-resistant *Leishmania donovani* clinical lines. *Parasitology*, *138*(11), 1392-1399. https://doi.org/10.1017/S0031182011001120

182. Kloehn, J., Saunders, E. C., O'Callaghan, S., Dagley, M. J., & McConville, M. J. (2015). Characterization of Metabolically Quiescent Leishmania Parasites in Murine Lesions Using Heavy Water Labeling. *PLOS Pathogens*, *11*(2), e1004683. https://doi.org/10.1371/journal.ppat.1004683

183. Croft, S. L., Sundar, S., & Fairlamb, A. H. (2006). Drug Resistance in Leishmaniasis. *Clinical Microbiology Reviews*, *19*(1), 111-126. https://doi.org/10.1128/CMR.19.1.111-126.2006

184. Dumetz, F., Cuypers, B., Imamura, H., Zander, D., D'Haenens, E., Maes, I., Domagalska, M. A., Clos, J., Dujardin, J.-C., & De Muylder, G. (2018). Molecular Preadaptation to Antimony Resistance in Leishmania donovani on the Indian Subcontinent. *mSphere*, *3*(2). https://doi.org/10.1128/mSphere.00548-17

185. Ashutosh, Sundar, S., & Goyal, N. (2007). Molecular mechanisms of antimony resistance in Leishmania. *Journal of Medical Microbiology*, *56*(2), 143-153. https://doi.org/10.1099/jmm.0.46841-0

186. Denton, H., McGregor, J. C., & Coombs, G. H. (2004). Reduction of anti-leishmanial pentavalent antimonial drugs by a parasite-specific thiol-dependent reductase, TDR1. *The Biochemical Journal*, *381*(Pt 2), 405-412. https://doi.org/10.1042/BJ20040283

187. Zhou, Y., Messier, N., Ouellette, M., Rosen, B. P., & Mukhopadhyay, R. (2004). Leishmania major LmACR2 Is a Pentavalent Antimony Reductase That Confers Sensitivity to the Drug Pentostam. *Journal of Biological Chemistry*, *279*(36), 37445-37451. https://doi.org/10.1074/jbc.M404383200

188. Mukhopadhyay, R., Bisacchi, D., Zhou, Y., Armirotti, A., & Bordo, D. (2009). Structural Characterization of the As/Sb Reductase LmACR2 from Leishmania major. *Journal of Molecular Biology*, *386*(5), 1229-1239. https://doi.org/10.1016/j.jmb.2008.07.056

189. Marquis, N., Gourbal, B., Rosen, B. P., Mukhopadhyay, R., & Ouellette, M. (2005). Modulation in aquaglyceroporin *AQP1* gene transcript levels in drug-resistant *Leishmania*: *AQP1* and drug resistance in *Leishmania*. *Molecular Microbiology*, *57*(6), 1690-1699. https://doi.org/10.1111/j.1365-2958.2005.04782.x

190. Wyllie, S., Cunningham, M. L., & Fairlamb, A. H. (2004). Dual Action of Antimonial Drugs on Thiol Redox Metabolism in the Human Pathogen *Leishmania donovani*. *Journal of Biological Chemistry*, *279*(38), 39925-39932. https://doi.org/10.1074/jbc.M405635200

191. Berman, J. D., Waddell, D., & Hanson, B. D. (1985). Biochemical mechanisms of the antileishmanial activity of sodium stibogluconate. *Antimicrobial Agents and Chemotherapy*, *27*(6), 916-920. https://doi.org/10.1128/AAC.27.6.916

192. Sereno, D., Holzmuller, P., Mangot, I., Cuny, G., Ouaissi, A., & Lemesre, J.-L. (2001). Antimonial-Mediated DNA Fragmentation in Leishmania infantum Amastigotes. *Antimicrobial Agents and Chemotherapy*, *45*(7), 2064-2069. https://doi.org/10.1128/AAC.45.7.2064-2069.2001

193. Imamura, H., Downing, T., Van den Broeck, F., Sanders, M. J., Rijal, S., Sundar, S., Mannaert, A., Vanaerschot, M., Berg, M., De Muylder, G., Dumetz, F., Cuypers, B., Maes, I., Domagalska, M., Decuypere, S., Rai, K., Uranw, S., Bhattarai, N. R., Khanal, B., … Cotton, J. A. (2016). Evolutionary genomics of epidemic visceral leishmaniasis in the Indian subcontinent. *eLife*, *5*. https://doi.org/10.7554/eLife.12613

194. Tavakoli, R., Sharifi, I., Afgar, A., Kareshk, A. T., Asadi, A., Heshmatkhah, A., Bamorovat, M., Jafarzadeh, A., Mohammadi, M. A., & Daneshvar, H. (2018). Unresponsiveness to meglumine antimoniate in anthroponotic cutaneous leishmaniasis field isolates: Analysis of resistance

biomarkers by gene expression profiling. *Tropical Medicine & International Health*, *23*(6), 622-633. https://doi.org/10.1111/tmi.13062

195. Haimeur, A., Guimond, C., Pilote, S., Mukhopadhyay, R., Rosen, B. P., Poulin, R., & Ouellette, M. (1999). Elevated levels of polyamines and trypanothione resulting from overexpression of the ornithine decarboxylase gene in arsenite-resistant Leishmania. *Molecular Microbiology*, *34*(4), 726-735. https://doi.org/10.1046/j.1365-2958.1999.01634.x
196. Mookerjee Basu, J., Mookerjee, A., Banerjee, R., Saha, M., Singh, S., Naskar, K., Tripathy, G., Sinha, P. K., Pandey, K., Sundar, S., Bimal, S., Das, P. K., Choudhuri, S. K., & Roy, S. (2008). Inhibition of ABC Transporters Abolishes Antimony Resistance in Leishmania Infection. *Antimicrobial Agents and Chemotherapy*, *52*(3), 1080-1093. https://doi.org/10.1128/AAC.01196-07
197. Mandal, G., Sarkar, A., Saha, P., Singh, N., Sundar, S., & Chatterjee, M. (2009). Functionality of drug efflux pumps in antimonial resistant Leishmania donovani field isolates. *Indian Journal of Biochemistry & Biophysics*, *46*(1), 86-92.
198. Leprohon, P., Fernandez-Prada, C., Gazanion, É., Monte-Neto, R., & Ouellette, M. (2015). Drug resistance analysis by next generation sequencing in Leishmania. *International Journal for Parasitology: Drugs and Drug Resistance*, *5*(1), 26-35. https://doi.org/10.1016/j.ijpddr.2014.09.005
199. do Monte-Neto, R. L., Coelho, A. C., Raymond, F., Légaré, D., Corbeil, J., Melo, M. N., Frézard, F., & Ouellette, M. (2011). Gene Expression Profiling and Molecular Characterization of Antimony Resistance in Leishmania amazonensis. *PLoS Neglected Tropical Diseases*, *5*(5), e1167. https://doi.org/10.1371/journal.pntd.0001167
200. Patino, L. H., Muskus, C., & Ramírez, J. D. (2019). Transcriptional responses of Leishmania (Leishmania) amazonensis in the presence of trivalent sodium stibogluconate. *Parasites & Vectors*, *12*(1). https://doi.org/10.1186/s13071-019-3603-8
201. Decuypere, S., Rijal, S., Yardley, V., De Doncker, S., Laurent, T., Khanal, B., Chappuis, F., & Dujardin, J.-C. (2005). Gene Expression Analysis of the Mechanism of Natural Sb(V) Resistance in Leishmania donovani Isolates from Nepal. *Antimicrobial Agents and Chemotherapy*, *49*(11), 4616-4621. https://doi.org/10.1128/AAC.49.11.4616-4621.2005
202. Iantorno, S. A., Durrant, C., Khan, A., Sanders, M. J., Beverley, S. M., Warren, W. C., Berriman, M., Sacks, D. L., Cotton, J. A., & Grigg, M. E. (2017). Gene Expression in *Leishmania* Is Regulated Predominantly by Gene Dosage. *mBio*, *8*(5). https://doi.org/10.1128/mBio.01393-17
203. Brotherton, M.-C., Bourassa, S., Leprohon, P., Légaré, D., Poirier, G. G., Droit, A., & Ouellette, M. (2013). Proteomic and Genomic Analyses of Antimony Resistant Leishmania infantum Mutant. *PLoS ONE*, *8*(11), e81899. https://doi.org/10.1371/journal.pone.0081899
204. Rastrojo, A., García-Hernández, R., Vargas, P., Camacho, E., Corvo, L., Imamura, H., Dujardin, J.-C., Castanys, S., Aguado, B., Gamarro, F., & Requena, J. M. (2018). Genomic and transcriptomic alterations in Leishmania donovani lines experimentally resistant to antileishmanial drugs. *International Journal for Parasitology: Drugs and Drug Resistance*, *8*(2), 246-264. https://doi.org/10.1016/j.ijpddr.2018.04.002
205. Mukherjee, A., Boisvert, S., Monte-Neto, R. L. do, Coelho, A. C., Raymond, F., Mukhopadhyay, R., Corbeil, J., & Ouellette, M. (2013). Telomeric gene deletion and intrachromosomal amplification in antimony-resistant *Leishmania*: Gene rearrangements in *Leishmania*. *Molecular Microbiology*, *88*(1), 189-202. https://doi.org/10.1111/mmi.12178

206. Reis-Cunha, J. L., Rodrigues-Luiz, G. F., Valdivia, H. O., Baptista, R. P., Mendes, T. A. O., de Morais, G. L., Guedes, R., Macedo, A. M., Bern, C., Gilman, R. H., Lopez, C. T., Andersson, B., Vasconcelos, A. T., & Bartholomeu, D. C. (2015). Chromosomal copy number variation reveals differential levels of genomic plasticity in distinct Trypanosoma cruzi strains. *BMC Genomics*, *16*(1). https://doi.org/10.1186/s12864-015-1680-4
207. Torres, E. M., Sokolsky, T., Tucker, C. M., Chan, L. Y., Boselli, M., Dunham, M. J., & Amon, A. (2007). Effects of Aneuploidy on Cellular Physiology and Cell Division in Haploid Yeast. *Science*, *317*(5840), 916-924. https://doi.org/10.1126/science.1142210
208. Quero, S., Párraga-Niño, N., García-Núñez, M., & Sabrià, M. (2016). Proteómica en enfermedades infecciosas. *Enfermedades Infecciosas y Microbiología Clínica*, *34*(4), 253-260. https://doi.org/10.1016/j.eimc.2014.07.015
209. Chawla, B., Jhingran, A., Panigrahi, A., Stuart, K. D., & Madhubala, R. (2011). Paromomycin Affects Translation and Vesicle-Mediated Trafficking as Revealed by Proteomics of Paromomycin – Susceptible –Resistant Leishmania donovani. *PLoS ONE*, *6*(10), e26660. https://doi.org/10.1371/journal.pone.0026660
210. Biyani, N., Singh, A. K., Mandal, S., Chawla, B., & Madhubala, R. (2011). Differential expression of proteins in antimony-susceptible and -resistant isolates of Leishmania donovani. *Molecular and Biochemical Parasitology*, *179*(2), 91-99. https://doi.org/10.1016/j.molbiopara.2011.06.004
211. Dong, G., Filho, A. L., & Olivier, M. (2019). Modulation of Host-Pathogen Communication by Extracellular Vesicles (EVs) of the Protozoan Parasite Leishmania. *Front. Cell. Infect. Microbiol.*, *9*, 100. https://doi.org/10.3389/fcimb.2019.00100
212. Vergnes, B., Gourbal, B., Girard, I., Sundar, S., Drummelsmith, J., & Ouellette, M. (2007). A Proteomics Screen Implicates HSP83 and a Small Kinetoplastid Calpain-related Protein in Drug Resistance in Leishmania donovani Clinical Field Isolates by Modulating Drug-induced Programmed Cell Death. *Molecular & Cellular Proteomics*, *6*(1), 88-101. https://doi.org/10.1074/mcp.M600319-MCP200
213. Kumar, A., Sisodia, B., Misra, P., Sundar, S., Shasany, A. K., & Dube, A. (2010). Proteome mapping of overexpressed membrane-enriched and cytosolic proteins in sodium antimony gluconate (SAG) resistant clinical isolate of Leishmania donovani: Overexpressed proteins in SAG resistant L. donovani. *British Journal of Clinical Pharmacology*, *70*(4), 609-617. https://doi.org/10.1111/j.1365-2125.2010.03716.x
214. Lima, B. S. de S., Esteves, B. B., Fialho-Júnior, L. C., Mendes, T. A. de O., Pires, S. da F., Chapeourouge, A., Perales, J., & de Andrade, H. M. (2020). Study of the differentially abundant proteins among Leishmania amazonensis, L. braziliensis, and L. infantum. *PLoS ONE*, *15*(10), e0240612. https://doi.org/10.1371/journal.pone.0240612
215. Matrangolo, F. S. V., Liarte, D. B., Andrade, L. C., de Melo, M. F., Andrade, J. M., Ferreira, R. F., Santiago, A. S., Pirovani, C. P., Silva-Pereira, R. A., & Murta, S. M. F. (2013). Comparative proteomic analysis of antimony-resistant and -susceptible Leishmania braziliensis and Leishmania infantum chagasi lines. *Molecular and Biochemical Parasitology*, *190*(2), 63-75. https://doi.org/10.1016/j.molbiopara.2013.06.006
216. Saboia-Vahia, L., Cuervo, P., Wiśniewski, J. R., Dias-Lopes, G., Pinho, N., Padrón, G., De Pilla Varotti, F., & Murta, S. M. F. (2022). In-Depth Quantitative Proteomics Characterization of In Vitro Selected

Miltefosine Resistance in Leishmania infantum. *Proteomes*, *10*(2), 10. https://doi.org/10.3390/proteomes10020010

217. Gutierrez Guarnizo, S. A., Karamysheva, Z. N., Galeano, E., & Muskus, C. E. (2021). Metabolite Biomarkers of Leishmania Antimony Resistance. *Cells*, *10*(5), 1063. https://doi.org/10.3390/cells10051063
218. Gutierrez Guarnizo, S. A., Tikhonova, E. B., Zabet-Moghaddam, M., Zhang, K., Muskus, C., Karamyshev, A. L., & Karamysheva, Z. N. (2021). Drug-Induced Lipid Remodeling in Leishmania Parasites. *Microorganisms*, *9*(4), 790. https://doi.org/10.3390/microorganisms9040790
219. Madusanka, R. K., Karunaweera, N. D., Silva, H., & Selvapandiyan, A. (2024). Antimony resistance and gene expression in *Leishmania*: Spotlight on molecular and proteomic aspects. *Parasitology*, *151*(1), 1-14. https://doi.org/10.1017/S0031182023001129
220. Pinho, N., Wiśniewski, J. R., Dias-Lopes, G., Saboia-Vahia, L., Bombaça, A. C. S., Mesquita-Rodrigues, C., Menna-Barreto, R., Cupolillo, E., De Jesus, J. B., Padrón, G., & Cuervo, P. (2020). In-depth quantitative proteomics uncovers specie-specific metabolic programs in Leishmania (Viannia) species. *PLOS Neglected Tropical Diseases*, *14*(8), e0008509. https://doi.org/10.1371/journal.pntd.0008509
221. Alvar, J., Yactayo, S., & Bern, C. (2006). Leishmaniasis and poverty. *Trends in Parasitology*, *22*(12), 552-557. https://doi.org/10.1016/j.pt.2006.09.004
222. OMS/OPS. (2009). *ELIMINACIÓN DE LAS ENFERMEDADES DESATENDIDAS Y OTRAS INFECCIONES RELACIONADAS CON LA POBREZA*. https://www.paho.org/hq/dmdocuments/2009/CD49.R19%20(Esp.).pdf
223. Klohe, K., Amuasi, J., Kaducu, J. M., Haavardsson, I., Bogatyreva, E., Onarheim, K. H., Harrison, W., Kristensen, F., Prazeres da Costa, C., & Winkler, A. S. (2019). The 2017 Oslo conference report on neglected tropical diseases and emerging/re-emerging infectious diseases – focus on populations underserved. *Infectious Diseases of Poverty*, *8*(1), 40. https://doi.org/10.1186/s40249-019-0550-8
224. WHO/PAHO. (2016). *PLAN OF ACTION FOR THE ELIMINATION OF NEGLECTED INFECTIOUS DISEASES AND POST-ELIMINATION ACTIONS 2016-2022*. https://www.paho.org/hq/dmdocuments/2016/CD55-R9-e.pdf
225. da Rocha, I. C. M., dos Santos, L. H. M., Coura-Vital, W., da Cunha, G. M. R., Magalhães, F. do C., da Silva, T. A. M., Morais, M. H. F., Oliveira, E., Reis, I. A., & Carneiro, M. (2018). Effectiveness of the Brazilian Visceral Leishmaniasis Surveillance and Control Programme in reducing the prevalence and incidence of Leishmania infantum infection. *Parasites & Vectors*, *11*(1), 586. https://doi.org/10.1186/s13071-018-3166-0
226. Cabaniel, G., Rada, L., Blanco, J., Rodriguez-Morales, A., & Escalera Antezana, J. P. (2005). Impacto de Los Eventos de El Niño Southern Oscillation (ENSO) sobre la Leishmaniosis Cutánea en Sucre, Venezuela, a través del Uso de Información Satelital, 1994-2003. *Revista Peruana de Medicina Experimental y Salud Publica*, *22*, 32-37.
227. Ghatee, M. A., Fakhar, M., Derakhshani-Niya, M., Behrouzi, Z., & Hosseini Teshnizi, S. (2020). Geo-climatic factors in a newly emerging focus of zoonotic visceral leishmaniasis in rural areas of north-eastern Iran. *Transboundary and Emerging Diseases*, *67*(2), 914-923. https://doi.org/10.1111/tbed.13416

228. Alexander, B., Agudelo, L. A., Navarro, J. F., Ruiz, J. F., Molina, J., Aguilera, G., Klein, A., & Quiñones, M. L. (2009). Relationship between coffee cultivation practices in Colombia and exposure to infection with Leishmania. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *103*(12), 1263-1268. https://doi.org/10.1016/j.trstmh.2009.04.018

229. Suarez, J., Carreño, L., Paniz-Mondolfi, A., Marco-Canosa, F., Freilij, H., Riera, J., Risquez, A., Rodriguez-Morales, A., Hernández-Rincón, E., Socarras, J., Contreras, G., Cabrera, M., Navas, R., Diaz-Quijano, F., Rosales, T., Barbella, R., Villamil gomez, W., Díaz, M., Carrero, Y., & Rodriguez-Morales, A. (2018). *Infectious Diseases, Social, Economic and Political Crises, Anthropogenic Disasters and Beyond: Venezuela 2019 – Implications for Public Health and Travel Medicine*. *1*, 73-93.

230. Sandhya, R., Rakesh, P. S., & Dev, S. (2019). Emergence of visceral leishmaniasis in Kollam district, Kerala, southern India. *International Journal Of Community Medicine And Public Health*, *6*(3), 1350. https://doi.org/10.18203/2394-6040.ijcmph20190639

231. Siriwardana, H. V. Y. D., Karunanayake, P., Goonerathne, L., & Karunaweera, N. D. (2017). Emergence of visceral leishmaniasis in Sri Lanka: A newly established health threat. *Pathogens and Global Health*, *111*(6), 317-326. https://doi.org/10.1080/20477724.2017.1361564

232. Berrozpe, P. E., Lamattina, D., Santini, M. S., Araujo, A. V., Torrusio, S. E., & Salomón, O. D. (2019). Spatiotemporal dynamics of *Lutzomyia longipalpis* and macro-habitat characterization using satellite images in a leishmaniasis-endemic city in Argentina. *Medical and Veterinary Entomology*, *33*(1), 89-98. https://doi.org/10.1111/mve.12334

233. Casaril, A. E., Alonso, D. P., Franco, K. G., Alvarez, M. V. N., Barrios, S. P. G., Fernandes, W. de S., Infran, J. de O. M., Rodrigues, A. C. M., Ribolla, P. E. M., & Oliveira, A. G. de. (2019). Macrogeographic genetic structure of Lutzomyia longipalpis complex populations using Next Generation Sequencing. *PLOS ONE*, *14*(10), e0223277. https://doi.org/10.1371/journal.pone.0223277

234. de Castro Ferreira, E., Pereira, A. A. S., Silveira, M., Margonari, C., Marcon, G. E. B., de Oliveira França, A., Castro, L. S., Bordignon, M. O., Fischer, E., Tomas, W. M., Dorval, M. E. C., & Gontijo, C. M. F. (2017). Leishmania ( V .) braziliensis infecting bats from Pantanal wetland, Brazil: First records for Platyrrhinus lineatus and Artibeus planirostris. *Acta Tropica*, *172*, 217-222. https://doi.org/10.1016/j.actatropica.2017.05.012

235. Šlapeta, J., Saverimuttu, S., Vogelnest, L., Sangster, C., Hulst, F., Rose, K., Thompson, P., & Whittington, R. (2017). Deep-sequencing to resolve complex diversity of apicomplexan parasites in platypuses and echidnas: Proof of principle for wildlife disease investigation. *Infection, Genetics and Evolution*, *55*, 218-227. https://doi.org/10.1016/j.meegid.2017.09.007

Universidad del Rosario

CIMBIUR
CENTRO DE INVESTIGACIONES EN MICROBIOLOGÍA Y BIOTECNOLOGÍA DE LA UNIVERSIDAD DEL ROSARIO

VNiVERSiDAD Đ SALAMANCA

Cietus
Enfermedades Tropicales
UNIVERSIDAD de SALAMANCA

# ANÁLISIS EPIDEMIOLÓGICO, GENÓMICO Y PROTEÓMICO DE *Leishmania infantum* EN COLOMBIA