Genómica y transcriptómica comparativa en cepas de *Leishmania* de Colombia

Luz Helena Patiño Blanco

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Estudiante

Luz Helena Patiño Blanco

Bacterióloga, Universidad Colegio Mayor de Cundinamarca

Magister en Microbiología, Universidad Nacional de Colombia

Director

Juan David Ramírez González. PhD

Director Doctorado en Ciencias Biomédicas y Biológicas

Director Grupo de Investigaciones Microbiológicas-UR (GIMUR)

Facultad de Ciencias Naturales y Matemáticas. Universidad del Rosario

Co-Director

Paula Ximena Pavia Velandia. Bac, MSc. PhD

Hospital Militar Central (HOMIC)

DOCTORADO EN CIENCIAS BIOMÉDICAS Y BIOLÓGICAS

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1. **Patino LH,** Mendez C, Rodriguez O, Romero Y, Velandia D, Alvarado M, Pérez J, Duque MC, Ramírez JD. Spatial distribution, Leishmania species and clinical traits of Cutaneous Leishmaniasis cases in the Colombian army. PLoS Negl Trop Dis. 2017 Aug 29;11(8):e0005876. doi: 10.1371/journal.pntd.0005876. eCollection 2017 Aug.

2. Herrera G, Higuera A, **Patiño LH**, Ayala MS, Ramírez JD. Description of Leishmania species among dogs and humans in Colombian Visceral Leishmaniasis outbreaks. Infect Genet Evol. 2018 Oct; 64:135-138. doi: 10.1016/j.meegid.2018.06.023. Epub 2018 Jun 21.

3.2 Capítulo 2: Diferencias genómicas y transcriptómicas de cepas de referencia de *Leishmania (Viannia) panamensis* y *Leishmania (Viannia) braziliensis,* sensibles y con resistencia inducida al antimoniato de N-metil glucamina (Glucantime®).

1. **Patino LH**, Ramírez JD. RNA-seq in kinetoplastids: A powerful tool for the understanding of the biology and host-pathogen interactions. Infect Genet Evol. 2017 Apr;49:273-282

2. **Patino LH**, Imamura H, Cruz-Saavedra L, Pavia P, Muskus C, Méndez C, Dujardin JC, Ramírez JD. Major changes in chromosomal somy, gene expression and gene dosage driven by Sb^{III} in *Leishmania braziliensis* and *Leishmania panamensis*. Sci Rep. 2019 Jul 1;9(1):9485. doi: 10.1038/s41598-019-45538-9.

3. **Patino LH,** Muskus C, Ramírez JD. Transcriptional responses of *Leishmania (Leishmania) amazonensis* in the presence of trivalent sodium stibogluconate. Parasit Vectors. 2019 Jul 12;12(1):348. doi: 10.1186/s13071-019-3603-8.

4. **Artículo sometido en la revista Acta Trópica**

   Luz H. Patino, Carlos Muskus, Marina Muñoz, Juan David Ramírez. Comparative genomics reveals moderate levels of ploidy, high heterozygosity and structural variations in *Leishmania (Leishmania) amazonensis.*

3.3 Capítulo 3: Descripción de la arquitectura genómica intra-específica de aislamientos clínicos de *Leishmania (Viannia) panamensis* y *Leishmania (Viannia) braziliensis*

Artículo en preparación

Luz H. Patino, Marina Muñoz, Hideo Imamura, Carlos Muskus, Claudia Mendez, Paula Pavia, Juan David Ramírez. Variación intra-especie en *Leishmania (Viannia) braziliensis* y Leishmania (*Viannia panamensis*) aisladas de pacientes con Leishmaniais Cutánea en Colombia.

4. LISTA DE ABREVIATURAS

Abreviatura	Término
LCL	Leishmaniasis Cutánea Localizada
LC-Difusa	Leishmaniasis Cutánea Difusa
LC- Diseminada	Leishmaniasis Cutánea Diseminada
LC-Atípica	Leishmaniasis Cutánea Atípica
LM	Leishmaniasis Mucosa
LV	Leishmaniasis Visceral
Sb^{III}	Antimoniato Trivalente
PCR	Siglas en inglés de Reacción en cadena de la polimerasa, de: Polymerase Chain Reaction.
ADN	Acído Desoxirribonucléico
SNPs	Polimorfismos de nucleótido simple
VNC	Variación en el número de copias
AQP1	Aquagliceroporina-1
INDELS	Inserciones/deleciones
Cytb	Citocromo b
HSP-70	Proteína de choque térmico 70
γGCS	Gamma-glutamylcysteine synthetase
MRPA	multidrug resistance protein A
NGS	secuenciación de última generación

5. RESUMEN

La Leishmaniasis es una enfermedad tropical ocasionada por parásitos protozoarios del género *Leishmania*. Actualmente, en Colombia esta enfermedad representa un grave problema de salud pública, debido no solo al elevado número de casos reportados anualmente y a la cantidad de especies encontradas, sino también a la aparición de cepas resistentes, principalmente frente a los medicamentos de primera línea: antimoniales (antimoniato N-metil glucamina (Glucantime®)), incrementando así el número de casos asociados a falla terapéutica. Aunque es bien conocido que dicha falla terapéutica es un proceso multifactorial, identificar los mecanismos utilizados por los parásitos para contrarrestar el efecto de los medicamentos, así como evaluar el comportamiento genómico intra-específico, permitirían a futuro generar alternativas conjuntas que promuevan una adecuada respuesta terapéutica.

Hasta el momento, varios estudios utilizando técnicas de secuenciación de última generación (NGS) y basados en herramientas como las "omicas" (genómica, transcriptómica y proteómica) han permitido la identificación de blancos terapéuticos y han proporcionado información crucial con respecto a los mecanismos de resistencia y adaptación utilizados por diferentes especies de *Leishmania* frente a los antimoniales. Sin embargo, este conocimiento en especies de *Leishmania* del nuevo mundo son escasos, así como estudios que permitan describir la arquitectura genómica intra-especifica en estas especies. Teniendo en cuenta lo anterior, este estudio evaluó el comportamiento genómico y transcriptómico de las principales especies de *Leishmania* que circulan en el territorio colombiano, bajo la presión del antimoniato N-metil glucamina (Glucantime® (Sb^{III}), así como la arquitectura genómica en estas especies, obtenidas a partir de diferentes aislamientos clínicos.

Este estudio inicia con la identificación de las principales especies de *Leishmania* circulantes en población militar colombiana. Análisis moleculares utilizando como blanco genes codificantes para las proteínas citocromo b (Cytb) y choque térmico de 70 KDa (HSP-70), permitieron identificar que *Leishmania (Viannia) braziliensis* y *Leishmania (Viannia) panamensis* son las especies con mayor frecuencia de aparición y sugerir la existencia de variabilidad genética intra-específica. Identificadas las especies blanco de estudio, el siguiente paso fue realizar el análisis genómico (ADN-seq) y transcriptómico (ARN-seq) de estas especies, cuando son sometidas (*in vitro*) a presión frente al antimonial trivalente (Sb^{III}) y finalmente, mediante ADN-seq describir la arquitectura genómica de estas especies aisladas a partir de diferentes aislamientos clínicos.

Los análisis genómicos y transcriptómicos permitieron determinar que *Leishmania (Viannia) braziliensis* y *Leishmania (Viannia) panamensis* usan la plasticidad de su genoma para regular la dosis genética y además estimulan la expresión de ciertos genes como mecanismo de adaptación a condiciones de estrés (Sb^{III}). El estudio permitió identificar diferencias en la somía, variación en el número de copias en algunos genes relacionados con la resistencia a los antimoniales y variaciones a gran escala (deleciones y duplicaciones) en cromosomas sin cambio en la somía, así como la expresión de ciertos genes involucrados no solo en la resistencia sino también en la virulencia y procesos biológicos utilizados por el parásito.

Finalmente, se evaluó la arquitectura genómica intra-especie, utilizando para este caso aislamientos clínicos proveniente de pacientes con Leishmaniasis cutánea ocasionada por *Leishmania (Viannia) braziliensis* o *Leishmania (Viannia) panamensis*. Los resultados obtenidos permitieron determinar poca variabilidad genética intra-específica en términos de somía, variación en el número de copias en genes locales y polimorfismos de nucleótido simple (SNPs), sin embargo durante dicho análisis dos aislamientos clínicos llamaron la atención. Uno de ellos proveniente de un paciente infectado con *Leishmania (Viannia) braziliensis* el cual presentó un extraño comportamiento en el número de copias cromosomales, sugiriendo un posible evento de recombinación, resultado que deberá ser confirmado con estudios adicionales y un aislamiento clínico infectado con *Leishmania (Viannia) panamensis,* el cual presentó un elevado número de SNPs, la mayoría de los cuales se presentaron en bloque a lo largo del cromosoma 20.

Los resultados producto de esta tesis permitieron describir que **(i)** la distribución de las especies de *Leishmania* a nivel urbano/selvático es diferente, **(ii)** los cambios genómicos y transcriptómicos en respuesta a los antimoniales trivalentes observados en especies de *Leishmania* del viejo mundo, es una característica compartida también es especies del nuevo mundo y finalmente **(iii)** describir la baja variación estructural encontrada en *Leishmania (Viannia) braziliensis* y *Leishmania (Viannia) panamensis,* lo cual podría estar asociada a un posible evento de adaptación de estas especies al hospedero humano. A pesar de los resultados obtenidos, consideramos que estudios en los cuales se evalúe el genoma y el transcriptoma durante la interacción hospedero-parásito (macrófago/amastigote y vector/promastigote) bajo el efecto del antimonial trivalente, son necesarios para seguir ampliando el conocimiento acerca de los mecanismos de adaptación que caracterizan a *Leishmania.*

Finalmente, es importante mencionar que este es el primer estudio realizado en Colombia, en donde utilizando ADN-seq y ARN-seq, se evalúa el comportamiento de especies de *Leishmania* del nuevo mundo bajo condiciones de estrés (Sb^{III}).

6. ESTADO DEL CONOCIMIENTO

6.1 Leishmaniasis

La Leishmaniasis constituye un espectro de enfermedades causadas por diferentes especies del protozoo flagelado *Leishmania*, este microorganismo es considerado un parásito intracelular obligado del humano y otros mamíferos, el cual produce lesiones a nivel visceral, mucoso y cutáneo [1, 2]. La enfermedad es una zoonosis que afecta humanos y a otras especies de mamíferos entre ellos los caninos, que son los principales reservorios domésticos de esta parasitosis [3]. Sin embargo, animales silvestres tales como liebres, zarigüeyas, coatíes y jurumíes, también actúan como reservorios del parásito [4].

6.2 Manifestaciones clínicas

Actualmente existen alrededor de 20 especies, las cuales pueden causar diferentes manifestaciones clínicas en el humano, dentro de las que se incluyen: Leishmaniasis Visceral (LV), Leishmaniasis Mucosa (LM), Leishmaniasis Cutánea Localizada (LCL), Leishmaniasis Cutánea Difusa (LC-Difusa), Leishmaniasis Cutánea Diseminada (LC-Diseminada) y Leishmaniasis Cutánea atípica (LC-Atípica) [5-7]

6.2.1 Leishmaniasis Visceral.

También conocida como Kala-zar, es la forma más severa de la enfermedad y se caracteriza por presentar diferentes manifestaciones clínicas como fiebre prolongada, debilidad, anorexia, pérdida de peso, hepatomegalia, esplenomegalia, hipergamaglobulinemia y pancitopenia. Sin el tratamiento adecuado la enfermedad puede progresar y presentar enfermedades sistémicas, sangrado, infecciones secundarias e incluso la muerte [1, 8]. La LV es causada principalmente por especies pertenecientes al subgénero *Leishmania (Leishmania)*, incluyendo: *Leishmania donovani* y *Leishmania infantum/chagasi* [9-12].

6.2.2 Leishmaniasis Mucosa.

Se caracteriza por producir lesiones en mucosa nasal, faringe, laringe, paladar o labio. Al examen físico se puede encontrar eritema y edema; y en estados más avanzados ulceración, perforación, destrucción de tabique y mutilaciones [1]. Generalmente, esta enfermedad ocurre meses o años después de que las lesiones cutáneas (LC) se han resuelto, aunque en algunos casos la lesión en piel y mucosa coinciden [13].

Aproximadamente el 90% de los pacientes con LM han presentado previamente LC, aunque en algunas ocasiones la lesión en la mucosa se presenta en pacientes sin previa lesión cutánea [14-16]. Cerca del 90% de los casos de LM se producen en Bolivia, Brasil y Perú y se asocia con mayor frecuencia a especies como *Leishmania (Viannia) braziliensis, Leishmania (Viannia) guyanensis* y *Leishmania (Leishmania) amazonensis*, aunque otras especies como *Leishmania infantum* pueden estar involucradas [12, 15, 17-19].

6.2.3 Leishmaniasis Cutánea.

Se caracteriza por lesiones cuyo espectro va desde pápulas, placas, ulceras y nódulos localizadas en cualquier área de la superficie corporal. Esta manifestación clínica se puede presentar como una Leishmaniasis Cutánea Localizada (LCL), Leishmaniasis Cutánea Difusa (LC-Difusa), Leishmaniasis Cutánea Diseminada (LC-Diseminada) o Leishmaniasis cutánea atípica (LC-Atípica) [6].

La Leishmaniasis Cutánea Localizada se caracteriza por la presencia de lesiones ulcerativas generalmente en el sitio de la picadura, frecuentemente indoloras redondeadas, con bordes elevados, eritematosos, con compromiso linfangítico y adenopatías regionales [7] (Figura 1A).

La Leishmaniasis Cutánea Difusa se caracteriza por la presencia de múltiples lesiones nodulares no ulcerativas que lenta pero irremediablemente se extienden para cubrir todo el cuerpo, con la excepción del cuero cabelludo, axilas, pliegue inguinal, palmas de las manos y plantas de los pies [6] (Figura 1B).

Para el caso de la Leishmaniasis Cutánea Diseminada esta se caracteriza por la presencia de múltiples lesiones cutáneas papulares (a veces más de 700) en diferentes regiones anatómicas, tales como la superficie del cuerpo, la cara, las extremidades y el tronco, las cuales pueden ir acompañadas de afectación de la mucosa y demuestran una tendencia a la cronicidad y la recaída. A diferencia de la LC-Difusa, en esta manifestación clínica se pueden observar lesiones ulcerativas necrotizantes y compromiso de la mucosa [1, 6] (Figura 1C).

Por otra parte, la LC-Atípica ha sido reportada en países de Centroamérica (Nicaragua, Honduras, Costa Ricas y El Salvador) y en América del sur en Venezuela; esta forma clínica se manifiesta por producir lesiones circunscritas no ulceradas y de evolución crónica [20] (Figura 1D).

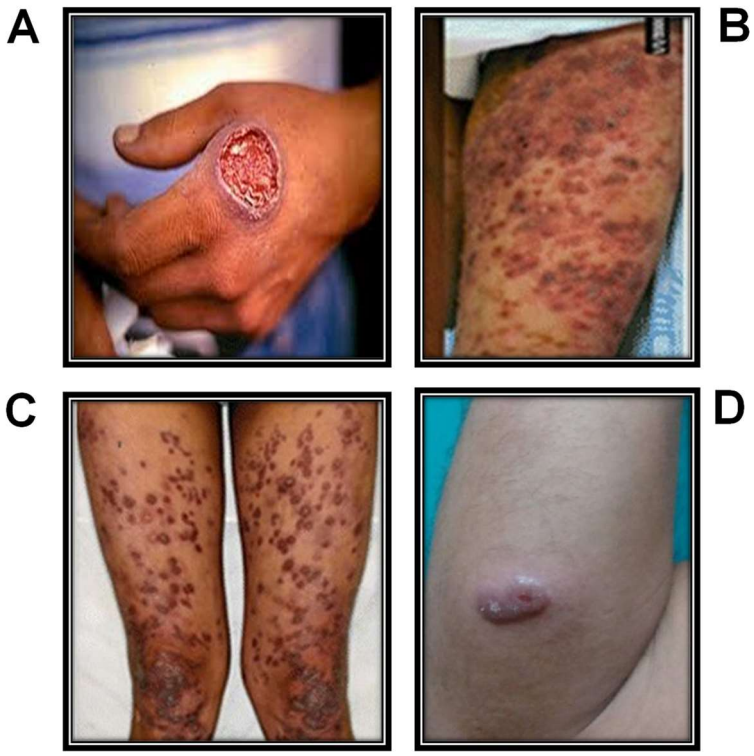

Figura 1. Formas de presentación clínica de la Leishmaniasis Cutánea**.** A: Leishmaniasis Cutánea Localizada. B: Leishmaniasis Cutánea Difusa. C: Leishmaniasis Cutánea Diseminada. D: Leishmaniasis Cutánea Atípica.

Estas manifestaciones clínicas se asocian a una variedad de especies de *Leishmania.* Dentro de las especies del viejo mundo, implicadas en la patología, se describen: *Leishmania major, Leishmania tropica, Leishmania aethiopica* y *Leishmania infantum* [21, 22] y dentro de las especies del nuevo mundo se han descrito: *Leishmania (Viannia) panamensis* (54-80% casos), seguido por *Leishmania (Viannia) braziliensis* (10-30% de los casos), y otras especies menos frecuentes *Leishmania (Viannia) guyanensis* (1-3%), *Leishmania amazonensis* (2-8%) y *Leishmania mexicana* (1-5%) [22-26].

6.3 Epidemiología

La Leishmaniasis es considerada una enfermedad infecciosa desatendida, asociada a la pobreza y la marginalidad según la Organización Mundial de la Salud (OMS). Esta enfermedad se encuentra distribuida en todos los continentes, siendo endémicas en 98 países, especialmente en el norte de África, Asia, el Mediterráneo y América Latina [5, 11, 12, 22, 27, 28]. En las Américas, la Leishmaniasis representa un problema en salud pública debido a su alta morbilidad. Datos epidemiológicos describen que las LC y LM son endémicas en 18 países de la región, las cuales se distribuyen desde México hasta

Argentina (a excepción de Chile y Uruguay) [29] y aproximadamente el 96% de los casos de LV, se presentan principalmente en el norte de Brasil [27, 28, 30] (Figura 2).

Se describe que Colombia, ocupa el segundo lugar en incidencia de Leishmaniasis en América. Esta enfermedad se encuentra distribuida en todo el territorio Nacional excepto en San Andrés Islas y Bogotá [31]. Alrededor del 98.5% de todos los casos de Leishmaniasis corresponden a LC, el 1.27% a LM y el 0.22% a LV [32]. Colombia no solo es uno de los países de América Latina endémicos para la enfermedad, sino también es el país que presenta el mayor número de especies de *Leishmania* que afectan a los seres humanos (Diez en total) [24].

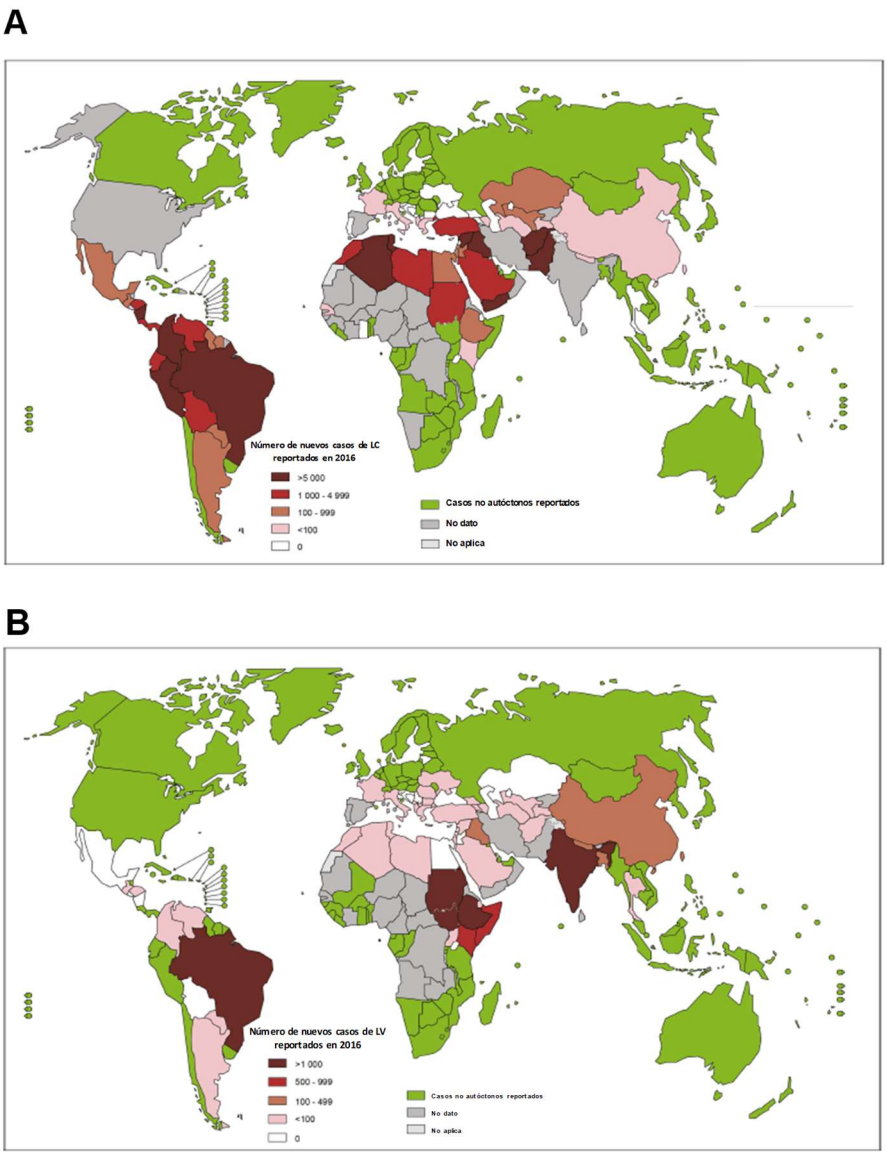

Figura 2. Epidemiología de la Leishmaniasis Cutánea (A) y Leishmaniasis Visceral (B) alrededor del mundo. 2016 https://www.who.int/leishmaniasis/burden/en/.

6.4 Taxonomía

Aproximadamente 53 especies de *Leishmania* han sido descritas, las cuales se han agrupado en cinco subgéneros: *Leishmania (Sauroleishmania), Leishmania (Leishmania)* y *Leishmania (Viannia), Leishmania (Paraleishmania)* y *Leishmania (Mundinia)* [33]. De estas especies, 31 son parásitos de mamíferos y al menos 23 son consideradas patógenas para el humano [34-36] (Figura 3).

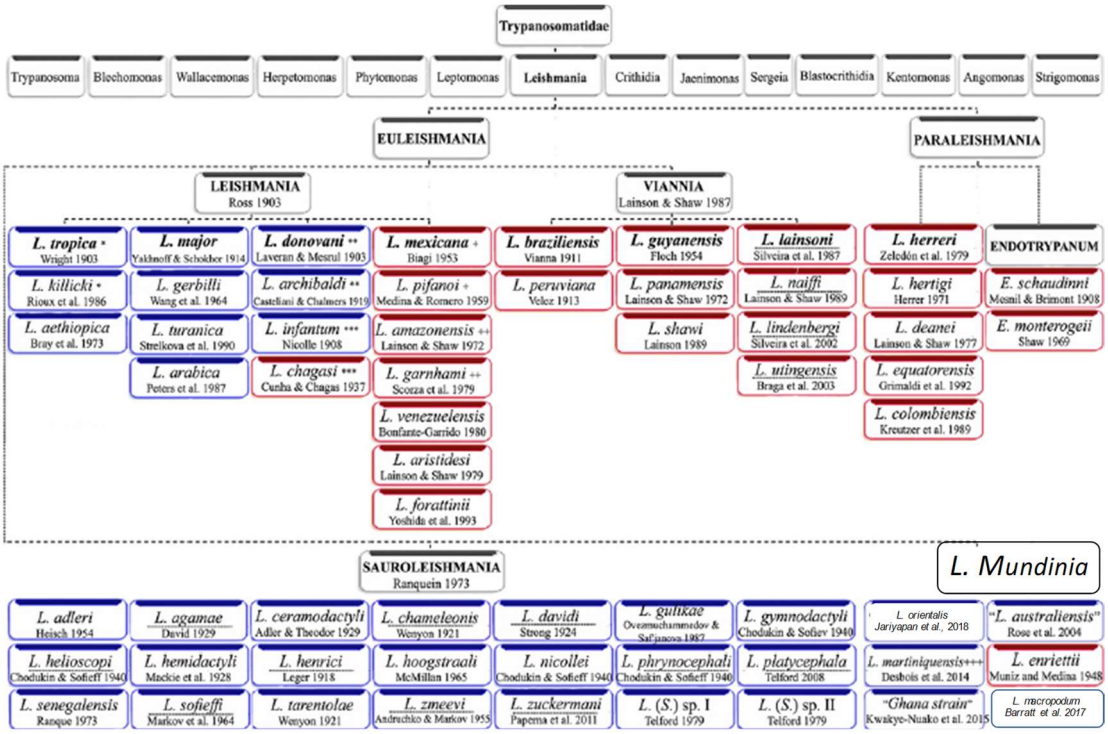

Figura 3. Clasificación taxonómica de las especies de *Leishmania* [33, 34].

6.5 Diagnóstico

Los métodos que se utilizan para realizar el diagnóstico de Leishmaniasis son diversos y su utilidad depende por lo general de la forma clínica. Para el caso de la LC, las pruebas de laboratorio rutinariamente utilizadas son los métodos parasitológicos (examen directo), biopsia de piel (para estudio histopatológico) y cultivo en medio NNN (Novy–MacNeal–Nicolle) o Schneider, cuyo objetivo es el aislamiento e identificación del parásito [12, 16, 37].

Adicionalmente, se han implementado una serie de pruebas que no solo permiten identificar al parásito sino también determinar la especie de *Leishmania* asociada. Hasta el momento, la técnica considerada como "*estándar de oro*" para la caracterización y discriminación de las especies de *Leishmania,* ha sido la electroforesis enzimática multilocus ó MLEE [38, 39]. Sin embargo, diferentes técnicas basadas en Reacción en

cadena de la polimerasa: PCR o la Reacción en cadena de la polimerasa-Polimorfismos de longitud de fragmentos de restricción: PCR-RFLP, han permitido dicha caracterización [37, 40, 41]. Por otra parte, teniendo en cuenta la variabilidad genética inter e intra especie observada en algunas especies de *Leishmania* y considerando el bajo poder de discriminación que tienen algunas de estas pruebas moleculares, las técnicas de secuenciación de última generación en donde se logra analizar el genoma completo del parásito, se han convertido en la herramienta más efectiva para cumplir dicho propósito [42, 43].

6.6 Tratamiento

Debido a la ausencia de una vacuna eficaz para controlar esta parasitosis, el tratamiento farmacológico se convierte en la principal herramienta para combatirla. Aunque hasta el momento, existen cerca de 25 compuestos que muestran efecto anti-leishmanial, sólo unos pocos de ellos son usados en humanos [44].

6.6.1 Antimoniales pentavalentes (Sb^{V})

Los antimoniales pentavalentes han sido considerados, desde hace más de 70 años como el tratamiento de primera elección para todas las formas de Leishmaniasis en América del sur, el norte de África, Turquía, Bangladesh y Nepal [45]. Estos medicamentos se encuentran actualmente disponibles en dos formulaciones: estibogluconato de Sodio o también denominado Pentostam® y antimoniato de N-metil glucamina (Glucantime®), los cuales se consideran equivalentes en términos de eficacia clínica, efectos secundarios, farmacocinética y mecanismos de acción [46]. Según la OMS la dosificación recomendada es 20/mg/kg/día por un periodo de administración de 20 días para LC y 28 días para LM y LV [47-49].

Una de las características de estos medicamentos, es que actúan como una pro-droga, lo cual significa que la forma pentavalente requiere ser reducida a su forma activa trivalente (Sb^{V} a Sb^{III}) [50]. Se ha descrito que estas inducen indirectamente el estrés oxidativo y nitrosativo en amastigotes y promastigotes y a su vez estimula a los macrófagos infectados para que generen especies reactivas de oxígeno (ROS) y óxido nítrico (NO), lo cual altera el balance redox del parásito llevándolo a la muerte [47, 51]. Hasta el momento, el mecanismo de acción de estos antimoniales no se conoce por completo, sin embargo, se ha observado que bajo la actividad del medicamento, hay modificaciones en algunas vías metabólicas como la glicólisis, la oxidación de ácidos grasos y la formación de ATP. Adicionalmente, estudios sugieren que el Sb^{III} causa

alteraciones en el potencial redox tripanotion y glutatión, por la liberación de los tioles intracelulares y por la inhibición de la enzima tripanotion reductasa (TryR) [47, 52, 53]. De igual manera, se ha observado que el antimonial destruye al parásito por un proceso que involucra la fragmentación del ADN y la externalización de la fosfatidilserina sobre la superficie de la membrana externa [54, 55]. Así mismo, se ha demostrado que este medicamento puede unirse al modelo péptido dedos de zinc CCHC, promoviendo la liberación del Zn (II), el cual está involucrado en la estructura, reparación y replicación del ADN [56-58] (Figura 4).

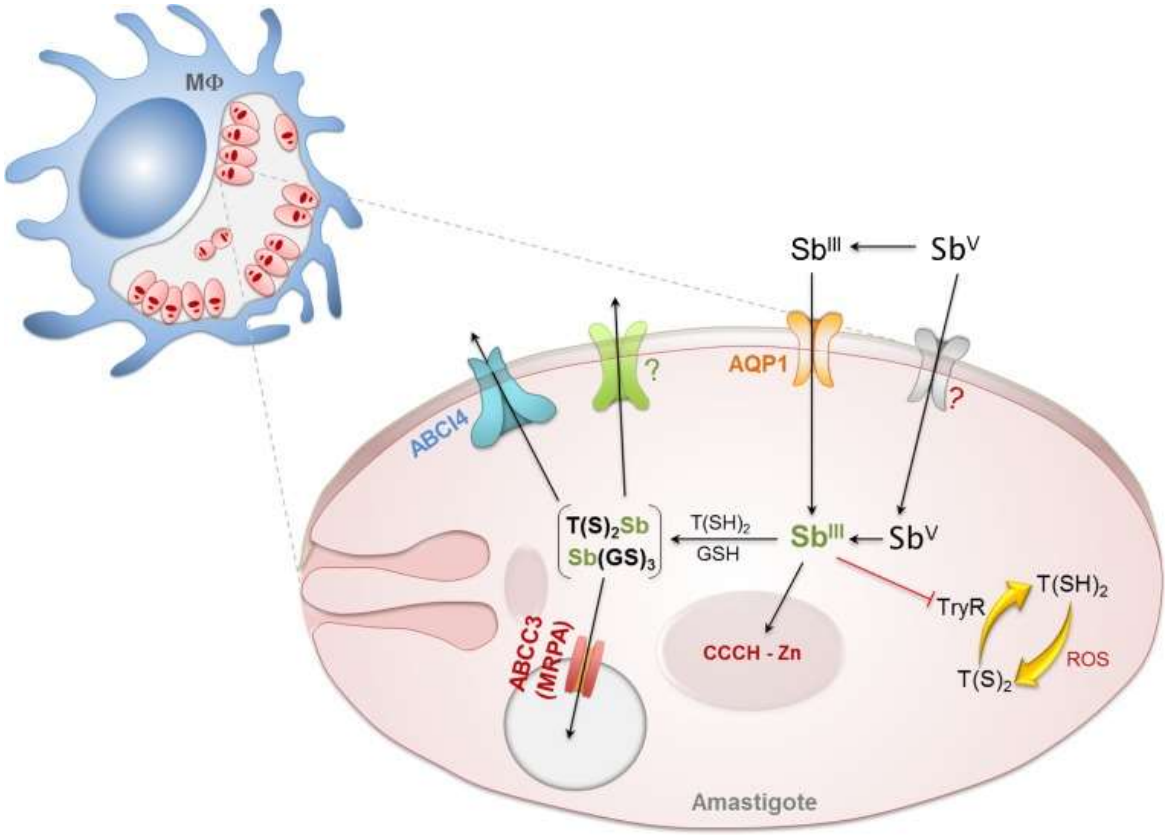

Figura 4. Mecanismo de acción de los antimoniales pentavalentes [59].

6.6.2 Anfotericina B

También conocida comercialmente como Fungizone®, ha sido usada en algunos países, como el fármaco de segunda línea, para el tratamiento de Leishmaniasis [60]. Es un antimicótico poliénico y fungicida de amplio espectro. La posología es de 1 mg/kg/día [61]. Debido a su gran afinidad por el Ergosterol es selectivo frente a hongos y parásitos tales como *Leishmania* y *Trypanosoma* [60]. Al igual que los antimoniales, presenta diferentes efectos secundarios y ha sido asociado con miocarditis y nefrotoxicidad [61]. Con el objetivo de reducir los efectos secundarios y mejorar la biodisponibilidad y

farmacocinética se ha generado la formulación liposomal (AmBisome®), esta ha sido considerada como el mejor fármaco para el tratamiento de la LV y es usada como el tratamiento de primera línea en Europa y Estados Unidos [61-63]. A pesar de ser menos tóxica, esta formulación tiene ciertas limitaciones para ser usada en países sub-desarrollados debido a su alto costo y su inestabilidad a temperaturas altas [63].

6.6.3 Pentamidina

Fue usada en los años 80s, para el tratamiento de la LV en India sin embargo, su uso fue descontinuado debido a su toxicidad y baja eficacia [64]. Su mecanismo de acción implica la inhibición en la síntesis del ADN, afecta la morfología del kinetoplasto y el potencial de membrana mitocondrial [65, 66]. Produce importantes efectos adversos tales como dolor, induración en el sitio de la inyección, náuseas, vómito, mialgia, dolor de cabeza hipotensión, hiper e hipoglicemia [61, 66, 67]. Este medicamento no es frecuentemente utilizado debido a la rápida aparición de cepas resistentes. Generalmente es recomendada cuando se usa combinada con otros protocolos terapéuticos [66, 68].

6.6.4 Miltefosina

La miltefosina (hexadecilfosfocolina) es un medicamento inicialmente desarrollado para el tratamiento del cáncer y en el año 2005 aprobado en Colombia para el tratamiento de la LC en niños y en adultos [69], sigue siendo en la actualidad el único medicamento oral eficaz para tratar todos los tipos de Leishmaniasis. Este medicamento fue introducido como primera línea para el tratamiento de la LV en India [70] y ha sido aprobado por la FDA (Food Drug Administration) para tratar infecciones ocasionadas por *Leishmania (Viannia) braziliensis*, *L. (Viannia) panamensis* y *L.(Viannia) guyanensis* [71], en el caso de infecciones por especies del viejo mundo su uso aún no ha sido aprobado [72]. A pesar de que la miltefosina ha demostrado una eficacia comparable a los antimoniales pentavalentes [73], presenta ciertas desventajas tales como su elevado costo, su larga vida media y la presencia de cepas resistentes, lo que hacen que su uso sea limitado [61, 69, 74, 75].

6.7 Falla terapéutica frente a los antimoniales

Actualmente, la falla terapéutica frente a los antimoniales pentavalentes ha alcanzado proporciones epidémicas en algunas regiones del mundo como Bihar (India), en donde entre el 35 al 65% de los pacientes con LV, no responden al tratamiento con Sb^V [76], en Irán por ejemplo, se ha observado un porcentaje de resistencia cercano al 11% [77] y existen reportes que demuestran falla terapéutica en varios países de América Latina,

como Bolivia con un porcentaje de falla terapéutica cercano al 7% [78], Brasil con un reporte de falla entre el 25-50% [79-81], Perú con un porcentaje cercano al 24% [82-84] y Colombia con cifras de falla terapéutica entre el 15-39% [69, 85-87].

Los mecanismos de resistencia frente a los antimoniales han sido ampliamente estudiados y han sido considerados multifactoriales. Se describe que la pérdida de la susceptibilidad es asociada a factores inherentes al hospedero (edad, sistema inmune, farmacocinética, farmacogenética y adherencia al tratamiento), factores propios al medicamento (formulación y farmacodinámica) y características propias del parásito (diferencias bioquímicas y moleculares intra e inter especies que hacen que estos organismos generen resistencia frente al mismo) [69]. Con respecto a este último, diferentes estudios han demostrado que la resistencia es debida a la propiedad que tiene el parásito para: **I**. Disminuir el ingreso del medicamento al interior de este, **II.** Aumentar la salida del medicamento al espacio extracelular, **III.** Inducir cambios cualitativos y cuantitativos en el blanco del medicamento o **IV.** Mediante la combinación de dichos factores [56]. Estos mecanismos de resistencia están principalmente asociados a variación en el número de copias de ADN o variación en el número de copias (VNC) tanto en cromosomas completos como en genes locales, amplificación o deleción génica, polimorfismos en un solo nucleótido o SNPs (Por su sigla en inglés: Single-Nucleotide Polymorphisms), inserciones o deleciones [88], así como alteraciones en la expresión génica y proteica que sufren estos agentes infecciosos. Por lo tanto, se han adelantado algunos estudios basados en genómica, transcriptómica y proteómica no solo para entender los procesos de resistencia en algunas especies de *Leishmania* [45, 89-93], sino también para conocer un poco más acerca de la biología del parásito así como su variabilidad inter e intraespecífica y su posible relación con los mecanismos de susceptibilidad [42, 43, 92, 94, 95].

6.8 “Omicas” en el estudio de la variabilidad genética en *Leishmania*

Hasta el momento, varios métodos moleculares han sido utilizados para identificar poblaciones en diferentes especies de *Leishmania* (secuenciación de loci ribosomales, MLST, DNA-RFLP, kDNA, entre otros) [96-99], sin embargo y a pesar de que estos métodos son capaces de discriminar genotipos, son poco efectivos en el momento de evaluar variaciones inter e intra especie en estos parásitos, debido al alto grado de conservación en el contenido y arquitectura de los genes [100] y a la aparición de poblaciones parasitarias que evolucionan rápidamente, las cuales son relativamente homogéneas.

Actualmente, varios estudios basados en el análisis del genoma completo (ADN-seq) han permitido documentar esta diversidad genética a una escala mucho más fina. Estos estudios han proporcionado información útil acerca de la estructura poblacional, las diferencias genéticas observadas en parásitos circulantes en la misma y en distinta zona geográfica [42, 101], la identificación de genes involucrados en diversos procesos biológicos [92, 101, 102] así como la identificación de variantes genéticas (somia cromosomal, dosis genéticas, SNPs, Indels) en aislamientos clínicos de la misma especie (*L. donovani, L. major, L. braziliensis, L. panamensis*) [42, 43, 92, 95], en especies relacionadas al mismo complejo (*L. braziliensis* y *L. peruviana*) [94] y entre parásitos pertenecientes a diferente especie [102]. Describiendo de esta manera la estrecha relación existente entre la variabilidad genética con los diferentes mecanismos de patogenicidad, virulencia, tropismo, severidad de la enfermedad, así como la variación frente a la respuesta terapéutica [42, 95].

6.9 "Omicas" en el entendimiento de la resistencia frente a los antimoniales

Genómica y transcriptómica en el estudio de la resistencia frente a los antimoniales**.**

Los parásitos del género *Leishmania* se caracterizan, entre otras cosas, por su manera particular de modular la expresión genética. En ausencia de regulación transcripcional y bajo condiciones de estrés ambiental (presión a medicamentos) estos parásitos modulan la dosis de sus genes alterando los niveles de ARN mensajero. Uno de los mecanismos utilizados para lograr dicho propósito, es produciendo un cambio en la dosis genética ya sea a través de duplicación, deleción génica y/o cambios en la somia. Así mismo se ha observado que el parásito puede generar polimorfismos de nucleótido simple (SNPs) en blancos terapéuticos o transportadores, lo cual favorece su adaptación sin necesidad de alterar la expresión de genes [93, 103].

En los últimos años, la secuenciación de última generación (NGS), utilizando como herramientas las "omicas" (genómica/transcriptómica) ha permitido no solo evaluar los mecanismos de adaptación de *Leishmania* frente a los medicamentos, sino también ha contribuido en la identificación de marcadores asociados a resistencia [104]. Estas técnicas han revelado que las alteraciones genómicas, transcriptómicas y la plasticidad que caracteriza a este parásito, se correlacionan con la resistencia a los antimoniales [92]

Hasta el momento, un gran número de estudios comparativos entre líneas sensibles y resistentes a los antimoniales han sido llevado a cabo utilizando esta tecnología de secuenciación de última generación; secuenciación de ADN (ADN-seq) y

secuenciación de ARN (ARN-seq), tanto en especies de *Leishmania* del viejo mundo (*L. donovani, L. tropica, L major* y *L. infantum*) [28, 89, 93, 105], así como del nuevo mundo (*L. guyanensis*) [106]. Los resultados obtenidos en estos estudios han revelado una plétora de mecanismos de adaptación usados por estos parásitos para sobrevivir o contrarrestar estas condiciones de estrés. Uno de los principales mecanismos es modulando la dosis genética a través de la VNC, la cual involucra cromosomas completos o regiones genómicas específicas ya sea a través de elementos intra o extracromosomales [88, 102, 104, 107].

Entre los estudios que confirman dicho mecanismo de adaptación frente al antimonial trivalente (Sb^{III}), se resaltan aquellos llevados a cabo por Brotherton *et al*., en donde analizando el genoma completo de cepas mutantes de *L. infantum* revela aneuplodia en 8 cromosomas [89], o el estudio realizado por Rastrojo *et al*., en el cual observa cambios en 3 de los 36 cromosomas de *L. donovani* [93], por su parte Mukherjee *et al*., no solo describe cambios de somia en cepas mutantes de *L. major*, sino también identifica una deleción terminal en el cromosoma 31, lo cual promueve tanto la disminución en el número de copias como la expresión del gen codificante para la Aquagliceroporina 1 (AQP-1), principal ruta de entrada del trivalente en *Leishmania* así como la amplificación intracromosomal de un locus en la región subtelomérica del cromosoma 34, promoviendo de esta manera la expresión de enzimas tales como la Glucosa 6 fosfato deshidrogeasa (G6PDH) y la ascorbato dependiente de la peroxidasa (APX), las cuales promueven la protección del parásito frente a las especies reactivas de oxígeno (ROS) [105]. Otro de los estudios fue realizado por Monte.neto *et al*, en el cual analizando cepas mutantes de *L. guyanensis*, demuestra cambios en la somia, así como amplificaciones subteloméricas en los cromosomas 19 y 23, este último albergando un gen codificante para una proteína transportadora: Proteína A resistente a multidrogas (MRPA) [106]

Otro de los mecanismos de adaptación desarrollados por *Leishmania* bajo la presencia de antimoniales, es generando SNPs y/o deleciones en genes codificantes para proteínas relacionadas con la resistencia a estos medicamentos. Entre ellos se describen los SNPs identificados en genes codificantes para proteín kinasas presentes en cepas de *L. infantum* [89], la inserción de 2 pares de bases identificadas en el gen codificante para la AQP1 en *L. donovani* [103] y la deleción terminal de aproximadamente 20 kb de siete genes en el cromosoma 31 incluyendo genes codificantes para la AQP1 en cepas mutantes de *L. guyanensis* [106]

Finalmente, se describe que la sobre-expresión de ciertos genes también es un mecanismo que puede favorecer la adaptación frente a los antimoniales. Uno de los principales estudios que describe el comportamiento transcriptómico en cepas de *L. donovani* resistentes versus cepas sensibles al Sb^{III} es el publicado por Rastrojo *et al*., el cual revela un elevado número de genes expresados diferencialmente entre ambas líneas; principalmente genes codificantes para proteínas transportadoras (ion/zinc, pteridina), Histonas, antígenos de superficie (PSA-2) así como aquellos codificantes para proteínas involucradas en la biosíntesis del tripanotion, tales como la γGCS (*Gamma-glutamylcysteine synthetase*) y la METK2 (*S-adenosylmethionine synthetase*) [93]

7. OBJETIVOS

7.1 Objetivo General

Describir el comportamiento genómico (ADN-seq) y transcriptómico (ARN-seq) de cepas de *Leishmania* en Colombia

7.2 Objetivos Específicos

1. Identificar las especies de *Leishmania*, que circulan en población militar colombiana.

2. Establecer las diferencias genómicas y transcriptómicas de cepas de referencia de *Leishmania (Viannia) panamensis* y *Leishmania (Viannia) braziliensis,* sensibles y con resistencia inducida al antimoniato de N-metil glucamina (Glucantime®).

3. Describir la arquitectura genómica intra específica de aislamientos clínicos de *Leishmania (Viannia) panamensis* y *Leishmania (Viannia) braziliensis.*

8. INTRODUCCIÓN A LOS CAPÍTULOS

La leishmaniasis es un grupo de enfermedades tropicales consideradas un grave problema en salud pública. Actualmente, existen una amplia gama de opciones terapéuticas y medicamentos desarrollados con el objetivo de controlar la enfermedad, sin embargo y a pesar de los esfuerzos realizados hasta el momento, el porcentaje de pacientes que no responden frente a los medicamentos cada vez es mayor.

Frente a este panorama, identificar los mecanismos que llevan a un paciente a presentar dicha falla terapéutica se ha convertido en el reto de muchos investigadores y aunque es bien conocido que la respuesta frente tratamiento es un proceso multifactorial [69, 76], el objetivo de muchos de ellos han sido identificar los mecanismos usados por el parásito para sobrevivir o contrarrestar la acción de medicamentos [104, 108, 109] y de esta manera poder identificar blancos terapéuticos.

Gracias a estas investigaciones y al uso de las técnicas de secuenciación de última generación (NGS), como la genómica: ADN-seq y la transcriptómica: ARN-seq, se ha logrado no solo identificar algunos de estos mecanismos los cuales facilitan la adaptación a los anti-leishmaniales: antimoniato de N-metil glucamina (Glucantime®), tanto en especies de *Leishmania* del viejo mundo (*L. donovani, L. tropica, L major* y *L. infantum*)) [28, 88, 92, 103, 110, 111] como del nuevo mundo (*L. amazonensis L. guyanensis*) [106, 112], sino también determinar que la variabilidad genética inter e intra específica [94] juega un papel muy importante en la respuesta terapéutica [42, 95].

Sin embargo, y a pesar del conocimiento obtenido hasta el momento, poco se conoce acerca de esta variabilidad genética intra-especie y de los mecanismos que conducen a la resistencia frente a los antimoniales, en las especies de *Leishmania* que circulan con mayor frecuencia en nuestro país.

Con base en lo descrito anteriormente y teniendo en cuenta el vacío en el conocimiento, el presente estudio fue diseñado con tres enfoques. El primer enfoque (**Capítulo 1**) permitió identificar las principales especies de *Leishmania* que circulan en población militar colombiana, teniendo conocimiento que esta población es la más vulnerable de adquirir la infección ya que, por sus actividades laborales deben estar continuamente en zonas endémicas para la enfermedad y con alta circulación del insecto vector, así como enriquecer la base de datos que permita seguir ampliando el conocimiento acerca de la distribución de especies de *Leishmania* en el país, el segundo enfoque (**Capítulo 2**) se basó en determinar mediante ADN-seq y ARN-seq la arquitectura genómica y evaluar el

comportamiento transcriptómico de cepas de referencia de las principales especies de *Leishmania* que circulan en Colombia (*Leishmania (Viannia) braziliensis* y *Leishmania (Viannia) panamensis*) bajo presión (*in vitro*) al antimoniato de N-metil glucamina (Glucantime®) en su forma trivalente: Sb^{III}. Adicionalmente en este estudio se evaluaron las características genómicas de *Leishmania (Leishmania) amazonensis*, así como el comportamiento transcriptómico de esta especie bajo la presión al Sb^{III}, y el último enfoque (**Capítulo 3**) permitió describir mediante DNA-seq la arquitectura genómica intra específica de *Leishmania (Viannia) braziliensis* y *Leishmania (Viannia) panamensis,* aisladas de pacientes con LC.

Capítulo 1: Para cumplir con dicho objetivo, se realizó un análisis molecular en 273 biopsias provenientes de militares con LC. Los resultados obtenidos en este análisis permitieron identificar las principales especies que circulan con mayor frecuencia en población militar colombiana, sugerir la existencia de variabilidad genética intra-específica en algunas de estas especies, confirmar que *Leishmania (Viannia) braziliensis* es la especie más frecuente en dicha población [87] y alimentar el mapa de distribución de especies de *Leishmania* en el país.

Por otra parte, y paralelo a este estudio participamos de manera activa en el análisis descriptivo de las especies de *Leishmania* circulantes en perros y humanos provenientes de un brote de LV en Colombia. Los resultados obtenidos nos permitieron determinar que *Leishmania infantum chagasi*, *Leishmania braziliensis* y *Leishmania amazonensis* fueron las especies involucradas en la enfermedad, así mismo identificamos variabilidad genética en una de las especies de *Leishmania infantum chagasi* circulante.

Como producto de este capítulo se adjuntan los siguientes artículos científicos:

1. **Patino LH,** Mendez C, Rodriguez O, Romero Y, Velandia D, Alvarado M, Pérez J, Duque MC, Ramírez JD. Spatial distribution, Leishmania species and clinical traits of Cutaneous Leishmaniasis cases in the Colombian army. PLoS Negl Trop Dis. 2017 Aug 29;11(8):e0005876. doi: 10.1371/journal.pntd.0005876. eCollection 2017 Aug.
2. Herrera G, Higuera A, **Patiño LH**, Ayala MS, Ramírez JD. Description of Leishmania species among dogs and humans in Colombian Visceral Leishmaniasis outbreaks. Infect Genet Evol. 2018 Oct; 64:135-138. doi: 10.1016/j.meegid.2018.06.023. Epub 2018 Jun 21.

Capítulo 2: Una vez se identificaron las especies de mayor circulación en población militar y con base en lo previamente reportado en población civil [24], se evaluó el comportamiento genómico y transcriptómico de estas especies con y sin inducción (*in vitro*) al antimoniato de N-metil glucamina (Glucantime®) en su forma trivalente: Sb^{III}, así como el comportamiento genómico de *Leishmania (Leishmania) amazonensis*, y el

comportamiento transcriptómico de esta especie bajo la presión al Sb^{III}. Los resultados nos permitieron determinar que *Leishmania (Viannia) braziliensis* y *Leishmania (Viannia) panamensis* usan la plasticidad de su genoma para regular la dosis genética (Somia y/o, VNC, SNPs) y la expresión de ciertos genes como mecanismo de adaptación a condiciones de estrés, como ha sido previamente descrito en otras especies de *Leishmania* del viejo y nuevo mundo [28]. Por otra parte, el análisis genómico y transcriptómico realizado en *Leishmania (Leishmania) amazonensis*, nos permitió identificar cambios en términos de ploidia, VNC y SNPs así como el aumento en la expresión de genes codificantes para proteínas involucradas en diferentes procesos biológicos incluidos adhesión, metabolismo, ciclo celular, organización estructural, respuesta al estrés y autofagia.

Adicionalmente, durante el desarrollo de este capítulo se realizó una revisión bibliográfica con el propósito de conocer como la tecnología de secuenciación de ARN (ARN-seq), ha contribuido en el entendimiento de la biología e interacción hospedero-patógeno en los Kinetoplástidos.

Como producto de este capítulo se adjuntan los siguientes artículos científicos:

1. **Patino LH**, Ramírez JD. RNA-seq in kinetoplastids: A powerful tool for the understanding of the biology and host-pathogen interactions. Infect Genet Evol. 2017 Apr;49:273-282

2. **Patino LH**, Imamura H, Cruz-Saavedra L, Pavia P, Muskus C, Méndez C, Dujardin JC, Ramírez JD. Major changes in chromosomal somy, gene expression and gene dosage driven by Sb^{III} in *Leishmania braziliensis* and *Leishmania panamensis*. Sci Rep. 2019 Jul 1;9(1):9485. doi: 10.1038/s41598-019-45538-9.

3. **Patino LH,** Muskus C, Ramírez JD. Transcriptional responses of *Leishmania (Leishmania) amazonensis* in the presence of trivalent sodium stibogluconate. Parasit Vectors. 2019 Jul 12;12(1):348. doi: 10.1186/s13071-019-3603-8.

Artículo sometido en la revista Acta Trópica

4. **Luz H. Patino**, Carlos Muskus, Marina Muñoz, Juan David Ramírez. Comparative genomics reveals moderate levels of ploidy, high heterozygosity and structural variations in *Leishmania (Leishmania) amazonensis.*

Capítulo 3: Evaluado el comportamiento genómico y transcriptómico de cepas de referencia sometidas a condiciones de estrés (Sb^{III}), se determinó mediante ADN-seq la arquitectura genómica de 27 aislamientos clínicos provenientes de pacientes con LC, infectados con *Leishmania (Viannia) braziliensis* o *Leishmania (Viannia) panamensis*. Al analizar los resultados obtenidos entre los aislamientos clínicos provenientes de ambas

especies, se evidenció baja diferencia estructural en términos de somía, dosis genética y polimorfismos de nucleótido simple, sin embargo, dos de estos aislamientos clínicos mostraron resultados interesantes; en uno de ellos (asociado a *Leishmania (Viannia) braziliensis*) se evidenció un posible evento de recombinación y el otro hallazgo fue identificado en una aislamiento de *Leishmania (Viannia) panamensis,* en el cual se observó en el cromosoma 20 un elevado número de SNPs distribuidos en bloque. Estos resultados serán confirmados con posteriores análisis.

Como producto de este capítulo se escribió el siguiente artículo: “Variación intra-especie en *Leishmania (Viannia) braziliensis* y Leishmania (*Viannia panamensis*) aisladas de pacientes con Leishmaniais Cutánea en Colombia“, el cual será próximamente sometido a una revista de primer cuartil de Scimago (Q1)

8.1 CAPITULO 1

Identificación de las especies de *Leishmania*, que circulan en población militar colombiana

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Spatial distribution, *Leishmania* species and clinical traits of Cutaneous Leishmaniasis cases in the Colombian army

Luz H. Patino[1], Claudia Mendez[2]*, Omaira Rodriguez[2], Yanira Romero[2], Daniel Velandia[2], Maria Alvarado[2], Julie Pérez[2], Maria Clara Duque[2], Juan David Ramírez[1]

1 Grupo de Investigaciones Microbiológicas-UR (GIMUR), Programa de Biología, Facultad de Ciencias Naturales y Matemáticas, Universidad del Rosario, Carrera 24# 63C-69, Bogotá, Colombia, **2** Laboratorio de Referencia e Investigación en Enfermedades Tropicales, Dirección de Sanidad Ejército, Ejército Nacional de Colombia. Avenida Carrera 7 No 52–48. Bogotá, Colombia

* Claudia.mendez@ejercito.mil.co

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Abstract

In Colombia, the cutaneous leishmaniasis (CL) is the most common manifestation across the army personnel. Hence, it is mandatory to determine the species associated with the disease as well as the association with the clinical traits. A total of 273 samples of male patients with CL were included in the study and clinical data of the patients was studied. PCR and sequencing analyses (Cytb and HSP70 genes) were performed to identify the species and the intra-specific genetic variability. A georeferenced database was constructed to identify the spatial distribution of *Leishmania* species isolated. The identification of five species of *Leishmania* that circulate in the areas where army personnel are deployed is described. Predominant infecting *Leishmania* species corresponds to *L. braziliensis* (61.1%), followed by *Leishmania panamensis* (33.5%), with a high distribution of both species at geographical and municipal level. The species *L. guyanensis*, *L. mexicana* and *L. lainsoni* were also detected at lower frequency. We also showed the identification of different genotypes within *L. braziliensis* and *L. panamensis*. In conclusion, we identified the *Leishmania* species circulating in the areas where Colombian army personnel are deployed, as well as the high intra-specific genetic variability of *L. braziliensis* and *L. panamensis* and how these genotypes are distributed at the geographic level.

Author summary

Colombia is one of the countries with the highest incidence of Cutaneous Leishmaniasis in the world and the army population is the most vulnerable population. Herein, we identified the infecting *Leishmania* species (*L. braziliensis*, *L. panamensis*, *L. guyanensis*, *L. mexicana* and *L. lainsoni*). We also showed the high intra-specific genetic variability of *L. braziliensis* and *L. panamensis* and how these genotypes are distributed at the geographic level.

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Introduction

Leishmaniasis is a group of tropical diseases caused by parasites of the genus *Leishmania*. This parasite is transmitted to humans through the bite of infected female insects of the family Psychodidae.[1, 2] There are about 20 species of *Leishmania*, which can cause different clinical manifestations in humans, including Cutaneous Leishmaniasis (CL), Mucocutaneous Leishmaniasis (ML) and Visceral Leishmaniasis (LV).[2, 3] These diseases are a major public health problem in 98 countries around the world, where 12 million people are infected, more than 350 million people at risk of infection and 1.3 million new cases per year occur.[2] CL is the most common clinical manifestation; between 0.7 and 1.3 million new cases of CL are reported annually and about 90% of them occur in countries such as Afghanistan, Algeria, Brazil, Iran, Syria and Colombia.[2, 3]

Colombia occupies the second place in incidence of Leishmaniasis in America after Brazil.[4] In 2016, about 10,743 new cases of Leishmaniasis were reported in the national territory (2,493 more than the year immediately preceding), of which 98% were associated with CL.[5] The increase in the number of cases has been attributed to different factors such as the colonization of vectors into new geographical areas, the urbanization of the disease [4] and the human activities that expose immune populations to infection, such as traveling, migration, civil conflict and military operations. [4, 6–8] Regarding the latter, the army population shows the highest incidence of the disease and constitute the most vulnerable group, due to the continuous deployment of troops to areas of high endemicity and high circulation of the insect vector.[8] Several CL outbreaks in the army population have been reported in different countries of the world including Colombia.[4, 9–12] These outbreaks not only affect the foot of force, the cessation of military operations, the quarantine throughout the battalion in which the disease occurs, but also the risks to which the military must be subjected by the use of antileishmanial treatments (which have a high degree of toxicity).

Epidemiological data generated by the Public Health Surveillance System of the Military Health Service of Colombia (Salud Operacional DISAN Ejercito) report a high number of cases associated with Leishmaniasis during the years 2011 to 2017 (17.796 cases of CL and 254 cases of MCL). However, very few studies report circulating species. The most recent study, conducted in a small army population (43 individuals), describes that *Leishmania (Viannia) braziliensis* is the predominant species (95.4%), followed by *Leishmania (Viannia) guyanensis* (2.3%).[13] Contrary to what was reported in populations living in urban areas where the most frequent species are *Leishmania (Viannia) panamensis* (61.3%) and *Leishmania (Viannia) braziliensis* (27.1%).[14] In spite of the existing data, to date there are no studies where using a larger army population are described the main species of *Leishmania* as well as the geographical distribution within the national territory. Similarly, there are very few studies reporting the genetic variability of the infecting species in relation to the geographical distribution.

Therefore, the objectives of this study were to evaluate the clinical traits, distribution and genetic variability of *Leishmania* species that circulate in the areas where army personnel are deployed using clinical samples (imprint in filter paper) from patients with CL.

Materials and methods

Study population

A total of 273 samples from male patients belonging to the Colombian National Army were collected. The selection of the patients was made by selective and stratified sampling and carried out on individuals with positive diagnosis of CL in 12 army units located throughout the

country (which reported the major number of cutaneous leishmaniasis cases during 2013).The collection of the samples was pretty accurate at department level (Colombian administrative subdivisions). Sampling was stratified by the number of cases reported by each unit divided by the total number of cases.

As inclusion criteria, patients had to be male, over 18 years of age, with clinical and parasitological diagnosis of CL; with lesions of minimum one month and maximum three months of evolution and without anti-leishmanial treatment for at least two months prior sampling. Only patients with a positive result for at least one direct smear or PCR-skin biopsy were included in the study. Those patients with lesions in face, genitals or mucosa and secondary infected lesions were not sampled. A summary of the demographic and clinical characteristics of patients included in this study are presented in the Table 1.

Ethics statement and sample collection

Once the adult patients accepted their participation in the study and after reading and signing written informed consent, a survey was conducted which included information associated with demographic factors (age, place of birth, site of possible acquisition of Leishmaniasis, personal protection measure to avoid insect bite (use of mosquito repellents, repellents and/or uniform use impregnated with Permethrin) and clinical factors (previous Leishmaniasis, clinical presentation, number and diameter of lesions of current leishmaniasis, time of evolution of

Table 1. Demographic and clinical characteristics of patients.

Characteristics	N: 221	Estimate	95%, CI
Age, median (p25-p75)	23	(22–26)	23–24
Skin type			
Brown	109	49.3%	42.5–56.1
White	96	43.4%	36.7–50.2
Black	11	4.9%	1.88–8.07
ND	5	-	-
Infected lesion	34	15.4%	10.4–20.4
Previous leishmaniasis	41	19.5%	14.1–24.9
Cutaneous	40/41	97.5%	80.9–98.5
Mucocutaneous	1/41-	2.5%	0.06–12.3
Scar	33/41	80.5%	69.5–95.5
Head/Neck	8/33-	24.2%	8.11–40.4
Trunk	5/33-	15.2%	5.11–31.9
Upper limbs	20/33	60.6%	42.4–78.8
Lower limbs	9/33-	27.3%	10.6–43.9
Actual leishmaniasis			
Cutaneous	220	99.6%	97.5–99.9
Mucocutaneous	1	0.45%	0.01–2.49
Species associated with the actual leishmaniasis			
L. braziliensis	135	61.1%	54.4–67.7
L. panamensis	74	33.5%	27.0–39.9
L. guyanensis	8	3.6%	0.93–6.30
L. mexicana	3	1.4%	0.28–3.91
L. lainsoni	1	0.4%	0.01–2.49

Age: expressed in years, **ND:** not data.

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the disease, lymphadenopathy and previous anti-leishmanial treatment)). Subsequently, the samples were obtained by imprint in filter paper of the site of the lesion (in order to completely cover a quarter of filter paper per patient); the numbers of imprints taken varied from patient to patient according to the size of the lesion. The imprints were stored at 4˚C until use. Once the samples were taken, all patients were treated with meglumine antimoniate (Glucantime), according to Colombia's treatment guide for Leishmaniasis.

All protocols applied in the study were approved by the Ethics Committee of the Central Military Hospital of Colombia, in accordance with the principles established in the Declaration of Helsinki and Resolution 008430 of October 4, 1993 of the Ministry of Health.

DNA extraction and PCR amplification for the identification of *Leishmania* species

DNA extraction was performed using the commercial kit ISOLATE II Genomic DNA kit (Bioline), following the protocol described by the manufacturer. Subsequently, species identification was performed using direct sanger sequencing of the genes coding for the cytochrome b (Cytb) molecules and the heat shock protein (HSP70), as described by Ramírez et al, 2016 [14] and Hernandez et al., 2014. [15] The PCR reaction was carried out at a final volume of 25 μl containing 12.5 μl GoTaq Green Master Mix (Promega, Madison, Wi, US), 0.5 μM of each primer and 1.25 μL DNA (Concentration <250 ng). PCR conditions for the amplification of the Cytb gene are described below: Initial denaturation at 95˚C for 5 min, 40 denaturation cycles at 95˚C for 1 min, annealing at 58˚C for 1 min, extension at 72˚C for 1 min, and final extension at 72˚C for 10 min. For the HSP70 gene, the following conditions were used: initial denaturation 94˚C for 5 min, 40 denaturation cycles at 94˚C for 1 min, annealing at 60˚C for 1 min, extension at 72˚C for 1 min and Final extension at 72˚C for 10 min. Amplification and size of the amplicon was verified by agarose gel electrophoresis stained with SYBR Safe DNA Gel Stain (Life Technologies, Carlsband, Ca, US) and a molecular weight marker (Promega). The amplification products were purified with EXOSAP (Affymetrix, USA) and sequenced by the dideoxy-terminal method, in an automated capillary sequencer (AB3730, Applied Biosystem). Subsequently the sequences were subjected to BLASTn to search similarity with the *Leishmania* sequences deposited on GenBank. [14]

Phylogenetic reconstruction, haplotype and nucleotide diversity analyses

The sequences obtained were edited in MEGA 5.0 and aligned with CLUSTAL W, using the reference sequences for the Cytb gene of *Leishmania donovani donovani* (AB095957), *Leishmania garnhami* (AB095965), *Leishmania mexicana* (AB095936), *Leishmania amazonensis* (AB095964), *Leishmania garnhami* (AB095965), *Leishmania mexicana* (AB095960), *Leishmania braziliensis* (AB095966), *Leishmania panamensis* (AB095968), *Leishmania guyanensis* (AB095969), *Leishmania equatoriensis* (AB434687), *Leishamnia pifanoi* (EF579907), *Leishmania lainsoni* (AB43 3280), *Leishmania colombiensis* (KF302738) and *Leishmania peruviana* (AB433282). For The HSP70 gene the reference sequences of *L. major* (HF586403.1), *L. donovani* (JX312712.1), *L. tropica* (HF586409.1), *L. peruvian*a (HF586368.1), *L. aethiopica* (HF586411. 1), *L. garnhami* (EU599092.1), *L. chagasi* (FN395037.1), *L. mexicana* (XM 003877072.1), *L. amazonensis* (L14 605.1), *L. braziliensis* (AF291716.1), *L. guyanensis* (EU599093.1), *L. infantum (chagasi)* (XM 003392632.1), *L. panamensis* (FN395055.1) and *L. lainsoni* (FN395049.2) were employed. A maximum composite likelihood (MCL) analysis using a Tamura-3 parameter was run in RaxML Phylogeny.fr platform. To evaluate the robustness of the nodes in the resulting phylogenetic tree, 1000 bootstrap replicates were performed. In addition to MCL analyzes, a Nexus matrix was

constructed for haplotype network analysis in Network 2.0 using a median-joining model based on 1000 iterations with default parameters. The purpose of this analysis was to determine the number of alleles across the population and determine the biological and geographical distribution of the alleles depicted for two species (*L. panamensis* and *L. braziliensis*). In order to analyze the distribution of species and haplotypes at the geographic level, the 32 Colombian departments were divided into five eco-geographical regions (Orinoquia, Amazonian, Andean, Atlantic and Pacific). Lastly, sequence genetic diversity was estimated for Cytb and HSP70 genes fragments by the most frequent species set. Π and θ nucleotide diversity indexes and haplotype diversity were calculated in DNAsp v.5.0.

Spatial distribution patterns

To address the spatial distribution of *Leishmania* parasites isolated, a georeferenced database was constructed. Data on human isolates belong to the posible site of infection acquisition, as reported by the patient. Using ArcGIS10.3 we extracted values of Ecoregions [16] and Colombian Ecosystems [17] in order to describe parasite distribution by Ecosystems and land´s use coverage.

Statistical analyses

The relationship between the clinical-demographic variables and the infecting *Leishmania* species was analyzed using the statistical packages XLSTAT (Version 2014.5.03), Minitab (Version 17) and SPSS Modeler (Version 18). Quantitative data were expressed in medians, qualitative in proportions (95% CI), the confidence interval to proportion (CI95%) was calculated using the next equations: 1. Upper limit of confidence Interval, $P+ 1.96^{*}(\sqrt{p(1-p)/n})$. 2. Lower limit of confidence Interval, $P- 1.96^{*}(\sqrt{p(1-p)/n})$ and comparisons between variables were performed with non-parametric statistics. A p value <0.05 was established to determine statistical significance. With a mesh chart (SPSS Version 18) we explored the strength of relationships between species of *Leishmania* and specific characteristics that previously demonstrated a statistically significant relationship (Chi squared, X2).

Results

Identification of *Leishmania* species and phylogenetic reconstruction

Sequencing analysis performed on a fraction of the Cytb and HSP70 genes allowed the identification of *Leishmania* species in 221 of the 273 samples analyzed (81%). Seventy-one of them were identified with Cytb and 150 with HSP70. The remaining 52 samples could not be analyzed due to the low amount of DNA of the parasite present in the sample. The 221 sequences edited, were submitted to Blastn, in search of similarity with the sequences deposited on the Genebank database. In general, the sequences had an average identity of 97%. *Leishmania braziliensis* (61.1%, 95% CI, 54.4–67.7), *Leishmania panamensis* (33.5%, 95% CI, 27.0–39.9), *Leishmania guyanensis* (3.6%, IC95%, 0.93–6.30), *Leishmania mexicana* (1.4%, IC95%, 0.28–3.91), and *Leishmania lainsoni* (0.4%, IC95%, 0.01–2.49), were identified in the final consensus of the Cytb and HSP70 typing (**Table 1**). The sequences obtained from the HSP-70 gene were aligned and a robust phylogenetic reconstruction was carried out (Boostrap greater than or equal to 85%). The results allowed the identification of four fully differentiated clusters corresponding to *L. braziliensis*, *L. panamensis*, *L. mexicana* and *L. lainsoni*, confirming the results obtained. It was also possible to show that within the clusters of *L. braziliensis* and *L. panamensis*, different genotypes occur (**Fig 1**).

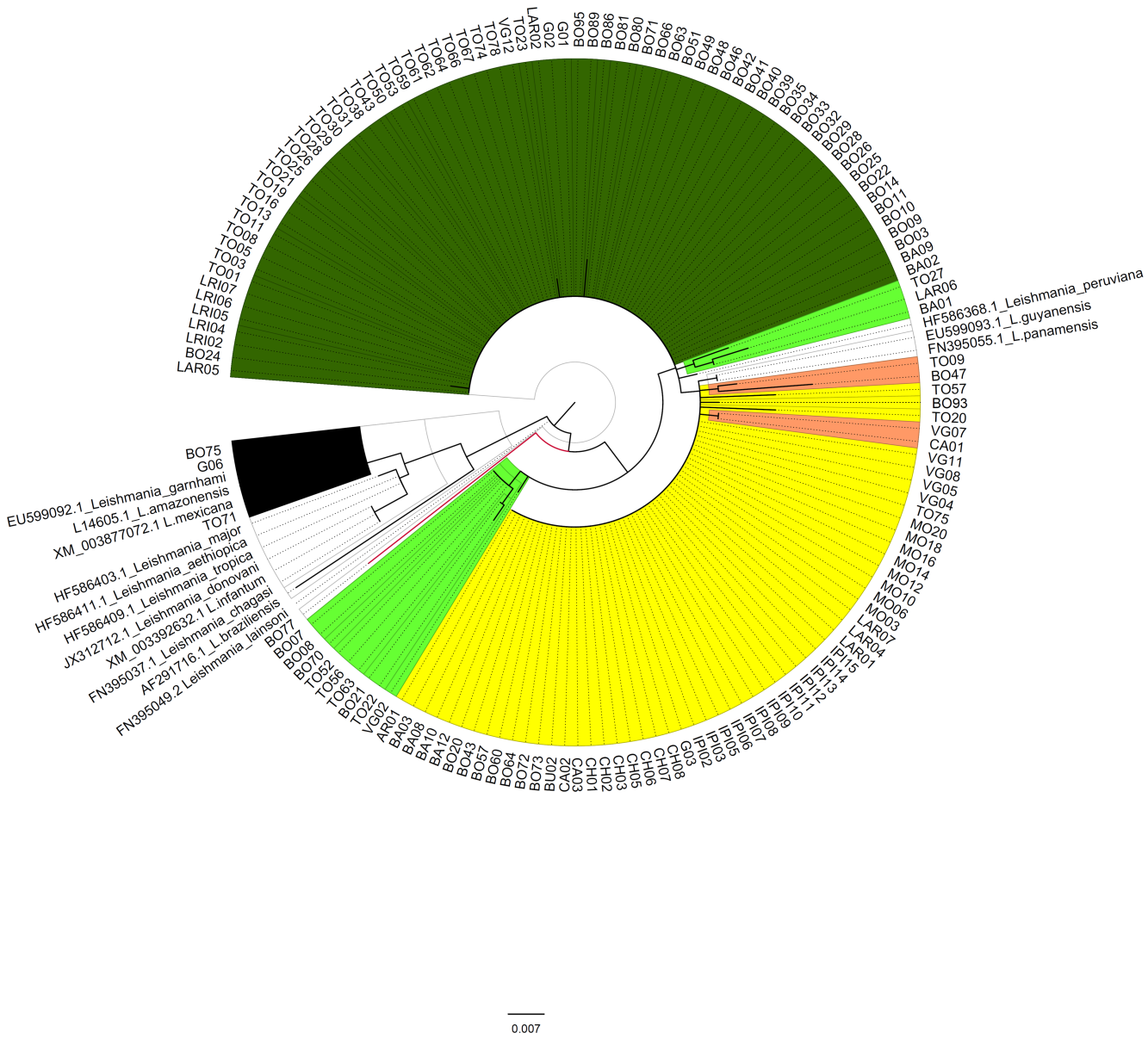

Fig 1. Phylogenetic reconstruction of HSP70 sequences. Phylogenetic reconstruction of 150 HSP70 gene sequences obtained from direct samples of CL patients and the reference strain retrieved from GenBank. *L. braziliensis* (green); *L. panamensis* (yellow); *L. mexicana* (black) and *L. lainsoni* (red). Futhermore, different genotypes associated with *L. braziliensis* (light green) and *L. panamensis* (light pink) were identified. The numbers represent the coding of each sample.

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Clinical and epidemiological traits

We analyzed 221/273 records of patients with median age (p25-p75) 23 years (22–26), mostly brown skin (49.3%). Nineteen point five percent (95% CI, 14.1–24.9) of the patients reported having previously presented leishmaniasis, of which 97.5% were associated with CL, the 80.5% (95% CI, 69.5–95.5%) of them presented sequelae of scars with a predominantly upper extremity compromise (60.6%, 95% CI, 42.4–78.8) (Table 1). The majority of patients (93%) were treated with meglumine antimoniate (mean dose of 20 mg antimony / kg / day), with a duration between 2–45 days of treatment (median of 20 days). The patients who received the

Table 2. Relationship between the clinico-epidemiology features and the infectious *Leishmania* species.

Variables	*L. braziliensis* n: 135	*L. panamensis* n: 74	*L. guyanensis* n: 8	*L. mexicana* n: 3	*L. lainsoni* n: 1	p-value
History	26 (19.3)	16 (21.6)	-	1 (33.3)	-	**0.000**
Scars	21 (15.6)	14 (18.9)	-	-	-	**0.000**
Semiology						
Papule	21 (17.4)	7 (12.3)	1 (16.7)	-	-	0.742
Ulcer	96 (79.3)	38 (66.7)	5 (83.3)	1 (50)	1 (100)	0.187
Nodule	11 (9.1)	8 (14.0)	-	1 (50)	-	0.354
Plaque	4 (3.3)	4 (7.0)	-	1 (50)	-	**0.002**

NA: not applicable, **-:** not events. Kruskall Wallis test was used to compare medians (IQR), Chi squared (X^2) was used to compare frequencies, **History**: previous leishmaniasis, **Scars**: scars secondary to leishmaniasis infections.

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treatment for less or more time than the standardized protocol (twenty days) was due to interruption of drug administration as consequence of altered clinical exams that compromise patient safety and health or due to more than one treatment cycle for an unfavorable response. Finally, we identified a 31.67% (95%CI, 19.06–44.27) of new ocurrence of leishmaniasis (probably associated with resistance to treatment, reactivation or reinfection). This value was calculated according to the data generated of the visual confirmation of scars and the described by the patients (which may not be very conclusive).

Regarding the current disease, it was observed that despite the use of personal protection elements to avoid insect bites (repellents, insect repellents or Permethrin impregnated uniform), all the patients included in the study presented clinical or epidemiological criteria positive for Leishmaniasis. Clinical evaluation criteria showed that in more than 95% of the cases, the lesions presented were localized and of cutaneous variety, with the upper extremities being the most affected body region (59.8%, 95% CI, 62.6–66.9%), with a number of lesions ranging from 1–3 (95% of patients), of which 50% had a diameter of 1.2 cm^2 (p75, 3 cm^2).

On the other hand, we evaluated the relationship between the collected clinical-epidemiological data and the infecting *Leishmania* species. We identified that certain parameters had a strong relationship with the *Leishmania* species. The results obtained in the mesh graph showed the strong relationship between *L. braziliensis* and *L. panamensis* with the absence of previous leishmaniasis and recurrent lesions, and with the clinical presentation (strong association with ulcerative and weak lesions with papular, nodular lesions and the presence of plaques (defined as an elevated lesion of the skin of more than 2 centimetres in diameter formed by the union of several papules or nodules)). Contrary to what happened with the other species identified, where a weak association with the evaluated parameters was observed (**Table 2**; **Fig 2**).

Nucleotide diversity analyses

The diversity analysis performed for the Cytb gene, in 61 sequences analyzed, showed a total of 315 polymorphic sites and 431 mutations for *L. braziliensis*, *L. panamensis* and *L. guyanensis*. Based on the haplotype (Hd) and nucleotide diversity indexes (π), for each species, *L. guyanensis* showed a marked genetic (Hd = 1) and nucleotide diversity (π = 0.20557), contrary to what was observed with *L. braziliensis* and *L. panamensis* which, despite having a high genetic diversity (Hd = 0.964 and 0.962, respectively) showed a moderate nucleotide diversity (π = 0.03929 and 0.04752, respectively) (**Table 3**). Regarding the HSP70 gene, a total of 38 polymorphic sites and 40 mutations for *L. braziliensis*, *L. panamensis* and *L. mexicana* were observed in 145 analyzed sequences. Haplotypic and nucleotide diversity indexes revealed that *L. mexicana* was

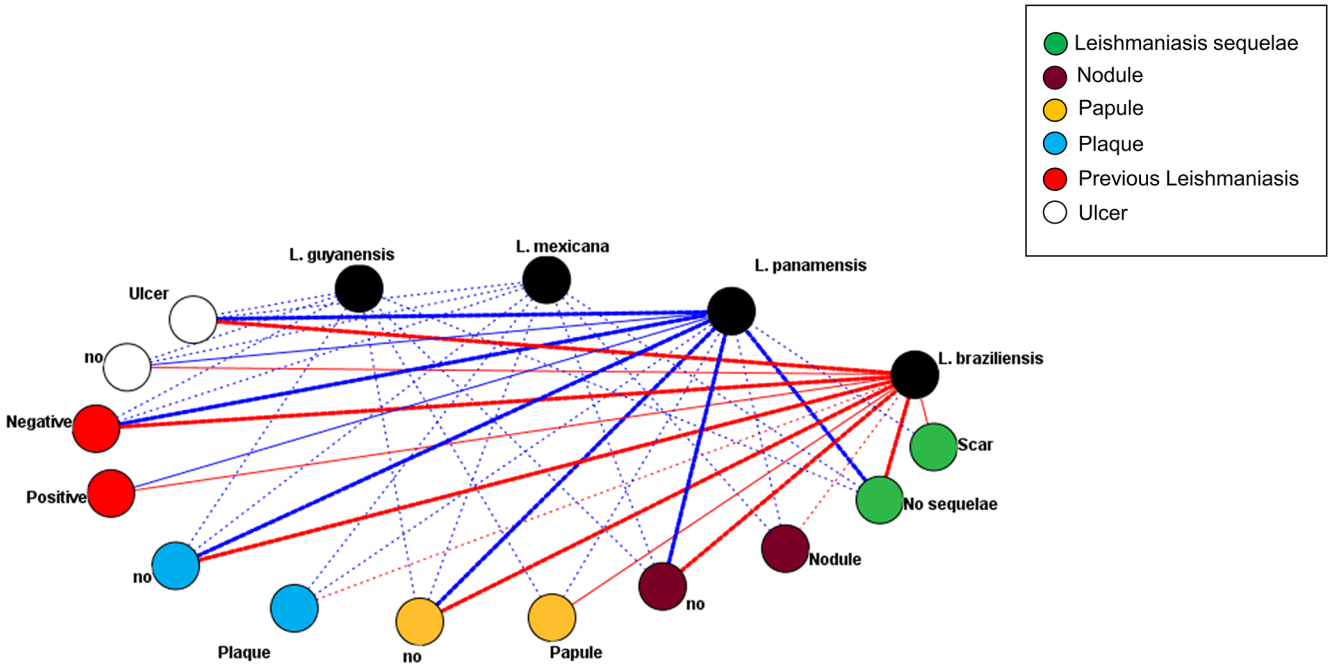

Fig 2. Relationship between clinical-epidemiological data and infecting *Leishmania* species. The frequency of linkages between species of *Leishmania* and the specific characteristics were divided into three levels: **1.** Thickness lines,> 35 links, **2.** Intermediate thickness lines, 15–35 links and **3.** Semi-dotted lines of weak thickness, <15 links. The links with *L. braziliensis* were highlighted in red because of the high frequency of identified cases. "no": it refers to absence of clinical manifestation.

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the species with the highest values (Hd = 0.667, π = 0.00473), compared to the other two *Leishmania* species analyzed (**Table 3**).

Patterns of spatial distribution

When the analysis of species at the departmental level was made, the patterns of geographic distribution showed that *L. braziliensis* and *L. panamensis* were distributed differently, while 136 clinical samples of *L. braziliensis* were distributed in 14 departments (predominance in the Orinoquia and Amazon regions), 74 samples of *L. panamensis* were distributed in 12 departments (with a greater predominance in the pacific region), which reflects the wide geographical distribution of *L. panamensis* at national level. Contrary to the other *Leishmania* species identified in the study, whose geographical distribution was limited to certain departments, such as *L. guyanensis* distributed in the departments of Meta, Tolima, Putumayo and Córdoba. *L. mexicana* in the departments of Meta and Guaviare and *L. lainsoni* in the department of Meta (**Fig 3A**). When we performed the analysis of abundance of species at the departmental level, we observed that the departments with the greatest number of positive samples were Meta, Guaviare and Nariño, of which Meta was the department that provided the largest number of cases of CL in the Colombian army population (48%) during November 2014 to June 2015 (*L. braziliensis*: 74/120, *L panamensis*: 14/66, *L. guyanensis*: 3/8 and *L. mexicana*). In this study, all the species identified (*L. braziliensis*, *L. panamensis*, *L. guyanensis*, *L. mexicana and L. lainsoni*) were circulating in the Meta department (**Fig 3B**).

Regarding the distribution of species at the municipal level, we could observe that in some municipalities, two or more species of *Leishmania* are circulating at the same time, as is the case of La Uribe (Meta) in which 4 of the five-species identified circulate (*L. braziliensis*, *L. panamensis*, *L. guyanensis* and *L. mexicana*), in Puerto Caicedo (Putumayo) and Vista Hermosa (Meta)

Table 3. Genetic diversity parameters of *Leishmania* Cytb and HSP70 genes sequences.

Cytb gene						
Species	**N**	**S**	**Eta**	**Hd**	**π**	**K**
L. braziliensis	46	204	257	0,962	0,04752	24,379
L. panamensis	8	77	82	0,964	0,03929	21,179
L. guyanensis	7	258	305	1	0,20557	99,905
All species	61	315	431	0,978	0,06979	32,521
HSP70 gene						
Species	**N**	**S**	**Eta**	**Hd**	**π**	**K**
L. braziliensis	82	14	14	0,33	0,00281	0,787
L. panamensis	60	15	15	0,25	0,00217	0,597
L. guyanensis	3	2	2	0,667	0,00473	1,333
All species	145	38	40	0,653	0,00608	1,666

N = Number of sequences. S = Number of polymorphic sites. Eta = Total number of mutations. Hd = Haplotype diversity. π = Nucleotide diversity. K = Average number of nucleotide differences.

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are circulating *L. braziliensis*, *L. panamensis* and *L. guyanensis*, in Puerto Lleras (Meta) are circulating *L. braziliensis*, *L. guyanensis* and *L. mexicana*; and in municipalities such as San Jose (Guaviare), Rioblanco (Tolima), Tierra Alta (Córdoba) Apartadó (Antioquia), La Macarena (Meta),

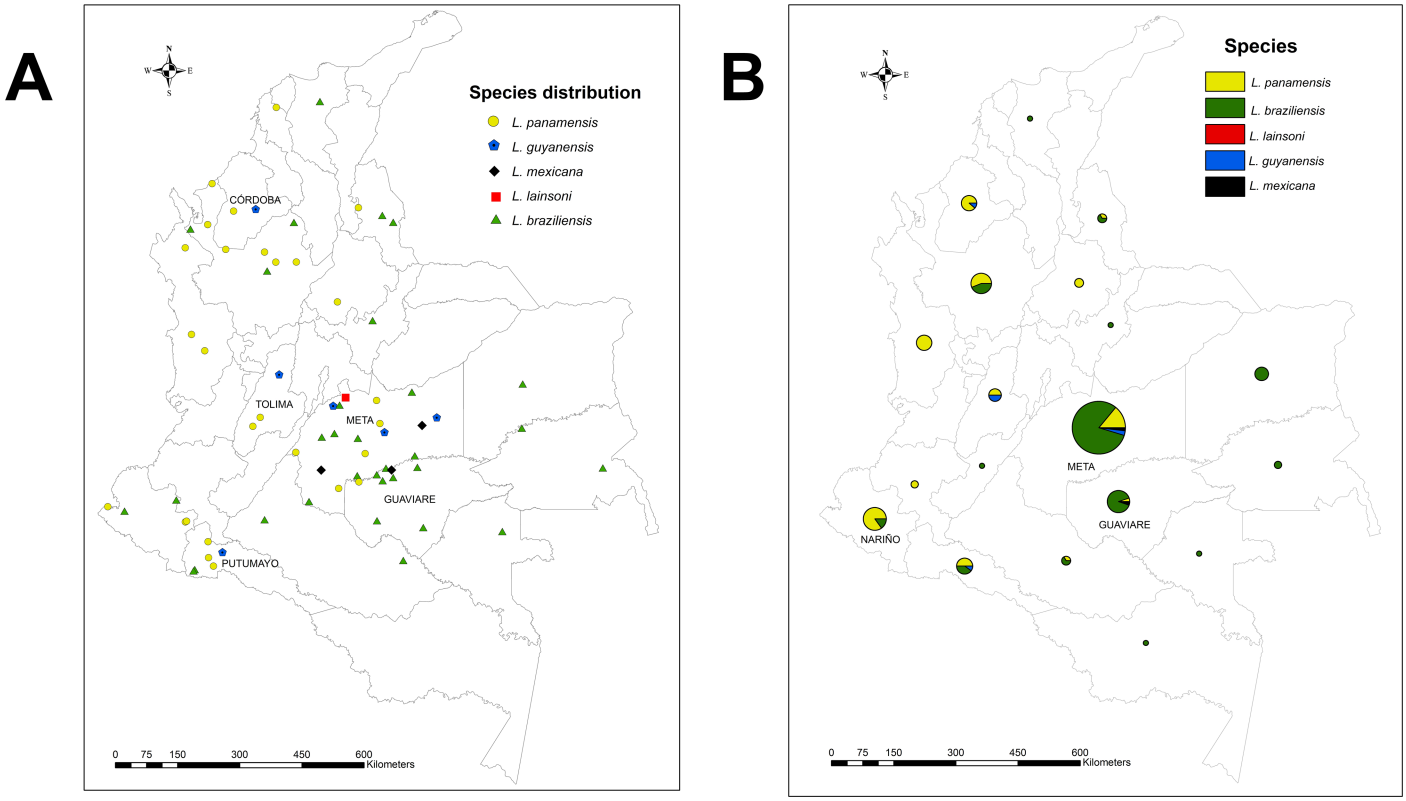

Fig 3. Geographical distribution of 222 *Leishmania* isolates associated to CL in Colombia. GPS coordinates were used to build georeferenced maps of isolates location. The maps were built on ArcGIS10.3 using Esri Colombia PublicadorSIG layer. (http://www.arcgis.com/home/item.html?id=b051fbef7fba406fbb8e62b90925f365#overview) (**A**) Georeferenced isolates discriminated by species in the country. (**B**) Relative abundance of *Leishmania* species in each Colombian department. The size of the circle refers to the number of samples collected by department.

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Puerto Rico (Brazil), Puerto Cachicamo (San José del Guaviare), Puerto Validibia (Antioquia) and Tumaco (Nariño), are circulating two of the species identified (*L. braziliensis* / *L. panamensis* or *L. braziliensis* / *L. mexicana* or *L. panamensis* / *L. guyanensis*) (**Fig 3B and S1 Fig**).

Haplotype networks

The haplotype network showed the high intra-specific genetic variability of *L. braziliensis* and *L. panamensis*. For *L. braziliensis* 13 types of sequence were identified and for *L. panamensis* 11, distributed in the different geographical areas analyzed. This analysis was performed only with these two species, because they were the most frequent in our study. For *L. braziliensis*, two of the haplotypes found were identified in more than one individual and in more than one geographical area. The most dense of these haplotypes, was distributed mainly in the Orinoquia region, followed by the Amazon region and to a lesser extent in the Andean and Atlantic regions; the second most prevalent haplotype was distributed mainly in the Orinoquia and Amazon regions. The remaining haplotypes were considered independent and distributed mainly in the Amazon and Orinoquia regions. Interestingly, we observed that the alleles of the Amazon region were shared with the alleles of the Andean region (**Fig 4A**). Regarding *L. panamensis*, we found four haplotypes distributed in more than one geographical region, where the most abundant was distributed in all geographic regions analyzed in the study (Pacific, Andean, Orinoquia, Amazon and Atlantic regions). The second haplotype was distributed in the Pacific and Andean regions, and the third and fourth haplotypes distributed equally in the Andean and Amazon regions and Orinoquia and Pacific, respectively. The remaining haplotypes were found in a single individual and in a particular geographical area (**Fig 4B**).

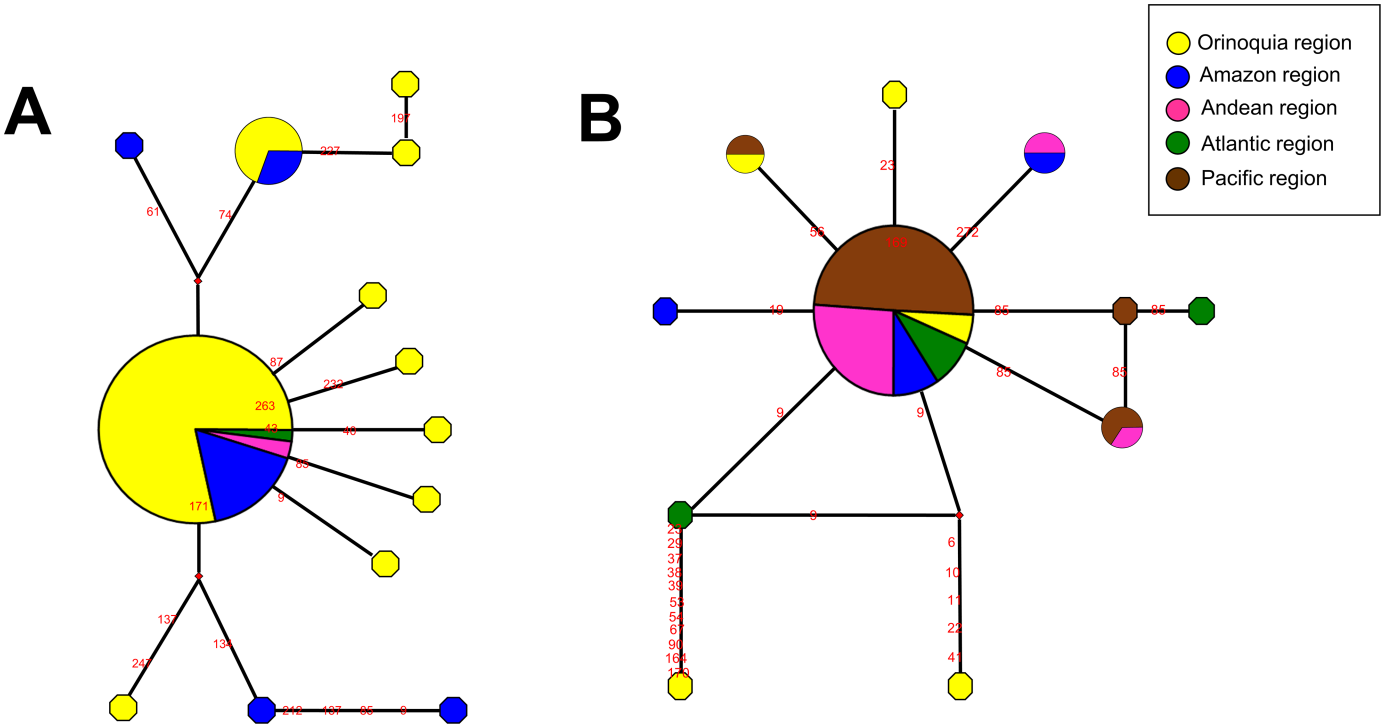

Fig 4. Network analysis of geographical distribution of *Leishmania* species. Alleles of the HSP70 gene were retrieved to construct the networks shown for each species as follows, the numbers on the lines specify the positions across the alignment where a nucleotide change occurred (**A**) *L. braziliensis* and (**B**) *L. panamensis*.

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Discussion

At present, it is well known that the main factor influencing the occurrence of outbreaks of Leishmaniasis, and other vector-borne diseases in the army population is the high number of personnel entering endemic areas with high circulation of the vector insect, which coincide with operational areas, due to Armed conflict in the country and the fight against drug dealing and the illegal minery. Between 2005 and 2009, approximately 45,000 cases of Leishmaniasis were reported, [4] and although the numbers have declined considerably during the years 2011 to 2017 (17.796 cases of CL and 254 cases of MCL) due to the recent Peace deal with the rebels of the FARC, the data remain alarming.[18] Previous studies have determined that the main clinical manifestation presented in the Colombian army population is CL, with ulcerative lesions considered the main forms of presentation. This work was carried out for several purposes: one of which was to expand the information currently available on the species of *Leishmania* that are circulating in areas where there is a greater military deployment and for the first time to determine the geographical distribution of these species in our country. The clinical-epidemiological analyzes carried out in the present study confirm that 97% of cases were associated with CL, and additionally, despite the use of personal protection elements to avoid insect bites (repellents and uniforms impregnated with Permethrin), the army continues to present a high infection rate; About 90% of the patients included in the present study (positive for leishmaniasis) reported using one or more of these elements. Although the 31% of patients positive for leishmaniasis mentioned having presented the disease previously, the data obtained did not allow us to determine if this was associated with resistence to treatment, reactivation or reinfection.

Although, the data reported so far in the army population correlate with the most common clinical form of the disease (CL), the information associated with the species involved is still scarce. To date, there have been several studies in which mitochondrial (Cytb) and nuclear (HSP-70) gene sequencing have been used to identify species of the genus *Leishmania*. [14, 19–22] In our case, the direct sequencing of these molecular markers was successful and sufficiently sensitive for the typing of *Leishmania* species from clinical samples. We observed that HSP70 gene was more sensitive than CytB in the detection of parasite DNA from clinical samples, due to differences in copy number and because this marker has major power resolution (64 SNPs in total) than Cytb. However, around 19% of samples positive by microscopy could not be identified. These samples were PCR negative by kDNA. This was unexpected and might be explained due to the presence of potential inhibitors of the samples or unlikely manipulation of the sample. Also, a possible mistake during the DNA extraction that did not allow the molecular detection of *Leishmania*. Lastly, it is important to highlight the limitation of the imprint that could explain the lack of congruence between microscopy and PCR results. Unfortunately, we did not have additional sample to repeat the process and rule out these assumptions. The 81% of the samples were identified showing five species of *Leishmania*, which are circulating in the areas where Colombian army personnel are deployed. *L. braziliensis* was the most frequently occurring species (61.1%), confirming the reported by Perez-Franco et al. 2016, where 95.4% of the 45 samples of patients with CL, belonging to the Colombian National Army were associated with *L. braziliensis*. [13] When analyzing a larger population group, we were able to identify other species of *Leishmania* such as *L. panamensis*, *L. guyanensis*, *L. mexicana* and *L lainsoni* (**Table 1**). The latter species recently identified in the departments of Putumayo and Antioquia in Colombia.[14] In addition, the analysis of the HSP70 gene allowed the identification of different genotypes within *L. braziliensis* and *L. panamensis* (**Fig 1**) in accordance with the reported by Van der Auwera et al., 2013 and 2015 suggesting the existance of intraspecific genetic variability using this locus. [22, 23]

When comparing these results with those reported in the urban population, we observed that the proportion and species identified in rural areas are the same as those that are circulating in urban areas. [14, 24] In general, we confirm that *L. braziliensis* and *L. panamensis* are not only the species most frequently associated with CL in Colombia, as described by several authors,[14, 24, 25] but they are also the main species that are circulating more frequently in all our Colombian territory. Additionally, when we performed the analysis of species at the municipal level, we could observe that there are municipalities (mainly from the department of Meta), in which more than one species of *Leishmania* is circulating at the same time (**Fig 3B**). Our findings have important implications, since the existence of two or more species circulating in the same geographical area increases the risk of reinfections, creates inconvenients at the moment of *Leishmania* species identification associated with the disease, in the selection of antileishmanial therapy (due to the influence of the species on the clinical outcome after treatment). [26–29] and the possibility of increasing the risk of resistance to anti-leishmanial therapy.

On the other hand, all studies conducted so far in the army population, describe the distribution of species at the geographical level but none analyzes the genetic diversity. [8, 13] So far, only one study using samples from urban population is reported.[14] Our study is the first to describe, in samples from the rural area the distribution and genetic diversity of *Leishmania* species. Herein and by sequencing the two previously described markers, we identified that *L. mexicana* and *L. guyanensis* are the most diverse species (Hd = 0.66, S = 3 and Hd = 1, S = 7, respectively) (**Table 3**). However, we believe that these findings should be confirmed with a higher number of positive samples for these species, which was out of reach, due to the low frequency in our population. In the cases of *L. braziliensis* and *L. panamensis*, we observed that these species are equally diverse (Hd = 0.33 and 0.25 respectively for the HSP-70 gene) and (Hd = 0.962 and 0.964 respectively, for the Cytb gene) (**Table 3**), which was confirmed by the haplotype network, in which 11 to 13 different sequence types were observed for each species (**Fig 4A and 4B**). One limitation of these assumptions is that we only used two genetic markers to unravel the intra-species genetic diversity. However, we did not have access to the *Leishmania* isolate of the patient. Therefore, the only option was to use two sensitive markers to pull out potential and informative SNP´s for these calculations. For further studies, it is mandatory to conduct Multilocus Sequence Analyses or Whole Genome Sequencing to obtain a suitable picture of intra-species variability.

Although, our analyzes determined that *L. braziliensis* is the most frequently encountered species, most of the haplotypes were limited to two geographic areas in particular (Orinoquia region and Amazon region), contrary to *L. panamensis*, where was observed a lower frequency but a broader geographical distribution (**Fig 4A and 4B**). These results vary regarding to what was observed until 2005 in urban areas, where *L. panamensis* was the species with the highest frequency of occurrence [14, 24] and *L. braziliensis* the species with broadest distribution.[14] Our results also allowed to determine not only the high intra-specific genetic variability of *L. braziliensis* and *L. panamensis* in the Colombian army population but also to observe how these genotypes/haplotypes are being distributed at the geographic level. One of the main reasons for the geographic shift in species and genotypes of *Leishmania* in most of the Colombian territory is due to the armed conflict in our country, which has caused an increase in the phenomena of displacement of the population towards endemic areas as well as movement of armed groups to and from these areas which has changed the epidemiology of the disease and consequently the distribution of the species. One of the species in which this phenomenon has been clearly observed is *L. guyanensis*, which was a prevalent species on the shores of the Orinoco and Amazon rivers[30] and in recent years has been detected in two different habitats;

Both in the Andean region (Department of Tolima) [14] as in the Caribbean region (Department of Sucre) of the country.[24]

In conclusion, the results obtained from this study allowed to determine that currently five species of *Leishmania* are circulating in the areas where the Colombian army personnel are deployed, of which *L. braziliensis* is the species with the highest frequency of occurrence. We also determined the wide geographical distribution of these species in the national territory and identified that in some departments there is not only a high prevalence of cases but also more than one species of *Leishmania* is circulating at the same time. Likewise, this study allowed to identify the high intra-specific genetic variability of *L. braziliensis* and *L. panamensis* and how these genotypes are distributed at the geographic level.

Supporting information

S1 Fig. Distribution of *Leishmania* species at the municipal level. The diagram shows the number and percentage of cases in which each species of *Leishmania* was identified in the different municipalities.
(TIF)

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Author Contributions

Conceptualization: Luz H. Patino, Claudia Mendez, Juan David Ramírez.

Data curation: Luz H. Patino, Omaira Rodriguez, Juan David Ramírez.

Formal analysis: Luz H. Patino, Yanira Romero, Daniel Velandia, Maria Alvarado, Julie Pérez, Maria Clara Duque, Juan David Ramírez.

Funding acquisition: Claudia Mendez, Juan David Ramírez.

Investigation: Luz H. Patino, Claudia Mendez, Omaira Rodriguez, Yanira Romero, Daniel Velandia, Julie Pérez, Maria Clara Duque, Juan David Ramírez.

Methodology: Luz H. Patino, Claudia Mendez, Omaira Rodriguez, Yanira Romero, Daniel Velandia, Julie Pérez, Maria Clara Duque, Juan David Ramírez.

Project administration: Claudia Mendez.

Resources: Claudia Mendez.

Supervision: Claudia Mendez, Juan David Ramírez.

Validation: Juan David Ramírez.

Visualization: Juan David Ramírez.

Writing – original draft: Luz H. Patino, Maria Clara Duque, Juan David Ramírez.

Writing – review & editing: Luz H. Patino, Claudia Mendez, Omaira Rodriguez, Yanira Romero, Daniel Velandia, Maria Alvarado, Julie Pérez, Maria Clara Duque, Juan David Ramírez.

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Short communication

Description of *Leishmania* species among dogs and humans in Colombian Visceral Leishmaniasis outbreaks

Giovanny Herrera[a], Adriana Higuera[a], Luz Helena Patiño[a], Martha S. Ayala[b], Juan David Ramírez[a,*]

[a] Grupo de Investigaciones Microbiológicas-UR (GIMUR), Programa de Biología, Facultad de Ciencias Naturales y Matemáticas, Universidad del Rosario, Bogotá, Colombia
[b] Grupo de Parasitología, Instituto Nacional de Salud, Bogotá, Colombia

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ABSTRACT

We report the species detected in dogs and humans from outbreaks of visceral leishmaniasis in Colombia. In this study, 91 sera from patients (n = 38) and dogs (n = 53) diagnosed with visceral leishmaniasis using IFAT were analyzed to determine the causative species. DNA extraction, PCR amplification, DNA sequencing and species identification was performed. Results were obtained with 13 of the sera. A phylogenetic tree and a network of haplotypes were constructed. *Leishmania infantum chagasi* (11/13), *Leishmania braziliensis* (1/13) and *Leishmania amazonensis* (1/13) were identified as the circulating species and genetic variability in one of the *L. infantum chagasi* strains was demonstrated. This is the first study describing *Leishmania* species in outbreaks of visceral leishmaniasis in Colombia.

1. Introduction

Visceral leishmaniasis (VL) is the most serious and deadly clinical form of leishmaniasis. Leishmaniases are a set of neglected tropical diseases transmitted by vectors that affect humans and other mammals, causing lesions in the skin, mucous membranes and organs of the reticuloendothelial system (WHO, World Health Organization., 2010). VL is considered a public health problem in various regions of the world including South America, Asia and Africa and it is estimated that between 50 and 90 thousand new cases occur globally every year, with about 2 million people at risk of contracting the disease (Alvar et al., 2012). In the American continent, 3668 cases were reported between 2003 and 2008, of which 3481 of these cases occurred in Brazil, which is considered the only endemic country in the region (Alvar et al., 2012). In Colombia, 181 cases of VL were reported between 2008 and 2016 (Alvar et al., 2012; República de Colombia, 2014). However, it is estimated that this number may be even higher due to under-reporting and the difficulties in correctly diagnosing the disease in this country. Consequently, most cases captured by the health system and epidemiological surveillance correspond to outbreaks in particular endemic foci in the country (Alvar et al., 2012).

In recent years, there has been an increase in the number of cases of VL in urban areas, where the presence of different vectors (sandflies) and reservoirs (opossums) have facilitated the transmission and emergence of outbreaks (Zambrano-Hernandez et al., 2015). Dogs (*Canis lupus familiaris*) also play a fundamental role in the urban transmission of VL. Dogs are a known reservoir of the parasite and are in close contact with humans. Seroprevalence rates between 3.8% and 50.2% have been detected in dogs in different areas of the country (Alvar et al., 2004; Romero et al., 2008). Nevertheless, serological tools are not able to discriminate *Leishmania* species and only molecular tools are available to do so. Molecular tools have been used to diagnose VL in humans and dogs with variable values of sensitivity and specificity (Fissore et al., 2004; Bagues et al., 2018). Therefore, during outbreaks, health authorities carry out sampling of patients and all the possible reservoirs that may be facilitating the appearance of VL (República de Colombia, 2014). However, routine surveillance of dogs is not carried out in Colombia and therefore their role in the infection remains to be fully elucidated.

Several species of *Leishmania* have been associated with VL, mainly species belonging to the *Leishmania donovani* complex (Chappuis et al., 2007). However, it is believed that any species that causes cutaneous leishmaniasis can invade the organs of the reticuloendothelial system, depending on the immune status of the infected individual (Ready, 2014). Several studies have identified *Leishmania braziliensis* and *Leishmania amazonensis* as causative agents of VL in canines and felines, which demonstrated that species other than the so-called viscerotropic species can cause disease (Carvalho et al., 2015; Tolezano et al., 2007). This has been reported only in Brazil, a country considered endemic for VL, but there are not reports available in non-endemic countries in

* Corresponding author.
E-mail address: juand.ramirez@urosario.edu.co (J.D. Ramírez).

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Latin America. In Colombia, no studies have been reported to date on the species involved in VL in any human or animal host, and subsequently the genetic variability and resistance profiles of *Leishmania* species also remain unknown in our country.

The objective of the present investigation was to use molecular methods in serum samples to determine the species involved in VL among dogs and humans in different outbreaks of VL in Colombia using conventional PCR and direct DNA sequencing of *Leishmania* positive serum samples.

2. Materials and methods

2.1. Sample collection

Ninety-one serum samples were obtained from humans and dogs from different outbreaks of VL in Colombia. A set of 53 corresponded to sera obtained from dogs and the remaining 38 from humans from six different geographical regions across Colombia that were collected between 2006 and 2016 by the Instituto Nacional de Salud as the national reference laboratory and maintained on its biobank.

The samples were submitted to an in house Indirect Immunofluorescence Assay Test (IFAT) as described in the Manual of the World Health Organization and the manual of the World Organization for Animal Health. The antigen was prepared from promastigotes of *L. infantum* from Instituto Nacional de Salud (Bogotá, Colombia). Anti-*Leishmania* antibodies were detected using anti-human and anti-dog IgG conjugated to fluorescein isothiocyanate (Sigma-Aldrich, USA). Samples were classified as positive if promastigote cytoplasmatic or membrane fluorescence was observed at a serum dilution of 1/32 or higher (IFAT In – House) (Instituto Nacional de Salud, 2016).

2.2. Ethical statement

The samples used in the present study belong to the cryobank of the Instituto Nacional de Salud, which is the National Reference Laboratory in Colombia, where the mandatory notification of diseases (VL is one of them) are confirmed. The identity of the patients was protected using codifications for the samples. The use of the samples was authorized by the Grupo de Parasitologia from the Instituto National de Salud responsibly for the custody of serum specimens.

2.3. DNA extraction, PCR amplification and Leishmania species identification

The 91 sera were subjected to DNA extraction using a High Pure PCR Template Preparation Kit ™ (Roche) following the manufacturer's protocol. Then, amplification of the gene encoding heat shock protein (HSP70) was performed using the primers HSP70F (5′ AGG TGA AGG CGA CGA ACG 3′) and HSP70R (5′ CGC TTG TCC ATC TTT GCG TC 3′) using the amplification conditions described by Patiño et al. (Patino et al., 2017). The amplified products were purified by EXOSAP (Affymetrix, USA) and sequenced by the dideoxy-terminal method, in an automated capillary sequencer (AB3730, Applied Biosystem). Further, the sequences were subjected to similarity analysis with *Leishmania* sequences deposited in the GenBank database using BLASTn (Reference strains used: JX312712.1 *L. donovani*, FN395037.1 *L. chagasi*, XM 003392632.1 *L. infantum*, FN395030.1 *L. archibaldi*, HF586411.1 *L. aethiopica*, HF586409.1 *L. tropica*, HF586403.1 *L. major*, HF586356.1 *L. turanica*, HF586355.1 *L. gerbili*, XM 003877072.1 *L. mexicana*, MF344859.1 *L. amazonensis*, EU599092.1 *L. garnhami*, KP244368.1 *L. martiniquensis*, JX852709.1 *L.* sp. *siamensis*, FN395049.2 *L. lainsoni*, MF344844.1 *L. braziliensis*, HF586368.1 *L. peruviana*, FN395055.1 *L. panamensis*, EU599093.1 *L. guyanensis*). Finally, a distribution map of the identified species was constructed and compared with the Alvar et al. (2012) report using the ArcGis Pro 2.0.0 program (https://pro.arcgis.com/es/pro-app/).

2.4. Phylogenetic reconstruction and determination of the haplotype network

The sequences obtained were edited using the MEGA 7.0 software and aligned with the built-in option MUSCLE in the same software using reference sequences for the HSP70 gene from *Leishmania* species. Subsequently, a phylogenetic tree was constructed using the maximum likelihood method with 1000 replicates using the same software. Finally, to determine the number of haplotypes in the population, a Fasta matrix was constructed for haplotype network analysis using the DNA alignment software (available at http://www.fluxus-engineering.com/align.htm) and a median-joining model based on 1000 iterations with default parameters using the Network 5.0 software (available at http://www.fluxus-engineering.com/sharenet.htm; Bandelt et al., 1999).

3. Results

All the 91 samples were positive for IFAT highlighting the presence of anti-*Leishmania* antibodies. PCR amplification was observed only in 13 (14.3%) of the 91 samples and the species involved in the infection could be correctly determined by BLAST. Eleven samples (85%) were identified as *L. infantum*, one sample (7.5%) corresponded to *L. amazonensis* and the remaining sample was identified as *L. braziliensis*. Seven (54%) of the samples came from the department of Bolívar, two (15%) from Huila and the remaining four came from the departments of Caldas, Sucre, Córdoba and Putumayo (Table 1, Supplementary Fig. 1). The phylogenetic tree shows the grouping of the samples with the reference strains. Three major groups were identified, two of which belonged to the subgenus *Leishmania* (*L. donovani* and *L. mexicana* complexes) and the other to the subgenus *Viannia* (Fig. 1a). Haplotype analysis revealed four different haplotypes. One of the samples identified as *L. infantum* (sample code 14156) formed a different haplotype to that of organisms from the same species (Fig. 1b).

4. Discussion and conclusions

In the present investigation, the *Leishmania* parasites were detected from sera samples from different humans that showed hepatomegaly as consequence of VL. In the dogs, a veterinary assessment showed the presence of clinical signs at physical examination and/or clinicopathologic abnormalities consistent with clinical leishmaniasis. These clinical manifestations clearly depict the status of sick individuals (Dogs and humans) showing that the found species could be agents of VL in the country (Table 1) but further studies are required to confirm this premise. On this sense, those species commonly associated with Cutaneous Leishmaniasis, have been described as causative agents of VL in other studies conducted in canines in Brazil, where several species of *Leishmania* have been found to cause infections in these animals, specially *L. amazonensis* that has been recently marked as an important causative specie of VL in dogs, which due to their close contact with the human population has favored the dispersion and urbanization of the disease (Alvar et al., 2004; Carvalho et al., 2015; Tolezano et al., 2007; Valdivia et al., 2017). The samples used in this study were collected from departments within regions characterized as endemic foci for VL (Frequent presence of outbreaks). It should be noted that the department of Bolivar has the highest incidence of VL in the country, as described by Alvar et al. (2012), which explains the concentration in this area of most of the samples used in this investigation (Table 1, Supplementary file 1). It is important to mention that Ferro et al. in 2015 reported the presence of different *Leishmania* vectors on the departments where the samples were collected, and estimates its probability of expansion to other zones of the country, that could increase the risk of spread of the disease to contiguous departments (Ferro et al., 2015).

Table 1
Source of samples and species identified.

Sample code	Source[a]	Geographical provenance	Serological titers	Species
1521	H	Bolívar	1/32	*L. infantum*
1525	H	Bolívar	1/32	*L. infantum*
1610	D	Bolívar	1/64	*L. infantum*
1617	H	Bolívar	1/128	*L. infantum*
1620	H	Caldas	1/256	*L. infantum*
1628	H	Bolívar	1/128	*L. infantum*
1630	H	Bolívar	1/128	*L. infantum*
1652	H	Sucre	1/256	*L. infantum*
14083	H	Córdoba	1/128	*L. infantum*
14127	H	Putumayo	1/64	*L. infantum*
14156	H	Bolívar	1/64	*L. infantum*
1689	D	Huila	1/32	*L. braziliensis*
1695	D	Huila	1/32	*L. amazonensis*

The table presents the source and geographical origin of the samples, as well as the titers of the IFAT and the identified species.
[a] (H: Human, D: Dog).

These elements are congruent with the occurrence of an epizootic cycle of transmission and highlights the importance of dogs as true reservoirs of VL in Colombia.

It is important to mention that the small number of samples with a positive result by PCR could be due to low parasite loads on the patient's serum or the low number of copies of the HSP70 gene used as amplification target (Drini et al., 2016). Other studies have demonstrated the usefulness of the PCR for the detection of *Leishmania* in VL cases, but those reports have used another molecular target as kinetoplastid DNA or different samples as spleen for parasite detection (Fissore et al., 2004; Bagues et al., 2018). In contrast, all samples were positive by IFAT, that is the method used by the National Reference Laboratory to confirm the diagnosis of VL in Colombia. This is an in-house method that allow the detection of antibodies using the crude extract from promastigotes of *L. infantum,* and has demonstrated good sensitivity and specificity values in humans and dogs samples (Camargo et al., 2010).

At the intraspecies level, the haplotype network constructed in this study demonstrated the variability among *L. infantum* strains circulating in Colombia (Fig. 1b). The clustering showed by the samples identified as *L. infantum* could be biased because the reduced genetic diversity of the HSP70 gene could not allow to discriminate the true intraspecies differences as described elsewhere (Nemati et al., 2017). However, this variability has also been demonstrated in other studies carried out in Tunisia, Algeria and Brazil, highlighting the importance of analyzing genetic variants to better understand the dissemination and epidemiology of the disease (Amro et al., 2013; Carvalho et al., 2017). In this sense, Multilocus Sequence Typing (MLST) analyses can be a valuable tool for further epidemiological surveillance as previously reported in our country with *L. panamensis* and *L. braziliensis* (Herrera et al., 2017).

This is the first study to explore the species involved in VL in Colombia. It was possible to demonstrate that there are different species associated to clinical cases of the disease in this country, as well as the possible variability that exists within the circulating species of *L. infantum*. These findings are novel in the country and contribute to the understanding of VL in South America. Despite the low sensitivity of the PCR compared to the serology, the findings across this low set of

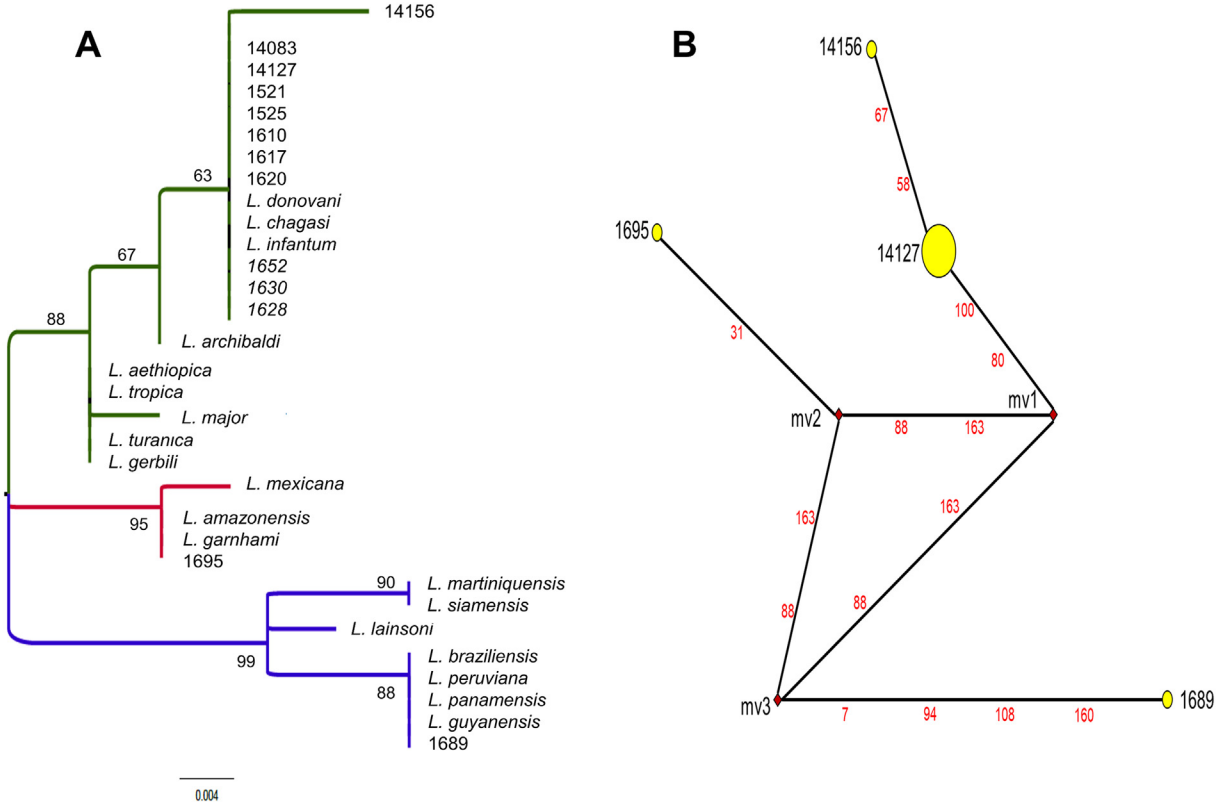

Fig. 1. Phylogenetic tree and network of the samples tested. A) Phylogenetic tree of the samples and reference strains. The tree shows the grouping of the species into three groups: the green branches represent species of the *L. donovani* complex, the red branches represent species of the *L. mexicana* complex and the blue branches represent samples of the subgenus *Viannia*. B) Haplotype network. The network shows the genetic variability of one of the samples identified as *L. infantum* (Sample code 14156), as well as the differentiation of the strains with other species. The red numbers represent the nucleotide positions where the changes among sequences are present. mv: median vector. (mv is a hypothesized (often ancestral) sequence which is required to connect existing sequences within the network with maximum parsimony. Without the median vector, there would be no shortest connection between the data set's sequences). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

samples is pivotal to have a comprehensive picture of VL in the country as well as the understanding of dogs as true reservoirs of VL. Our results highlight the need for further studies on both the epidemiology of VL and the genetic variability of circulating *Leishmania* species in the national territory to strengthen surveillance networks in public health and establish adequate policies for the control of the disease.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2018.06.023.

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8.2 CAPITULO 2

Diferencias genómicas y transcriptómicas de cepas de referencia de *Leishmania (Viannia) panamensis* y *Leishmania (Viannia) braziliensis*, sensibles y con resistencia inducida al antimoniato de N-metil glucamina (Glucantime®)

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Major changes in chromosomal somy, gene expression and gene dosage driven by Sb^{III} in *Leishmania braziliensis* and *Leishmania panamensis*

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Luz H. Patino[1], Hideo Imamura[2], Lissa Cruz-Saavedra[1], Paula Pavia[3], Carlos Muskus[4], Claudia Méndez[5], Jean Claude Dujardin[2,6] & Juan David Ramírez[1]

Leishmania braziliensis **and** ***Leishmania panamensis*** **are two species clinically and epidemiologically important, among others because of their relative resistance to first-line drugs (antimonials). The precise mechanism underlying the ability of these species to survive antimony treatment remains unknown. Therefore, elucidating the pathways mediating drug resistance is essential. We herein experimentally selected resistance to trivalent antimony (Sb^{III}) in the reference strains of** ***L. braziliensis*** **(MHOM/BR75/M2904) and** ***L. panamensis*** **(MHOM/COL/81L13) and compared whole genome and transcriptome alterations in the culture promastigote stage. The results allowed us to identify differences in somy, copy number variations in some genes related to antimony resistance and large-scale copy number variations (deletions and duplications) in chromosomes with no somy changes. We found mainly in** ***L. braziliensis*****, a direct relation between the chromosomal/local copy number variation and the gene expression. We identified differentially expressed genes in the resistant lines that are involved in antimony resistance, virulence, and vital biological processes in parasites. The results of this study may be useful for characterizing the genetic mechanisms of these** ***Leishmania*** **species under antimonial pressure, and for clarifying why the parasites are resistant to first-line drug treatments.**

Leishmaniases are a group of parasitic diseases caused by Protozoa belonging to the genus *Leishmania*[1,2]. These diseases are characterized by a broad spectrum of clinical manifestations, including cutaneous leishmaniasis (CL), mucosal leishmaniasis (ML), and visceral leishmaniasis (VL)[3]. Being endemic in 87 countries, CL is the most common form of leishmaniasis with an estimated of 0.6–1.0 million cases annually[4]. These clinical manifestations have been associated with diverse *Leishmania* species, including Old World species such as *L. major*, *L. tropica*, *L. aethiopica* and *L. infantum*[5,6]; and New World species such as, *L. braziliensis*, *L. amazonensis L. mexicana*, *L. panamensis* and *L. guyanensis*[7–10].

L. braziliensis and *L. panamensis* are very important in clinical and epidemiological terms, not only because these species are widely distributed in Latin America[7,11] but also because infections by these species, especially *L. braziliensis*, have a substantially greater potential to manifest as ML[12,13], and for the relatively unresponsiveness to first-line drugs [e.g., antimonials like sodium stibogluconate (SSG)]. Previous studies revealed that in some Latin American countries (e.g., Brazil, Peru, Guatemala, and Colombia), the therapeutic failure rates are between 25–40% for patients with CL caused by *L. braziliensis*[14–16] and 15–31% for patients with CL caused by *L.*

[1]Grupo de Investigaciones Microbiológicas-UR (GIMUR), Programa de Biología, Facultad de Ciencias Naturales y Matemáticas, Universidad del Rosario, Bogotá, Colombia. [2]Molecular Parasitology Unit, Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium. [3]Unidad de Investigación Científica, Subdirección de Docencia e Investigación, Hospital Militar Central, Bogotá, Colombia. [4]Programa de Estudio y Control de Enfermedades Tropicales (PECET), Facultad de Medicina, Universidad de Antioquia, Medellín, Colombia. [5]Dirección de Sanidad Militar, Ejercito Nacional de Colombia, Bogotá, Colombia. [6]Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium. Correspondence and requests for materials should be addressed to J.D.R. (email: juand.ramirez@urosario.edu.co)

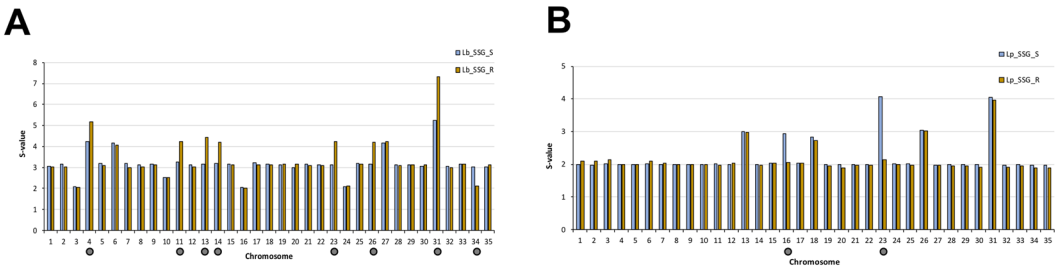

Figure 1. Dynamics of ploidy in *L. braziliensis* and *L. panamensis*. Comparisons of the chromosomal copy number between lines sensitive and resistant to Sb^{III} in *L. braziliensis* (left) and *L. panamensis* (right). The grey points indicate the chromosomes that underwent a change in somy.

panamensis[10,17–19]. However, numerous factors influence the final therapeutic outcome of antimonial treatments, related to the host, the drug and the parasite[20,21]. Moreover, the biological features of parasites might play an important role in this process[21–24].

To date, various approaches have been applied, including next generation sequencing techniques (genomics, transcriptomics), proteomic and metabolomic analysis, to characterize and establish the relationship between some *Leishmania* species and the mechanisms underlying antimony resistance[25–39]. These studies have demonstrated that under drug pressure and in the absence of transcriptional regulation, *Leishmania* uses several adaptive mechanisms to modulate the gene dosage of therapeutic targets or other determinants of resistance, such as the generation of episomal amplicons[34], aneuploidy and/or local gene copy number variation (CNV)[32,33,40], and single-nucleotide polymorphisms (SNPs) in drug targets or transporters[35].

These adaptive mechanisms have been mainly studied in Old world species, such as *L. donovani*[35,41], *L. tropica*[42] and *L. infantum*[27,28]. Comparative genomic and transcriptomic analysis (DNA-seq and RNA-seq) of lines sensitive and resistant to trivalent antimony (Sb^{III}) have revealed that these species alter the copy number of particular genes either by a local copy number variation or by modulating a copy number of the whole chromosome[25,42]. These copy number variations affected specific genes like genes encoding transport proteins, such as ABC transporter MRPA[34,42], aquaglyceroporin 1 (AQP1)[28,35]; genes encoding proteins essential for virulence, such as amastin and GP63[37,38]; or genes encoding proteins associated with the trypanothione biosynthesis pathway, such as gamma-glutamylcysteine synthetase[38].

Despite the advances that have enabled to characterize the mechanisms underlying the drug resistance of *Leishmania* species, the genome and transcriptome level changes occurring in New World *Leishmania* parasitic species (mainly in the subgenus *Viannia*) in response to stresses (e.g., drug treatments) remain not well characterized. Therefore, in this study, we performed comparative genomic and transcriptomic analyses of the experimentally selected Sb^{III} resistant strain and wild type sensitive strain of two of the main clinically and epidemiologically important *Leishmania* species in Latin America, namely *L. braziliensis* and *L. panamensis*.

Results

Induction of Sb^{III} resistance in *L. braziliensis* and *L. panamensis* lines. Initially, we selected *in vitro* populations of *L. braziliensis* and *L. panamensis* that were resistant to Sb^{III}. The selection process was initiated in quadruplicates, starting with 1.5 and 1.8 μg/mL Sb^{III} for *L. braziliensis* and *L. panamensis* respectively, with six rounds of selection to each species as described in materials and methods. The selection dynamics for both species was similar; for *L. braziliensis*, two replicates did not survive the second (3 μg/mL Sb^{III}) and fourth (3 μg/mL Sb^{III}) rounds of selection, respectively and two replicates were successfully selected to survive the highest Sb^{III} concentration (48 μg/mL). In *L. panamensis* the behavior was similar, two replicates did not survive the third (7.2 μg/mL Sb^{III}) and fifth (28.8 μg/mL Sb^{III}) rounds of selection, respectively and two replicates were successfully selected to survive the highest Sb^{III} concentration (57.6 μg/mL). When we evaluated the time of resistance, which was defined as the time needed for each line to display a similar growth curve compared to the parental line in the presence of 48 μg/mL Sb^{III} for *L. braziliensis*, and 57,6 μg/mL Sb^{III} for *L. panamensis*. We observed that the time estimated of resistance for *L. braziliensis* was 14 weeks and for *L. panamensis* was 18 weeks. Finally, we evaluated the stability of the resistance phenotype, in which all the Sb^{III}-resistant lines were maintained for 4 weeks in medium without Sb^{III}. The results obtained demonstrated that the index of resistance of each line remained, suggesting that the *in vitro* selected drug resistance phenotype was stable.

Chromosome/gene copy number variations. *Chromosome copy number variations.* For each chromosome, the median somy value S within each sample, its corresponding median absolute deviation across reads of that chromosome, and the statistical significance of differences in S values between samples were calculated as described in materials and methods. When we compared the somy levels in the SSG_R and SSG_S lines, we observed that the S values of eight chromosomes in the Lb_SSG_R line changed regarding to Lb_SSG_S line. In seven chromosomes, the S values increased significantly: chromosomes 11, 13, 14, 23, and 26 (trisomic to tetrasomic), chromosome 4 (tetrasomic to pentasomic), and chromosome 31 (pentasomic to heptasomic); and in one chromosome: chromosomes 34 the S values decreased (trisomic to disomic), the rest of the karyotype remained unchanged (trisomic) (Fig. 1A). In the Lp_SSG_R line, we observed that the S values of chromosomes 16 and 23 decreased significantly: chromosome 16 (trisomic to disomic) and chromosome 23 (tetrasomic to disomic), and

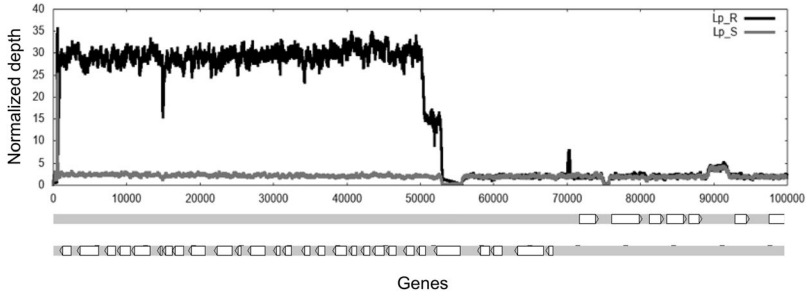

Figure 2. Intrachromosomal duplication in Sb^{III}-resistant *L. panamensis* mutants. Raw read depth for chromosome 27 of the Sb^{III}-resistant and -sensitive lines. The black and gray lines show the raw depth in Lp_SSG_S and Lp_SSG_R lines, respectively. The figure below is a representation of genes located in this region.

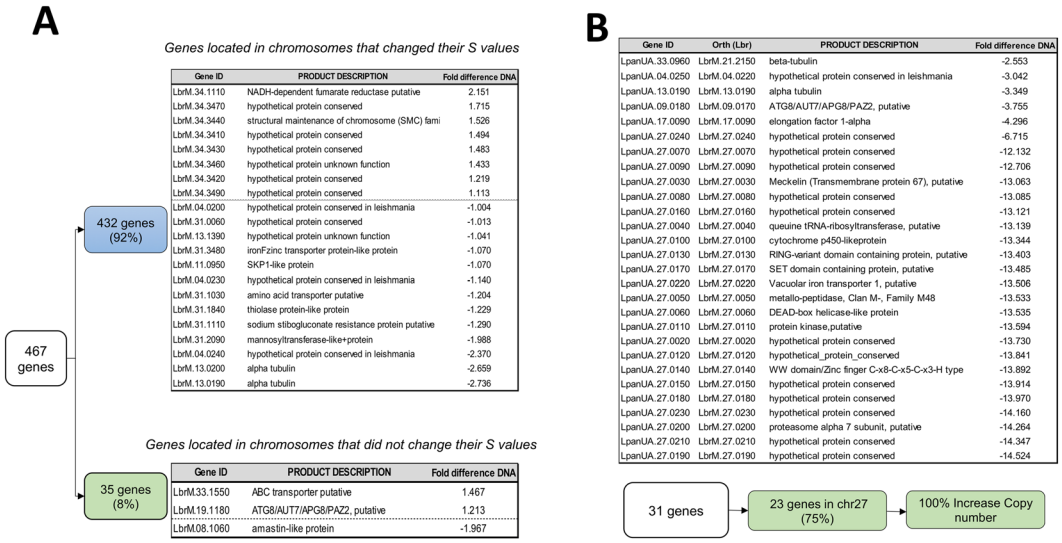

Figure 3. Gene copy number variation between sensitive and resistant lines of *L. braziliensis* and *L. panamensis*. Selection of genes with different copy numbers in the SSG_S and SSG_R lines (difference >1.0) in (**A**) *L. braziliensis* and (**B**) *L. panamensis*. The genes in *L. panamensis* were listed along with their corresponding *L. braziliensis* ortholog genes.

the rest of the karyotype remained unchanged (disomic) (Fig. 1B). Additionally, a uniform increase in read depth was observed for the first 50,000 bp of chromosome 27 in the Lp_SSG_R line (Fig. 2). This concerned 23 genes that were amplified between 8–19 times more regarding to Lp_SSG_S line (Supplementary Table S1).

The S-Values of *L. braziliensis* and *L. panamensis* were consistent with the somy values predicted based on the alternative allele frequency profile. The allele frequency counts for each predicted heterozygous SNP did not exhibit a discordance between read depth and allele frequencies, confirming the accuracy of the previously described somy profiles.

Gene copy number variation (CNV). We examined the gene CNVs between the SSG_S and SSG_R lines and found that in *L. braziliensis*, the changes in the chromosome copy number matched the gene copy numbers for the same chromosomes, as expected. Specifically, a total of 467 genes presented CNV between the Lb_SSG_S and Lb_SSG_R lines (Z score >2, equivalent to $p < 0.05$). Of these genes, 432 (92%) were located on eight chromosomes whose somy had changed, whereas the remaining 35 genes (8%) were located on chromosomes whose somy remained the same (Supplementary Table S2). After applying a stricter difference cut off (gene depth difference between Lb_SSG_R and Lb_SSG_S greater than 1.0), we detected in the chromosomes with change in the somy, 13 genes with higher copy number and 8 genes with lower copy number in the Lb_SSG_R than in the Lb_SSG_S line (Fig. 3A), likewise, 3 genes with variation in the copy number between both lines in the chromosomes without change in the somy (Fig. 3A).

Most of the genes with a lower copy number in the Lb_SSG_R line were annotated as hypothetical proteins. Of the genes with a higher copy number in the Lb_SSG_R line, the following five genes appeared functionally intriguing: two genes encoding an alpha-tubulin, with three extra copies (LbrM.13.0190 and LbrM.13.0200), two genes encoding transporter proteins, with one extra copy (amino acid transporter: LbrM.31.1030 and iron-zinc

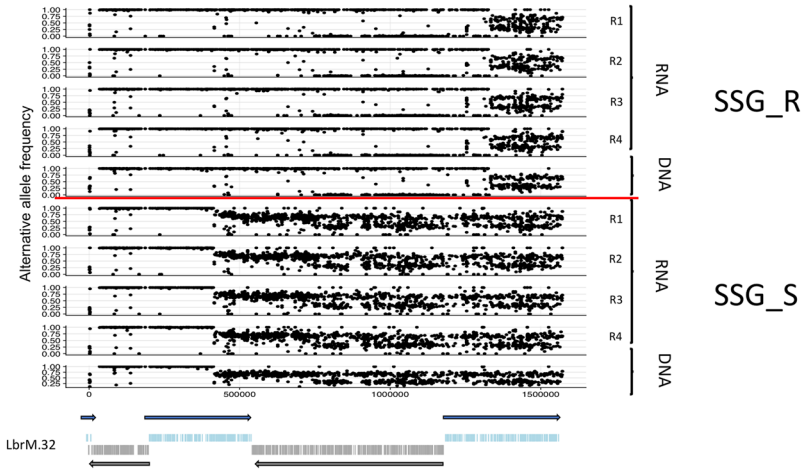

Figure 4. Single nucleotide polymorphism (SNPs) in the chromosome 32 of *L. braziliensis* sensitive and resistant to Sb^{III}. The figure shows the SNPs (DNA/RNA) found in the chromosome 32, comparing the resistant and sensitive lines to Sb^{III}. The figure below is a representation of the gene transcription in chr 32.

Gene ID	a	b	c	d	Product description	Lb_SSG_S DNA	Lb_SSG_R DNA	Lb_SSG_S RNA	Lb_SSG_R RNA
LbrM.32.2610	5642	C/G	Glu-Gln	308	aquaporin-like protein	0.326	0.000	0.3838	0.0025
LbrM.32.3540	51288	A/C	Gln-Pro	800	mitogen-activated protein kinase, putative	0.691	1.000	0.6630	0.9988
LbrM.32.3540	44169	G/T	Ala-Ser	792	mitogen-activated protein kinase, putative	0.680	1.000	0.6495	0.9958
LbrM.32.3540	44274	A/G	Lys-Arg	914	mitogen-activated protein kinase, putative	0.639	1.000	0.6440	0.9950
LbrM.32.2270	28477	T/C	ILe-Met	387	ABC transporter-like protein	0.557	1.000	0.7053	1.0000

Table 1. List of mainly SNPs found in the chromosome 32. [a]SNP position on the gene, [b]Change of nucleotide respect to reference (Ref/Alt), [c]Change in the amino acid and [d] SNP position on the protein.

transporter: LbrM.31.3480), and one gene encoding a protein associated with antimony resistance, with one extra copy (sodium stibogluconate resistance protein: LbrM.31.1110) (Fig. 3A).

In contrast, we detected fewer differences in the gene copy numbers per chromosome between the Lp_SSG_S and Lp_SSG_R lines. We observed that 31 genes had different copy numbers between the two lines (Z score >2, equivalent to $p < 0.05$). Of these 31 genes, 23 (74%) were located on chromosome 27, most strikingly, the copy number for many of the genes was more than 10 times higher in the Sb^{III}-resistant line than in the Sb^{III}-sensitive line (Fig. 3B). As for the potential mechanism of this amplification, it spanned between the base position between 1 and 53055 bp of chromosome 27, ending in a protein kinase gene (LpanUA.27.0110/LbrM 27.0110) that contains a gap in the middle. We identified 472 bases, for which there were 4 direct and 6 reverse paralogs. These repeats are not a part of a known transposon, but they may be potentially involved in the formation of this duplication.

Single nucleotide polymorphisms (SNPs). The comparison with the reference *L. braziliensis* sequence identified 35,878 SNPs in the Lb_SSG_S line and 35,689 SNPs in the Lb_SSG_R line. We evaluated the differences in the allele frequencies between the Lb_SSG_S and Lb_SSG_R lines and found that 176 heterozygous SNPs exhibited an allele frequency shift greater than 0.33 (Supplementary Table S3). Of these SNPs, remarkably 155 (88%) were located on chromosome 32, and the remaining 21 were located on other chromosomes. Of the SNPs on chromosome 32, we determined that 34 were present in the Lb_SSG_S line, but not in the Lb_SSG_R line (Fig. 4, Supplementary Table S3). Most of the SNPs found only in the Lb_SSG_S line were located in genes encoding hypothetical proteins, except one in a gene encoding an aquaporin-like protein (position 5642), leading to a change in the amino acid sequence (glutamine to glycine). Interestingly, this change was not observed in the Lb_SSG_R line (Table 1). Additionally, of SNPs on chromosome 32 detected in both Lb_SSG_S and Lb_SSG_R lines, four of them were located in genes encoding proteins associated with antimony resistance, one SNP in a gene encoding an ABC transporter protein (LbrM.32.2270) and the other three SNPs located in different positions of a gene encoding a mitogen-activated protein kinase (Table 1).

In contrast to *L. braziliensis*, we did not identify many SNPs in *L. panamensis* or no major differences in the allele frequency between the Lp_SSG_S and Lp_SSG_R lines. Notably, on the highly duplicated region of chromosome 27 of Lp_SSG_R line descibed above, a major shift in the allele frequency between the Lp_SSG_S and Lp_SSG_R lines were observed. Specifically, average allele frequency differences between the Lp_SSG_S and

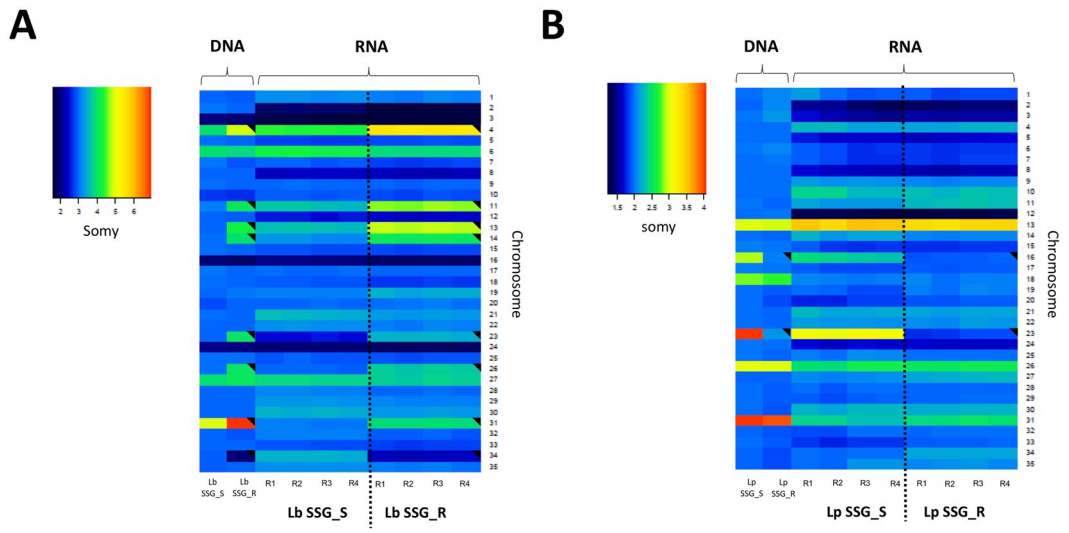

Figure 5. Relationship between chromosomal copy number variations and gene expression levels in the SSG_S and SSG_R lines of *L. braziliensis* and *L. panamensis*. The heatmaps show median normalized read depths of 35 chromosomes (y axis) found in *L. braziiensis* (Left) and *L. panamensis* (right) sensitive and resistant to Sb^{III} (x axis) The color key indicates the somy value (S), which ranged from 1 to 5 as follows: monosomy, $S < 1.5$; disomy, $1.5 \leq S < 2.5$; trisomy, $2.5 \leq S < 3.5$; tetrasomy, $3.5 \leq S < 4.5$; and pentasomy, $4.5 \leq S < 5$[40]. A black triangle in an upper right corner indicates a significant change in S value. R (replicates).

Lp_SSG_R lines were 0.38 ± 0.08 and 0.06 ± 0.07 on the 44 heterozygous SNPs on duplicated region and on the 214 heterozygous SNPs on the non-duplicated region, respectively, where a standard deviation was shown with $\pm$. The significance of separation of these differences was $p = 3.6 \times 10^{-96}$ measured by a two-sided t-test. Little or no indels were observed in both *L. braziliensis* and *L. panamensis*.

Finally, we constructed a neighbor-joining network to observe genetic distances based on the SNPs between the Sb^{III}-sensitive and -resistant lines, including RNA and DNA. For both species (Supplementary Fig. S1), the results demonstrated a clear separation between the Sb^{III}-sensitive and -resistant lines. The RNA samples showed higher base difference with respect to their corresponding DNA samples mainly due to their depth variation not transcriptional strand biases because these were not observed based on the alternative allele frequency data.

Relationships among the somy, gene dosage (CNV), and gene expression levels. For each chromosome, we computed the median transcript level and compared this with DNA-based S values. The results obtained allow us to observe that in Lb_SSG_R and Lp_SSG_R lines, all chromosomes that presented changes in S values showed alterations of RNA in the same direction (Fig. 5A,B). This demonstrates that most of the gene expression changes were concordant with the chromosomal somy changes. Later, we investigated the dependency of gene expression on somy by regression analysis, between the relative chromosomal somy and the average relative gene expression levels of the SSG_R and SSG_S lines. Regarding the Lb_SSG_R *vs* Lb_SSG_S comparison, we detected a significant correlation, with a Pearson correlation $R^2 = 0.911$ ($p = 6.13 \times 10^{-19}$) and a slope of 1.00. In contrast, for the Lp_SSG_R *vs* Lp_SSG_S comparison, the R^2 and slope were 0.885 ($p = 4.66 \times 10^{-17}$) and 0.97, respectively.

We also evaluated the impact of the gene CNVs on gene expression levels. In *L. braziliensis*, we observed that of the genes with different copy numbers between the Lb_SSG_S and Lb_SSG_R lines (difference >1.0), 54% of them were differentially expressed in the two lines (greater than 1.5-fold difference). The genes exhibiting upregulated expression in the Lb_SSG_R line were mainly located on chromosomes 4, 8, 11, 13, and 31, whereas the downregulated genes were mostly on chromosomes 34 and only one in chromosome 33 (Supplementary Fig. S2).

Among upregulated genes in the Lb_SSG_R line we highlight three of them whose differential expression was associated with the gene CNV: LbrM.31.3480 (an iron-zinc transporter like-protein), LbrM.08.1060 (amastin-like protein), and LbrM.13.0190 (alpha-tubulin). Among the downregulated genes in the Lb_SSG_R line, two of them were LbrM.34.1110 (putative NADH-dependent fumarate reductase) and LbrM.33.1550 (putative ABC transporter) (Supplementary Fig. S2).

Even though Sb^{III} did not substantially affect the local gene/chromosome CNVs in *L. panamensis*, we observed that among the 31 genes with different copy numbers between the Lp_SSG_S and Lp_SSG_R lines, 28 (90%) were differentially expressed (by more than 1.5-fold). Thirteen of these genes encode hypothetical proteins without a known function. The other 15 genes encode known proteins and interestingly, all these genes exhibited upregulated expression in the Lp_SSG_R line. Specifically, LpanUA.33.0960/LbrM.21.2150 (beta tubulin), LpanUA.13.0190/LbrM.13.0190 (alpha-tubulin) and LpanUA.27.0140/LbrM.27.0140 (WWW domain/zinc finger C-x8-C-x5-C-x3-H-type protein). Finally, we observed the latter gene presented an expression level 100-fold higher in the Sb^{III}-resistant line than in the Sb^{III}-sensitive line (Supplementary Fig. S2).

Although the transcription levels were concordant with the somies and with local CNV in most of the chromosomes, we observed that the transcription of some specific genes do not follow this overall trend. A potential mismatch between transcriptional level and the local CNV was observed (Supplementary Fig. S3).

Differentially expressed genes between SSG_S and SSG_R *L. braziliensis* and *L. panamensis* lines. The data for *L. braziliensis* revealed 844 differentially expressed genes between the SSG_S and SSG_R lines, among which 103 had a fold-change ≥2 in the Lb_SSG_R line, with 44 and 59 genes exhibiting upregulated and downregulated expression, respectively (Supplementary Table S4). In *L. panamensis*, 803 genes were differentially expressed, and 148 genes had a fold-change ≥2 in the Lp_SSG_R line, with 72 and 76 genes exhibiting upregulated and downregulated expression, respectively (Supplementary Table S5). A Venn diagram illustrated the number of significantly upregulated/downregulated genes and the number of differentially expressed genes in those two species (Fig. 6A). Moreover, the genes that were differentially expressed among the experimental lines were compared, and the log fold-changes in expression levels were calculated (Tables of Fig. 6B,C).

Among the differentially expressed genes, with high expression in the Lb_SSG_R line, we highlight those genes that encode transporter proteins and that have been described in previous studies. For example, a zinc transporter 3 (LbrM.28.2110), an iron/zinc transporter protein-like protein (LbrM.31.3490), and a putative pteridine transporter (LbrM.06.1250) (Fig. 6B and Supplementary Table S4). Two genes encode histone 4 (LbrM.35.0020 and LbrM.35.0070), one gene encodes a putative heat shock protein 90 (LbrM.29.0780), and one gene encodes a putative 60 S ribosomal protein L28 (LbrM.11.0910), whose function in the SSG-resistant *Leishmania* species is still unknown (Fig. 6B and Supplementary Table S4).

We also observed downregulated expression in the Lb_SSG_R line, including one gene encoding a putative superoxide dismutase (LbrM.32.2860), six genes encoding members of the amastin family (LbrM.08.0680, LbrM.18.0470, LbrM.24.1590, LbrM.20.4290, LbrM.20.4300, and LbrM.20.1080), five genes encoding transporter proteins, three of which representing a putative folate/biopterin transporter (LbrM.10.0380, LbrM.10.0370, and LbrM.34.5090) and two of which encoding a putative pteridine transporter and an ABC transporter (LbrM.10.1460 and LbrM.33.1550, respectively) (Fig. 6B and Supplementary Table S4).

A Gene Ontology enrichment analysis of the genes differentially expressed between *L. braziliensis* SSG_S and SSG_R lines (fold-change >2) indicated the enriched terms among the upregulated genes were mainly associated with phosphate ion transport. In contrast, the enriched terms among the genes exhibiting downregulated expression were mainly related to deoxynucleoside catabolism (Fig. 6B).

Although many of the differentially expressed genes in the *L. panamensis* lines encode hypothetical proteins with unknown function, some of these genes with significantly upregulated or downregulated expression are mainly associated with the mechanisms underlying antimony resistance that have been described in other *Leishmania* species. Here, *L. panamensis* genes were listed along with their corresponding *L. braziliensis* ortholog genes that were used for this Gene Ontology enrichment analysis since *L. panamensis* genes were not yet integrated in TritrypDB at the time of this analysis.

Of the genes that exhibited upregulated expression in the Lp_SSG_R line, four (LpanUA.27.0220/LbrM.27.0220, LpanUA.10.0410/LbrM.10.0370 LpanUA.14.1530/LbrM.14.1530, and LpanUA.27.1050/LbrM.02.0350) were annotated as encoding a putative vacuolar iron transporter 1, a putative folate/biopterin transporter, a pteridine transporter, and an ABC1 transporter, respectively. Additionally, we observed that LpanUA.27.0140/LbrM.27.0140, encoding a putative WW domain/zinc finger C-x8-C-x5-C-x3-H-type protein, had the highest CNV and was the most differentially expressed gene in the Sb^{III}-resistant line (4-fold higher expression in the Sb^{III}-resistant line than in the Sb^{III}-sensitive line) (Fig. 6C and Supplementary Table S5).

The genes that exhibited downregulated expression in the Sb^{III}-resistant line included LpanUA.23.0490/LbrM.23.0490 and LpanUA.23.1530/LbrM.23.0490, which respectively encode trypanothione synthetase and a succinate dehydrogenase-fumarate reductase iron-sulfur protein. The expression levels of these genes were approximately 2-fold lower in the Sb^{III}-resistant line than in the Sb^{III}-sensitive line (Fig. 6C and Supplementary Table S5).

Finally, the Gene Ontology enrichment analysis of *L. panamensis* revealed that among the genes exhibiting upregulated expression, the enriched terms were mainly associated with the regulation of the microtubule cytoskeleton organization, whereas the genes with downregulated expression were mainly enriched with terms associated with the anchoring of microtubules (Fig. 6C).

Discussion

This study is the first whole genome scale investigation of the impact of Sb^{III} on the genomes and transcriptomes of two of the most important *Leishmania* species in Latin America. We identified significant genomic and transcriptomic differences between the sensitive and resistant lines in both species. The main findings obtained in this study were: (i) Somy changes were observed in both species, being more prominent in the Lb_SSG_R line than in the Lp_SSG_R line. (ii) A high copy number variation affecting 23 genes was observed in the chromosome 27 of Lp_SSG_R line. (iii) The most striking SNPs changes were seen over the long middle section of chromosome 32. (iv) The transcriptional changes were mainly driven by the corresponding genomic copy number changes.

Among the chromosomes that presented somy change, we highlight the chromosomes 11, 13, 23, and 31 in the Lb_SSG_R line and the chromosome 23 in the Lp_SSG_R line due to that similar somy changes were observed in the same chromosomes of the Sb^{III}-resistant *L. guyanensis*[43], *L. infantum*[27], *L major*[28], and *L. donovani*[38] mutants. Although we could not conclude that the observed somy changes were directly responsible for the Sb^{III}-resistance of the subgenera/species, we believe that the gene dosage effects caused by chromosome copy number changes are likely critically linked to Sb^{III} resistance phenotype. Despite that the aneuploidy is usually lethal or result in severe abnormalities in some eukaryotes, in others such as *Candida albicans* or *Cryptococcus neoformans*[44] and in some trypanosomatids (*L. major*[45], *L. donovani*[41,45], *L. tropica*[45], and *T. cruzi*[46]), have been identified to be beneficial trait that provides survival advantages. Particularly aneuploidy has been shown to play a key role in environmental

A

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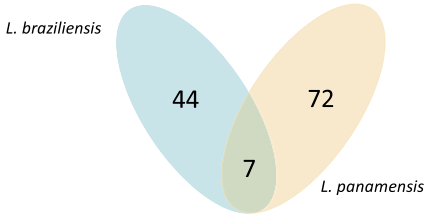

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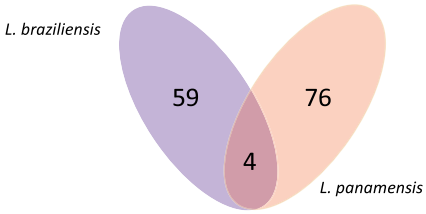
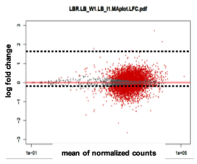
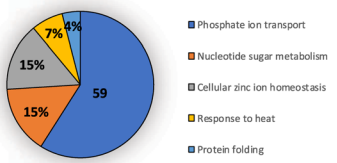


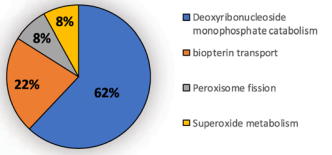
B

Gene ID	Product description	log2FoldChange (R/S)
LbrM.04.1250	Actin	1.038
LbrM.06.1250	pteridine transporter, putative	2.189
LbrM.09.0080	RNA-binding protein 5, putative	1.707
LbrM.10.0030	phosphate-Repressible Phosphate Permease-like protein	1.314
LbrM.10.1450	phosphate-Repressible Phosphate Permease-like protein	1.338
LbrM.10.tRNA2	tRNA-Asn	1.918
LbrM.11.0910	60S ribosomal protein L28, putative	2.060
LbrM.11.tRNA5	tRNA-Arg	1.440
LbrM.13.0270	ALBA-domain protein 1	1.026
LbrM.18.0770	citrate synthase, putative	1.301
LbrM.23.0120	GDP-mannose pyrophosphorylase	1.208
LbrM.23.0810	RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain), putative	1.122
LbrM.23.1350	Protein of unknown function (DUF3250), putative	1.029
LbrM.23.1420	lathosterol oxidase-like protein	1.407
LbrM.24.2310	hypothetical predicted multi-pass transmembrane protein	1.618
LbrM.26.0990	protein kinase, putative	1.168
LbrM.28.2110	Zinc transporter 3, putative	2.920
LbrM.29.0780	heat shock protein 90, putative	1.038
LbrM.30.1670	ferric reductase transmembrane protein, putative	1.106
LbrM.31.1400	cytochrome b5-like Heme/Steroid binding domain containing protein,	1.313
LbrM.31.1430	cytochrome b5-like Heme/Steroid binding domain containing protein,	1.084
LbrM.31.2940	calreticulin, putative	1.182
LbrM.31.3490	iron/zinc transporter protein-like protein	1.514
LbrM.31.tRNA1	tRNA-Ala	1.181
LbrM.31.tRNA2	tRNA-Gly	1.406
LbrM.31.tRNA3	tRNA-Glu	1.345
LbrM.31.tRNA4	tRNA-Phe	3.280
LbrM.33.tRNA4	transfer RNA-Lys	1.185
LbrM.35.0020	histone H4	1.017
LbrM.35.0060	phosphoglycan beta 1,3 galactosyltransferase 4	1.715
LbrM.35.0070	histone H4	2.977
LbrM.35.tRNA2	transfer RNA-Gly	2.989



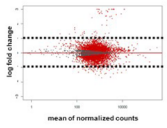
Gene ID	Product description	log2FoldChange (R/S)
LbrM.34.2480	Double RNA binding domain protein 9	-1.000
LbrM.34.2990	glycerol kinase, glycosomal, putative	-1.005
LbrM.34.5090	biopterin transporter, putative	-1.006
LbrM.33.1560	expression-site associated gene (ESAG3), putative	-1.014
LbrM.34.1420	NLI interacting factor-like phosphatase/Zinc finger C-x8-C-x5-C-x3-H ty	-1.020
LbrM.34.1650	dynein-associated roadblock protein-like protein	-1.023
LbrM.33.3550	delta-12 fatty acid desaturase	-1.030
LbrM.25.1020	Ribonuclease 3, putative	-1.031
LbrM.34.3670	Peroxisomal biogenesis factor 11 (PEX11), putative	-1.033
LbrM.27.0360	protein kinase, putative	-1.036
LbrM.34.1250	thioredoxin, putative	-1.037
LbrM.08.0680	amastin-like protein	-1.040
LbrM.34.0250	Protein of unknown function (DUF1242), putative	-1.058
LbrM.10.1460	pteridine transporter, putative	-1.084
LbrM.18.0470	amastin-like surface protein	-1.093
LbrM.34.4380	Snf7, putative	-1.093
LbrM.33.1550	ABC transporter, putative	-1.099
LbrM.34.5280	Dynein heavy chain and region D6 of dynein motor/Ankyrin repeats (3	-1.119
LbrM.34.3000	Dnaj domain containing protein, putative	-1.165
LbrM.24.1590	amastin-like surface protein-like protein	-1.168
LbrM.34.4480	anaphase promoting complex subunit protein, putative	-1.195
LbrM.10.0370	folate/biopterin transporter, putative	-1.206
LbrM.20.4290	amastin-like surface protein, putative	-1.216
LbrM.34.2310	Eukaryotic protein of unknown function (DUF1764), putative	-1.216
LbrM.34.1950	ankyrin repeat protein, putative	-1.241
LbrM.34.4250	Present in the outer mitochondrial membrane proteome 6	-1.256
LbrM.20.1050	tuzin-like protein	-1.268
LbrM.20.1460	ubiquitin-conjugating enzyme E2, putative	-1.281
LbrM.35.3820	cysteine synthase	-1.294
LbrM.34.3730	Cullin family, putative	-1.339
LbrM.20.1110	ubiquitin-conjugating enzyme E2, putative	-1.396
LbrM.34.snoRNA1	LM35C3C1	-1.425
LbrM.29.0510	thymine-7-hydroxylase, putative	-1.476
LbrM.23.1820	Nucleoside 2-deoxyribosyltransferase, putative	-1.523
LbrM.20.4300	amastin-like surface protein, putative	-1.534
LbrM.18.0010	TATE DNA Transposon	-1.796
LbrM.33.1000	beta-tubulin (pesudogene)	-1.974
LbrM.10.0380	folate/biopterin transporter, putative	-2.282
LbrM.20.1080	amastin-like surface protein, putative	-2.299
LbrM.30.snoRNA1	LM30C4C1A	-3.045
LbrM.32.2860	superoxide dismutase, putative	-5.870



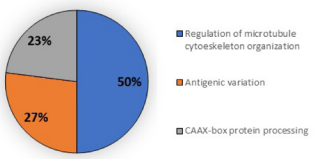


C

Gene ID	Orth (Lbr)	Product description	log2FoldChange (R/S)
LpanUA.07.1250	LbrM.07.0240	phosphoglycan beta 1,3 galactosyltransferase 1	2.645
LpanUA.09.0080	LbrM.09.0080	RNA-binding protein 5, putative	1.527
LpanUA.10.0410	LbrM.10.0370	folate/biopterin transporter, putative	1.396
LpanUA.13.0520	LbrM.13.0520	kinesin, putative	1.519
LpanUA.14.1530	LbrM.14.1530	pteridine transporter	1.255
LpanUA.15.0005	LbrM.07.0240	phosphoglycan beta 1,3 galactosyltransferase 1	2.564
LpanUA.17.0180	LbrM.17.0180	peptidase t, putative	1.339
LpanUA.20.0630	LbrM.20.0630	Uncharacterized conserved protein (DUF2039), putative	1.171
LpanUA.20.0960	LbrM.20.0960	amastin-like protein	1.584
LpanUA.20.2160	LbrM.20.2160	acyl-CoA binding protein, putative	1.167
LpanUA.20.2830	LbrM.20.2830	Alpha-keto-acid decarboxylase, putative	1.385
LpanUA.20.4110	LbrM.20.4110	Histone-lysine N-methyltransferase, H3 lysine-76 specific	1.034
LpanUA.21.0010	LbrM.07.0240	phosphoglycan beta 1,3 galactosyltransferase 1	2.569
LpanUA.26.1360	LbrM.26.1360	DNA ligase k alpha, putative	1.016
LpanUA.27.0080	LbrM.27.0080	Mecketin (Transmembrane protein 67), putative	3.058
LpanUA.27.0040	LbrM.27.0040	queuine tRNA-ribosyltransferase, putative	2.801
LpanUA.27.0050	LbrM.27.0050	metallo- peptidase, Clan M- Family M48	2.544
LpanUA.27.0060	LbrM.27.0060	DEAD-box helicase-like protein	3.725
LpanUA.27.0100	LbrM.27.0100	cytochrome p450-like protein	3.205
LpanUA.27.0110	LbrM.27.0110	protein kinase, putative	3.093
LpanUA.27.0130	LbrM.27.0130	RING-variant domain containing protein, putative	2.841
LpanUA.27.0140	LbrM.27.0140	WW domain/Zinc finger C-x8-C-x5-C-x3-H type (and similar), putative	3.607
LpanUA.27.0170	LbrM.27.0170	SET domain containing protein, putative	2.761
LpanUA.27.0200	LbrM.27.0200	proteasome alpha 7 subunit, putative	2.386
LpanUA.27.0220	LbrM.27.0220	Vacuolar iron transporter 1, putative	2.531
LpanUA.27.0990	LbrM.27.0990	Pep3/Vps18/deep orange family/Region in Clathrin and VPS, putative	1.344
LpanUA.27.1000	LbrM.27.1000	Protein of unknown function (DUF1295), putative	1.258
LpanUA.27.1050	LbrM.02.0350	ABC1 transporter, putative	1.099
LpanUA.29.0510	LbrM.29.0510	thymine-7-hydroxylase, putative	1.163
LpanUA.29.1440	LbrM.29.1440	RNA-binding protein, putative	1.068
LpanUA.30.2040	LbrM.30.2040	alcohol dehydrogenase, putative	1.324
LpanUA.31.1400	LbrM.31.1400	cytochrome b5-like Heme/Steroid binding domain containing protein, putative	1.969
LpanUA.31.3010	LbrM.31.3010	glutamamyl carboxypeptidase (pseudogene), putative	1.494
LpanUA.31.3070	LbrM.31.3070	Regulator of chromosome condensation (RCC1) repeat, putative	2.116
LpanUA.33.1720	LbrM.33.1720	RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain), putative	1.582
LpanUA.33.2020	LbrM.33.2020	macrophage migration inhibitory factor-like protein	1.283
LpanUA.34.1091	LbrM.27.0360	protein kinase, putative	1.045
LpanUA.35.6110	LbrM.35.6110	Nitroreductase family, putative	2.278



Gene ID	Orth (Lbr)	Product description	log2FoldChange (R/S)
LpanUA.23.0620	LbrM.23.0620	oxidoreductase-like protein	-1.004
LpanUA.23.1530	LbrM.23.1530	succinate dehydrogenase and fumarate reductase	-1.006
LpanUA.23.0490	LbrM.23.0490	trypanothione synthetase, putative	-1.007
LpanUA.23.1160	LbrM.23.1160	HIT domain containing protein, putative	-1.015
LpanUA.13.0150	LbrM.13.0150	protein kinase A regulatory subunit	-1.019
LpanUA.30.0030	LbrM.30.0030	cyclophilin 9, putative	-1.024
LpanUA.23.0621	LbrM.23.0621	unspecified product	-1.027
LpanUA.23.1440	LbrM.23.1440	Ycel-like domain containing protein, putative	-1.036
LpanUA.23.1540	LbrM.23.1540	palmitoyl acyltransferase 12, putative	-1.040
LpanUA.13.0870	LbrM.13.0870	Dpy-30 motif containing protein, putative	-1.043
LpanUA.23.0560	LbrM.23.0560	SET domain containing protein, putative	-1.044
LpanUA.23.0570	LbrM.23.0570	ribosomal RNA methyltransferase-like protein	-1.048
LpanUA.23.0520	LbrM.23.0520	5'-AMP-activated protein kinase subunit beta, putative	-1.050
LpanUA.23.1150	LbrM.23.1150	5-formyltetrahydrofolate cyclo-ligase family, putative	-1.053
LpanUA.23.1570	LbrM.23.1570	inositol hexakisphosphate, putative	-1.058
LpanUA.16.1430	LbrM.16.1430	small GTPase, putative	-1.065
LpanUA.26.1590	LbrM.26.1590	thimet oligopeptidase, putative	-1.070
LpanUA.23.0580	LbrM.23.0580	acetyl-CoA synthetase, putative	-1.073
LpanUA.16.0620	LbrM.16.0620	meiotic recombination protein SPO11, putative	-1.082
LpanUA.23.0720	LbrM.23.0720	MYCBP-associated protein family, putative	-1.102
LpanUA.24.2060	LbrM.24.2060	STOP axonemal protein, putative	-1.110
LpanUA.16.0030	LbrM.16.0030	AAA domain containing protein, putative	-1.118
LpanUA.23.1830	LbrM.23.1830	histone H4	-1.167
LpanUA.23.0690	LbrM.23.0690	PAP2 superfamily, putative	-1.184
LpanUA.10.1380	LbrM.10.1380	Protein of unknown function (DUF1861), putative	-1.203
LpanUA.23.1690	LbrM.23.1690	Tumour suppressor, Mitostatin, putative	-1.229
LpanUA.10.0180	LbrM.10.0180	leucine-rich repeat protein, putative	-1.292
LpanUA.23.1600	LbrM.23.1600	Meckel syndrome type 1 protein, putative (pseudogene)	-1.314
LpanUA.23.0810	LbrM.23.0810	RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain), putative	-1.329
LpanUA.29.2890	LbrM.29.2890	RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain), putative	-1.345
LpanUA.26.2500	LbrM.26.2500	protein kinase, putative	-1.367
LpanUA.23.1820	LbrM.23.1820	Nucleoside 2-deoxyribosyltransferase, putative	-1.430
LpanUA.23.1420	LbrM.23.1420	lathosterol oxidase-like protein	-1.494



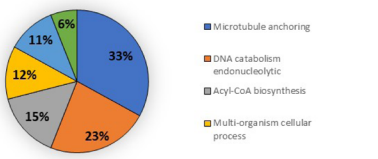


Figure 6. Transcriptional profile of the SSG_R and SSG_S lines in *L. braziliensis* and *L. panamensis*. (**A**) Venn diagram showing transcripts down- and up-regulated more than 2-fold in the resistant lines regarding the expression levels found in the parental sensitive line. The number of transcripts having significantly altered levels (up and down regulated) and the number of transcripts with DE when both species were compared (middle of each diagram) (**B**,**C**) Differentially expressed transcripts between the experimental conditions. B *(L. braziliensis*). C (*L. panamensis*). The graphic in the middle represents the MA plot constructed based on the DESeq2 results. Red points indicate significance at a 10% adjusted p-value, up-regulation and down-regulation respectively. Grey triangles indicate transcripts that showed no change. The lower figures present the results of the Gene Ontology enrichment analyses for biological process of up (left) and down (right) regulated transcripts. The transcripts included had an expression value higher than 2-fold change in both comparisons. The number of transcripts with a GO term is indicated in the corresponding pie slice.

adaptation[41], virulence[47] and acquisition of Sb^{III} drug resistance[32,34]. Thus, we believe that the gene dosage effects caused by chromosome copy number changes facilitate the acquisition of Sb^{III} resistance phenotype.

A second major genomic change observed in our experimental lines that may be associated with the adaptation of the species to Sb^{III} was related to local CNVs, which were not affected by somy changes. Specifically, the amplified genes in the Lb_SSG_R line and the telomeric amplification of more than 10 fold, involving 23 genes for chromosome 27, in Lp_SSG_R line (Fig. 2) confirmed the results previously reported[43,48,49]. This last finding observed in the Lp_SSG_R line results were interesting, since *Leishmania* subgenus *Viannia* species have never been observed to form episomes because of RNAi mechanisms. Previous studies suggested that *Leishmania* species and other parasites (*P. falciparum* and *T. cruzi*) use CNVs at specific loci to develop drug resistance[25,28,31,35,43,50,51], to promote the tissue tropism[40] and enable adaptations to culture conditions[52]. In this study, we observed that *L. braziliensis* and *L. panamensis* took advantage of CNVs to modulate the gene dosage in response to Sb^{III}, similar to other species such as *L. major*[28], *L. tropica*[42] and *L. guyanensis*[43].

Likewise, our results demonstrated the relationship between aneuploidy and gene CNVs in *L. braziliensis*, but not in *L. panamensis*. The 92% of genes that presented copy number variation in Lb_SSG_R line, were present in chromosomes with change in the somy, while in Lp_SSG_R line, the genes that presented copy number variation were in chromosomes where the somy was not affected (Fig. 3A,B). These genomic analyses revealed that *L. braziliensis* and *L. panamensis*, like other *Viannia* species (*L. guyanensis)*, use genome structural changes (aneuploidy and/or gene CNVs) as a response to Sb^{III} pressure. However, it remains unclear whether the observed genomic changes are related to Sb^{III} resistance or they are just an adaptive mechanism to overcome external stresses. Resolving this uncertainty will require comprehensive analyses of naturally-occurring antimony-resistant clinical isolates.

Another genomic strategy used by *Leishmania* species to circumvent the effects of drug pressure involves the generation of nucleotide-level variations (SNPs and indels). Some SNPs have been identified as related to the antimony resistance phenotype (e.g., a SNP detected in a gene encoding a protein kinase in *L. infantum* or in the *AQP1* gene of *L. donovani* and *L. guyanensis* strains[27,35,43]). Despite that we did not observe several changes to SNPs level and Indels in the analyzed *L. panamensis* and *L. braziliensis* lines (Sb^{III}-sensitive and -resistant lines), the SNPs on chromosome 32 of *L. braziliensis*, which did not lead to changes in the gene dosage and chromosomal somy (Fig. 4), suggest that this species can generate nucleotide-level changes associated with drug resistance without the need for alteration to the genomic structure and gene expression (Table 1). Another interesting finding regarding chromosome 32 was the presence of 32 SNPs in the Sb^{III}-sensitive line, but not in the Sb^{III}-resistant line. Most of these 32 SNPs were detected in genes encoding hypothetical proteins, but one was in the gene encoding a transmembrane protein (aquaporin) (Table 1), which is reportedly involved in multiple physiological processes in the parasite, such as nutrient absorption and end-product efflux[53]. Although the reason for these genomic changes is unknown and will need to be further investigated, we believe that the behavior observed in our Lb_SSG_R line may be associated to an unconventional recombination event, which can be considered as an analogous mechanism like somy change in a partial chromosome scale.

The pairwise comparison herein reported demonstrates how differences in chromosome numbers and intrachromosomal amplifications or deletions directly affect gene expression in the Lb_SSG_R line (Fig. 5A. Supplementary Fig. S2). However, in the Lp_SSG_R line, we detected extreme gene CNVs on chromosome 27 that were not associated with somy changes (Figs 1 and 2). The increased copy numbers directly resulted in the overexpression of some genes described as molecular targets of Sb^{III} (e.g., a gene encoding the WW domain/zinc finger protein)[54], as well as genes involved in virulence and in vital biological processes such as parasite growth and survival (elongation factor, alpha- and beta-tubulin)[47] (Supplementary Fig. S2). Similar synchronized changes between genomic and transcriptomic level were also found critical in other *Leishmania* species and others parasites (e.g., *Plasmodium falciparum*)[55] that undergoes intrachromosomal amplifications to influence transcript abundance. We believe that our study would have benefited from the inclusion of more than one Sb^{III}-resistant mutant line per species. Moreover, we think that additional studies, such as quantitative real-time PCR assays, cloning, and transfections and gene editing, will enable to test the observed overexpression of the genes in the Lp_SSG_R line. These future studies would also clarify the contribution of this gene to the antimony resistance phenotype. Additionally, we believe that the observed drastic changes to chromosome 27 are biologically important, and should be investigated in greater detail (e.g., analyses of infected animals and the effects of resistance or virulence).

Similar to the results of previous studies regarding other parasites, such as *P. falciparum*[55], we observed that our Sb^{III}-resistant *L. braziliensis* and *L. panamensis* lines carried genes with CNVs that did not affect the transcript level (e.g. a gene encoding mannosyl transferase-like protein or encoding putative polyubiquitin present in Lb_SSG_R and Lp_SSG_R lines, respectively) (Supplementary Fig. S3). We also detected varying transcript levels that were not related to differences in gene copy numbers (e.g. a gene encoding 40S ribosomal protein S15A putative or encoding cysteine peptidase, Clan CA, family C2, putative present in Lb_SSG_R and Lp_SSG_R lines, respectively) (Supplementary Fig. S3).

Interestingly, some of these genes were shown to be associated with the resistance of *L. donovani*, *L. tarentolae*, *and L. major* to antimonials[38,56]. Increases in gene expression levels without gene amplifications or vice versa may be explained by a lack of transcriptional regulation, which has been described for *Leishmania* species. Various studies have determined that this behavior is probably due to co-amplification of another nearby gene, or an increase in RNA stability, which promotes expression, or a mutation in a promoter-like element of an upstream gene[55,57,58]. These explanations are also likely applicable to our results. Finally, the pairwise comparison of genes differentially expressed in the SSG_S and SSG_R lines suggested that gene expression confers a degree of plasticity to these species under Sb^{III} pressure. For example, these lines may undergo changes to the expression of genes mediating antimony resistance as well as genes related to virulence and vital biological processes, including parasite growth and survival (Fig. 6A–C).

One limitation of our study was the use of the promastigote stage rather than intracellular amastigote stage, which has been considered as the gold standard for *in vitro Leishmania* drug discovery research and evaluation of resistance[59,60], and despite that experimental evidence (using mainly microarrays) have suggested that the transcriptomic behavior between amastigote and promastigote stages is different[61,62]. We consider that the molecular changes observed can be used for future WGS studies using amastigotes stage. Our findings represent the baseline for future studies that conduct genomic and transcriptomic analyses on amastigotes and depict the molecular features associated to the Sb^{III} response. Likewise, and taken into account that in *Leishmania* parasites, the expression of individual genes is regulated post-transcriptionally, due to the absence of promoter-mediated regulation of transcription initiation of nuclear genes[42,63], which can result in a variable correlation between gene and protein expression level[64]. We believe that future analysis should consider how the transcriptomic findings observed in this study could have a relationship with the final protein dosage. We also recommend conducting comprehensive and robust proteomic studies to unravel the true proteins associated in response to drug pressure.

In conclusion, this whole genome scale DNA-seq and RNA-seq study highlighted the importance of gene dosage effects of genomic and transcriptional levels as the coping mechanisms against the antimony exploited by *L. braziliensis* and *L. panamensis*. These two species still remain to be enigmatic parasites which can cause disfiguring mucocutaneous leishmaniasis. Our data would serve as a first step towards the better understanding of the genomic and transcriptomic changes caused under the SSG stress *in vitro* and should also provide the basis for future studies examining the applicability and commonality of the genomic and transcriptomic changes observed in the study to the parasites encountered in the clinical setting (i.e., strains with natural resistance). It is our hope that the further sequencing and molecular analyses on experimental and clinical SGG resistance will be able to contribute to the identification of new therapeutic targets in the near future.

Methods

Culture conditions and development of drug-resistant *Leishmania braziliensis* and *Leishmania panamensis* promastigotes. Promastigotes of *L. braziliensis* (MHOM/BR75/M2904) and *L. panamensis* (MHOM/COL/81/L13) sensitive to Sb^{III} (SSG_S) and resistant to Sb^{III} (SSG_R) were axenically maintained in RPMI 1640 medium (Sigma-aldrich) supplemented with 10% (v/v) heat inactivated fetal bovine serum (Invitrogen) and culture at 26 °C with 5% CO_2.

The Sb^{III}-resistant population, [*L. braziliensis* (Lb_SSG_R) and *L. panamensis* (Lp_SSG_R)] were obtained from wild-type sensitive *L. braziliensis* (Lb_SSG_S) and *L. panamensis* (Lp_SSG_S) via the continuous stepwise increase in drug pressure with Sb^{III}, as described by Liarte and Murta[65]. For each species, selection of resistant parasites was initiated in quadruplicates. Briefly, 10^6 logarithmic-phase promastigotes were incubated with 1.5 μg/ml Sb^{III} (*L. braziliensis)* or 1.8 μg/ml Sb^{III}, (*L. panamensis)*. This concentration corresponds to the effective concentration that inhibits growth by 50% (EC_{50}). The drug concentration was increased in a stepwise manner only when the drug-exposed parasites had a growth rate similar to that of the parental parasites. Selection rounds were performed successively at 2-fold increase with 1.5, 3, 6, 12, 24 and 48 μg/ml Sb^{III} for *L. braziliensis* and 1.8, 3.6, 7.2, 14.4, 28.8 and 57.6 μg/ml Sb^{III} for *L. panamensis*. This increment was continued until the maximum concentration allowing parasite growth was reached. After this period, the SSG_R lines were maintained for 3 weeks at the final drug concentration. To verify that the observed drug resistance phenotypes were stable, we cultivated the Sb^{III}-resistant lines for 4 weeks in the absence of Sb^{III}. The Sb^{III}-sensitive *Leishmania* species were cultured in parallel, but without any drug pressure. At the end of this period, the susceptibility of the sensitive and resistant lines to Sb^{III} was determined by calculating the EC_{50} in an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay, as previously described[66]. The corresponding absorbance values were obtained and the EC_{50} was calculated using the Graph Pad Prism 5.0. The assays were performed three times in triplicate. Differences in the data were considered significant when the resistance index was $\geq$10-fold different between the Sb^{III}-resistant and -sensitive lines.

Isolation of RNA and DNA. Approximately 1×10^6 promastigotes (sensitive and resistant to Sb^{III}) in the late logarithmic growth phase were cultured and harvested by centrifugation. The resulting pellets were divided in two equal parts, for DNA and RNA extraction.

Total RNA was extracted from four independent replicates (two technical and two biological replicates) of each Sb^{III}-resistant and -sensitive line, each originating from a separate culture. The RNA was extracted with the RNeasy Mini Kit (Qiagen, USA), and the DNA was extracted from one replicate of each Sb^{III}-resistant and -sensitive line with the High Pure PCR Template Preparation Kit (Roche Life Science). The RNA and DNA concentrations were determined with the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA). The RNA quality and integrity were assessed with the 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. The DNA quality and integrity were determined by 1% agarose gel electrophoresis. All samples had an A_{260}/A_{280} ratio greater than 2.0.

Genome and transcriptome sequencing. The mRNA, cDNA libraries and the extracted whole genome DNA were prepared and sequenced with the HiSeq X-Ten system (Illumina) by Novogene Bioinformatics Technology Co., Ltd, Beijing, China. Paired reads of 75 nucleotides were obtained for the mRNA libraries, whereas 2 × 100 bp reads length were obtained for the cDNA libraries. Sequence quality metrics were assessed with FastQC (Illumina platform, PE 150, Q30 ≥80%; 250–300 bp insert cDNA library). Additionally, the 20 million raw reads/sample rRNA depleted was completed according to the poly(A) magnetic beads capture protocol, with the Strand-specific TruSeq RNA-seq Library Prep kit (Illumina), which was also used to prepare libraries.

To whole genome DNA, the mate-paired libraries constructed by end repair (350-bp insert size) were subjected to paired-end sequencing (2 × 150-bp read length).

Genome data analysis. Paired-end Illumina reads were mapped to the reference MHOM/BR75/M2904 *L. braziliensis* genome sequence and the UA946 *L. panamensis* genome sequence assembly with the SMALT program (version 0.7.4) (www.sanger.ac.uk/resources/software/smalt/). The mapping involved the following parameters: exhaustive search option (−x and −y 0.8); a reference hash index of 13 bases; and a sliding step of 3. An identity threshold of y = 0.8 prevented the mapping of non-*Leishmania* reads to the reference sequences because SMALT can trim reads before mapping them to the reference sequence. The read file merging, sorting, and elimination of PCR duplicates were implemented with SAMtools (version 0.1.18) and Picard (version 1.85)[35].

For the chromosomal somy estimation, the median read depth of each chromosome was initially calculated (d_i). All positions with a read depth >1 standard deviation away from this initial median were then removed, and the d_i was recalculated. This approach removed depth outliers due to assembly errors, local CNVs, or spurious high-coverage regions influencing the final median. Subsequently, the median depth of the 35 chromosomes (d_m) for *L. braziliensis and L. panamensis* was calculated, and the somy (*s*-value) of each chromosome was obtained with the following formula: $s = 3 \times d_i/d_m$ (for *L. braziliensis*) and $s = 2 \times d_i/d_m$ (for *L. panamensis*)[37]. Among our samples, the shorter chromosomes did not tend to deviate from the expected somy[41]. The somy values calculated from sequencing data are averages across the potentially variable somy of these cells. For this reason, somy values may be noninteger values, representing the mean value of a mixed population. The range of monosomy, disomy, trisomy, tetrasomy, and pentasomy was then used to define the full cell-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]. Because the depth of samples was sufficient, we did not test other normalization factors, such as various percentile depths or a statistically weighted normalization factor. To evaluate the CNVs at the gene level, we defined an average haploid depth per gene without the somy effect as $\overline{d}_{HG}$, and defined the full cell depth with the somy effect as $\overline{d}_{FG}$. Their relationship was defined as $\overline{d}_{FG} = S \cdot \overline{d}_{HG}$.

Two criteria were used to evaluate whether differences in the gene or chromosome copy number between the Sb^{III}-sensitive and -resistant lines were biologically and statistically significant. The first requirement was that the absolute difference in the gene/chromosome copy number between the Sb^{III}-sensitive and -resistant lines should be at least 0.5[41]. Second, the false discovery rate (FDR) adjusted p-value (Student's *t*-test and Benjamini–Hochberg correction) had to be lower than 0.05. Heatmaps were created using the Heatmap3 package in R[67]. All gene IDs reported herein for *L. panamensis* were based on the orthologous genes in the *L. braziliensis* genome.

To detect the single nucleotide polymorphisms and insertions/deletions, the reads were aligned to the reference MHOM/BR75/M2904 *L. braziliensis* genome sequence or the UA946 *L. panamensis* genome sequence assembly, 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]. The SNPs and insertions/deletions (indels) shorter than 15 bp were called with the population-based Unified Genotyper method in the Genome Analysis Toolkit (GATK) (version 3.4; https://software.broadinstitute.org/gatk/). DNA regions with more than three SNPs within 10 bases of each other were marked as SNP clusters and were maintained for subsequent analyses. Low-quality SNPs were filtered by GATK Variant Filtration with QD < 2.0 || MQ < 40 || FS > 60.0 || ReadPosRankSum < −8.0. To avoid false negatives, the SNP quality cut-off was set to 300. All candidate SNPs were visually inspected in the Integrative Genomic Viewer (IGV_2_3_47)[41] and SAMtools to avoid false positives. The SnpEff program (version v4.1)[41] was used to classify all SNPs and indels based on their functional impact. The SNPs and indels were compiled in a population genetic variation vcf file. From this vcf file, alternative allele and depth information was extracted for further analysis.

The SNPs and small indels were considered significantly different between the Sb^{III}-sensitive and -resistant lines when the allele shift difference was at least 0.25 for *L. panamensis* and at least 0.33 for *L. braziliensis*[68] with a Mann–Whitney U test p-value < 0.05. Allele shifts larger than 0.80 were considered homozygous variants.

Transcriptome data analysis. Transcript abundance was quantified by assessing read depth, as previously described[25,35] and multiply mapped reads were kept in the analysis to quantify repetitive genes. For each chromosome, the average transcript depth was used to compute an RNA-based relative somy value, (i.e., RNA-S). The correlation between DNA and RNA depth, namely between S and RNA-S, was calculated and visualized with SciPy[69]. For differential expression analysis, STAR (2.5.2) was used for mapping and read counting per gene with default parameters where multiply mapped read were marked and ignored. DEseq2 (version 1.18.1) was then used to normalize the read counts and evaluate the statistical significance of differentially expressed genes. Here the following criteria were used: a fold-change cut off ≥2 and a Benjamini–Hochberg adjusted p-value < 0.05. The percentage of differentially expressed genes per chromosome was defined as follows: (number of differentially expressed genes per chromosome)/(number of total genes per chromosome) × 100.

Gene Ontology enrichment analyses were performed using Tritrypdb tools (http://tritrypdb.org) with Fisher exact test used to maintain the FDR below 0.05. The GO terms were submitted to REVIGO[70].

Finally, Venn diagram was constructed using a program provided by the Bioinformatics and Evolutionary Genomics group of the University of Gent and the VIB institute (http://bioinformatics.psb.ugent.be/webtools/Venn/).

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Author Contributions

L.H.P. conceived and designed the study, analysed and interpreted the data and prepared the manuscript. H.I. carried out the bioinformatic analysis. L.C.S., P.P., C.M., C.M. and J.C.D. critically revised the manuscript and made important suggestions. J.D.R. conceived and designed the study, prepared and revised the manuscript. All authors have reviewed and approved the manuscript.

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Parasites & Vectors

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Transcriptional responses of *Leishmania* (*Leishmania*) *amazonensis* in the presence of trivalent sodium stibogluconate

Luz H. Patino[1], Carlos Muskus[2] and Juan David Ramírez[1*]

Abstract

Background: In the last decade, resistance to antimonials has become a serious problem due to the emergence of drug-resistant strains. Therefore, understanding the mechanisms used by *Leishmania* parasites to survive under drug pressure is essential, particularly for species of medical-veterinary importance such as *L. amazonensis*.

Methods: Here, we used RNA-seq technology to analyse transcriptome profiles and identify global changes in gene expression between antimony-resistant and -sensitive *L. amazonensis* promastigotes.

Results: A total of 723 differentially expressed genes were identified between resistant and sensitive lines. Comparative transcriptomic analysis revealed that genes encoding proteins involved in metabolism (fatty acids) and stress response, as well as those associated with antimony resistance in other *Leishmania* species, were upregulated in the antimony-resistant line. Most importantly, we observed upregulation of genes encoding autophagy proteins, suggesting that in the presence of trivalent stibogluconate (Sb^{III}) *L. amazonensis* can activate these genes either as a survival strategy or to induce cell death, as has been observed in other parasites.

Conclusions: This work identified global transcriptomic changes in an *in vitro*-adapted strain in response to Sb^{III}. Our results provide relevant information to continue understanding the mechanism used by parasites of the subgenus *Leishmania* (*L. amazonensis*) to generate an antimony-resistant phenotype.

Keywords: Resistance, Diffuse leishmaniasis, DEG, Hierarchical cluster analysis (HCA), Principal components analysis (PCA), Transcript

Background

Leishmaniasis is a complex of tropical diseases caused by protozoan parasites of the genus *Leishmania*, characterised by a broad spectrum of clinical manifestations that have been classified into five categories: localised cutaneous leishmaniasis (CL); diffuse CL (DCL); disseminated CL (DL); mucocutaneous leishmaniasis (MCL); and visceral leishmaniasis (VL). Over 30 *Leishmania* species have been identified to date, and classified into four subgenera: *Leishmania* (*Leishmania*), *Leishmania* (*Viannia*), *Leishmania* (*Sauroleishmania*) and *Leishmania* (*Mundinia*) [1, 2]. Of the species belonging to subgenus *Leishmania*, *L. amazonensis* has particular clinical and epidemiological importance, especially in Latin America. *Leishmania amazonensis* is the main etiological agent of DCL, is implicated in borderline disseminated cutaneous leishmaniasis [3] and is responsible for 8% and 3% of CL cases in Brazil and Colombia, respectively [4, 5]. Additionally, several studies have identified *L. amazonensis* as a causative agent of VL in humans and animals (canines and felines), demonstrating its importance in both clinical and veterinary medicine [6–9].

To date, and in the absence of an available vaccine, chemotherapy is the only option for treatment of leishmaniasis. Although several different drugs are available, antimonials (e.g. sodium stibogluconate and meglumine

*Correspondence: juand.ramirez@urosario.edu.co
[1] Grupo de Investigaciones Microbiológicas-UR (GIMUR), Programa de Biología, Facultad de Ciencias Naturales y Matemáticas, Universidad del Rosario, Bogotá, Colombia
Full list of author information is available at the end of the article

antimoniate) remain standard treatment and the drugs of choice for treatment of all forms of leishmaniasis in different endemic areas (particularly Latin American). However, in the last decade there has been a large-scale increase in therapeutic failure of antimonials [10]. Although the incidence of therapeutic failure in patients infected with *L. amazonensis* is unclear, a percentage of patients who subsequently develop DCL (caused by a failure of the immune response) show a poor response to antimonials [11].

Numerous factors impact the final therapeutic outcome of antimonial treatment [12], with factors associated with the parasite itself. Several studies have focused on determining the mechanisms used by the parasite to survive under drug pressure using next-generation sequencing techniques [genomics, transcriptomics (RNA-seq), proteomics and metabolomics]. Some of these studies, mainly using strains of *L. donovani*, *L. major* and *L. infantum*, have demonstrated that, under drug pressure, *Leishmania* uses several adaptative mechanisms to modulate the gene dosage of therapeutic targets or other determinants of resistance. Some of these mechanisms include the generation of episomal amplicons, changes in ploidy of the whole chromosome and/or generation of local gene copy number variation, production of single-nucleotide polymorphisms in drug targets or upregulating proteins that may play a role in intracellular survival [13–18].

Recently, RNA-seq technology has emerged as a powerful tool in the study of *Leishmania* species. It has been used to determine the transcriptomic profiles of different species of *Leishmania* (*L. major*, *L. donovani*, *L. infantum*, *L. mexicana*, *L. amazonensis* and *L. braziliensis*), expanding our knowledge about parasite biology and their interactions with vertebrate and invertebrates hosts [19–23]. In addition, RNA-seq has been used to study the transcriptomic response to different stress conditions, and to identify genes associated with resistance to antimonials, mainly in strains of the *L. donovani* complex [15, 24, 25].

RNA-seq-based analyses have also been used in New World *Leishmania* species, including *L. amazonensis*, *L. braziliensis* and *L. mexicana*, to analyse transcriptional behaviour under specific conditions [20, 22, 26, 27]. However, none of these studies have focused on identifying transcriptional changes that occur in these parasites under stress conditions (such as drug pressure), as has been described for Old World *Leishmania* species. These data are particularly lacking for *L. amazonensis*, a species that is emerging as a pathogen of medical-veterinary importance in Latin America. Therefore, the purpose of this study was to conduct a comprehensive transcriptome profiling using RNA-seq to identify global changes in gene expression that occur in *L. amazonensis* in response to Sb^{III} exposure, and to obtain a general picture of the mode of action in which this species regulates *in vitro* gene expression under drug pressure. Our results contribute to the understanding of *in vitro* Sb^{III}-resistance phenotypes and help to determine the global transcriptional effects of Sb^{III}. This is also the first report providing transcriptome data for *L. amazonensis* submitted to a specific drug pressure.

Methods

Culture conditions and development of drug-resistant *L. amazonensis* promastigotes

Promastigotes of *L. amazonensis* [obtained from one patient with clinical CL symptoms from Medellin (Colombia) and named UA301] sensitive to Sb^{III} (Sb^{III}-S) and resistant to Sb^{III} (Sb^{III}-R) were axenically maintained in RPMI 1640 medium from Sigma-Aldrich (St. Louis, MO, USA) supplemented with 10% (v/v) heat inactivated fetal bovine serum from Thermo Fisher Scientific (Boston, MA, USA) and cultured at 26 °C with 5% CO_2. DNA extraction and subsequent species identification, which was performed by direct Sanger sequencing of the cytochrome b (*cytb*) and heat-shock protein (*hsp70*) gene fragments, was carried out as described by Ramirez et al. [5].

The Sb^{III}-resistant population, *L. amazonensis* (La-Sb^{III}-R) promastigotes were obtained from wild-type sensitive *L. amazonensis* (La-Sb^{III}-S) *via* the continuous stepwise increase in drug pressure with Sb^{III}, as described previously [28], with slight modifications. The selection of resistant parasites was initiated in quadruplicates. Briefly, 10^6 logarithmic-phase promastigotes were incubated with different concentrations of Sb^{III}. The drug concentration was increased in a stepwise process only when the drug-exposed parasites had a growth rate similar to that of the parental parasites. Selection rounds were performed successively with 2-fold increase with 1.0, 2.0, 4.0, 8.0, 16, 32, 64 and 128 µg/ml Sb^{III}. This incrementation was continued until the maximum concentration of parasite growth. After this period, the Sb^{III}-R line was maintained for 3 weeks at the final drug concentration. To verify that the observed drug-resistant phenotype was stable, we cultivated the Sb^{III}-resistant line for 4 weeks in the absence of Sb^{III}. The Sb^{III}-sensitive *L. amazonensis* was cultured in parallel, but without any drug pressure. At the end of this period, the susceptibility of the sensitive and resistant lines to Sb^{III} was determined by calculating the EC_{50} in an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay, as previously described [29]. The reduction of MTT to its insoluble form formazan was evaluated in a Tecan GENios Microplate Reader (Biotek, Winooski, VT, USA), with an emission of 570 nm. The corresponding

absorbance values were obtained from the spectrofluorometric reading and the EC_{50} was calculated using Graph Pad Prism v.5.0 software. The assays were performed three times in triplicate. Differences in the data were considered significant when the resistance index was $\geq$ 10-fold different between the Sb^{III}-resistant and -sensitive lines. Once the parasites were selected (Sb^{III}-resistant and sensitive), they were cloned from culture into 96-well plates containing RPMI medium supplemented, *via* limiting dilution as described previously [30].

RNA isolation

Approximately 1×10^6 promastigotes (sensitive and resistant to Sb^{III}) in the middle logarithmic growth phase were cultured and harvested by centrifugation. The resulting pellets were used to conduct the RNA extraction. Total RNA was extracted from four independent replicates (two technical and two biological replicates) of each Sb^{III}-resistant and -sensitive line, each originating from a separate culture. The RNA was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA concentrations were determined with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and the quality and integrity with a 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturers' instructions.

Transcriptome sequencing and data analysis

The mRNA and cDNA library were prepared and sequenced with the HiSeq X-Ten system (Illumina, San Diego, CA, USA) by Novogene Bioinformatics Technology Co., Ltd, Beijing, China. Paired reads of 75 nucleotides were obtained for the mRNA libraries, whereas 2×100 bp length of reads were obtained for the cDNA libraries. Sequence quality metrics were assessed with FastQC (Illumina platform, PE 150, Q30 $\geq$ 80%; 250–300 bp insert cDNA library). Additionally, 20M raw reads/sample rRNA depletion was performed by poly(A) magnetic beads capture protocol, using Strand-specific TrueSeq RNA-seq Library Prep (Illumina), according to the manufacturer's instruction.

Reads were mapped to the *L. mexicana* reference genome (MHOM/GT/2001/U1103) obtained from TriTrypDB (www.tritrypdb.org) using Smalt v.7.4 (http://www.sanger.ac.uk/science/tools/smalt-0). The *L. mexicana* genome was used as the *L. amazonensis* genome is not completely annotated. The amounts of each of the transcripts were quantified by assessing read depth, as described previously [31, 32]. For differential expression analysis, STAR v.2.5.2 was used for mapping and read counting per gene with default parameters where multiply mapped reads were marked and ignored. DEseq2 v.1.18.1 was then used to normalize the read counts and evaluate the statistical significance of differentially expressed genes. Here the following criteria were used: a fold-change cut-off of $\geq$ 2 and a Benjamini–Hochberg adjusted *P*-value < 0.05. The percentage of differentially expressed genes (DEGs) per chromosome was defined as follows: (number of differentially expressed genes per chromosome)/(number of total genes per chromosome) $\times$ 100.

In the initial data exploration, we constructed a principal components analysis (PCA) and hierarchical cluster analysis (HCA) to test whether both conditions (sensitive and resistant) could be clustered separately. The PCA was performed in R directly and was based on the variant stabilized count of each sample. The HCA was performed by applying the Euclidean distance measure and Ward's algorithm. The Euclidean distance was calculated over the rlog-transformed count using DESeq2 and plotted using the *pHeatmap* R package (https://cran.r-project.org/). The four replicates of each condition (La-Sb^{III}-S and La-Sb^{III}-R) were used.

Gene Ontology enrichment analyses were performed using Tritrypdb tools (http://tritrypdb.org) with Fisher's exact test used to maintain the FDR below 0.05. The GO terms were submitted to REVIGO, which is a web server that takes long lists of GO terms and summarizes them in categories and clusters of differentially expressed genes by removing redundant entries [33]. Finally, a Venn diagram was constructed using an online program provided by the Bioinformatics and Evolutionary Genomics group of the University of Gent and the VIB institute (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Results

Induction of Sb^{III} resistance in *L. amazonensis* line

Initially, we selected *in vitro* populations of *L. amazonensis* that were resistant to Sb^{III}. In the selection dynamics, two replicates did not survive; the third (4.0 µg/ml Sb^{III}) and fourth (8.0 µg/ml Sb^{III}) rounds of selection and two replicates were successfully selected to survive to seven rounds (64 µg/ml). At the highest Sb^{III} concentration (128 µg/ml), the parasites died (see Additional file 1: Figure S1). Likewise, when we evaluated the stability of the resistance phenotype (64 µg/ml Sb^{III} for 4 weeks), we observed that the resistance index of each line remained, suggesting that the *in vitro* selected drug resistance phenotype was stable.

Differentially expressed transcripts between the Sb^{III}-resistant and -sensitive *L. amazonensis* lines

As a first data exploration of the variation in our dataset, we performed a principal component analysis (PCA) and hierarchical cluster analysis (HCA). The results observed

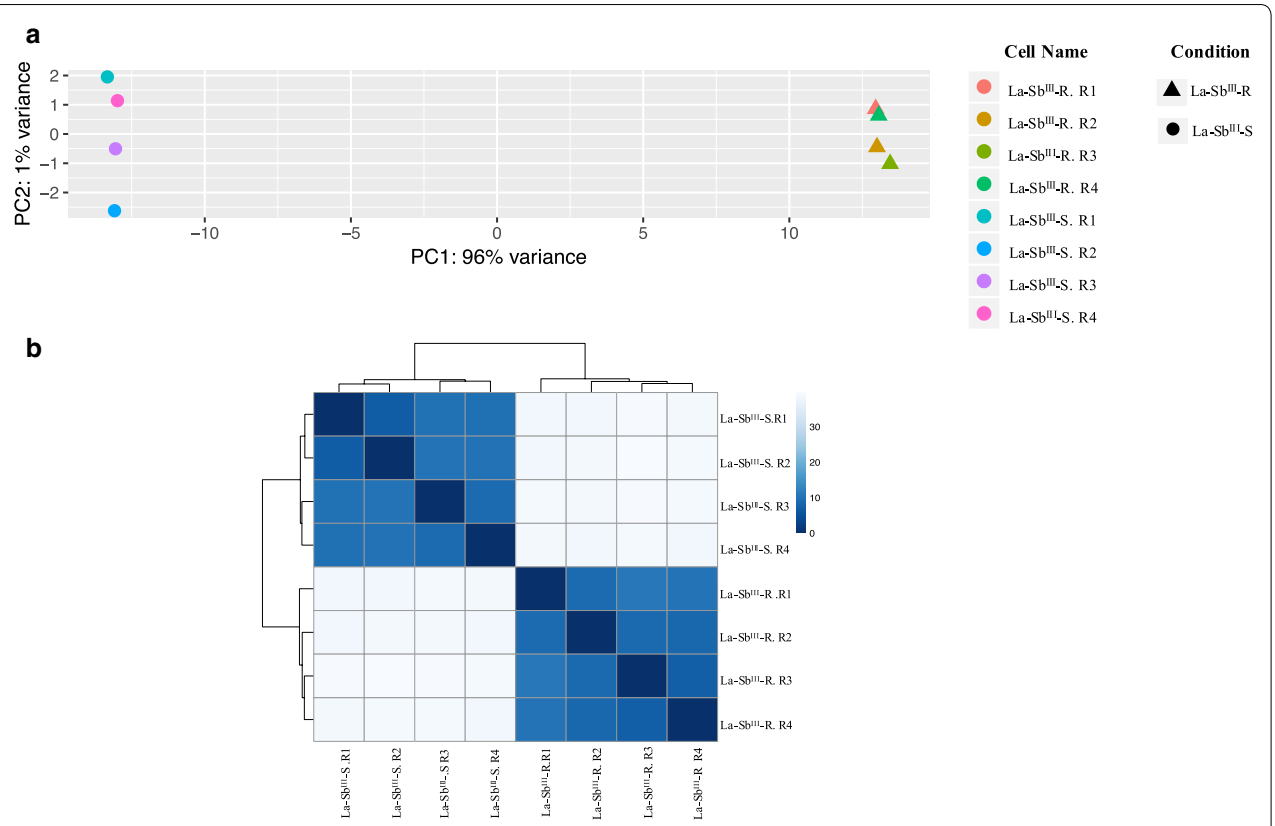

Fig. 1 La-Sb^{III}-S and La-Sb^{III}-R lines discriminations. **a** Principal component analysis based on the variant stabilized counts from four individual replicates (La-Sb^{III}-S and La-Sb^{III}-R). **b** Hierarchical clustering analysis, which was based on data from four individual replicates (La-Sb^{III}-S and La-Sb^{III}-R lines) and plotted using the pHeatmap R package (https://cran.r-project.org/). Stronger relationships between variables are indicated by shorter distances in the dendrograms and darker blue color in the heatmap

in the PCA showed that the first principal component explained 96% of the total variation in our experimental lines and clearly separated the La-Sb^{III}-S from La-Sb^{III}-R lines (Fig. 1a). Likewise, in the HCA, when Euclidean distance between samples was computed and used to create a heatmap colour image and dendrogram depicting the relatedness between samples, a clear separation between resistant and sensitive lines was observed (Fig. 1b).

Later, we evaluated the expression profile of *L. amazonensis* under drug pressure, performing differential gene expression analysis of Sb^{III}-sensitive and Sb^{III}-resistant *L. amazonensis* lines (La-Sb^{III}-S and La-Sb^{III}-R, respectively). We identified a total of 723 genes that were differentially expressed between the two lines (*P*-value cut-off of < 0.05 and fold-change difference ≥ 2), 330 upregulated and 393 downregulated in the La-Sb^{III}-R line (see Additional file 2: Table S1). Additionally, these genes were visualised using an MA plot showing the relationship between mean expression and fold-change for each gene (Fig. 2). Of the 723 genes that were significantly up/downregulated in the La-Sb^{III}-R line, 46% (335/723) were annotated as hypothetical proteins, with the remaining gene products associated with various biological functions in the parasite (surface proteins, virulence, metabolism, cell cycle, autophagy, cytoskeletal and stress response).

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed genes (DEGs)

To better analyse the DEGs, we performed GO and KEGG enrichment-based analyses. The 723 DEGs were categorised into three functional GO groups: biological process; molecular function; and cellular component. Within the biological processes GO group, the genes upregulated in the La-Sb^{III}-R line were mainly predicted to be involved in regulation of the cell cycle and organelle organisation but were also associated with stress response and divalent metal ion transport. However, the downregulated genes were involved in nucleotide biosynthesis and carbohydrate transport (Fig. 3a). For the molecular function group, genes upregulated in the La-Sb^{III}-R line mainly encoded binding proteins and proteins with enzymatic activity, while the downregulated genes

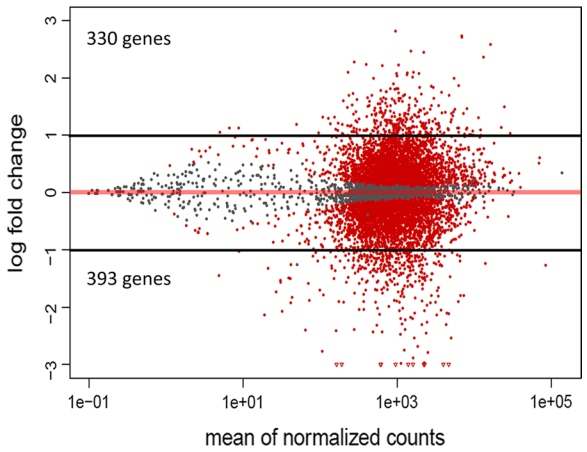

Fig. 2 Graphical representation of genes differentially expressed between La-Sb^{III}-S and La-Sb^{III}-R. The figure represents the MA plot constructed based on the DESeq2 results, showing the relationship between mean expression (normalised counts) and fold-change for each gene. Each point represents one gene. Gray dots indicate the genes that were not differentially expressed and the red dots, located above and below of black discontinuous lines (cut-off for the fold-change (log fold-change > 1 and < − 1), represent differentially expressed genes with abs [log2(FC)] > 1 and an adjusted *P*-value < 0.01, between La-Sb^{III}-S and La-Sb^{III}-R

mainly encoded carbohydrate transporters and proteins with peptidase activity (Fig. 3b). Finally, within the cellular components group, the up- and downregulated genes in the La-Sb^{III}-R line encoded proteins localised mainly in the nuclear component and in the axoneme, respectively (Fig. 3c).

KEGG enrichment analysis revealed that genes upregulated in the La-Sb^{III}-R line were involved in pyrimidine metabolism, while the downregulated genes were involved in ubiquinone biosynthesis, glycine, serine and threonine metabolism, ascorbate and aldarate metabolism, drug metabolism-cytochrome P450 and glycosaminoglycan degradation (Fig. 4, Table 1).

Surface molecules

Thirteen transcripts encoding surface proteins were downregulated in the La-Sb^{III}-R line compared with the La-Sb^{III}-S line. Eight of these encoded surface antigen-like protein (PSA), four were expressed in tandem in the chromosome 4, two encoded proteophosphoglycan ppg3/ppg1 and the remaining transcripts encoded lipophosphoglycan (LPG), surface membrane protein gp46-like and major surface protease gp63 (GP63, or leishmanolysin). The most strongly downregulated transcripts in the

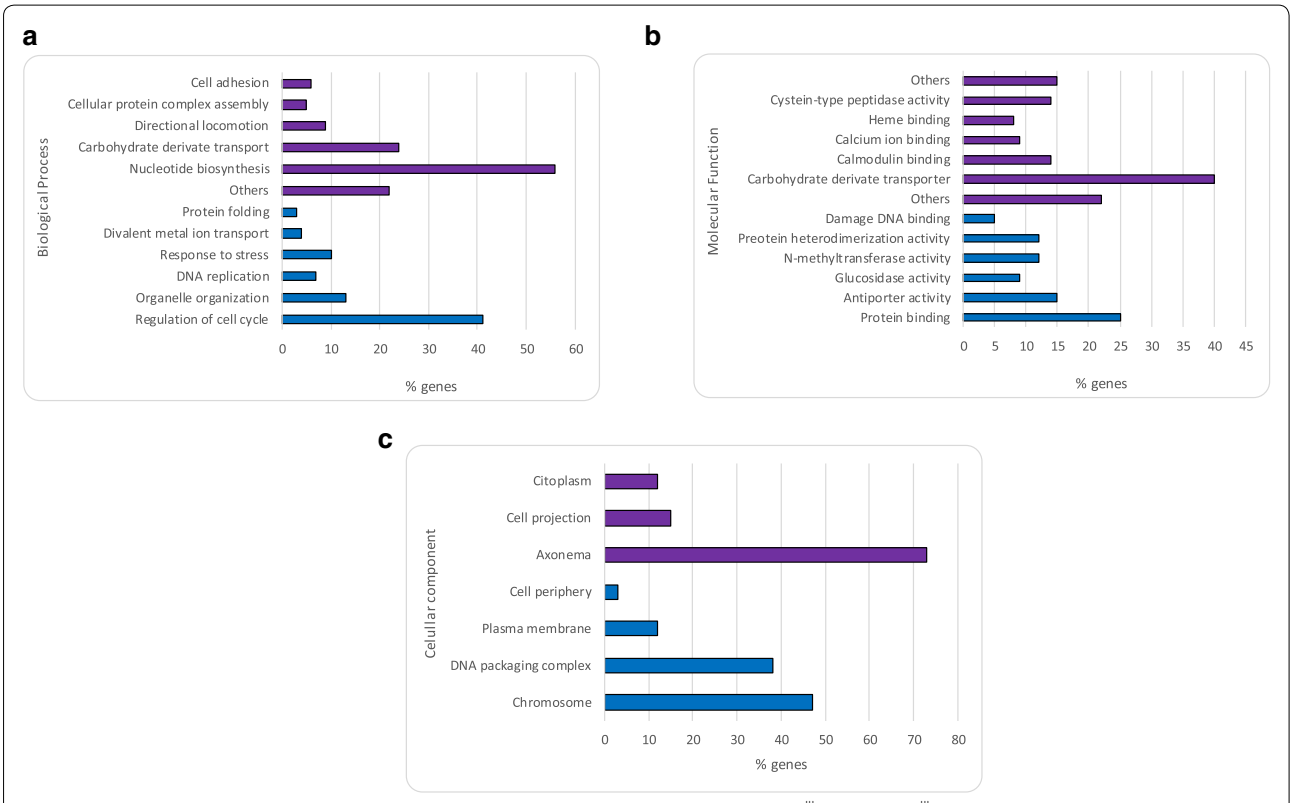

Fig. 3 Gene Ontology (GO) predictions for the genes differentially expressed between La-Sb^{III}-S and La-Sb^{III}-R. The bar graphs show the most represented functions within three categories: biological process (**a**), molecular function (**b**) and cellular component (**c**). The blue and purple bars represent the up- and downregulation, respectively, of genes in the resistant line compared with the sensitive line

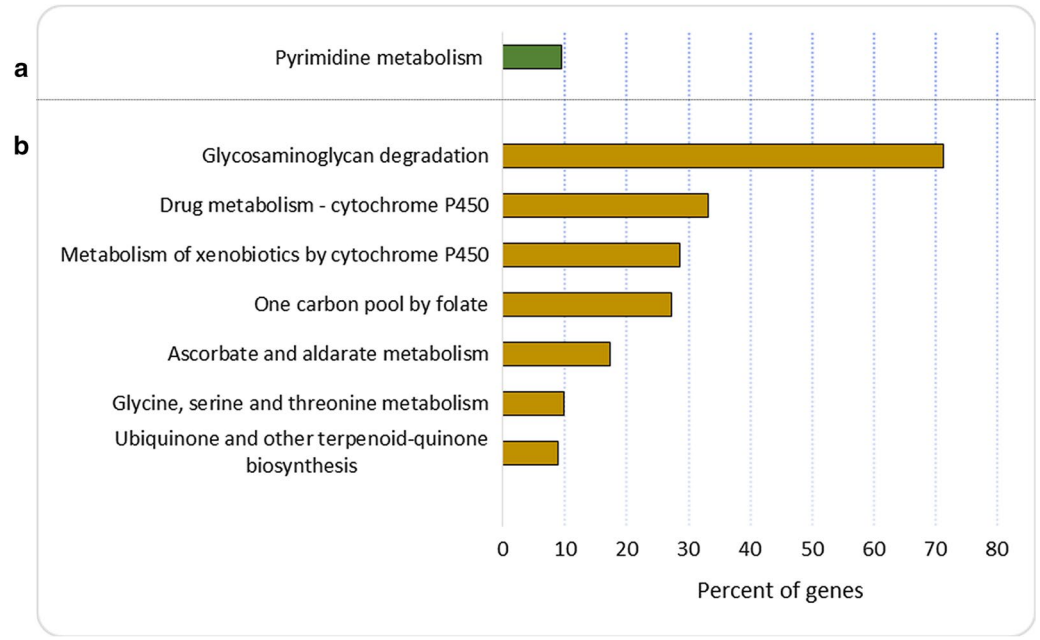

Fig. 4 Kyoto Encyclopedia of Genes and Genomes enrichment analysis for the genes differentially expressed between La-Sb^{III}-S and La-Sb^{III}-R. The bar graphs show the pathways regulated by upregulated (**a**) and downregulated (**b**) genes in the La-Sb^{III}-R line compared with the La-Sb^{III}-S line

Table 1 KEGG enrichment analysis of the up- and downregulated genes in the La-Sb^{III}-R line

Pathway ID	Map name	Genes
Upregulated genes		
ec00240	Pyrimidine metabolism	LmxM.13.1630, LmxM.18.1580, LmxM.21.1210, LmxM.23.0680, LmxM.28.0890, LmxM.28.1420, LmxM.28.1430, LmxM.33.0010, LmxM.34.1790
Downregulated genes		
ec00130	Ubiquinone and other terpenoid-quinone biosynthesis	LmxM.05.1100, LmxM.34.0500, LmxM.34.0500a, LmxM.34.0520b, LmxM.34.0540, LmxM.34.0550
ec00260	Glycine, serine and threonine metabolism	LmxM.10.0090, LmxM.14.1320, LmxM.29.2090, LmxM.32.0520, LmxM.36.3800
ec00053	Ascorbate and aldarate metabolism	LmxM.10.0090, LmxM.18.0160, LmxM.26.0160, LmxM.32.0520, LmxM.33.0010, LmxM.34.0500, LmxM.34.0500a, LmxM.34.0520b, LmxM.34.0540, LmxM.34.0550
ec00670	One carbon pool by folate	LmxM.14.1320, LmxM.36.3800, LmxM.36.6390
ec00980	Metabolism of xenobiotics by cytochrome P450	LmxM.29.2090, LmxM.32.0240
ec00982	Drug metabolism - cytochrome P450	LmxM.29.2090, LmxM.32.0240
ec00531	Glycosaminoglycan degradation	LmxM.34.0500, LmxM.34.0500a, LmxM.34.0520b, LmxM.34.0540, LmxM.34.0550

La-Sb^{III}-R line were homologous transcript described in *L. mexicana*, a close species related with *L. amazonensis*, LmxM.05.0900, LmxM.34.0500 and LmxM.28.0570, encoding PSA, proteophosphoglycan ppg3 and major surface protease gp63, respectively (Table 2). Despite mainly observing downregulation of surface molecules in the La-Sb^{III}-R line, five transcripts (LmxM.08.0720, LmxM.08.0730, LmxM.08.0740, LmxM.28.1400 and LmxM.33.1920) encoding amastin-like surface protein were upregulated. Three of these transcripts were expressed in tandem from chromosome 8.

Table 2 List of most highly differentially-expressed genes between the La-Sb^{III}-S and La-Sb^{III}-R lines (*P*-value cut-off < 0.05 and fold-change difference ≥ 2)

Biological functions	Transcript	Product description	Log_2 fold-change (R/S)
Surface proteins	LmxM.09.0580	Surface antigen-like protein	− 1.1603
	LmxM.05.1215	Surface antigen-like protein	− 1.2685
	LmxM.21.1170	Surface antigen-like protein	− 1.2882
	LmxM.04.0190	Surface antigen-like protein	− 1.8081
	LmxM.04.0210	Surface antigen-like protein	− 1.9894
	LmxM.04.0180	Surface antigen-like protein	− 2.0656
	LmxM.04.0200	Surface antigen-like protein	− 2.3355
	LmxM.05.0900	Surface antigen-like protein	− 3.7896
	LmxM.34.0550	Proteophosphoglycan ppg1	− 1.5215
	LmxM.34.0500	Proteophosphoglycan ppg3, putative	− 3.2111
	LmxM.33.3120	Lipophosphoglycan biosynthetic protein (lpg2)	− 1.2312
	LmxM.30.1450	Surface membrane protein gp46-like protein	− 1.3431
	LmxM.28.0570	Major surface protease gp63, putative	− 3.0596
	LmxM.08.0730	Amastin-like protein, putative	1.0151
	LmxM.28.1400	Amastin-like protein	1.1033
	LmxM.08.0720	Amastin-like protein, putative	1.1165
	LmxM.08.0740	Amastin-like protein, putative	1.2047
	LmxM.33.1920	Amastin-like surface protein, putative	1.7594
Metabolism	Metabolite transporters		
	LmxM.10.0350	Pteridine transporter ft5, putative	− 1.0251
	LmxM.24.0360	UDP-galactose transporter	− 1.0742
	LmxM.15.1230	Nucleoside transporter 1, putative	− 1.4466
	LmxM.32.0290	Glucose transporter/membrane transporter D2, putative	− 1.5513
	LmxM.15.1240	Nucleoside transporter 1, putative	− 1.9139
	LmxM.30.0320	Amino acid transporter, putative	− 1.9548
	Glycolysis		
	LmxM.10.0510	Glycerol-3-phosphate dehydrogenase [NAD+], glycosomal/mitochondrial	− 1.1530
	Proteolysis		
	LmxM.08.1080	Cathepsin L-like protease, putative	− 2.2280
	Fatty acid pathways		
	LmxM.30.2970	Acetyl-CoA carboxylase	1.0198
	LmxM.23.0710	Acetyl-CoA synthetase, putative	1.3180
	Other metabolic enzymes		
	LmxM.29.1940	Succinyl-coa:3-ketoacid-coenzyme a transferase- like protein	− 1.5541
	LmxM.26.1610	Proline dehydrogenase, mitochondrial	1.2112
	LmxM.27.0880	2-oxoglutarate dehydrogenase subunit, putative	1.1954

Table 2 (continued)

Biological functions	Transcript	Product description	Log_2 fold-change (R/S)
Cell cycle	LmxM.20.0030	Histone-lysine N-methyltransferase, H3 lysine-76 specific	2.4946
	LmxM.07.0025	Histone-lysine N-methyltransferase, putative	2.4788
	LmxM.10.0990	Histone H3	2.0457
	LmxM.10.0870	Histone H3	1.8415
	LmxM.25.0920	Histone RNA hairpin-binding protein	1.5715
	LmxM.20.0050	Histone chaperone ASF1A	1.3835
	LmxM.36.0020	Histone H4	1.2945
	LmxM.19.0050	Histone H2B	1.2791
	LmxM.30.3180	Histone H4	1.1477
	LmxM.34.1310	Histone H4	1.0938
	LmxM.19.0030	Histone H2B	1.0316
	LmxM.25.1470	Cyclin	2.3643
	LmxM.31.3320	Cyclin 6, putative	1.2431
	LmxM.28.1420	DNA polymerase kappa, putative	1.2764
	LmxM.28.1430	DNA polymerase kappa, putative	1.2214
	LmxM.34.1790	DNA polymerase epsilon subunit B, putative	1.1082
	LmxM.13.1630	Mitochondrial DNA polymerase I protein D, putative	1.0411
Autophagy	LmxM.27.0390	Autophagy protein APG9, putative	1.2217
	LmxM.23.1170	Membrane-bound acid phosphatase 2	1.5534
Cytoskeletal	LmxM.08.1230	β-tubulin	− 1.1278
	LmxM.21.1860	β-tubulin	− 1.8051
	LmxM.32.0792	β-tubulin	− 1.9300
	LmxM.05.0040	Paraflagellar rod component par4, putative	− 1.2326
	LmxM.16.1430	Paraflagellar rod protein 2	− 1.2388
	LmxM.09.1320	Paraflagellar rod component, putative	− 1.3933
	LmxM.08_29.1750	Paraflagellar rod protein 1D, putative	− 1.3995
	LmxM.36.4780	Paraflagellar rod component, putative	− 1.6234
	LmxM.16.1425	Paraflagellar rod protein 2C	− 1.9274
	LmxM.08_29.1760	Paraflagellar rod protein 1D, putative	− 1.9294
	LmxM.07.0310	Paraflagellar rod protein, putative	− 2.0882
	LmxM.32.0610	Paraflagellar rod component, putative	− 2.3516
	LmxM.36.4230	Paraflagellar rod component, putative	− 3.0082
Transporters associated with antimony response	LmxM.28.1930	Zinc transporter 3, putative	1.5220
	LmxM.30.3070	Ferrous iron transport protein	1.1716
	LmxM.23.0250	ABC-thiol transporter	1.1165
	LmxM.19.0180	Mitogen-activated protein kinase 9, putative	1.0732
	LmxM.13.0440	Mitogen-activated protein kinase kinase 2	1.0641
	LmxM.30.1290	Multidrug-resistance protein, copy 1-like protein	1.1374
H-locus	LmxM.23.0230	Hypothetical protein, conserved	1.0664
	LmxM.23.0240	Terbinafine resistance locus protein (yip1)	1.3429
	LmxM.23.0250	ABC-thiol transporter	1.1165
	LmxM.23.0260	Argininosuccinate synthase, putative	1.2138

Table 2 (continued)

Biological functions	Transcript	Product description	Log_2 fold-change (R/S)
Chaperones and stress proteins	LmxM.36.2030	Chaperonin HSP60, mitochondrial precursor	1.1311
	LmxM.28.2780	Heat-shock protein hsp70, putative	2.6066
	LmxM.32.0312	Heat-shock protein 83-1	2.3109
	LmxM.32.0316	Heat-shock protein 83-1	2.1008
	LmxM.32.0314	Heat-shock protein 83-1	2.0412
	LmxM.18.1370	Heat-shock protein, putative	1.3501
	LmxM.28.2770	Heat-shock protein hsp70, putative	1.1175

Metabolism

We then analysed and compared the expression of transcripts associated with the transport of sugar, nucleobases and amino acids between the La-Sb^{III}-S and La-Sb^{III}-R lines. Most of the transcripts associated with these processes were downregulated in the resistant line, including six transcripts (LmxM.10.0350, LmxM.24.0360, LmxM.15.1230, LmxM.32.0290, LmxM.15.1240 and LmxM.30.0320) annotated as coding for a pteridine transporter, a UDP-galactose transporter, a nucleoside transporter 1, a glucose transporter/membrane transporter D2, a nucleoside transporter 1 and an amino acid transporter, respectively.

Several genes encoding proteins associated with various metabolic pathways (glycolytic pathway, tricarboxylic acid cycle and proteolysis) were also differentially expressed between the sensitive and resistant lines. Downregulated genes in the La-Sb^{III}-R line included LmxM.10.0510, encoding a glycerol-3-phosphate dehydrogenase [NAD+] enzyme that not only catalyses the interconversion of dihydroxyacetone phosphate and L-glycerol-3-phosphate during the glycolysis, but also is important in both lipid and carbohydrate metabolism; LmxM.08.1080, encoding cathepsin-L protease, which is putatively involved in proteolysis; and LmxM.29.1940, encoding succinyl-CoA:3-ketoacid-coenzyme, a transferase involved in the catabolism of ketone bodies. These three transcripts were approximately 1.1-fold, 2.2-fold and 1.5-fold less abundant, respectively, in La-Sb^{III}-R than in susceptible line La-Sb^{III}-S.

Analysis of genes involved in the fatty acids pathway, which is associated with the intracellular amastigote stage and with promastigotes in culture, revealed upregulation in the La-Sb^{III}-R line, of transcripts encoding a putative acetyl-CoA carboxylase (LmxM.30.2970), a putative acetyl-CoA synthetase (LmxM.23.0710), a mitochondrial proline dehydrogenase (LmxM.26.1610) and a 2-oxoglutarate dehydrogenase subunit (LmxM.27.0880), which is associated with the TCA cycle (Table 2).

Cell cycle and autophagy

Among the upregulated cell cycle-associated genes in the La-Sb^{III}-R line, histone protein-coding genes were particularly enriched. Eleven transcripts (LmxM.10.0990, LmxM.10.0870, LmxM.36.0020, LmxM.19.0050, LmxM.30.3180, LmxM.34.1310, LmxM.19.0030, LmxM.20.0030, LmxM.07.0025, LmxM.25.0920 and LmxM.20.0050) encoding histone-family or associated proteins, including histone 3, histone 4 and histone 2B, were upregulated in the resistant line. Additionally, transcripts coding for proteins related to cellular replication were also upregulated in the La-Sb^{III}-R line, including those encoding cyclin and cyclin 6 (LmxM.25.1470 and LmxM.31.3320), along with various polymerases (LmxM.28.1420, LmxM.28.1430, LmxM.34.1790 and LmxM.13.1630), such as DNA polymerase kappa, DNA polymerase epsilon subunit B and mitochondrial DNA polymerase I protein D.

We also observed upregulation of transcripts encoding autophagy protein APG9 (LmxM.27.0390) and membrane-bound acid phosphatase 2 (MBAP2) (LmxM.23.1170) in La-Sb^{III}-R, both of which have been associated with the recycling of proteins under stress conditions and/or while undergoing a differentiation process (Table 2).

Cytoskeleton

Our analysis also identified differential expression of transcripts encoding proteins associated with the cytoskeleton between the La-Sb^{III}-R and La-Sb^{III}-S lines. We observed that three transcripts encoding β-tubulin and 10 transcripts encoding paraflagellar rod protein 1D were between 1.2-fold and 3.0-fold less

abundant in the La-Sb^{III}-R line than in susceptible line La-Sb^{III}-S (Table 2).

Antimonial resistance and stress response

Some of the genes previously associated with antimonial resistance mechanisms in *Leishmania* species were shown to be differentially expressed between La-Sb^{III}-R and La-Sb^{III}-S. In La-Sb^{III}-R, upregulated genes included LmxM.28.1930 (zinc transporter 3), LmxM.30.3070 (ferrous iron transport protein), LmxM.23.0250 (ABC-thiol transporter), LmxM.19.0180 and LmxM.13.0440 (mitogen-activated protein kinase 9/2) and LmxM.30.1290 [multidrug resistance protein, copy 1-like protein (MDR1)]. In addition, amplicons derived from the H locus were also upregulated in La-Sb^{III}-R, including transcripts coding for a hypothetical protein (LmxM.23.0230), HTB or terbinafine-resistance locus protein (Yip1) (LmxM.23.0240), an ABC-thiol transporter (MRPA) (LmxM.23.0250) and a putative argininosuccinate synthase (LmxM.23.0260).

Genes encoding several heat-shock proteins of different molecular masses were also upregulated in the La-Sb^{III}-R line. Seven transcripts coding for heat-shock protein family members HSP70, HSP83-1 and HSP60 (LmxM.28.2780, LmxM.32.0312, LmxM.32.0316, LmxM.32.0314, LmxM.18.1370, LmxM.28.2770 and LmxM.36.2030) were approximately 2-fold more abundant in the resistant line (Table 2).

Discussion

RNA-seq technology was used to characterise alterations in gene expression of *L. amazonensis* resulting from experimental induction of Sb^{III} resistance compared with an uninduced strain. *Leishmania amazonensis* is tremendously important in public health terms in Brazil and Colombia because of its association with CL and, more recently, VL in both humans and domestic animals (cats and dogs) [34, 35]. This association not only indicates the severity of *L. amazonensis* infection, but also the possible emergence of a domestic cycle and an increased risk of disease transmission. Until now, different approaches have been used with the purpose of understanding the transcriptomic behaviour of different species of *Leishmania* against antimonials; however, to our knowledge, this is the first attempt to elucidate and demonstrate the global gene expression profile of *L. amazonensis* under Sb^{III} pressure through RNA-seq. Herein, we identified a large number of genes showing differential expression between the sensitive and resistant lines (Fig. 2). Among these were transcripts encoding proteins associated with various biological processes, including adhesion, metabolism, cell cycle, autophagy, structural organisation and stress response (Fig. 3a).

Transcriptomic analysis of the different membrane-related proteins revealed differences between the La-Sb^{III}-S and La-Sb^{III}-R lines. Five transcripts encoding amastin proteins were overexpressed in La-Sb^{III}-R (Table 2). The amastins are surface glycoproteins whose expression has been noted in other parasites such as *Trypanosoma cruzi* and *Trypanosoma brucei* (amastigotes and epimastigotes) [36, 37], as well as in two related insect parasites, *Leptomonas seymouri* and *Crithidia* spp. [38] and had been involved in host-parasite interactions, with roles in both infection and survival [38]. The upregulation of genes encoding amastin in our resistant line is consistent with a previous report [15], and although the relationship between this surface protein and antimonial resistance has not previously been demonstrated in *Leishmania*, our results suggest that overexpression of genes encoding amastin could increase the resistance of the parasite to the cellular stresses elicited by Sb^{III}. In contrast, other surface protein-encoding genes, including those coding for PSA, proteophosphoglycan ppg3/ppg1, LPG, surface membrane protein gp46-like protein and major surface protease gp63/leishmanolysin, appeared to be downregulated in the resistant line (Fig. 3a, Table 2). Of these, only GP63 has previously been identified on the surface of *Leishmania* and other trypanosomatid species [39]. The downregulation of these genes under our study conditions suggests that *L. amazonensis* reduces the expression of some genes involved in virulence, interaction and survival in macrophages that are not necessary for survival under drug pressure. Future studies are needed in insect cell lines/macrophages to determine whether these genes are also downregulated during the *Leishmania* infection process.

On the other hand, most trypanosomatid species predominantly utilise glycolysis, amino acid metabolism and the fatty acid pathway (promastigotes maintained in culture) for energy generation [40–42]. Previous studies in *Leishmania* species have suggested that antimonials not only alter energetic metabolism by inhibiting glycolysis and fatty acids oxidation [10], but also cause changes in the transport of nutrients through the plasma membrane, as has been observed in Sb-resistant *Leishmania* strains [43]. Although we did not observe large variations in the expression of genes associated with metabolism between the sensitive and resistant lines, changes in the expression of genes encoding proteins associated with the glycolytic pathway or encoding glycolytic enzymes essentials in both lipid and carbohydrate metabolism and ATP production (downregulation of glucose transporter/membrane transporter D2 and glycerol-3-phosphate dehydrogenase [NAD+]) were consistent with previous reports in Sb-resistant *L. amazonensis* [44, 45] (Table 2). Additionally, GO analysis revealed a strong

downregulation of genes involved in carbohydrate transport (Fig. 3b), which suggests decreased formation of reactive oxygen species as a result of reduced glucose uptake, thereby aiding survival in the oxidative environment triggered by the drug [45].

In the present study, we observed the upregulation of 11 transcripts in the Sb^{III}-resistant line encoding histone proteins, namely H2B, H3 and H4 (Table 2). These proteins are associated with various biological processes in *Leishmania* and other trypanosomatids (*T. brucei* and *T. cruzi*) and are closely associated with transcription, DNA replication, recombination and repair [46–49], and likewise have been associated with antimony resistance in *Leishmania* parasites [15, 50]. GO analysis also confirmed a strong upregulation of genes involved in the regulation of the cell cycle (Fig. 3b), which agrees with data presented in a previous report [50]. These results reinforce the previously-noted association of histone proteins with resistance to antimonials found mainly in *L. donovani* [18, 50], and suggest similar behaviour in New World *Leishmania* species such as *L. amazonensis*.

Previous studies showed that the recycling of proteins by autophagic mechanisms is associated with metabolism in cells that are undergoing a differentiation process (metacyclogenesis) and/or under stress conditions [51, 52]. Our study identified upregulation of mRNA from chromosome 27 corresponding to the putative APG9 protein (Table 2), which is involved in autophagy and cytoplasm-to-vacuole transport (Cvt) vesicle formation, in the La-Sb^{III}-R line. This suggests that in the presence of Sb^{III}, *L. amazonensis* activates genes that induce autophagy, either as a survival strategy or as a form of cell death. This has also been observed in other parasites such as *T. brucei*, *T. cruzi*, *Leishmania donovani*, *Toxoplasma gondii* and *Plasmodium falciparum*, which activate different autophagy proteins (ATG3, ATG5, ATG7, ATG24 and PI3K) during nutrient starvation and under drug-induced stress as a mechanism of programmed cell death [53–55].

Another factor that may trigger protein recycling is purine starvation. *Leishmania*, *Trypanosoma* and *Toxoplasma* do not synthesise purines *de novo* and must scavenge them from the environment [56–58]. In response to this starvation, alterations are made to different metabolic processes, such as upregulation of purine salvage machinery. One of the most upregulated genes in purine-starved *Leishmania* parasites codes for membrane-bound acid phosphatase (MBAP2), which has a role in endosomal trafficking [52]. In the present study, we observed upregulation of the MBAP2 transcript in the La-Sb^{III}-R line (Table 2), suggesting an increase in lysosome-related recycling processes, as has been noted in *L. major* [52].

Additionally, studies have demonstrated that drug pressure produces changes at the cytoskeletal level (α- and β-tubulin proteins), provoking several mutations related to drug resistance. This phenomenon has been identified in *Leishmania* species, including *L. tarentolae* [59], and has also been present in the homologous genes from *T. cruzi*, *T. brucei* and *T. evansi* [18, 60]. In the present study, we observed downregulation of transcripts encoding β-tubulin and paraflagellar rod protein 1D in the Sb^{III}-resistant line (Table 2), as was recently observed in a resistant strain of *L. braziliensis* [61]. These results suggest that the development of antimony resistance may cause changes in cytoskeleton proteins as well.

Finally, several studies support the existence of a variety of resistance mechanisms in *Leishmania* parasites. One known mechanism of antimony resistance involves the reduction of drug accumulation by either reduced uptake or increased efflux through different membrane transporters, the most studied of which belongs to the ATP-binding cassette (ABC) protein superfamily [16, 62]. These protein transporters have been identified in other parasites including *T. brucei* and *T. cruzi*, and as in *Leishmania* species, their overexpression is implicated in resistance to different drugs [63–65]. In the present transcriptomic analysis, we observed upregulation of different transcripts encoding protein transporters in the La-Sb^{III}-R line (Table 2), all of which have previously been implicated in resistance to antimonials in other *Leishmania* species [15, 16]. These transporters included zinc transporter 3, ferrous iron transport protein and membrane transporters of the ABC superfamily (MDR1 and MRPA).

The *L. amazonensis* mdr1 gene, which has demonstrated to be 91 and 78% identical to the closely related ldmdr1 gene in *L. donovani* and lemdr1 gene in *L. enriettii*, respectively [66, 67], has been shown to be overexpressed in amphotericin B- and Sb-resistant strains of *L. donovani* [68–70], in a melarsoprol-resistant strain of *T. brucei* [71, 72] and in benznidazole-resistant epimastigotes of *T. cruzi* [64, 65]. Otherwise the gene encoding MRPA, which is one of three genes related to drug resistance identified within the H locus and which is amplified in extrachromosomal circles of DNA, was overexpressed in a number of *Leishmania* strains selected for resistance to Sb^{III}, Sb^{V} or the related metal [15, 73–76]. Additionally, overexpression of MRPA has been reported to decrease the influx of antimony rather than increase efflux [10]. The overexpression of genes that encode the MDR1 and MRPA transporters in our experimentally-induced Sb^{III}-resistant *L. amazonensis* strain suggests that active efflux/influx of Sb^{III} is a mechanism used by this species to survive in the presence of drug pressure, supporting previous reports in other species.

We also observed upregulation of genes coding for mitogen-activated protein kinases (MAPKs), which have

been associated with important cell processes such as proliferation, differentiation, cell shape, stress response, apoptosis and immune evasion in trypanosomatids [77, 78], and putatively with antimony resistance in *Leishmania* parasites [79]. Of the 17 MAPKs and MAPK-like kinases identified in *Leishmania* [80], only MAPK1 has previously been associated with antimony resistance. However, expression of the MAPK1 gene in resistant *L. donovani* appears variable, with some reports showing consistent upregulation in resistant isolates [50] and others showing downregulation in antimony-resistant field isolates [79, 81]. Although genes coding for MAPK2 and MAPK9 were upregulated in our resistant line, neither of these proteins have previously been reported in Sb^{III}-resistant strains, suggesting that their association with antimony resistance should be further studied.

Other genes overexpressed in the resistant *L. amazonensis* line were those encoding heat-shock proteins (HSPs). HSPs are a family of proteins whose function is to protect the cell from toxic external stimuli. Various *in vitro* studies have recorded the overexpression of different HSPs in drug-resistant *Leishmania* strains [15, 18, 82, 83]. However, although HSPs are the most abundant proteins in *T. cruzi* [84], their role in drug resistance remains unclear [85]. Of the HSPs identified in *Leishmania* parasites, HSP83 and HSP70 are involved in the activation of programmed cell death mediated by drugs, as they interfere with the mitochondrial membrane potential as has been observed in strains of *L. donovani* [83, 86]. In the present study, we observed the overexpression of transcripts encoding HSP70, HSP83 and HSP60 in the La-Sb^{III}-R line (Table 2). This supports previous findings [61] and reinforces the role of these proteins in resistance to antimony, both in Old and New World *Leishmania* species.

Conclusions

The transcriptomic analysis conducted in this study identified several transcripts that were differentially abundant between the antimony-resistant and -sensitive lines, several of which have previously been reported as potential therapeutic targets in Old World species as well as some New World species, including *L. braziliensis*, *L. guyanensis* and *L. panamensis.* Thus, we conclude that next-generation sequencing technologies are, and will continue to be, the gold standard techniques for understanding transcriptomic behaviour of a large number of organisms, increasing our knowledge of poorly understood species. Finally, although various studies propose intracellular amastigotes as the gold standard for *in vitro Leishmania* drug discovery research and evaluation of resistance [87, 88], we focused our molecular analysis on the promastigote stage for several reasons: the amastigote model is (i) time-consuming, (ii) laborious, (iii) difficult to manipulate in terms of inducing Sb^{III}-resistance [89, 90], and (iv) difficult to scale, thereby limiting its use in high-throughput screening approaches [91]. However, considering that the amastigote stage is the infectious form in the host, and that some of the genes with differential expression found in this study have been previously described by other researches using axenic amastigotes [22, 26], the results obtained here can be used in the future to guide targeted studies in this parasite infective stage. Future studies need to be conducted to validate the transcriptomic responses herein described.

Additional files

Additional file 1: Figure S1. Percent viability (Y-axis) of *L. amazonensis* promastigotes treated for 72 h with different concentrations of Sb^{III} (1.0 to 128.5 µg/ml), represented as $[Sb^{III}]$ Log_{10} (X-axis). The arrows show the IC50 reached by each line.

Additional file 2: Table S1. List of differentially expressed genes between La-SbIII-S and La-SbIII-R lines with a fold-change ≥ 2.

Abbreviations

La: *Leishmania amazonensis*; Sb^{III}: trivalent sodium stibogluconate; DEG: differentially expressed gene; HSP: heat-shock protein; RNA-seq: ribonucleic acid sequencing.

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Authors' contributions

LHP conceived and designed the study, analysed and interpreted the data and prepared the manuscript. CM critically revised the manuscript and made important suggestions. JDR conceived and designed the study and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Data supporting the conclusions of this article are included within the article and its additional files. The dataset generated during the present study was deposited at DDBJ/ENA/GenBank under the accession number PRJEB31417.

Ethics approval and consent to participate

This project was approved by the Ethics Committee of the Universidad de Antioquia (number VRI3445/2010). Written informed consent was obtained from the patient from which the strain was isolated.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details
[1] Grupo de Investigaciones Microbiológicas-UR (GIMUR), Programa de Biología, Facultad de Ciencias Naturales y Matemáticas, Universidad del Rosario, Bogotá, Colombia. [2] Programa de Estudio y Control de Enfermedades Tropicales (PECET), Facultad de Medicina, Universidad de Antioquia, Medellín, Colombia.

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Publisher's Note

Manuscript Details

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Title	Comparative genomics reveals moderate levels of ploidy, high heterozygosity and structural variations in Leishmania (Leishmania) amazonensis
Article type	Short Communication

Abstract

Leishmania amazonensis is one of the causative agents of the different forms of cutaneous leishmaniasis present in Latin America. This species has been isolated from humans and animals (canine/feline) in some endemic regions of Colombia. Therefore, L. amazonensis is of great relevance at the clinical and epidemiological levels in medicine and veterinary science. Until now, very few genomes from this species are available. Here, we report the complete genome sequence of a laboratory-adapted L. amazonensis strain isolated from a human patient with clinical manifestation of cutaneous leishmaniasis in Colombia. The genome sequence not only allowed inter and intra-species comparative analyses to be performed with the sequenced genomes of L. amazonensis strains from different geographical regions, but also increased our knowledge about the genomic behavior of this L. amazonensis Colombian strain. This isolate was also characterized in terms of single nucleotide polymorphisms, chromosome and gene copy number variations (CNVs). The results revealed moderate aneuploidy, CNVs in genes involved in the virulence, growth, and survival of the parasite, and in the distributions of the multicopy genes on some chromosomes, as well as a high level of heterozygosity. The data confirmed previous reports that identified unique variations in L. amazonensis, suggesting aneuploidy may not have a high fitness cost and may allow the rapid generation of diversity in Leishmania parasites growing normally.

Keywords	Genome sequencing, Aneuploidy, DNA-seq, Copy number variation, Parasites, Diversity.

Corresponding Author	Juan David Ramírez
Corresponding Author's Institution	UNIVERSIDAD DEL ROSARIO
Order of Authors	luz Patino, Marina Muñoz, Carlos Muskus, Juan David Ramírez

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Bogotá D.C., June 17th, 2019

Universidad del Rosario

Doctor
Felipe Guhl
Editor
Acta Tropica

Dear Felipe,

The leishmaniases are vector-borne diseases caused by the protozoan *Leishmania*, which exert a substantial burden on human populations since there are approximately 1.3 million cases per year, 98 countries are affected, and 350 million people are at risk of infection. Studies have reported that chromosome and copy number variation in *Leishmania*, produce a mechanism for the adaptation of this parasite to stress conditions. This demonstrates that this parasite presents a complex molecular machinery to circumvent stress conditions. However, some authors report that laboratory-adapted strains can also respond to genome changes.

Leishmania amazonensis is one of the causative agents of the different forms of cutaneous leishmaniasis present in Latin America. This species has been isolated from humans and animals (canine/feline) in some endemic regions of Colombia. Therefore, *L. amazonensis* is of great relevance at the clinical and epidemiological levels in medicine and veterinary science. Until now, very few genomes from this species are available. Here, we report the complete genome sequence of a laboratory-adapted *L. amazonensis* strain isolated from a human patient with clinical manifestation of cutaneous leishmaniasis in Colombia. The isolate was characterized in terms of single nucleotide polymorphisms, chromosome and gene copy number variations (CNVs). The results revealed moderate aneuploidy (disomic to tetrasomic), CNVs in genes involved in the virulence, growth, and survival of the parasite, and in the distributions of the multicopy genes on some chromosomes, as well as a high level of heterozygosity. The data confirmed previous reports that identified unique variations in L. amazonensis, suggesting aneuploidy may not have a high fitness cost and may allow the rapid generation of diversity in *Leishmania* parasites growing normally. The genome sequence that we constructed not only allowed inter and intra-species comparative analyses to be performed with the sequenced genomes of *L. amazonensis* strains from different geographical regions, but also will increase our knowledge about the genomic behavior of *this L. amazonensis* Colombian strain.

By means of this letter we intent to submit the manuscript entitled **"Comparative genomics reveals moderate levels of ploidy, high heterozygosity and structural variations in Leishmania (Leishmania) amazonensis"**

We believe this manuscript is of paramount importance for the scientific community and might be a good contribution for the wide-range audience ACTA TROPICA attracts.

Our sincere regards,

The authors

Grupo de Investigaciones Microbiológicas-UR (GIMUR)
Sede Quinta Mutis: Carrera 24 No. 63C-69
Tel: (571) 2970200 Ext. 4033
www.urosario.edu.co

Comparative genomics reveals moderate levels of ploidy, high heterozygosity and structural variations in *Leishmania (Leishmania) amazonensis*

Luz H. Patino[a], Carlos Muskus[b], Marina Muñoz[a], Juan David Ramírez[a]*

[a]Grupo de Investigaciones Microbiológicas-UR (GIMUR), Programa de Biología, Facultad de Ciencias Naturales y Matemáticas, Universidad del Rosario, Bogotá, Colombia.

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

*Corresponding author: Juan David Ramírez, Grupo de Investigaciones Microbiológicas-UR (GIMUR), Programa de Biología, Facultad de Ciencias Naturales y Matemáticas, Universidad del Rosario, Bogotá, Colombia. Tel: (571) 2970200 Ext. 4033. E-mail: juand.ramirez@urosario.edu.co

Data deposition:

The *L. amazonensis* assembled genome reported in this study was deposited at DDBJ/ENA/GenBank. It is openly available as BioSample SAMN10955936, being part of the BioProject PRJNA522806 with the accession number: CP040129-CP040162.

Abstract

Leishmania amazonensis is one of the causative agents of the different forms of cutaneous leishmaniasis present in Latin America. This species has been isolated from humans and animals (canine/feline) in some endemic regions of Colombia. Therefore, *L. amazonensis* is of great relevance at the clinical and epidemiological levels in medicine and veterinary science. Until now, very few genomes from this species are available. Here, we report the complete

genome sequence of a laboratory-adapted *L. amazonensis* strain isolated from a human patient with clinical manifestation of cutaneous leishmaniasis in Colombia. The genome sequence not only allowed inter and intra-species comparative analyses to be performed with the sequenced genomes of *L. amazonensis* strains from different geographical regions, but also increased our knowledge about the genomic behavior of this *L. amazonensis* Colombian strain. This isolate was also characterized in terms of single nucleotide polymorphisms, chromosome and gene copy number variations (CNVs). The results revealed moderate aneuploidy, CNVs in genes involved in the virulence, growth, and survival of the parasite, and in the distributions of the multicopy genes on some chromosomes, as well as a high level of heterozygosity. The data confirmed previous reports that identified unique variations in *L. amazonensis*, suggesting aneuploidy may not have a high fitness cost and may allow the rapid generation of diversity in *Leishmania* parasites growing normally.

Keywords: Genome sequencing, Aneuploidy, DNA-seq, Copy number variation, Parasites, Diversity.

1. Introduction

Leishmania amazonensis has emerged as a species of medical and veterinary importance particularly in Latin America, because of its strong association with the different forms of leishmaniasis: cutaneous leishmaniasis (Camara Coelho et al., 2011; Ramirez et al., 2016), diffuse and disseminated cutaneous leishmaniasis, (Silveira et al., 2009) and visceral leishmaniasis (Carvalho et al., 2015; Sanches et al., 2016; Tolezano et al., 2007), this latter mainly associated to infections in canines and felines (Herrera et al., 2018; Paz et al., 2018).

Due to the advances that have occurred during the last decade in the genomic area, mainly after the advent of high-throughput sequencing technology, the whole genome sequence of different trypanosomatids has been obtained; among them *T. cruzi, T. brucei, L. braziliensis, L. panamensis, L. mexicana, L. infantum, L. major* and *L. amazonensis* (Llanes et al., 2015; Real

et al., 2013; Tschoeke et al., 2014). In recent years, the whole genome sequence of *L. amazonensis* has been reported, and was found to be similar to the genome sequence of other *Leishmania* species with a high degree of conservation in gene content and architecture (Real et al., 2013). However, until now, few studies have analyzed the whole genome sequence of *L. amazonensis* isolates from endemic regions of Latin America. Recently, one comparative genomic analysis was conducted in Brazil, but in countries such as Colombia this information remains unknown. In this study, we performed genomic profiling to analyze the whole genome sequence of a laboratory-adapted strain of *L. amazonensis* isolated from a human patient with clinical manifestation of cutaneous leishmaniasis in the city of Medellin (Colombia).

2. Materials and Methods

2.1 Leishmania amazonensis culture conditions, DNA extraction, and species identification

Promastigotes of *L. amazonensis* (La_UA301) were axenically maintained in RPMI 1640 medium, supplemented with 10% (v/v) of fetal bovine serum and cultured at 26°C with 5% CO_2. The DNA extraction was conducted using the High Pure PCR Template Preparation Kit (Roche Life Science), according to manufacturer's instructions, later we did the species identification, which was performed using direct sanger sequencing of genes coding for the cytochrome b (Cytb) molecules and heat shock protein (HSP70), as described previously (Ramirez et al., 2016).

2.2 Inter- and intra-species phylogenetic inferences.

The inter-species analysis was done among *L. amazonensis* (La_UA301) and New and Old World *Leishmania* species (*L. braziliensis* (MHOM/BR/75/M2904), *L. panamensis* (MHOM/PA/94/PSC-1), *L. mexicana* (MHOM/GT/2001/U1103), *L. major* (Friedlin), *L. infantum* (JPCM5), and *L. donovani* (BPK282A1)) following the methodology proposed previously (Ocana and Davila, 2011). Briefly the amino acid sequence of 12 universal orthologs genes (UO) were clustered using CD-HIT (Fu et al., 2012)) and aligned using MAFFT v7.245

(Katoh and Standley, 2013). The phylogenetic tree was obtained using FastTree version 2.1.10 (Price et al., 2010). The intra-species analysis was done by comparing the publicly available draft genomes to *L. amazonensis* with the draft genome obtained in this study (La_UA301) through multiple genome alignment using progressive Mauve (Darling et al., 2010).

2.3 Genome sequencing and assembly

The extracted whole-genome DNA was sequenced on a HiSeq X-Ten system (Illumina) by the Novogene Bioinformatics Technology Co., Ltd (Beijing, 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 a read contained adapter contamination, >10% uncertain nucleotides, or >50% low-quality nucleotides (base quality <5) (Yan et al., 2013). Paired-end Illumina reads were mapped to the reference genome of *L. mexicana* MHOM/GT2001/U1103 (because the *L. amazonensis* genome is not completely annotated, and the genomes available until now do not present a good quality in the assembly) using SMALT v0.7.4 (www.sanger.ac.uk/resources/software/smalt/). The mapping involved the following parameters: exhaustive search option (−x and −y 0.8); a reference hash index of 13 bases; and a sliding step of 3. The read file merging, sorting and elimination of PCR duplicates were implemented with SAMtools (version 0.1.18) and Picard v1.85 (Dumetz et al., 2017; Imamura et al., 2016).

2.4 Evaluation of Chromosome and gene copy number variations (CNVs)

To obtain chromosomal read-depths, the sequence data of the sample was normalized using the median depth of 34 (*L. amazonensis*) chromosomes. The range of somy, the normalization of depths and the evaluation of local CNVs were performed as described previously (Dumetz et al., 2017). Heatmaps were created using the Heatmap3 package in R (Zhao et al., 2014). We evaluated the gene or chromosome copy number by considering their biological and statistical significance. Significance was set as z score cut off >2 and adjusted *p*-value (Student's t-test)

<0.05. Tandem gene arrays were defined as groups of homologous genes that were located contiguously in a chromosome.

2.5 Single nucleotide polymorphism (SNPs) analysis

To detect the SNPs, the reads were aligned to reference genome of *L. mexicana* MHOM/GT2001/U1103, using the Smalt program (version 0.7.4) (http://www.sanger.ac.uk/science/tools/smalt-0). SNPs were called using GATK program (version 3.4) (https://software.broadinstitute.org/gatk/). Low-quality SNPs were filtered by GATK Variant Filtration with QD < 2.0 || MQ < 40 || FS > 60.0 || ReadPosRankSum < −8.0.

3. Results and Discussion

3.1 Genome assembly

The assembly of our genome (La_UA301) was against *L. mexicana* MHOM/GT/2001/U1103, because this showed better assembly statistics (Table 1 and supplementary Table 1), that the reference genome of *L. amazonensis* (APNT01) (Real et al., 2013).

3.2 Phylogenetic analysis

The universal orthologous genes-based inter-species phylogeny showed that *L. amazonensis* (La_UA301) was closely related with *L. mexicana,* being these two species grouped in a nearby cluster to Old world *Leishmania* species (*L. infantum, L. donovani* and *L. major*). This reconstruction also revealed that *L. braziliensis* and *L. panamensis* belonging to *Viannia* subgenus, formed an independent cluster (Fig. 1A-left). The intra-species analysis revealed a close relationship between the currently available *L. amazonensis* sequences, whereas La_UA301 formed an independent node with RZOD01.1; this node was closely related with M2269 (Fig. 1A-Right). Although these results contribute in the intra-taxa description of *L. amazonensis*, analyses using a large number of genomes from different hosts and regions are necessary to confirm the relationship.

3.3 Chromosome copy number variations

Our results showed disomic behavior and strong diversity in the ploidy of *L. amazonensis,* 24 chromosomes disomic, 9 trisomic and 1 tetrasomic (Fig. 1B). These results confirmed previously published results (Imamura et al., 2016; Peacock et al., 2007; Real et al., 2013; Rogers et al., 2011; Valdivia et al., 2017), but contradicted those of Valdivia (Valdivia et al., 2017), who reported low or moderate ploidy in *L. amazonensis.*

Additionally, when we analyzed the chromosome copy number variation in La_UA301 line, we detected one extra copy in nine chromosomes and two extra copies in one chromosome (Fig. 1C). The results are consistent with those of previous studies, which demonstrates that the ploidy is unstable and may fluctuate in response to changes in the environmental conditions (Cuypers et al., 2018; Downing et al., 2011; Dumetz et al., 2017; Rogers et al., 2011).

3.4 Gene copy number variations

To evaluate the gene dosage per chromosome, we performed a read depth analysis and measured the copy number variation (CNV) for each gene. A total of 61 genes showed CNV (Z_score >2 adjusted p <0.05). Among these genes, 42% were associated with hypothetical proteins and the remaining 58% encoded proteins associated. The most expanded genes include those encoding an RNA helicase and putative pyroglutamyl peptidase I (PPI) (Supplementary Table 2). RNA helicase has been described in various organisms, but its biological function has yet to be established. PPI has been identified in *T. brucei* (Morty et al., 2006), where it is associated with protection against antimicrobial peptides, in *L. major*, where it seems to be a factor required for differentiation (Schaeffer et al., 2006) and in *L. amazonensis,* where it is also likely to act during the transition of metacyclic promastigotes (Valdivia et al., 2017). These results suggest that PPI may be an important virulence and differentiation factor in members of the Trypanosomatidae family.

Gene ontology (GO) analysis of the expanded genes was used to assign term under two main GO categories: biological process and molecular function. Under biological process, the

expanded genes were associated mainly with protein polymerization and regulation of organic transport and GTP catabolism. Under molecular function, the expanded genes were associated mainly with GTP binding, GTPase activity and carbohydrate derivate binding (supplementary Fig. 1).

In our analysis we found differences in the distribution of tandem genes arrays between the disomic and supernumerary chromosomes. We found five tandem arrays of genes encoding: surface antigen protein 2 (PSA2), elongation factor 1(EF-1α), ama1, heat shock protein 83-1, and beta tubulin that were distributed on disomic and trisomic chromosomes (Supplementary Table 2). This agree with previously reported results (Rogers et al., 2011; Valdivia et al., 2017; Valdivia et al., 2015), reaffirming the hypothesis that these proteins play an important roles in the adhesion, invasion, survival and stress response in laboratory-adapted isolated *L. amazonensis* promastigotes.

3.5 Single nucleotide polymorphism (SNPs)

We identified a total of 40,641 SNPs, between the reference genome sequence of *L. mexicana* and genome sequence of La_UA301 line. The high level of heterozygosity observed could in part be due to the supernumerary nature of some of its chromosomes (10 in total).

4. Conclusion

In summary, we identified the genomic profile in terms of ploidy, CNV and SNPs of laboratory-adapted *L. amazonensis* promastigotes from Colombia. The results obtained confirm previous reports which identified variations unique to this species (Valdivia et al., 2017) and suggest that the ploidy state could allow rapid generation of diversity in *Leishmania* parasites growing normally and is not only a condition present in strains under stress conditions.

Further analysis that allow to compare the genomic profile between laboratory-adapted *L. amazonensis* isolate and natural population are necessary, as well as transcriptomic data to determine if the CNVs found in this study can affect genome expression.

Figure Legends

Fig. 1. Description of phylogenetic intra e inter species relationship in *Leishmania amazonensis*. Phylogenetic reconstructions at inter-species level (left) based on 12 universal orthologous (UO) genes shared between six *Leishmania* species (*L. braziliensis*, *L. panamensis, L. mexicana, L. major*, *L. infantum* and *L. donovani*) and our experimental line (*L. amazonensis*). Blue dots represent the percentage of bootstrap values (≥ 90%). Phylogenetic relationship of *L. amazonensis* at the intra-species level (Right). La_UA301: Colombian *L. amazonensis* genome, M2269: *L. amazonensis* (MHOM/BR/71973/M2269); APNT01.1: *L. amazonensis* (APNT01.1); RZOD01.1: *L. amazonensis* strain 210-660; GDB: *Leishmania (L.) amazonensis* Genome DB. **B.** Dynamics of aneuploidy in *Leishmania. amazonensis*. Heatmap of the copy numbers of 34 chromosomes in the *L. amazonensis*. The color key indicates the somy value (S) as describe (Rogers et al., 2011). **C.** Chromosomal copy number variation in *Leishmania amazonensis*. The columns represent the estimated haploid copy number for each of the 34 chromosomes. Mean genome ploidy is indicated by a dotted red line.

Conflict of interest.

The authors declared that they have no conflict of interest.

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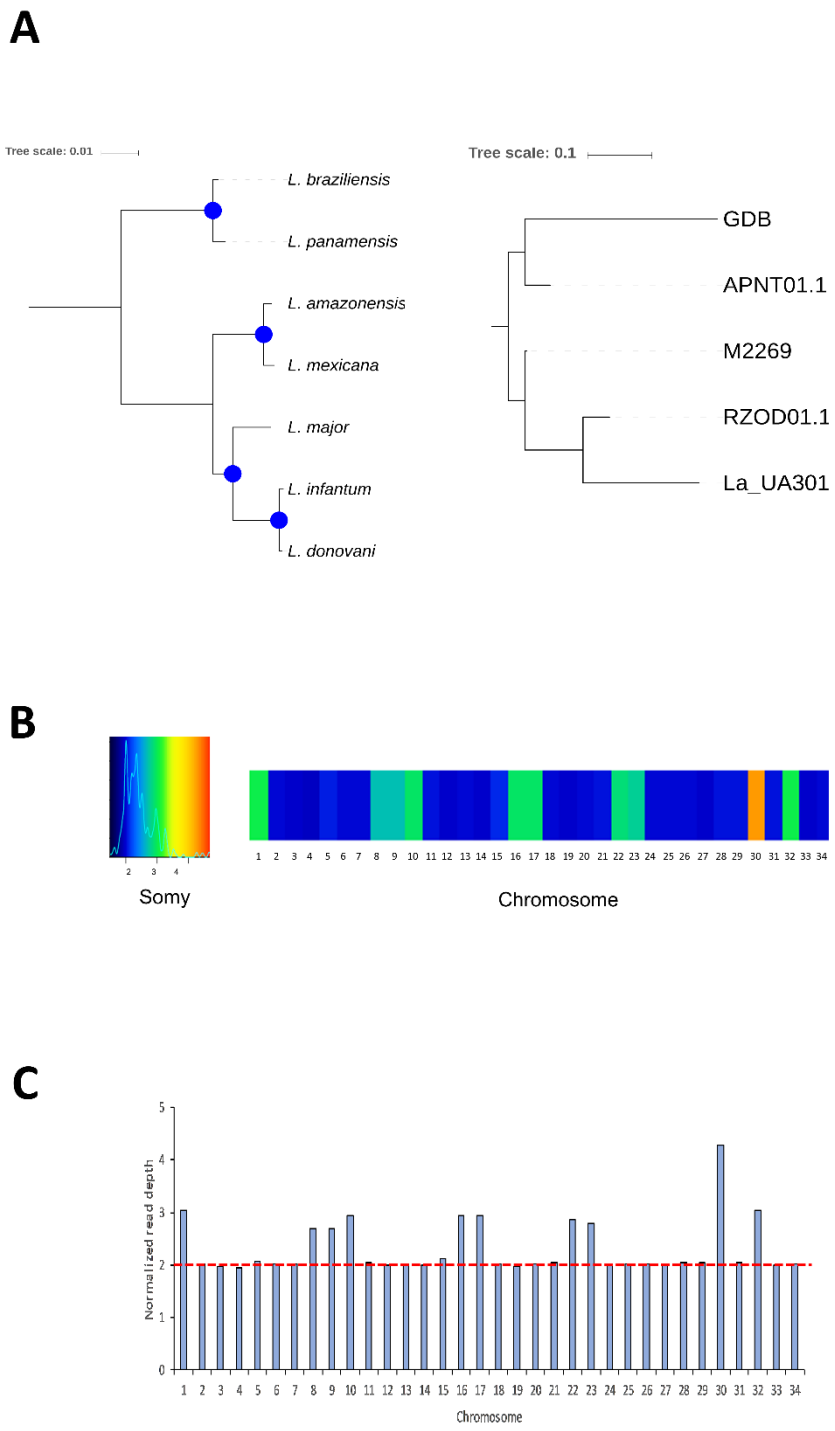
Table 1. Genome assembly results using as genomes of reference: *L. amazonensis* (APNT01) or *L. mexicana* (MHOM/GT/2001/U1103)

Variable	*L. amazonensis* (APNT01)	*L. mexicana* MHOM/GT/2001/U1103
Total of scaffolds	126	34*
average	10421	945778
Longest	58957	3336136
N50 (lenght)	19830	1135553
N50 (n)	23	9

* one per chromosome

Figure 1. Description of phylogenetic intra e inter species relationship in *Leishmania amazonensis*



Declaration of interests

 The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

LUZ H PATINO
MARINA MUÑOZ
CARLOS MUSKUS
JUAN RAMIREZ

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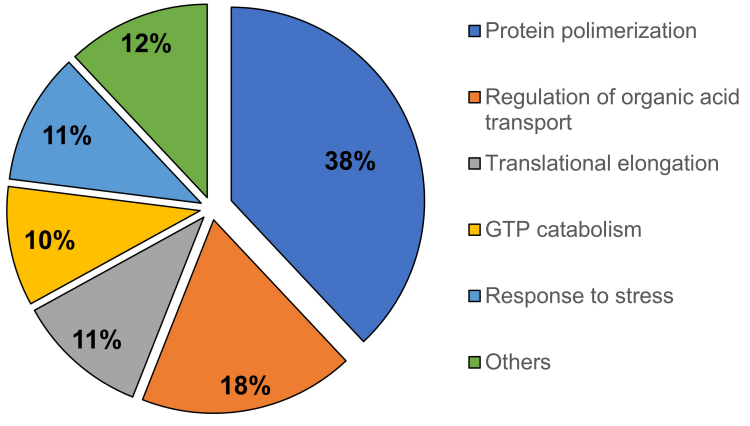
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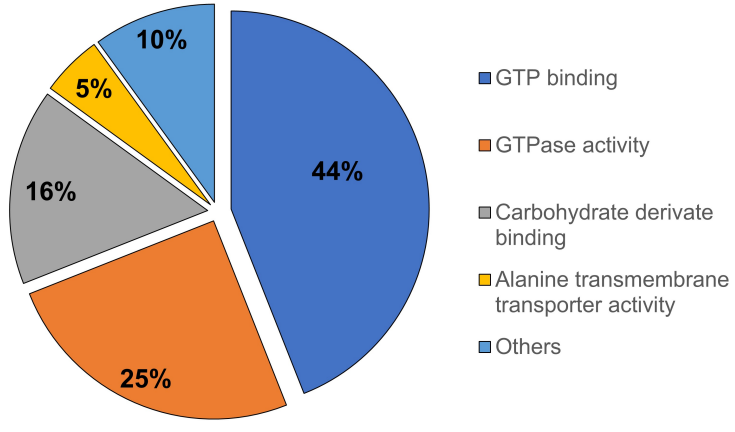
Translational elongation
GTP catabolism
Response to stress
Others

B


44%
25%
16%
5%
10%
GTP binding
GTPase activity
Carbohydrate derivate binding
Alanine transmembrane transporter activity
Others

Supplementary Table S1. Genome assembly statistics (Scaffolds) by chromosome

Sequence ID	Chromosome name	Length
La_UA301.01	1	272354
La_UA301.02	2	297518
La_UA301.03	3	375031
La_UA301.04	4	438102
La_UA301.05	5	449889
La_UA301.06	6	479116
La_UA301.07	7	567734
La_UA301.08	8	1668271
La_UA301.09	9	543028
La_UA301.10	10	515793
La_UA301.11	11	557223
La_UA301.12	12	505844
La_UA301.13	13	611478
La_UA301.14	14	609140
La_UA301.15	15	573732
La_UA301.16	16	687791
La_UA301.17	17	663912
La_UA301.18	18	691723
La_UA301.19	19	653729
La_UA301.20	20	3336136
La_UA301.21	21	735541
La_UA301.22	22	682569
La_UA301.23	23	743192
La_UA301.24	24	837376
La_UA301.25	25	882953
La_UA301.26	26	1027075
La_UA301.27	27	1042268
La_UA301.28	28	1135553
La_UA301.29	29	1320397
La_UA301.30	30	1435569
La_UA301.31	31	1521989
La_UA301.32	32	1404422
La_UA301.33	33	1649823
La_UA301.34	34	1964255

Supplementary Table 2. List of genes that presented CNV in UA301 line (z score cut off >2 and adjusted p-value (Student's t-test) <0.05.)

Gene ID	Product description	Z score
LmxM.08.1171	unspecified product	6.611
LmxM.10.0470	GP63 leishmanolysin	4.551
LmxM.10.0720	amino acid permease 24 putative	3.907
LmxM.10.1320	fatty acid desaturase putative	2.240
LmxM.12.0867	hypothetical protein conserved	11.192
LmxM.12.0890	surface antigen protein 2 putative	3.879
LmxM.12.0891	surface antigen protein 2 putative	4.888
LmxM.12.0990	surface antigen protein putative	4.551
LmxM.13.0280	alpha tubulin	6.064
LmxM.13.0290	unspecified product	3.066
LmxM.13.0300	unspecified product	6.121
LmxM.13.0390	alpha tubulin	6.218
LmxM.14.0400	hypothetical protein conserved	2.478
LmxM.15.0440a	unspecified product	13.378
LmxM.15.0440b	unspecified product	11.108
LmxM.15.1050	developmentally regulated protein putative	5.560
LmxM.15.1150	developmentally regulated protein putative	3.430
LmxM.15.1160	tryparedoxin peroxidase	6.331
LmxM.17.0080	elongation factor 1-alpha	5.112
LmxM.17.0081	elongation factor 1-alpha	6.288
LmxM.17.0082	elongation factor 1-alpha	5.967
LmxM.17.0084	elongation factor 1-alpha	5.840
LmxM.17.0085	elongation factor 1-alpha	4.579
LmxM.19.0850	ATG8FAUT7FAPG8FPAZ2 putative	2.086
LmxM.22.1290	ribonucleoside-diphosphate reductase small chain putative	4.355
LmxM.27.2020	D-lactate dehydrogenase-like protein	2.296
LmxM.28.2770	heat-shock protein hsp70 putative	2.716
LmxM.29.1410	ama1 protein putative	4.789
LmxM.29.1420	ama1 protein putative	3.150
LmxM.29.1500	p1Fs1 nuclease	3.430
LmxM.30.0430	unspecified product	17.944
LmxM.30.0440	unspecified product	19.220
LmxM.30.0440b	unspecified product	2.871
LmxM.30.0470	hypothetical protein conserved	3.710
LmxM.30.0470b	unspecified product	3.262
LmxM.30.0480	hypothetical protein conserved	12.831
LmxM.30.0480b	unspecified product	12.944
LmxM.30.0490	hypothetical+protein+unknown+function	6.148
LmxM.30.0930	sodium stibogluconate resistance protein putative	4.033
LmxM.30.0950	unspecified product	2.926

LmxM.30.0960	unspecified product	2.100
LmxM.30.1820	amino acid permease	2.926
LmxM.30.1890	peptidase m20Fm25Fm40 family-like protein	2.100
LmxM.30.3180	histone H4	3.753
LmxM.32.0312	heat shock protein 83-1	7.129
LmxM.32.0314	heat shock protein 83-1	7.101
LmxM.32.0316	heat shock protein 83-1	7.059
LmxM.32.0792	beta tubulin	6.232
LmxM.32.0794	beta tubulin	5.336
LmxM.33.0960	amastin-like surface protein putative	3.921
LmxM.33.1830	unspecified product	15.956
LmxM.33.1990	hypothetical protein conserved	21.433
LmxM.33.2000	pyroglutamyl-peptidase I	19.303
LmxM.33.2010	hypothetical protein conserved	14.947
LmxM.33.2020	hypothetical protein conserved	15.857
LmxM.33.2030	hypothetical protein conserved	14.737
LmxM.33.2040	hypothetical protein unknown function	21.391
LmxM.33.2050	RNA helicase putative	20.999
LmxM.33.2060	hypothetical protein conserved	20.691
LmxM.33.2070	hypothetical protein conserved	22.386
LmxM.34.1830a	unspecified product	2.071

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Review

RNA-seq in kinetoplastids: A powerful tool for the understanding of the biology and host-pathogen interactions

Luz Helena Patino, Juan David Ramírez *

Grupo de Investigaciones Microbiológicas-UR (GIMUR), Programa de Biología, Facultad de Ciencias Naturales y Matemáticas, Universidad del Rosario, Carrera 24# 63C-69, Bogotá, Colombia

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ABSTRACT

The kinetoplastids include a large number of parasites responsible for serious diseases in humans and animals (*Leishmania* and *Trypanosoma brucei*) considered endemic in several regions of the world. These parasites are characterized by digenetic life cycles that undergo morphological and genetic changes that allow them to adapt to different microenvironments on their vertebrates and invertebrates hosts. Recent advances in ´omics´ technology, specifically transcriptomics have allowed to reveal aspects associated with such molecular changes. So far, different techniques have been used to evaluate the gene expression profile during the various stages of the life cycle of these parasites and during the host-parasite interactions. However, some of them have serious drawbacks that limit the precise study and full understanding of their transcriptomes. Therefore, recently has been implemented the latest technology (RNA-seq), which overcomes the drawbacks of traditional methods. In this review, studies that so far have used RNA-seq are presented and allowed to expand our knowledge regarding the biology of these parasites and their interactions with their hosts.

Contents

1. Introduction

The kinetoplastids are flagellated protozoa, considered unicellular eukaryotic organisms. This group includes a number of parasites responsible for a number of diseases in humans and animals. The most relevant in causing disease in humans are *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania* (Archer et al., 2011; McCall and McKerrow, 2014; Murray et al., 2012).

Trypanosoma brucei, the causative agent of African trypanosomiasis, also known as sleeping sickness presents two clinical forms: The Gambian sleeping sickness (caused by *Trypanosoma brucei gambiense*) (Checchi et al., 2008) and Rhodesian sleeping sickness (caused by *Trypanosoma brucei rhodesiense*) (MacLean et al., 2010). The main

* Corresponding author at: Carrera 24 # 63C - 69, Bogotá, Colombia.
E-mail address: juand.ramirez@urosario.edu.co (J.D. Ramírez).

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mechanism of transmission is through the bite of the tsetse fly (genus *Glossina*) (Aksoy et al., 2003). However, other less frequent transmission routes could be associated: vertical transmission, accidental mechanical transmission (laboratory accidents), blood transfusions, organ transplants and possible sexual contact (Franco et al., 2014). This disease is mainly concentrated in African countries. It is estimated that 70 million people are at risk for African trypanosomiasis, of which 82% of the population is at risk of infection by *Trypanosoma brucei gambiense* and the remaining 17.8% are at risk of infection by *Trypanosoma brucei rhodesiense* (Simarro et al., 2012). Gambian African trypanosomiasis is distributed in 14 countries; the majority (97%) concentrated in five of them. In contrast, the Rodhesiense African trypanosomiasis is distributed in 13 countries in eastern and southern Africa (Welburn et al., 2016).

The genus *Leishmania* is transmitted to humans by the bite of female infected sandflies (Maroli et al., 2013; Van der Auwera and Dujardin, 2015). It is responsible for leishmaniasis, which is characterized by a broad spectrum of clinical manifestations, among which include: Cutaneous leishmaniasis (CL) (Azmi et al., 2012) mucosal leishmaniasis (ML) and, visceral leishmaniasis (VL) (Banuls et al., 2007; Lukes et al., 2007; Reithinger et al., 2007). The World Health Organization (WHO) estimates that 350 million people worldwide are at risk of infection, thus considering this disease as a public health problem. Recent data determined that approximately 0.2 to 0.4 million new VL cases and 0.7 to 1.2 million of new cases of CL are presented annually (Alvar et al., 2012; Banuls et al., 2007; Desjeux, 2004).

During their processes of differentiation in the vertebrate and invertebrate host, these parasites are subject to multiple environmental changes (Barak et al., 2005; Garlapati et al., 1999; Geiger et al., 2011; Zilberstein and Shapira, 1994), many of these environmental cues promote differentiation and gene expression, associated to important biochemical and morphological changes (Alcolea et al., 2010; Rochette et al., 2008). While some of these adaptations can be seen as morphological modifications and changes in the components of the cell surface, little is known about global changes occurring at the transcriptional level (Ambit et al., 2011; Dillon et al., 2015a; Matthews et al., 2004; Wheeler et al., 2011).

Recent advances in the technology of "omics" including genomics and transcriptomics have allowed to reveal important aspects associated with such molecular changes, thus allowing to expand the knowledge about the biology of the parasite, its interaction with the vertebrate and invertebrate hosts, the phenotype of the disease, the mechanisms of action of drugs conventionally used in the treatment and strategies of resistance that these parasites have developed against them (Cantacessi et al., 2015; Geiger et al., 2011; Kaur and Rajput, 2014; Pawar et al., 2014). As transcriptomics refers, various technologies have been developed for deducing and quantifying the level of gene expression (Geiger et al., 2011; Gerhard et al., 2004; Malone and Oliver, 2011; Harbers and Carninci, 2005; Kavak et al., 2010; Reinartz et al., 2002; Veitch et al., 2010), where the sequencing of RNA (RNA-seq) has been the technology recently developed and has presented several advantages over those conventionally used (Geiger et al., 2011; Wang et al., 2009).

This review allows an overview of studies that have used RNA-seq, in order to determine gene expression in the infective and non-infective stages of these parasites (*Trypanosoma brucei* and *Leishmania*) and the interaction between them and their vertebrates and invertebrates hosts. However, we included in this review only *Trypanosoma brucei*, and *Leishmania* due to the lack of information available about *Trypanosoma cruzi*. This review is not intended to describe all the studies that have used RNA-seq as a tool, to understand the molecular characteristics of the trypanosomatids, this review aims to show that RNA-seq has been a fundamental tool to determine gene expression in the infective and non-infective stages of these parasites (*Trypanosoma brucei* and *Leishmania*) and the interaction between them and their vertebrates and invertebrates hosts. The results contribute to not only clarify aspects still uncertain about their biology but also contribute to the implementation of strategies for diagnosis, treatment and prophylaxis.

2. RNA-seq as a tool in the knowledge of gene expression of trypanosomatids

One of the main objectives of the techniques used in the analysis of the transcriptome is to capture many genes and identify the precise sequence of nucleotides transcripts (Fiebig et al., 2015; McGettigan, 2013). This is particularly important in the Trypanosomatidae where regulation in gene expression occurs post-transcriptionally (Haile and Papadopoulou, 2007) and also where it has been shown that the differences in non-coding regions (UTR) 5′ and 3′ of the same gene are associated with differential expression of transcripts in the different phases of the life cycle of the parasites (Fiebig et al., 2015; Mishra et al., 2003; Murray et al., 2007). While the UTRs sequences have been a valuable resource for many researchers, the lack of precise techniques has hindered the ability of analysis of these fragments which may be involved in the regulation of individual genes. Such is the case of the analysis of gene expression based on microarrays which have been used to compare the differential expression of genes at different stages of the life cycle of these parasites (Akopyants et al., 2004; Cohen-Freue et al., 2007; Diehl et al., 2002; Holzer et al., 2006; Jensen et al., 2009; Kabani et al., 2009; Queiroz et al., 2009; Saxena et al., 2003). This approach has certain limitations, such as artifacts in hybridization, loss of sensitivity, need for large quantities of RNA and the inability to detect UTR regions in the 3′ and 5′ ends (Geiger et al., 2011). These limitations result in the identification of an incomplete list of genes that show significant changes in mRNA levels at different stages of the life cycle of the parasite (Dillon et al., 2015a). Fortunately, the use of technologies of next generation sequencing as RNA-seq have helped to minimize these great difficulties and additionally have allowed not only to map the transcripts at the nucleotide level but also to provide a robust reading of the mRNA, thus revolutionizing the way wherein the transcriptomes are analyzed (Haydock et al., 2015; Rastrojo et al., 2013).

Up next are shown the studies that so far have been conducted and that using RNA-seq technology have allowed not only an approach of gene expression of some species of Trypanosomatidae but also in some stages of their life cycle, and also understand how this differential expression allows adaptation and host-parasite interaction.

2.1. Gene expression profiles in different parasite stages

So far, several studies which used different technologies (Microarrays, SAGE, DGE) to analyze the gene expression profile in the different stages of the life cycle of Trypanosomatidae parasites (*Leishmania* and *T. brucei*) conclude that only a small percentage of genes show significant changes in mRNA levels in some of these species (Brems et al., 2005; Diehl et al., 2002; Haydock et al., 2015; Kabani et al., 2009; Koumandou et al., 2008; Queiroz et al., 2009; Veitch et al., 2010). However, as we shall see below, when next-generation sequencing (RNA-seq) for the analysis of these transcripts is used, not only increases in the percentage of genes with differential expression but also the identification of new genes, non-coding RNAs (ncRNA), small nucleolar RNA (snoRNA) and UTR 3′ and 5′ analyses.

2.1.1. Leishmania

One of the first species of *Leishmania* in which a complete characterization of the transcriptome using next-generation sequencing for analysis of RNA (RNA-seq) was conducted is *Leishmania major*, whose gene sequence was completed in 2005 (Ivens et al., 2005). This analysis was conducted by Rastrojo et al. This group conducted the first score of the transcriptome in promastigotes, covering nearby 91% of the genome by identifying and determining the level of relative expression of 10,285 transcripts (Fig. 1). Among the most abundant transcripts identified in the promastigotes are those coding for heat shock protein 70 (HSP70), ribosomal proteins, transport proteins nucleosides, histones associated proteins, lipophosphoglycans (major glycoprotein cell surface of promastigotes), peptidases, transcripts coding for hypothetical proteins

Sample Used	Objective of the study	Findings	Reference
Prom. *L. major* (Clone V1 MHOM/IL/80/Friedlin)	Characterize the transcriptome poly-A on promastigotes	10285 trnascripts (1884 new genes and 410 genes that had been poorly annotated).	Rastrojo et al., 2013
	Map UTR regions 5 ' and 3 ' and determine the heterogeneity in spliced leader (SL) and polyadenylation sites.	9530 and 4531 major alternative sites, addition of SL and 3718 main polyadenylation sites and 1238 alternative.	
Prom. *L. major* (Clone V1 MHOM/IL/80/Friedlin)	Resequencing and the novo assembly	Identification of 7 lost regions, leading to the discovery of six new genes and correction in the annotation of two genes.	Alonso et al., 2016
Prom.P vs Prom.M. *L major* (clone V1 MHOM/IL/80/Friedlin)	Determine changes in gene expression when *L. major* changes from Prm-P to Prm-M	3138 differentially expressed genes.	Dillon et al., 2015a
	Quantify events of alternative RNA processing (quantification of alternative RNA processing events)	8777/8981 genes (~98%) using more than one trans-splicing site and 8391/8841 genes (~95%) show alternative polyadenylation.	
Prom y Ama. *L. mexicana* (MNYC/BZ/62/M379)	Characterize the transcriptome of promastigotes and amastigotes	9169 encoding genes (937 new genes).	Fiebeg et al., 2015
	Compare the transcriptome between promastigotes and amastigotes	3.832 genes (41%) were differentially expressed.	
	Identify the genomic positions of spliced leader sites and polyadenilation sites	Genomic position of the sites SL: 21.249. Genomic position of polyadenylation sites: 96.522.	
No information	Analyze the small nucleolar RNA repertoire (snoRNA)	snoRNA guide the modification of ribosomal RNA (rRNA) and nuclear RNA (snRNA).	Eliaz et al., 2015

Sample Used	Objective of the study	Findings	Reference
Prom. *L. major* (Clone V1 MHOM/IL/80/Friedlin). Murine macrophages	Evaluate global changes in gene expression in murine macrophages, in the course of infection (during 72hpi)	6872, 931, 1813 and 1460 differentially expressed genes at 4, 24, 48 and 72 hpi.	Dillon et al., 2015b
	Evaluate global changes in gene expression in the parasite, in the course of infection (during 72hpi)	2962 genes differentially expressed between Prm-M and Ama 4hpi, 301 in amastigotes between 4-24hpi and none in the other time points analyzed.	
Tissue fragments obtained from patients with cutaneous and mucocutaneous leishmaniasis caused by *L. braziliensis*	Compare and characterize the transcriptome of primary cutaneous lesions in patients with CL and MCL and the *L. braziliensis* transcriptome (RNA-seq dual)	Overexpression of associated recruitment and activation of immune cells and inflammatory response regulation of genes. Low level of expression in *L. braziliensis* transcripts.	Maretti-Mira et al., 2012
L. major (clone VI,MHOM/IL/80/Friedlin and *L. amazonensis* (IFLA/BR/67/PH8) and human Macrophages	Evaluate the transcriptome of human macrophages, in the course of an intracellular infection (4-72hi) (RNA-seq dual)	5713, 4846, 4188 and 2142 genes with differential expression were detected in macrophages infected with *L. major* at 4, 24, 48 and 72 hpi respectively and 3964 genes in macrophages infected with *L. amazonensis.*	Fernandes et al., 2016
	Assess individual gene expression during the transition from Prm-M to Ama, *L. major* and *L. amazonensis*, in the course of an intracellular infection (4-72hpi) (RNA-seq dual)	3224, 356, 3 and 0 genes with differential expresson in *L. major* during 4, 4-24, 24-48 and 48-72 hpi respectively and 3896, 577, 3 and 0 genes differentially expressed in *L. amazonensis* during 4, 4-24, 24-48 and 48-72 hpi respectively.	
Cutaneous leishmaniasis (CL) patients in an area endemic to *L. braziliensis*	Capture the transcriptomic of both the parasite and the human host during *L. braziliensis* localized cutaneous infection.	477 and 241 differentially expressed genes in PTpos and PTneg, respectively and high degree of uniformity in *L. braziliensis* transcript expression in all patient lesions.	Christensen et al., 2016

Fig. 1. Principal findings retrieved using RNA-seq for a deeper analysis of *Leishmania* biology and its interaction with the vertebrate host. Prm.P: procyclic promastigote, Prm.M: metacyclic promastigote, Ama: amastigote, Macr: macrophage. PTpos: parasite transcripts positive. PT neg: parasite transcripts negative.

and tubulin. In addition, transcriptome analysis allowed mapping the 3′ and 5′ UTR regions. Regarding the 5′ region, the results demonstrated the existence of a remarkable heterogeneity in the sites of the spliced leader: SL (mini-exon of 39 nucleotides), and in sites of polyadenylation (Fig. 1). Finally, it was observed that about 50% of the genes presented multiple transcripts that differ in the length of UTR, which could modify the stability of RNA and/or influence the efficiency of translation of RNA (Rastrojo et al., 2013; Rogers et al., 2011). Subsequent studies by the same group using the same technology (RNA-seq), carry out a *de novo* assembly to rebuild some genetic regions, which had not been assembled in previous studies. During initial analysis 163,714 reads (1.1%), of a total of 14,656,121 could not be aligned with the genome sequence available

(Friedlin *L. major*). This new assembly allowed to identify lost seven regions, which led to the discovery and the correction of new genes (Fig. 1) (Alonso et al., 2016).

Another study that analyzed the level of gene expression by RNA-seq of promastigotes of *L. major* was conducted by Dillon et al. In this study, they performed the characterization of changes in gene expression and RNA processing events when *Leishmania major* promastigotes become procyclical (not infective form) to metacyclic promastigote (infective form), events occurring within the invertebrate host (vector). The results showed that 3138 genes are differentially expressed during this transformation (Fig. 1) 60% (1829/3138) which were annotated as hypothetical proteins. Additionally, the list of differentially expressed genes was used along with Gene Ontology analysis to identify functions and cellular processes that are carried out during the metacyclogenesis of *L. major*. The results showed a reduction in the number of cellular processes including DNA replication, assembly of nucleosome related transcription (initiation and elongation), protein metabolism and energy metabolism activities, while genes that were over-regulated in metacyclic promastigotes indicate an increase in cell signaling and response to stress. In this study, the authors describe the major genes that showed down-regulation in metacyclic promastigotes: LIT1 (iron transporter), histones (H2, H2B, H4 and similar to H1 protein), cyclin A, β-tubulin and genes associated with the metabolism of purines (adenine aminohydrolase). While genes that were over-expressed were those associated with the synthesis of ascorbate peroxidase, casein kinase 1 and the p27 gene which encodes a membrane-associated mitochondrial protein. As in other studies, Dillon et al., identified sites of spliced leader and polyadenylation but unlike these, they quantitated alternative splicing processes (Fig. 1) (Dillon et al., 2015a).

Leishmania mexicana has been one of the species of *Leishmania* used for transcriptome analysis whose genome was completed in 2011 (Rogers et al., 2011). This species was the object of the study of Fiebig et al., who by RNA-seq, characterized and compared the gene expression profile in promastigotes and amastigotes, the resulted obtained allowed to identify genes of which had not been previously described (Fig. 1). The comparative analysis between the two forms revealed that 3832 genes (41%) showed a statistically significant change in the amount of mRNA from promastigotes and amastigotes when they are exposed to different conditions. 126 new transcripts were 2 times more abundant in amastigotes compared to promastigotes. Many genes that were over-expressed included cell surface proteins, carrier proteins and peptidases, whereas those genes associated with motility (flagellum) showed low expression. Of 936 genes identified as new, 293 were not characterized. Similarly, the differential expression of the entire genome revealed aneuploidy on chromosome 30 (tetrasomic) in the amastigote form, which could be the first evidence of a link between the events of gene duplication and adaptation to the vertebrate host. Additionally, this group defined by RNA-seq the genomic position of splice leader and polyadenylation sites (Fig. 1) (Fiebig et al., 2015).

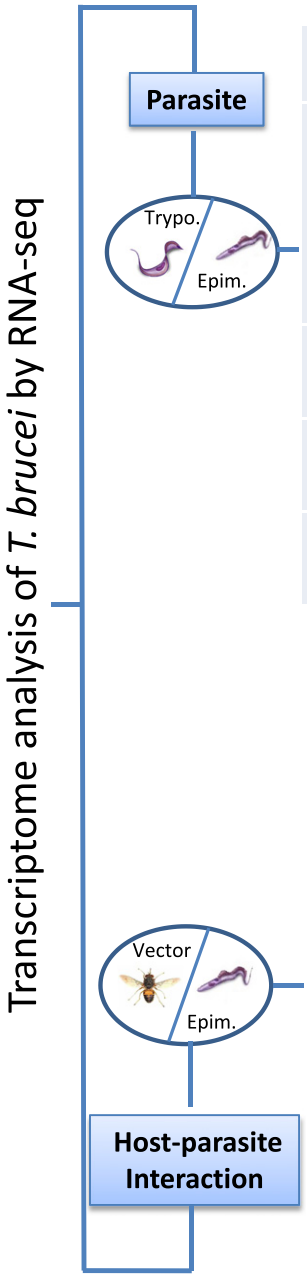
In contrast, what is described so far, RNA-seq has also been useful to analyze the full repertoire of small nucleolar RNA (snoRNA) of *L. major*. A study by Eliaz et al., using core proteins C/D snoRNA (SNU13) and H/ACA (NHP2) reveal that almost all snoRNA have great potential to guide the modification of ribosomal RNA (rRNA). However, in other studies, these same authors note that these snoRNA also guide the change in nuclear RNA (snRNA) suggesting that *Leishmania* species have the ability to use snoRNA H/ACA to guide more than a simple modification (Fig. 1). On the other hand, comparative analysis of snoRNA of *T. brucei* and *L. major* reveals that 80% of cases, the molecules snoRNA H/ACA and C/D are homologous between the two species guiding the same modification (Eliaz et al., 2015). Finally, RNA-seq has allowed to evaluate the gene expression profile when promastigotes of *L. amazonensis* and *L. donovani* are exposed to different growing conditions, especially with deprivation of essential nutrients revealing the importance of these components during the life cycle (Martin et al., 2014; Mittra et al., 2013), additionally has allowed to decipher the role of iron, iron superoxide dismutase and reactive oxygen species in differentiating *L. amazonensis* amastigotes (Mittra et al., 2013). Although so far there are very few studies that using RNA-seq allow to compare the transcriptomes of the stages of the *Leishmania* species during their life cycle, the results provide valuable information and which provide the basis for future comparative analysis with other stages of the life cycle or other *Leishmania* species (do Monte-Neto et al., 2011; Guimond et al., 2003; Rabhi et al., 2013).

2.1.2. Trypanosoma brucei

Trypanosoma brucei has been one of the members belonging to the Trypanosomatidae family whose transcriptome has been analyzed by RNA-seq. As mentioned above, this parasite presents several stages during its life cycle. However, the two stages most commonly studied in the laboratory have been circulatory forms present in mammalian hosts (BF: blood forms) and procyclic forms (PF: procyclic form) which differentiate in the midgut of the insect vector (tsetse fly). One of the studies that allowed by RNA-seq to evaluate the transcriptome of these parasitic forms was conducted by Siegel et al., who not only evaluated the gene expression level of the slender circulatory forms and procyclic forms but they also mapped the 3′ and 5′ UTR regions for 7000 genes (which represent 90% of the genome) (Fig. 2). Additionally they were able to identify introns in only two of the genes evaluated, which according to the authors could be due to its rare presence in the genome of *Trypanosoma* or that these introns represent a significant percentage in genes with low expression in the stages of the life cycle (Siegel et al., 2010).

Moreover Kolev et al., by RNA-seq managed mapping transcribed regions and sites of initiation of transcription by RNA polymerase II (Fig. 2), thereby enabling to confirm and/or correct previously annotated genes. 27 of these new transcripts containing ORF that were preserved and recorded in *T. cruzi* and *Leishmania major* and 23 polypeptides were preserved but not listed on *T. cruzi* and *Leishmania major*. Additionally, RNA-seq was useful to observe heterogeneity in *trans*-splicing sites and polyadenylation. Although the *trans*- and *cis*-splicing are related, the authors observe differences between these two processes. While the *cis*-splicing occurs within the ORF, the *trans*-splicing takes upstream location thereof. One consequence of the heterogeneity observed in both the *trans*-splicing and polyadenylation was the generation of a lot of mRNAs with the same coding potential but UTRs (3′ or 5′) of variable length (hundreds of nucleotides), which is unusual, especially for a parasite where regulation of gene expression occurs post-transcriptional level. Finally, the group estimated that 75% of *T. brucei* genes are expressed at levels between 1 and 10 mRNAs by procyclical cell (Kolev et al., 2010).

Subsequently, Siegel et al., suggest that variation in the length of the UTR described in previous studies can lead to the inclusion or exclusion of regulatory elements that influence the translational efficiency (Siegel et al., 2011). A year later (2012), Michaeli et al., evaluated the ability of RNA-seq to examine the repertoire of ncRNA small ribonucleoprotein particles present in (NTR) of *T. brucei* (Fig. 2). The results obtained might suggest that differential expression of ncRNA can contribute in gene regulation during the life cycle of this parasite (Michaeli et al., 2012). Another study by RNA-seq in *T. brucei* was conducted by Schulz et al. They determined using this technology, that the protein bromodomain maintains the circulatory stage of the parasite (BF) preventing their differentiation to procyclically shape (PF) (Fig. 2). The results showed that when proteins are inhibited or mutated, expression is increased in genes that reside only in the procyclic form (procyclins: EP1–3 and GPEET, genes associated with procyclins: PAG1, 2, 4, 5, procyclin surface antigen status: PSSA) and gene regulation is decreased, the expression is normally high in circulatory stage as invariant surface glycoprotein 64: ISG64, pyruvate kinase 1: PYK1 and glycosylphosphatidyl inositol specific phospholipase C: GPI-PLC. Additionally, it was shown that these proteins play an important role in the processes of immune evasion in the mammalian



Sample Used	Objective of the study	Findings	Reference
BF (MITat 1.2, clone 221a) and PF (Strain Lister 427)	Quantify the transcriptomes for BF and PF	221 genes upregulated >2-fold in BF and 204 upregulated more than 2-fold in PF	Siegel et al., 2010
	Map the 3' and 5' UTR regions	10.857 SAS for 6959 genes and 16.863 polyadenylation sites to 5948 genes.	
No Information	Map of transcribed regions and sites of initiation of transcription by RNA polymerase II	1114 new transcripts (12% of total), 103 of which were non-coding (ncRNA)	Kolev et al., 2010
PF strain 29–13	To examine the repertoire of ncRNAs present in small ribonucleoprotein particles (RNPs)	79 C/D and 63 H/ACA snoRNAs, expressed in the PF form	Michaeli et al., 2012
BF cells (strain Lister 427 antigenic type MITat1.2 clone 221a)	To determine if the bromodomain protein maintain to *T. brucei* in their BF and preventing differentiation to the PF	The bromodomain proteins maintains bloodstream stage identity and immune evasion.	Schulz et al., 2015

Sample Used	Objective of the study	Findings	Reference
No Information	To evaluate the profile of differential expression in vectors (tsetse flies) infected with *T. brucei.*	Increase in the expression of mRNAs associated with binding protein RNA-6 (RBP6) and several of VSGs glycoproteins	Kolev et al., 2012
Colony of *G. m. morsitans*	To compare the transcriptome of the salivary gland (SG) from uninfected and infected *G. m.* morsitans	Increase expression level of SG genes involved in immunity, stress tolerance, protein translation and cell division.	Telleira et al., 2014
BF *T. b. brucei* (RUMP 503 strain)	To compare the transcriptome of BF with the transcriptome of parasites obtained from the infected salivary gland.	About 30% of the transcripts are differentially regulated between the two states.	
Glossina palpalis gambiensis flies and the *T. b. gambiense* isolate T.b.g. S7/2/2	To analyze and compare the transcriptome of the midgut of *Glossina palpalis gambiensis* fed with uninfected and *T. b gambiense* infected blood	1372, 52 and 1025 genes differentially expressed between infected vectors vs uninfected identified during the post-feed 3, 10 and 20 days, respectively.	Hamidou Soumana et al., 2015
	To identify SNPs, insertions and deletions and to evaluate their relationships within the levels of gene expression in the three post-feeding moments.	SNPs could be assigned to 625, 17, and 480 annotated contigs from the 3-, 10-, and 20-day tsetse samples, respectively. Three hundred and ninety nine annotated contigs showing SNPs were identified both in 3 and 20-days tsetse samples.	

Fig. 2. Principal findings retrieved using RNA-seq for a deeper analysis of *T. brucei* biology and its interaction with the invertebrate host. BF: Bloodstream form PF: Procyclic form. NI: unidentified. Trypo: Trypomastigote. Epi: Epimastigote.

host as monoallelic expression of genes encoding the surface protein VSG and rapid internalization of VSG bound antibodies on the surface are blocked when this protein is inhibited. These results suggest the therapeutic potential possessing of the bromodomain protein (Schulz et al., 2015).

Finally, a recent study by Bühlmann et al., using RNA-seq allowed to demonstrate the increased expression of PAG transcripts (genes associated with procyclin) and down-regulation of genes associated with hypothetical proteins and conserved hypothetical proteins when the NMD3 depletion protein (protein involved in rRNA processing and export of) in procyclical cells (Buhlmann et al., 2015).

2.2. Analysis of gene expression profile associated with the vertebrate host-parasite interaction

One of the main characteristics of the members of the Trypanosomatidae family is the ability to live as obligate intracellular parasites within different cell types, including macrophages, the first line of host defense and primary target cell for replication. Several studies, mainly using technology based on microarray and SAGE have shown that these parasites suffer structural and genetic remodeling and also alter the expression of genes involved in life processes of the host cell to adapt to different microenvironments and multiply in these cells (Beattie et al.,

2013; Gregory et al., 2008; Guerfali et al., 2008; Li et al., 2011; Rabhi et al., 2012; Simo et al., 2015). However, these techniques have several limitations, so the next generation sequencing (RNA-seq) becomes the best option for the complete and accurate analysis not only associated with gene expression in the parasite but also cellular transcripts.

2.2.1. Leishmania

A study by Dillon et al., using RNA-seq revealed the global changes in gene expression both in the host (murine macrophages) and in the parasite (*L. major* metacyclic promastigotes) when the latter enters and remains within macrophages during the first 72 h post-infection (hpi). When the expression profile between infected macrophages *vs.* uninfected at different hpi, it was observed that the primary response to infection, macrophages appeared at 4 hpi, otherwise compared to what happened at 24, 48 and 72 hpi, whose gene expression values decreased significantly (Fig. 1) (Dillon et al., 2015b). The results showed that the 4 hpi (at which the main macrophage response occurs) high set of genes were over-expressed, (i) associated with anti-inflammation (*Csf1*, *Csf3*, *IL10*, *IL11r*, *IL1rn*, *Socs3*, *Hmox1*, *Egfr*, *Vegfy Figf*), demonstrating the immunoregulatory phenotype by macrophages, (ii) genes associated with pro-inflammatory response (*IL1*, *IL6*, *TNF*, *NOS2*, *IL1rap* and *IL18r1*) and (iii) genes associated with glycolysis, glycolytic enzymes mainly (hexokinase, enolase, glyceraldehyde 3-phosphate dehydrogenase, lactate dehydrogenase a), which indicates that after infection with *L. major*, macrophages undergo metabolic changes that result in increased glycolysis and ATP generation. Contrary to what happened with those associated with lipid metabolism genes, biogenesis, the biosynthesis of unsaturated fatty acids, Fc mediated phagocytosis gamma receptor, which had a low expression level (Dillon et al., 2015b). For analysis on gene expression profile in the parasite, this study evaluated the transcriptome of amastigotes, during the course of infection. The results showed a significant increase of genes differentially expressed between metacyclic promastigotes and amastigotes at 4 hpi, indicating that the main events of differentiation occur when the parasite enters the cell. However, few changes were observed in amastigotes, in the course of time (Fig. 1).

By analyzing the genes that showed a high or low level of expression or during the transformation of metacyclics to amastigote promastigotes, the authors revealed that over-expressed genes that are associated with offset or mitigate the effects of oxidative response to the immune system and heat shock proteins, particularly Hsp83, family members tryparedoxin peroxidase, cyclophilins, surface antigens (metalloproteinase GP63), 1,3 galactosyltransferase beta phosphoglycan were observed. As genes that showed a down-regulation, the authors described those associated with translation, cell signaling, fatty acid biosynthesis and flagellar structure (Dillon et al., 2015b). Finally, 58% of genes differentially expressed in the transition from metacyclic promastigote to amastigote and 49% of genes differentially expressed in the amastigote transition 4–24 hpi were considered as hypothetical proteins.

Moreover, it has been reported that RNA intracellular pathogens, as in the case of trypanosomatids can be sequenced simultaneously with the RNA of the host cell, in order to analyze the host-pathogen interaction within the same biological sample and prevent the processes of cell purification, which can affect gene expression patterns before extraction (dual RNA-seq) (Humphrys et al., 2013; Pittman et al., 2014; Tierney et al., 2012; Westermann et al., 2012). In the case of *Leishmania*, two investigations have allowed such analysis, one of these studies was conducted by Maretti-Mira et al., which characterized and compared the transcriptome, fragments of tissues obtained from patients with cutaneous and mucocutaneous leishmaniasis caused by *L. braziliensis*. The results of this analysis revealed a significant over-expression of genes associated with recruitment and activation of immune cells (lymphocytes, granulocytes, natural killer and antigen presenting cells) and the regulation of the inflammatory response in tissues of patients with cutaneous leishmaniasis, revealing the powerful immune response that should trigger the host on the site of the lesion (Fig. 1) (Maretti-Mira et al., 2012).

Fernandes et al., assessed the transcriptome of *L. major*, *L. amazonensis* and human macrophages in the course of an intracellular infection (4–72 hpi), in the context of dual biological system, in order to evaluate changes in gene expression and reprogramming events that occur during host-parasite interaction. The results showed increase in the number of genes with differential expression to 4 hpi and a gradual decrease in the number of genes during the course of infection. It was observed that most of the genes that overexpress at 4 hpi, in the infected cell, corresponded to genes associated with the immune response: Pro-inflammatory cytokines (IL1-β, TNF, IL-6), immunomodulators (prostaglandin-endoperoxide synthase 2, stimulator factor colonies 1 and 2, superoxide dismutase 2) and metallothioneins (Fig. 1). Additionally, it was determined that *Leishmania* species modify the individual expression of their genes, during the transition from metacyclic promastigotes to amastigotes during intracellular infection. The results showed many genes differentially expressed at 4 hpi and few changes observed during the course of infection (Fig. 1). The expression pattern observed in macrophages and parasites, suggests that the host-pathogen interaction is greater during internalization and the establishment of infection. Finally, this group observed difference in the transcriptome or the transcriptional response of each macrophage infected with *Leishmania* species (Fernandes et al., 2016).

Finally, a recent analysis carried out by Christensen et al., 2016 allowed to capture the transcriptomic of both the parasite and the human host during *L. braziliensis* localized cutaneous infection. Regarding the transcriptional response of the host, the authors identified unique differentially expressed genes in lesions where parasites were producing detectable transcripts, compared with those where parasite transcripts were undetectable. Much of these genes encoded immunoglobulin-related transcript, genes encoding proteins associated with the granulocyte migration (CXCL8), B-cell proliferation (IL-21) and with the cellular cytotoxicity (granulysin). This suggests that B cells and host IgG may be strong contributors to parasite persistence. Regarding the parasite transcriptome during infection, the authors found most highly-expressed transcripts including cysteine peptidases, cysteine synthase, a proteasome subunit, various hydrolase-like, and hypothetical proteins, additional amastin family genes and known virulence factors including GP63, heat-shock proteins 70 and 83, and cysteine. The identification and characterization of these proteins may shed new light on how this parasite establishes infection, persists within mammalian cells, or escapes these cells to spread disease (Fig. 1) (Christensen et al., 2016).

2.3. Analysis of gene expression profile associated with the invertebrate host-parasite interaction

The vertebrates and invertebrates host range members of the Trypanosomatidae family can infect and the distinct manifestations of the diseases have generally been attributed to the ability of these parasites to produce genetic changes in the vector (*Glossina* spp. (tsetse fly) for *Trypanosoma brucei* and Gender *Phlebotomus* in the Old World and *Lutzomyia* in the New World for *Leishmania*) (Dillon et al., 2006; Inbar et al., 2013; Lehane et al., 2003; Ramalho-Ortigao et al., 2007). These genetic changes have been evidenced, thanks to transcriptomics, which has allowed revealing differential gene expression in specific anatomic locations of certain vectors, infected with these parasites (Lehane et al., 2003; Lehane et al., 2008; McCarthy et al., 2013; Urwyler et al., 2005). Currently, different authors have conducted studies which have implemented the platforms using next-generation sequencing (RNA-seq), providing valuable information that has allowed the complete genome analysis and identification of isoforms and transcriptome analysis of some of the vectors associated with these species (Rinker et al., 2016).

2.3.1. Leishmania

So far, different studies have analyzed the transcriptome in the salivary glands in five of the twelve subgenera of the genus *Phlebotomus*,

including the subgenus *Phlebotomus* (*Phlebotomus papatasi* and *P. duboscqi*) *Larroussius* (*P. ariasi*, *P. perniciosus*, *P. orientalis*, and *P. tobbi*), *Euphlebotomus* (*Phlebotomus argentipes*), *Adlerius* (*Phlebotomus arabicus*) and *Paraphlebotomus* (*Phlebotomus sergenti*), and in two subgenres of gender *Lutzomyia*, including the subgenus *Lutzomyia* (*Lutzomyia longipalpis*) and *Helcocyrtomyia* (*Lutzomyia ayacuchensis*) (Abdeladhim et al., 2016), some of whom have used EST (Abrudan et al., 2013; Azevedo et al., 2012; Dillon et al., 2006), pyrosequencing (McCarthy et al., 2013) and cDNA libraries (Anderson et al., 2006; Dostalova et al., 2011). However, at present, no studies in using RNA-seq are available to analyze the difference in the expression profile of vectors infected and not infected with *Leishmania*. An approach to such analyzes is the study by Pretella et al., in which using RNA-seq and by *de novo* assembly analyzed the transcriptome and perform differential expression analysis of adult males and females of *Phlebotomus perniciosus* (Petrella et al., 2015).

2.3.2. Trypanosoma brucei

In contrast, *Trypanosoma brucei* studies have been reported in which using next-generation sequencing (RNA-seq) have evaluated the profile of differential expression in vectors (tsetse flies) infected with *T. brucei* (Fig. 2). The results obtained allowed to demonstrate not only the role of RNA binding proteins but also to provide a robust *in vitro* system for recapitulating the development of the parasite within the insect vector, and the production of infectious metacyclic forms (Kolev et al., 2012).

Two years later, Telleria et al., analyzed the transcriptome in salivary glands of uninfected and infected tse-tse flies. The results showed that infection of *T. brucei*, suppresses the expression level of several salivary proteins, secreted abundantly, changing the composition and functional activities in saliva, additionally compare the transcriptome of parasites in salivary glands with those from the bloodstream of rodents. The results demonstrate than these strains undergo changes in surface proteins and metabolic changes; reflecting the adaptation that should suffer the parasite to suit the nutritional and immune environment in different hosts (Fig. 2) (Telleria et al., 2014).

Finally, Hamidou et al., using *de novo* assembly and RNA-seq, analyzed and compared the transcriptome of the midgut of *Glossina palpalis gambiensis*, fed with uninfected and infected blood with *Trypanosoma brucei gambiense* (Fig. 2); additionally, they determined the SNPs (Single Nucleotide Polymorphisms) identified during the three post-feeding (3, 10 and 20) moments, were found in genes that are differentially expressed and that encode proteins important in the maintenance, survival and cellular transcription. Genes encoding proteases, antimicrobial peptides, associated with glucose metabolism, associated with nucleotide metabolism enzymes, chitinases, aquaporins, glutathione *S*-transferase, thrombin inhibitor, proteins involved in the transcription

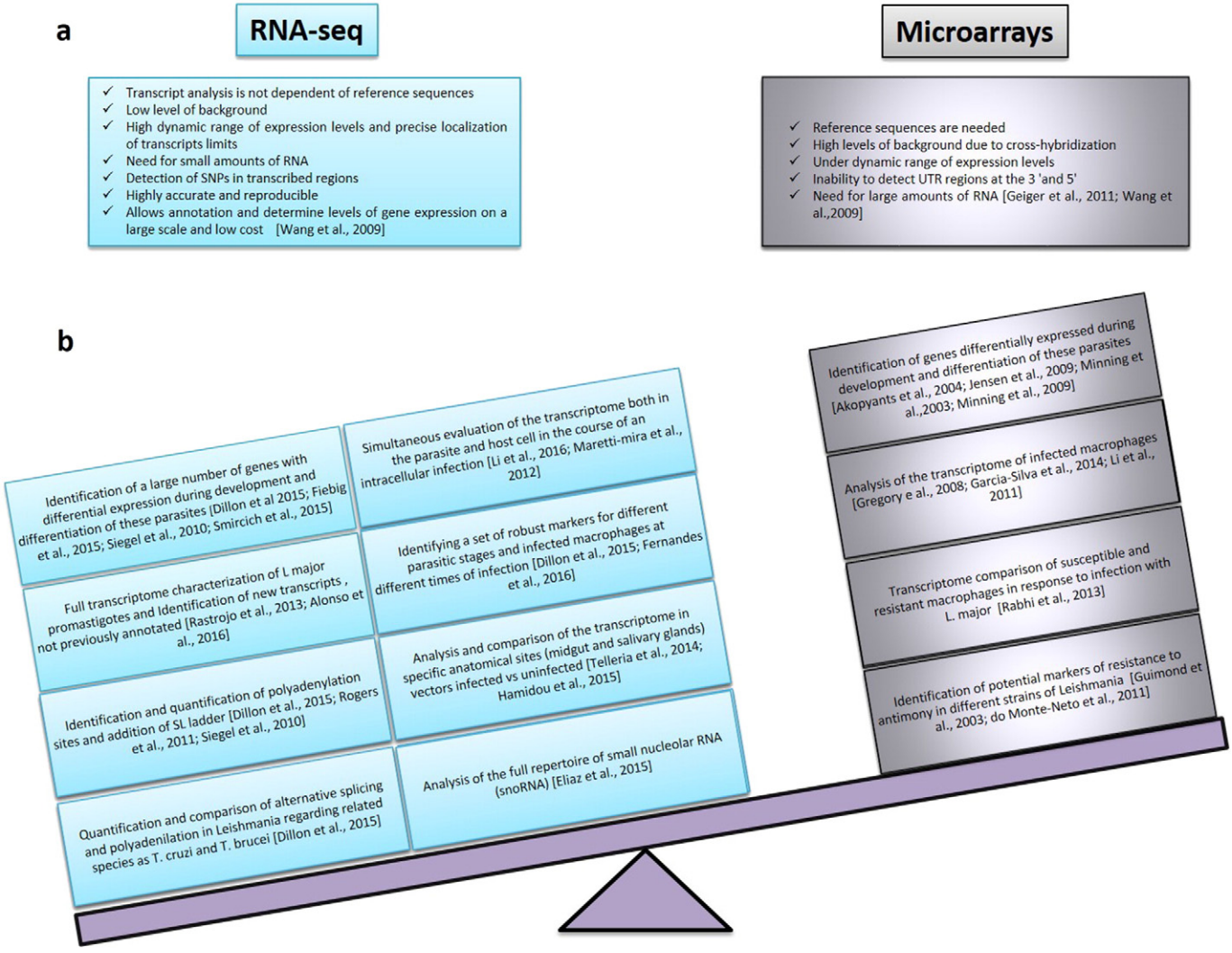


Fig. 3. Overall comparison of RNA-seq *vs.* microarrays for the understanding of the transcriptome of kinetoplastids a. Benefits that RNA-seq technology offers compared to microarrays. b. Comparative analysis of the main findings in parasites of the Trypanosomatidae family (*Trypanosoma brucei* and *Leishmania*).

process, enzyme systems detoxification, heat shock proteins, among others (Fig. 2) (Hamidou Soumana et al., 2015).

3. Conclusions

The vector-borne diseases have been, for a long time a major public health problem due to its high rate of morbidity and mortality; therefore, it is necessary to implement effective measures in diagnostic, prophylactics and therapeutics to control and treat the infections. To achieve this purpose is necessary not only to understand the biology of the parasite, but also all those associated with the response mechanisms and gene modulation when it interacts with its host (vertebrate and invertebrates). Different molecular tools have advanced this knowledge, mainly in Trypanosomatidae family parasites (*Trypanosoma brucei* and *Leishmania*). However, these techniques have certain disadvantages that limit their full analysis (Fig. 3), disadvantages which currently could be eliminated with the use of next generation sequencing (RNA-seq) platforms. This tool has not only allowed us to analyze and quantify the level of mRNA expression during the differentiation process and development of these parasites, as well as gene modulation generated during interaction with their hosts but also the analysis of processes such as the initiation of transcription, mRNA maturation, and more recently degradation in translation. Another major advantage of sequencing RNA (RNA-seq), is the ability of this technique to perform assembly and sequence analysis of RNA in the absence of reference genomes (*de novo* assembly), which is vitally important, mainly for species in which its genome has not been completely sequenced.

Finally, despite the advantages described and the great utility of this technique in the knowledge about trypanosomatids, the RNA-seq present some disadvantages, such as the high cost, the computational complexities associated with data analyses, the extremely sensitivity and the need of a very careful quality control for each wet laboratory step, arguments that are still considered when selecting the method of sequencing (Hitzemann et al., 2013; Hirsch et al., 2015). This review can prove that RNA-seq, has become a valuable tool that has allowed not only to extend the knowledge about the biology of these parasites, but also to know the molecular processes involved in the triad of infection (parasite-vector-host), thus contributing to the development of strategies to identify molecular targets to achieve interrupting the transmission of the disease.

Abbreviations

ML	mucosal leishmaniasis
CL	cutaneous leishmaniasis
VL	visceral leishmaniasis
WHO	World Health Organization
EST	Expressed Sequence Tag
SAGE	Serial Analysis of Gene Expression
MPSS	Massively Parallel Signature Sequencing
DGE	Digital Gene Expression
RNA-seq	sequencing of RNA
ncRNA	non-coding RNAs
snoRNA	small nucleolar RNA
HSP70	heat shock protein 70
SL	spliced leader
rRNA	ribosomal RNA
BF	blood forms
PF	procyclic form
hpi	hours post-infection
SNPs	Single Nucleotide Polymorphisms
GPCRs	G protein coupled receptors

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8.3 CAPITULO 3

Descripción de la arquitectura genómica intra-específica de aislamientos clínicos de *Leishmania (Viannia) panamensis* y *Leishmania (Viannia) braziliensis.*

Variación intra-especie en *Leishmania braziliensis y Leishmania panamensis* aisladas de pacientes con Leishmaniais Cutánea en Colombia

Luz H. Patino[a], Hideo Imamura[b] Marina Muñoz[a], Carlos Muskus[c], Claudia Méndez[d] Juan David Ramírez[a]*

[a] Grupo de Investigaciones Microbiológicas-UR (GIMUR), Programa de Biología, Facultad de Ciencias Naturales y Matemáticas, Universidad del Rosario, Bogotá, Colombia.

[b] Unidad de Parasitología Molecular, Departamento de Ciencia Biomédicas, Instituto de Medicina Tropical, Amberes, Bélgica.

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

[d] Dirección de Sanidad Militar, Ejercito Nacional de Colombia, Bogotá, Colombia.

Resumen

Leishmania braziliensis y *Leishmania panamensis* son las principales especies responsables de la leishmaniasis cutánea en América latina. Hasta el momento, muy pocos estudios han analizado el genoma completo de estas especies, con el fin de evaluar la variabilidad genética intra-especie, la cual ha demostrado estar estrechamente relacionada con las diferentes presentaciones clínicas, así como con el grado de virulencia del parásito y la variable respuesta frente a los anti-leishmaniales. Así mismo, ninguno de los estudios realizados, se ha enfocado en analizar dicho comportamiento en especies obtenidas a partir de aislamientos clínicos colombianos. Por lo tanto, el objetivo de este estudio fue evaluar la diversidad genética intra-especie en *Leishmania braziliensis* y *Leishmania panamensis* a partir de aislamientos clínicos de pacientes colombianos con leishmaniasis cutánea. El genoma completo de 20 aislamientos clínicos de *L. panamensis* y 7 aislamientos clínicos de *L. braziliensis* fue analizado mediante secuenciación de genoma completo y la variabilidad genética intra-especie entre cada aislamiento fue evaluada. Los resultados obtenidos en ambas especies demostraron bajo nivel de variabilidad, en términos de variación en el número de copias cromosomales/genes (CNV) así como en polimorfismos de nucleótido simple (SNPs) e inserciones/deleciones (Indels). Sin embargo, dos aislamientos clínicos mostraron resultados interesantes, uno de ellos infectado con *L. braziliensis* evidenció

duplicación génica en todos los cromosomas (excepto en el cromosoma 31) lo cual sugiere un posible evento de recombinación y el otro aislamiento infectado con *L. panamensis* mostró un elevado número de SNPs generados en bloque, ubicados a lo largo del cromosoma 20. Los resultados obtenidos en este estudio permiten no solo ampliar nuestro conocimiento acerca del comportamiento genómico intra-especie de aislamientos clínicos de *L. panamensis* y *L. braziliensis* colombianas, sino también sugerir que la baja variación estructural encontrada podría estar asociada a un posible evento de adaptación de estas especies al hospedero humano.

Keywords: Leishmaniasis cutánea, Resistencia, DNA-seq, Variación en el número de copias (CNV), polimorfismos de nucleótido simple (SNPs)

INTRODUCCIÓN

La Leishmaniasis cutánea (LC) es una enfermedad parasitaria endémica en muchas regiones del mundo. Esta enfermedad se encuentra distribuida en una amplia área geográfica, en regiones como África Sub-Sahariana, el Mediterráneo, el Oriente Medio, Asia central y meridional, así como en Sur y Centro América [1]. Se estima que 350 millones de personas están en riesgo de adquirirla y aproximadamente 12 millones de individuos alrededor del mundo se encuentran infectados [2, 3].

Esta enfermedad puede ser causada por una amplia variedad de especies del género *Leishmania*, incluyendo *Leishmania braziliensis, Leishmania amazonensis, Leishmania aethiopica, Leishmania mexicana, Leishmania guyanensis, Leishmania panamensis, Leishmania peruviana, Leishmania tropica* y *Leishmania major* [4]. Sin embargo, en algunos países de Suramérica, incluida Colombia, las principales especies responsables de esta enfermedad son *Leishmania braziliensis* y *Leishmania panamensis* [5, 6].

Diferentes estudios han demostrado que el amplio espectro de manifestaciones clínicas causadas por estas dos especies, las cuales han sido reportadas en humanos [1, 7] y en animales [7-9] así como la variable respuesta terapéutica frente a los diferentes anti-leishmaniales, están determinados por factores relacionados al parásito [10], el vector [11] y la respuesta inmune así como el background genético del hospedero [12].

Hasta el momento, varios estudios demuestran que la variabilidad genética del parásito es un factor fundamental tanto en el curso de la enfermedad, la patogénesis, la virulencia, así como en la respuesta terapéutica [13-17]. Varios polimorfismos han sido identificados, uno de ellos en genes como el *hsp70* los cuales han permitido determinar

la presencia de distintas cepas de *L. braziliensis* circulando en la misma zona geográfica y su asociación con diferentes manifestaciones clínicas [18], así como la variación genética observada en los espaciadores transcritos internos (ITS) de los genes del ARN ribosomal, los cuales han permitido la caracterización de 7 genotipos en aislamientos de *L. donovani* y la asociación de algunos de ellos con aspectos epidemiológicos [19]

La mayoría de estos polimorfismos han sido caracterizados mediante técnicas tales como PCR (*Polymerase chain reaction*) [9], MLST (Multilocus Sequence Typing) [20], MLEE (*Multi Locus Enzyme Electrophoresis*) [21] y RAPD (*random amplified polymorphism*) [22]. Sin embargo, diferencias genéticas a nivel del genoma completo tales como cambios en la somia, variaciones en el número de copias de genes, polimorfismo de nucleótido simple (SNPs), inserciones y/o deleciones (Indels), identificadas a partir de aislamientos clínicos y utilizando técnicas de secuenciación de última generación (NGS) como secuenciación de ADN, solo han sido estudiadas en un limitado número de especies (*L. major, L. donovani, L. panamensis* y *braziliensis*) [15, 23, 24].

Por lo tanto, el objetivo de este artículo es presentar los resultados obtenidos de un análisis genómico comparativo intra-especie de aislamientos clínicos de *Leishmania panamensis* y de *Leishmania braziliensis,* obtenidos de pacientes colombianos con Leishmaniasis cutánea. El interés de estudiar la variabilidad genética de estas especies en el territorio colombiano, radica en que Colombia no es solo uno de los países con el mayor número de casos asociados a Leishmaniasis cutánea, sino también se ha descrito que diferentes factores asociados a las características geográficas, condiciones políticas y socio-económicas de este país [25], así como la adaptación de algunos vectores a nuevos ecosistemas, la habilidad del parásito a infectar diferentes hospederos mamíferos y la capacidad de adaptarse a nuevos vectores, contribuyen a la expansión de la enfermedad [26]. Así este sería el primer estudio en Colombia, en el cual utilizando secuenciación de genoma completo se evalúe el comportamiento intra-específico en dos de las principales especies circulantes en Colombia.

MATERIALES Y MÉTODOS

Población de Estudio

Un total de 30 aislamientos clínicos provenientes de pacientes con Leishmaniasis cutánea atendidos en el Programa de Estudio y Control de Enfermedades Tropicales (PECET), Medellín- Colombia fueron colectados. La distribución geográfica de las

muestras incluidas en este estudio es presentada en la **Figura 1.** Cada uno de estos aislamientos fue cultivado en medio Schneider (Sigma-Aldrich S9895, United States, MO) suplementado con 10% de Suero Fetal Bovino (SFB) hasta obtener un cultivo en fase exponencial al cual posteriormente se le realizó extracción de ADN.

Obtención de ADN y PCR para la identificación de especies de *Leishmania*

El ADN de cada uno de los aislamientos clínicos fue obtenido usando el kit High Pure PCR Template Preparation kit (Roche Life Science). La pureza y la concentración del ADN obtenido fueron verificadas usando un espectofometro 2000 Nanodrop (Thermo Fisher Scientific Inc, United States, MA) y la calidad se evaluó mediante electroforesis en gel de agarosa al 1% teñidos con SYBR Safe DNA Gel Stain. Verificada la concentración, pureza y calidad del ADN obtenido, este fue dividido en dos fracciones, una de ellas para identificación de especie y la otra para secuenciación de genoma completo.

La identificación de especie fue realizada usando secuenciación directa por sanger de los genes codificantes para citocromo b (Cytb) y la proteína de choque térmico 70 (HSP-70), como se describe previamente [5, 6]. El producto amplificado fue purificado usando EXOSAP (Affymetrix, USA) y secuenciado por el método dideoxy-terminal, en un secuenciador capilar automatizado (AB3730, Applied Biosystem). Finalmente, las secuencias fueron sometidas a BLASTn para buscar similitud con las secuencias de *Leishmania* depositadas en GenBank [5].

Secuenciación de genoma completo y análisis de datos

Se construyeron bibliotecas genómicas “mate-paired”, con un tamaño de inserto de 350 pares de bases (pb), las cuales fueron posteriormente sometidas a secuenciación “paired-end”, con una longitud de lectura 2 x 150 pb, utilizando para ello la plataforma Illumina HiSeq X-Ten (Illumina) [27].

Mapeo de ADN

Lecturas paired-end fueron mapeadas al genoma de referencia de *Leishmania panamensis* (LpanUA) y *Leishmania braziliensis* (MHOM/BR75/M2904*)* y ensambladas usando el programa SMALT (version 0.7.4) (www.sanger.ac.uk/resources/software/smalt/). La fusión, clasificación y eliminación de lecturas duplicadas fue implementada con SAMtools (version 0.1.18) y Picard (version 1.85) [28].

Evaluación del número de copias cromosoma/gen (VNC)

Para la estimación de la somia cromosomal, se determinó inicialmente la profundidad de cada lectura (d_i), posteriormente, la profundidad media de los 35 cromosomas (d_m) para *L. braziliensis* y *L. panamensis* fue calculada y finalmente, la somia (*s*) de cada cromosoma fue obtenido siguiendo la siguiente fórmula: $s = 3 \times di/dm$ (para *L. braziliensis*) y $s = 2 \times di/dm$ (para *L. panamensis*) [29]. El rango de monosomia, disomia, trisomía, tetrasomia y pentasomia fue definido como se describe previamente [30]. Para evaluar la variación en el número de copias por gen, se definió una profundidad haploide media por gen sin el efecto de la somia (d_{HG}) y se definió la profundidad completa con el efecto de la somia (f_{HG}). Su relación fue definida como: $d_{FG} = S.\ d_{HG}$. Para seleccionar los genes que presentaron VNC, se tuvieron en cuenta aquellos que tuvieran un valor de Z Score mayor o igual a 2 y un valor *p* menor o igual a 0.05. Los Heatmaps fueron creados usando el paquete Heatmap 3 en R [31]. Los genes que presentaron variación en el número de copias fueron sometidos a análisis de ontología génica (GO), para clasificar los cambios dentro de categorías funcionales, para lo cual se utilizó la base de datos TriTrypDB (http://tritrypdb.org). Los términos del GO fueron sometidos a REVIGO [32].

Análisis de polimorfismos de nucleótido simple (SNPs)

Para detectar Polimorfismos de nucleótido simple (SNPs), inserciones/deleciones (Indels), las lecturas fueron alineadas al genoma de referencia de *Leishmania panamensis* (UA947) y *Leishmania braziliensis* (MHOM/BR75/M2904*)* usando el programa SMALT (version 0.7.4) (www.sanger.ac.uk/resources/software/smalt/). El programa Picard (version 1.85) (http://broadinstitute.github.io/picard/) fue usado para la fusión, clasificación de los archivos bam y para marcar las lecturas duplicadas, como se describe previamente [30]. Los SNPs e Indels menores a 15pb fueron llamados con el programa GATK (Unified Genotyper method in the Genome Analysis Toolkit) (version 3.4) (https:// software.broadinstitute.org/gatk/). Todos los candidatos a SNPs fueron visualizados utilizando el programa IGV (IGV_2_3_47) (Integrative Genomic Viewer) y SAMtools, para evitar falsos positivos. El programa SnpEff (Versión v4.1) [30] fue usado para clasificar todos los SNPs e indels de acuerdo con su impacto funcional. Los SNPs e indels fueron considerados significativos cuando la diferencia en la frecuencia alélica es al menos 0.33 para *L. braziliensis*, 0.25 para *L. panamensis* [33], con un valor p <0.05. Una vez se identificaron los SNPs a lo largo del genoma de los aislamientos clínicos para cada especie, se determinó la frecuencia por cromosoma; los resultados obtenidos fueron representados en una tabla dinámica.

Reconstrucción filogenética

Los SNPs identificados en el set de datos de interés, tanto del genoma nuclear como del genoma mitocondrial, fueron usados para la construcción de un alineamiento de genomas completos usando Mauve progresivo [34]. Con el objetivo de evitar el alineamiento forzado de secuencias no relacionadas, se tuvieron en cuenta parámetros personalizados de transición de homología basados en Modelos Ocultos de Markov. Estos parámetros fueron fijados en 0.001 para homólogos y de 0.000005 para secuencias no relacionadas. Como outgroup se utilizaron las secuencias de referencia de *L. braziliensis* y *L. guyanensis*. Así mismo, se incluyeron en los análisis dos secuencias de referencia de *L. panamensis* actualmente disponibles: LPPSC1 y LpanUA; las secuencias fueron obtenidas de la base de datos TriTrypDB (http://tritrypdb.org) y la secuencia *de* LpanUA de Urrea et al., 2018 [16]. Finalmente, el árbol obtenido fue visualizado en la herramienta web Interactive Tree Of Life V3 (http://itol.embl.de).

RESULTADOS

Identificación de especies de *Leishmania*

El análisis de secuenciación de la fracción Cytb y HSP-70 permitió la identificación de especie en 27 de las 30 muestras analizadas (90%), las tres muestras restantes (10%) no pudieron ser analizadas debido a la baja cantidad del ADN obtenido. De las 27 muestras 20 de ellas (74%) fueron identificadas como *L. panamensis* y las 7 restantes (26%) como *L. braziliensis.*

Variación en el número de copias por cromosoma/gen

En el presente estudio, se utilizaron las lecturas obtenidas de la secuenciación, para estimar el número de copias por cromosoma en todos los aislamientos clínicos de *L. panamensis* y *L. braziliensis* e investigamos posibles cambios de somía. Los resultados obtenidos permitieron confirmar lo previamente descrito en la literatura, en donde la mayoría de los cromosomas presentaron un comportamiento disómico para el caso de los aislamientos de *L. panamensis* y trisómico para *L. braziliensis*, a excepción del cromosoma 31 el cual presentó más de tres copias en todos los aislamientos clínicos de ambas especies (**Figura 2**). Finalmente, el valor de la somia obtenido fue consistente con el valor de la somia basado en el análisis de la frecuencia alélica, sin embargo, uno de los aislamientos de *L. brazileinsis* presento una discordancia en este análisis (aislamiento 8125), el cual evidenció una extraña duplicación en el número de copias de cromosomas a lo largo del genoma (excepto el cromosoma 31).

Al evaluar el número de copias por gen entre cada uno de los aislamientos para cada especie, observamos que el número de genes con VNC entre los aislamientos no difiere el uno del otro. Para el caso de los 20 aislamientos clínicos de *L. panamensis* el aislamiento que presentó el menor número de genes con VNC fue el aislamiento Lp8131 (79 genes) y el que presentó el mayor número de genes con VNC fue el aislamiento Lp8087 (104 genes) (**Figura 3A**). Al realizar el análisis de ontología génica basada en su función biológica, observamos que aquellos genes que presentaron VNC estaban asociados principalmente con la homeostasis del ion zinc, el transporte de glicerol y la detección de estímulos, un resultado particular fue el observado en el aislamiento Lp8036, en el cual el 100% de los genes que presentaron VCN estaban asociados a la síntesis del aminoacyl-prolyl tRNA (**Figura 3A**). Con respecto a los resultados obtenidos en los 7 aislamientos clínicos de *L. braziliensis*, observamos que el rango de genes que presentaron VNC estuvo entre 52 genes (aislamientos Lb 7616 y Lb7864) y 66 genes (aislamiento Lb7933), el análisis de ontología génica revelo que la mayoría de estos genes estaban asociados con la integración del ADN y la homeostasis del ion zinc (**Figura 3B**).

Polimorfismos de nucleótido simple (SNPs)

Después de mapear cada una de las lecturas contra el genoma de referencia respectivo, se realizó, para todos los aislamientos clínicos en las dos especies, la identificación de los SNPs y la correspondiente anotación.

Al comparar el número de SNPs entre cada uno de los aislamientos, tanto en *L. panamensis* como en *L. braziliensis,* se observó que los SNPs están homogéneamente distribuidos a lo largo del genoma en todos los aislamientos, observándose un mayor número en los cromosomas 20, 31, 34 y 35, probablemente debido al gran número de genes presentes en estos cromosomas (**Figura 4A y Figura 5**). La mayoría de SNPs aquí encontrados presentaron un moderado y alto impacto funcional (ganancia/pérdida en codones de parada) en genes codificantes para proteínas con importantes funciones biológicas. Adicionalmente, cabe la pena resaltar que al comparar el número total de SNPs entre *L. panamensis* versus *L. braziliensis*, esta última especie reportó mayor número de polimorfismos **(Figura 5**).

Por otra parte, un aislamiento clínico de *L. panamensis* llamó nuestra atención: aislamiento Lp8132 en el cual se observó no solo un número elevado de SNPs (7809 en total) a lo largo del genoma, siendo más representativo en el cromosoma 20 (**Figura 4A**), sino también que muchos de estos SNPs fueron identificados en bloque (**Figura 4B)**. Al realizar el análisis del cromosoma 20, observamos dos características

particulares, la primera de ella que algunos de los SNPs identificados son compartidos entre todos los aislamientos (**Figura 4B**), principalmente SNPs ubicados en genes codificantes para proteínas involucradas en la interacción hospedero-patógeno o en la degradación intracelular de proteínas, y la segunda característica se refiere a los SNPs identificados en bloque en el aislamiento Lp8132, los cuales se encontraron principalmente en genes codificantes para proteínas transportadoras (transportador ABC), proteínas de superficie (amastina) y algunas proteínas de unión (unión GTP/ unión zinc).

Inferencia filogenética basada en el análisis de SNPs

Con el objetivo de visualizar la relación entre los diferentes aislamientos clínicos de *L. panamensis*, un árbol filogenético basado en SNPs del genoma nuclear y un árbol filogenético basado en SNPs del genoma mitocondrial (maxicírculo) fueron construidos. Como se observa en la **Figura 6A,** al evaluar los SNPs del genoma nuclear, dos clusters completamente diferenciados fueron identificados, uno de los cuales conformado por la mayoría de los aislamientos clínicos lo que indica una baja variación genética entre ellos y un segundo cluster formado por el aislamiento Lp8132, que debido al elevado número de SNPs presentes, se agrupo de manera independiente. Igualmente, al evaluar la filogenia realizada con base en el maxicírculo (**Figura 6B),** se observa al aislamiento Lp8132 formando un cluster independiente y muy cercano al genoma de referencia de *L. braziliensis.*

DISCUSIÓN

Hasta el momento y gracias al uso de la secuenciación de última generación, varios estudios realizados en diferentes países y usando como modelo diferentes especies de *Leishmania*, (*L. donovani, L. major, L. braziliensis, L. panamensis*) han revelado una estrecha relación entre la variabilidad genética intra especie de estos parásitos con aspectos epidemiológicos asociados a la enfermedad, tales como localización geográfica, formas clínicas, patogenicidad, virulencia, resistencia a medicamentos y variación antigénica [15, 16, 23, 24]. Sin embargo y a pesar de la información existente, el comportamiento intra-especifico en dos de las principales especies que circulan en Colombia tales como *L. panamensis* y *L. braziliensis* [5, 6] obtenidos a partir de aislamientos clínicos, aún sigue siendo escaso. En este estudio analizamos, mediante análisis de genoma completo, la variabilidad genética intra-especie de 20 aislamientos clínicos de *L. panamensis* y 7 aislamientos clínicos de *L. braziliensis* obtenidos de pacientes colombianos con Leishmaniasis cutánea.

Diversos estudios, la mayoría de ellos analizando el ADN del kinetoplasto (kDNA), el cual es considerado ser la mejor herramienta para distinguir la heterogeneidad genética intra e inter específica en *Leishmania* [35, 36] y los estudios en los cuales se analiza el genoma completo del parásito, describen que *L. braziliensis* y *L. panamensis* presentan una sustancial variabilidad genética, la cual ha sido asociada con algunas características clínicas, geográficas y de virulencia [15, 16, 37]. Sin embargo, los resultados obtenidos en estos estudios contrastan con la homogeneidad observada en términos de somia, variación en el número de copias y SNPs, entre los 7 aislamientos clínicos de *L. braziliensis* y entre los 20 aislamientos clínicos de *L. panamensis* aquí analizados.

Hasta el momento varios estudios describen que algunas especies de *Leishmania (L. infantum, L. major, L. donovani, L. amazonensis, L. braziliensis* y *L. panamensis*) generan cambio en el número de sus cromosomas, como un mecanismo dependiente del ambiente, así como un proceso de adaptación en respuesta a condiciones de estrés [29, 30, 38-41], sin embargo y a pesar de que este mecanismo es compartido entre especies del viejo y del nuevo mundo, el cambio en el número de copias varía considerablemente entre especies. Los resultados obtenidos en este estudio confirman el elevado número de copias en el cromosoma 31, lo cual es característico en todas las especies de *Leishmania*, hasta ahora evaluadas [42], pero describe una somia homogénea a lo largo del genoma en cada uno de los aislamientos de *L. panamensis* y *L. braziliensis* (**Figura 2**). En vista de los resultados obtenidos en este estudio y teniendo en cuenta que en respuesta a cambios en las condiciones ambientales dentro del hospedero, *Leishmania* altera no solo cromosomas completos sino también regiones genómicas específicas [30, 43]; la baja variación estructural encontrada en estas especies, podría ser explicada como un posible evento de adaptación a los cambios ocurridos en el hospedero humano.

Por otra parte, durante el análisis de la somia basada en la frecuencia alélica, uno de los aislamientos de *L. braziliensis* (8025) presentó una rara duplicación en el número de copias por cromosoma a lo largo de todo el genoma (excepto el cromosoma 31), lo que nos hace pensar en un posible evento de recombinación, teniendo en cuenta que ya se han identificado en *Leishmania*, híbridos entre especies muy cercanas del nuevo mundo, entre especies muy cercanas del viejo mundo y entre especies muy divergentes del viejo mundo [44]. Partiendo de estos estudios y de aquellos que mencionan que el mosaicismo cromosomal es una característica de *Leishmania* [45], se hace necesario explorar este posible evento de recombinación partiendo del análisis genómico en una sola célula (clon), para lo cual la hibridización fluorescente in situ (FISH) podría ser la técnica más apropiada, ya que esta permite no solo evaluar la somia a nivel de célula

individuales, sino también determinar el número de cromosomas homólogos en una sola célula [46], igualmente este posible evento de recombinación deberá ser analizado realizando secuenciación del genoma completo del ADN obtenido en clones biológicos de la cepa estudiada.

Un segundo parámetro genómico evaluado correspondió a la variación en el número de copias locales (VNC). Resultados obtenidos en otros análisis describen que la VNC (amplificación/deleción) de genes específicos es una solución genómica de *Leishmania* para modular los niveles de transcritos y sus correspondientes productos [29], lo cual describe que la variación en el número de copias de ciertos genes presenta una importancia funcional. Los resultados obtenidos en este estudio los cuales permitieron analizar la VNC, demostraron un comportamiento similar entre cada uno de los aislamientos clínicos en las dos especies evaluadas, lo cual sigue confirmando la poca variabilidad estructural de algunas especies del subgénero *Viannia (L. braziliensis* y *L. panamensis*). Por otra parte, al analizar cada aislamiento clínico por separado observamos que muchos de los genes que presentaron VNC tanto en los aislamientos de *L. braziliensis* como de *L. panamensis,* estaban asociados a procesos tales como replicación del ADN, transporte, metabolismo, infección, supervivencia y virulencia **(Figura 3)**, lo cual describe la importancia de estos genes durante los diferentes procesos biológicos por los que cursa el parásito [47-49].

Los resultados obtenidos al evaluar los polimorfismos de nucleótido simple (SNPs) demostraron una muy baja variabilidad entre cada uno de los aislamientos clínicos de *L. braziliensis* y *L. panamensis* **(Figura 4A y Figura 5)**, lo cual indica un comportamiento genético homogéneo en cada una de estas especies. Sin embargo, al realizar la comparación inter-especies (*L. panamensis / L. braziliensis*), los resultados demostraron una significativa diferencia en términos de SNPs entre estas especies (~2.600 vs ~18.000 SNPs respectivamente), lo cual concuerda con previos estudios en donde mediante MLST (multilocus sequence typing) describen la baja diversidad de *L. panamensis* versus *L. braziliensis* [20], así como la alta diversidad de *L. braziliensis* con respecto a otras especies como *L. peruviana* [8]. A pesar de que los resultados obtenidos en este estudio contrastan con el elevado número de variantes identificadas en aislamientos clínicos provenientes de Brasil (~96.000 y 123.000 SNPs) [15], este estudio demuestra que *L. brazilensis* sigue siendo una especie con elevada variabilidad genética, en términos de SNPs comparada con otras especies del subgénero *Viannia.*

Por otra parte, al evaluar los SNPs entre cada uno de los aislamientos clínicos de *L. panamensis*, identificamos que uno de estos aislamientos (aislamiento Lp8132), no solo

presentó un número elevado de SNPs con respecto a los demás (**Figura 4)** sino también que estos SNPs fueron distribuidos en bloque a lo largo del cromosoma 20. El resultado obtenido resulta interesante, teniendo en cuenta que el análisis de SNPs ha sido ampliamente utilizado para caracterizar sub-estructuras poblacionales y/o determinar el origen geográfico ancestral de un individuo o un grupo poblacional [50, 51]. Así mismo, cuando la localización de estos SNPs es muy cercana permite que ellos se hereden de forma conjunta, de tal manera que si extrapolamos estos conceptos al resultado obtenido en este aislamiento podríamos pensar (i) que Lp8132 tiene un origen ancestral particular y (ii) que los SNPs ubicados a lo largo de este cromosoma tienen una importancia en el parásito que se ha venido heredando generación tras generación.

Sin embargo, estudios adicionales deben ser conducidos con el fin de aclarar las relaciones filogenéticas entre estos aislamientos. Para esto, uno de los abordajes podría ser la selección de un grupo informativo de SNPs, que puedan resultar informativo para determinar la historia evolutiva y el ancestro común mas cercano entre ellas, considerando los lineamientos propuestos por las teorías descritas por Sampson *et al*., [50], en su modelo de AIM-SNP (*ancestry informative markers (AIM) single nucleotide polymorphisms*). Alternativamente, se identifica la necesidad de desarrollar estudios que permitan determinar el reloj molecular de los aislamientos estudiados y los parámetros que conducen a su aislamiento genético [52]. En conjunto, este tipo de información (molecular y genómica) permitiría identificar conjuntos de datos útiles para estimar la diversidad de este aislamiento clínico y su historia evolutiva.

Sin embargo, estas hipótesis deberán ser confirmadas con estudios adicionales, tales como análisis de coalescencia o aplicando las teorías descritas por Sampson *et al*., [50] las cuales permiten seleccionar un grupo de SNPs que puedan predecir con precisión la ancestría de un individuo: AIM-SNP (*ancestry informative markers (AIM) single nucleotide polymorphisms*).

Finalmente, con el fin de visualizar mejor la relación entre los diferentes aislamientos clínicos de *L. panamensis*, un árbol filogenético basado en SNPs tanto del genoma nuclear como del genoma mitocondrial fue construido. Los resultados obtenidos al analizar el genoma nuclear revelan que el aislamiento Lp8132 formó un cluster independiente **(Figura 6A),** lo cual indica que este aislamiento presenta una menor relación genética con respecto a los demás aislamientos clínicos analizados. Con el propósito de soportar estos hallazgos, un análisis filogenético basado en el genoma mitocondrial fue realizado; teniendo en cuenta que los genes mitocondriales están

presentes en todos los taxones eucariotas, presentan una relativa conservación y estructura [53] y que en particular el maxicírculo representa un excelente marcador filogenético en *Leishmania* [54] un análisis basado en SNPs de esta región mitocondrial fue evaluado**.** Los resultados obtenidos de estos análisis revelan una clara congruencia con la filogenia obtenida del genoma nuclear **(Figura 6B)** y confirman la baja relación filogenética del aislamiento Lp8132 con relación a los otros aislamientos de la misma especie. Considerando los resultados obtenidos y en vista de que fue el único aislamiento que presentó dicho comportamiento, consideramos que se necesita ampliar el muestreo en la misma zona geográfica de donde esta muestra fue aislada, para poder plantear la posibilidad de que dos grupos filogenéticos de *L. panamensis* están circulando en territorio colombiano.

En conclusión, nuestro estudio revela la baja variación estructural intra especifica *en L. panamensis* y *L. braziliensis* aisladas de pacientes colombianos con leishmaniasis cutánea. Estos resultados concuerdan con lo previamente descrito en cepas colombianas de *L. panamensis* usando MLST [20] y es el primer estudio en el cual se observa la homogeneidad genética en *L. braziliensis* a partir de aislamientos colombianos. Así mismo, futuros estudios son necesarios para confirmar los hallazgos que resaltan esta investigación, tales como el posible evento de recombinación en *L. braziliensis* y la elevada variación en términos de SNPs en un aislamiento clínico de *L. panamensis*.

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Lista de Figuras

Figura 1. Distribución geográfica de los aislamientos clínicos incluidos en el estudio. En la parte izquierda de la figura se observa la localización geográfica de Colombia dentro de Suramérica y a la derecha, el mapa de Colombia el cual muestra los aislamientos clínicos georeferenciados discriminados por especies, en cada departamento, *L panamensis* (azul) y *L. braziliensis* (amarillo). Los mapas fueron construidos en QGIS 3.6.0 teniendo como base las coordenadas GPS de cada

aislamiento. (QGIS Geographic Information System. Open Source Geospatial Foundation Project. http://qgis.osgeo.org").

Figura 2. Dinámica de somia entre los aislamientos clínicos de *L. panamensis* y *L. braziliensis*. El heatmap muestra el número de copias para todos los cromosomas en *L. panamensis* (A) y *L. braziliensis*. (B). Basados en el panel de color ubicado en la parte superior izquierda, es posible identificar que la mayoría de los cromosomas en *L. panamensis* presentan dos copias (disómicos) y en *L. braziliensis* tres copias (trisómicos). El eje de las X presenta el número del aislamiento y el eje de las Y el número del cromosoma.

Figura 3. Evaluación del número de copias por gen y análisis de ontología génica. La figura describe el número de genes con VNC (gráfica de barras) en los aislamientos clínicos de *L. panamensis* (A) y *L. braziliensis* (B), las líneas rojas punteadas señalan el número de genes con menor o mayor VNC. A la derecha se observan los procesos biológicos en los que están asociados los genes con VNC, en cada uno de los aislamientos en las dos especies. Los colores describen el porcentaje de genes asociados en cada uno de los procesos.

Figura 4. Descripción de SNPs en *L. panamensis*. En el Panel A se observa la distribución de los SNPs a lo largo del genoma en cada uno de los aislamientos clínicos de *L. panamensis* analizados. Al final de la figura se describe el total de SNPs encontrado por cada aislamiento. Panel B: Análisis de SNPs realizado a lo largo del cromosoma 20 para cada aislamiento. Los asteriscos en rojo representan los SNPs que fueron compartidos entre todos los aislamientos y a la derecha la tabla de anotación de cada uno de ellos. El recuadro en rojo señala los genes en los que se identificaron los SNPs que fueron distribuidos en bloque en el aislamiento Lp8132.

Figura 5. Descripción de SNPs en *L. braziliensis*. En la figura se observa la distribución de los SNPs a lo largo del genoma en cada uno de los aislamientos clínicos de *L. braziliensis* analizados. Al final de la figura se describe el total de SNPs encontrado por cada aislamiento.

Figura 6. Relación filogenética entre los aislamientos clínicos de *L. panamensis*. Árbol filogenético construido con base en una matriz de distancia, derivada de los SNPs nucleares (A) y mitocondriales: maxicírculo (B) compartidos entre los veinte aislamientos de *L. panamensis.* Lb_W_D y Lg 9913: genomas de referencia de *L braziliensis* y *L. guyanensis,* respectivamente. LPPSC1 y LpanUA: Genomas de referencia de *L. panamensis.*

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Figura 1

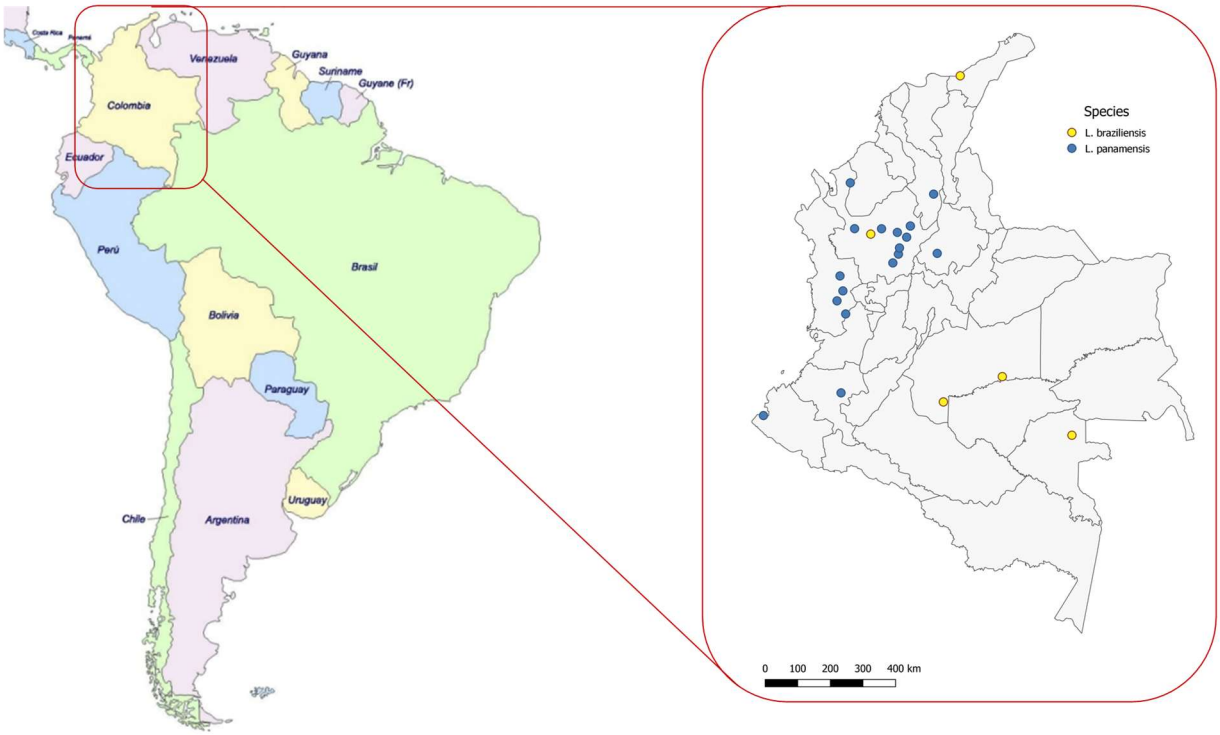
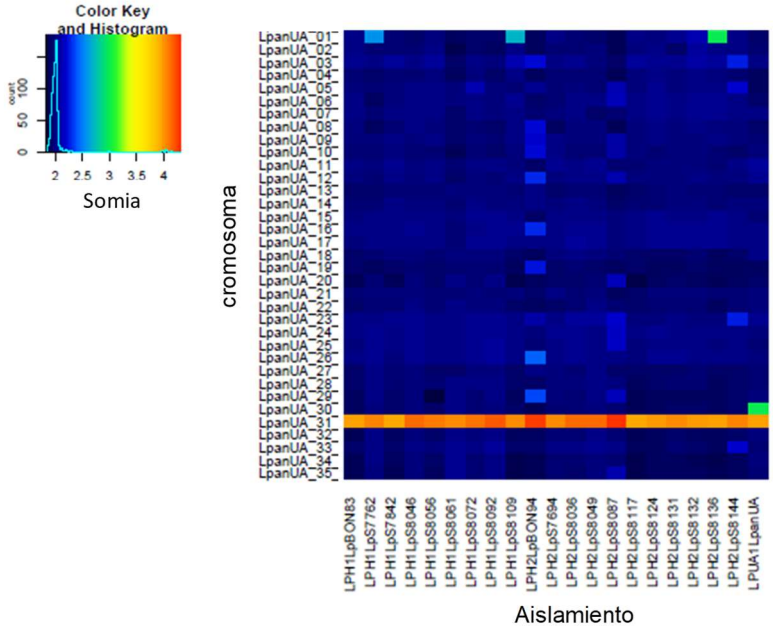

Costa Rica
Panamá
Venezuela
Guyana
Suriname
Guyane (Fr)
Colombia
Ecuador
Perú
Brasil
Bolivia
Paraguay
Chile
Argentina
Uruguay
Species
L. braziliensis
L. panamensis
0 100 200 300 400 km

Figura 2

A


Color Key and Histogram
count
0
50
100
2
2.5
3
3.5
4
Somia
cromosoma
LpanUA_01
LpanUA_02
LpanUA_03
LpanUA_04
LpanUA_05
LpanUA_06
LpanUA_07
LpanUA_08
LpanUA_09
LpanUA_10
LpanUA_11
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LpanUA_31
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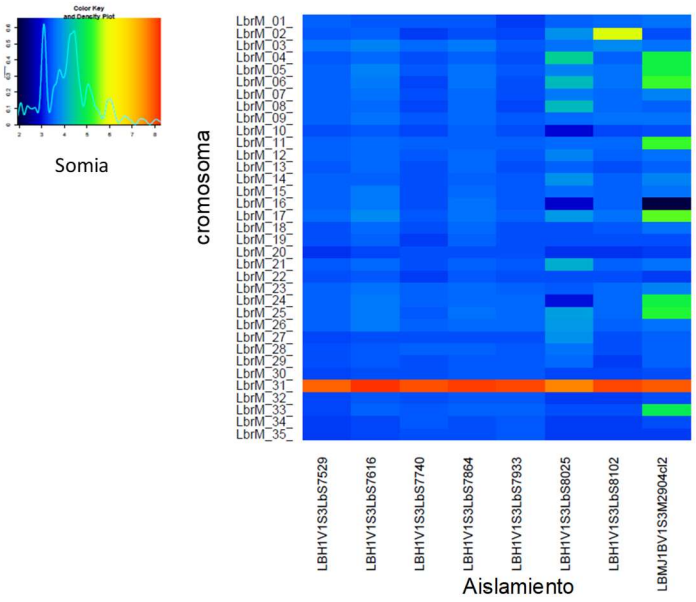
LpanUA_33
LpanUA_34
LpanUA_35
LPH1LpBON83
LPH1LpS7762
LPH1LpS7842
LPH1LpS8046
LPH1LpS8056
LPH1LpS8061
LPH1LpS8072
LPH1LpS8092
LPH1LpS8109
LPH2LpBON94
LPH2LpS7694
LPH2LpS8036
LPH2LpS8049
LPH2LpS8087
LPH2LpS8117
LPH2LpS8124
LPH2LpS8131
LPH2LpS8132
LPH2LpS8136
LPH2LpS8144
LPUA1LpanUA
Aislamiento

B


Color Key and Density Plot
Somia
cromosoma
LbrM_01
LbrM_02
LbrM_03
LbrM_04
LbrM_05
LbrM_06
LbrM_07
LbrM_08
LbrM_09
LbrM_10
LbrM_11
LbrM_12
LbrM_13
LbrM_14
LbrM_15
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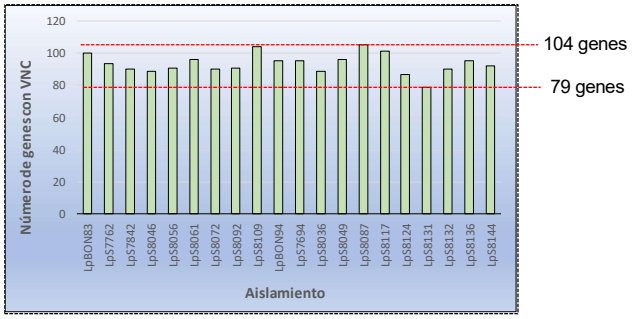
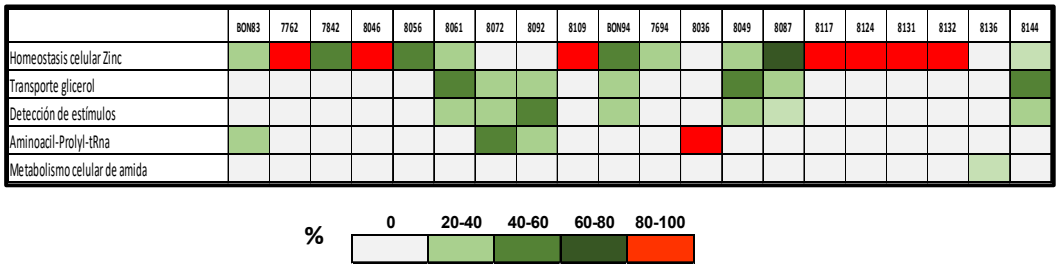
LbrM_29
LbrM_30
LbrM_31
LbrM_32
LbrM_33
LbrM_34
LbrM_35
LBH1V1S3LbS7529
LBH1V1S3LbS7616
LBH1V1S3LbS7740
LBH1V1S3LbS7864
LBH1V1S3LbS7933
LBH1V1S3LbS8025
LBH1V1S3LbS8102
LBMJ1BV1S3M2904dl2
Aislamiento

Figura 3

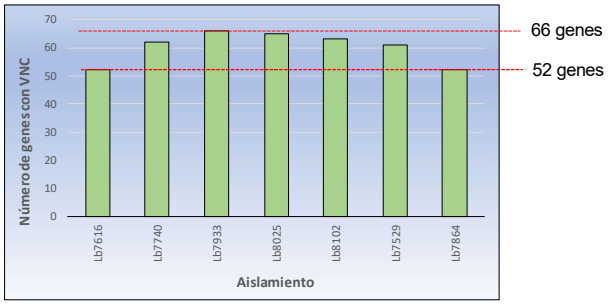
A



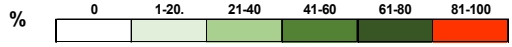
Z Score ≥ 2



B



	7616	7740	7933	8025	8102	7529	7864
Integración ADN							
Homeostasis Celular Ion Zinc							
Transporte Ion metal							
Metabolismo de carbohidratos							
Adhesion celular							
Catabolismo de Carbohidratos							
Metabolismo de Proteínas							



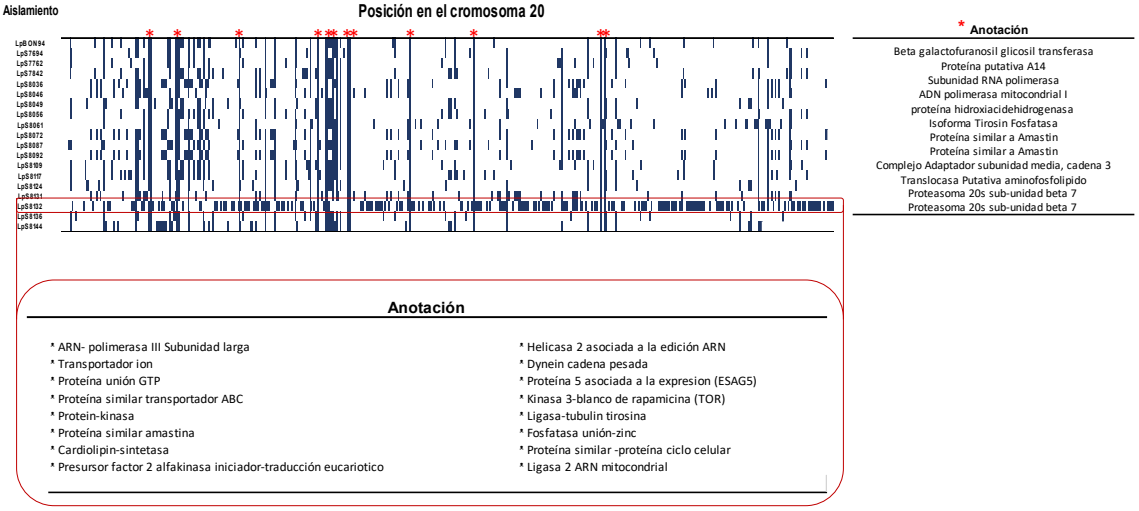
Z Score ≥ 2

Figura 4

A

Aislamiento	Cromosoma 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	Total
LpBON83	16	17	13	21	21	24	19	27	28	21	21	35	31	31	19	36	26	33	30	163	26	39	41	64	34	41	42	46	57	69	79	88	57	93	143	1551
LpBON94	14	12	12	22	21	21	21	29	26	29	23	31	34	31	19	39	28	39	23	150	24	52	42	80	40	40	45	44	46	70	84	87	72	81	128	1559
LpS7694	12	17	11	20	31	24	25	25	29	23	20	36	39	23	20	44	26	39	29	169	23	30	34	63	37	41	43	35	63	74	87	91	66	74	128	1551
LpS7762	14	9	7	23	29	21	28	24	21	30	22	33	30	20	17	33	31	25	26	143	22	47	41	66	36	39	43	31	43	63	80	75	54	72	121	1419
LpS7842	14	14	18	24	28	24	20	25	25	17	25	34	31	27	21	30	20	35	31	152	24	38	37	68	36	39	44	42	48	63	73	73	59	83	122	1464
LpS8036	27	28	18	43	23	58	35	27	63	33	34	42	60	45	39	71	62	56	40	242	49	42	52	93	81	103	78	88	97	71	139	128	130	163	233	2593
LpS8046	27	24	19	46	27	59	39	38	56	39	36	43	61	43	38	55	65	51	48	236	47	45	53	92	79	106	71	91	94	78	144	119	130	160	221	2580
LpS8049	11	13	6	18	26	15	16	17	14	15	20	30	21	22	11	31	13	28	25	143	22	29	23	53	29	32	34	35	40	67	77	53	41	82	106	1218
LpS8056	14	16	15	19	23	20	31	18	21	16	24	35	31	28	18	31	29	35	25	145	23	36	31	62	39	32	38	37	41	70	69	76	56	79	124	1407
LpS8061	28	26	23	30	39	42	41	32	53	31	28	45	58	40	39	57	52	59	32	212	38	50	51	85	73	81	68	87	79	87	125	134	114	146	190	2375
LpS8072	27	25	18	43	21	63	39	28	59	35	35	45	59	49	44	70	63	55	39	243	47	41	51	92	82	107	79	90	98	68	135	125	131	161	239	2606
LpS8087	13	15	10	25	34	22	22	25	31	17	23	30	23	31	22	42	24	34	21	151	25	40	30	67	36	51	48	34	59	67	85	82	52	84	111	1486
LpS8092	27	30	18	42	26	58	41	29	64	35	35	44	62	45	41	67	61	53	39	246	51	46	50	93	80	104	76	87	98	74	132	119	126	168	233	2600
LpS8109	14	19	18	21	25	23	30	29	33	26	20	36	45	30	11	36	28	39	30	168	27	38	41	67	44	44	41	42	62	66	89	105	77	90	129	1643
LpS8117	15	17	15	22	23	23	24	27	26	19	27	31	32	26	17	37	24	33	29	158	27	48	42	67	43	41	41	44	56	71	83	94	57	98	136	1573
LpS8124	10	13	7	14	25	14	16	20	22	19	19	29	18	24	11	26	20	28	29	144	20	34	26	57	30	38	34	27	46	63	92	55	40	65	110	1245
LpS8131	23	23	25	40	28	56	44	36	58	37	32	44	57	46	37	69	68	49	44	261	45	47	55	90	84	110	73	93	92	76	148	133	124	185	229	2661
LpS8132	76	87	111	117	100	213	144	177	152	158	92	127	144	169	150	151	232	101	167	843	181	122	193	323	284	219	225	242	224	268	385	299	290	377	358	7548
LpS8136	15	11	10	20	25	15	18	24	22	16	18	32	29	28	10	23	18	26	22	143	24	40	35	54	38	32	42	34	42	62	82	81	43	80	114	1328
LpS8144	5	7	10	13	11	12	12	9	17	15	18	18	13	11	12	15	21	3	12	107	23	23	18	43	21	23	12	15	31	50	57	44	37	43	72	853

B



Anotación

* ARN- polimerasa III Subunidad larga
* Transportador ion
* Proteína unión GTP
* Proteína similar transportador ABC
* Protein-kinasa
* Proteína similar amastina
* Cardiolipin-sintetasa
* Presursor factor 2 alfakinasa iniciador-traducción eucariotico
* Helicasa 2 asociada a la edición ARN
* Dynein cadena pesada
* Proteína 5 asociada a la expresion (ESAG5)
* Kinasa 3-blanco de rapamicina (TOR)
* Ligasa-tubulin tirosina
* Fosfatasa unión-zinc
* Proteína similar -proteína ciclo celular
* Ligasa 2 ARN mitocondrial

Figura 5

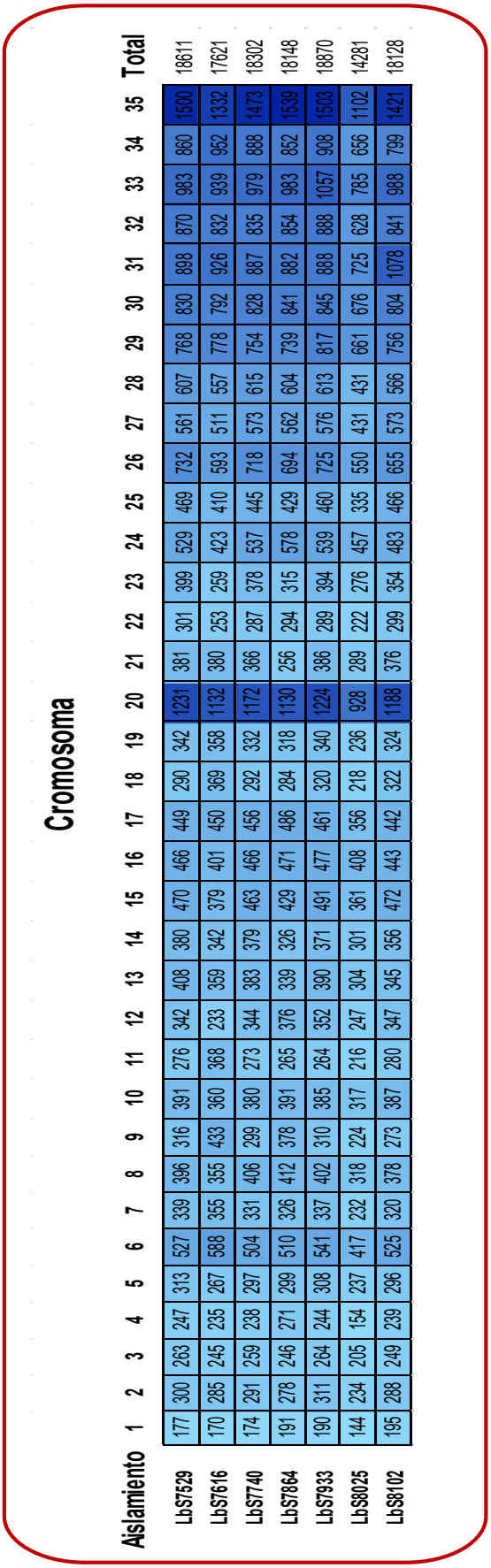
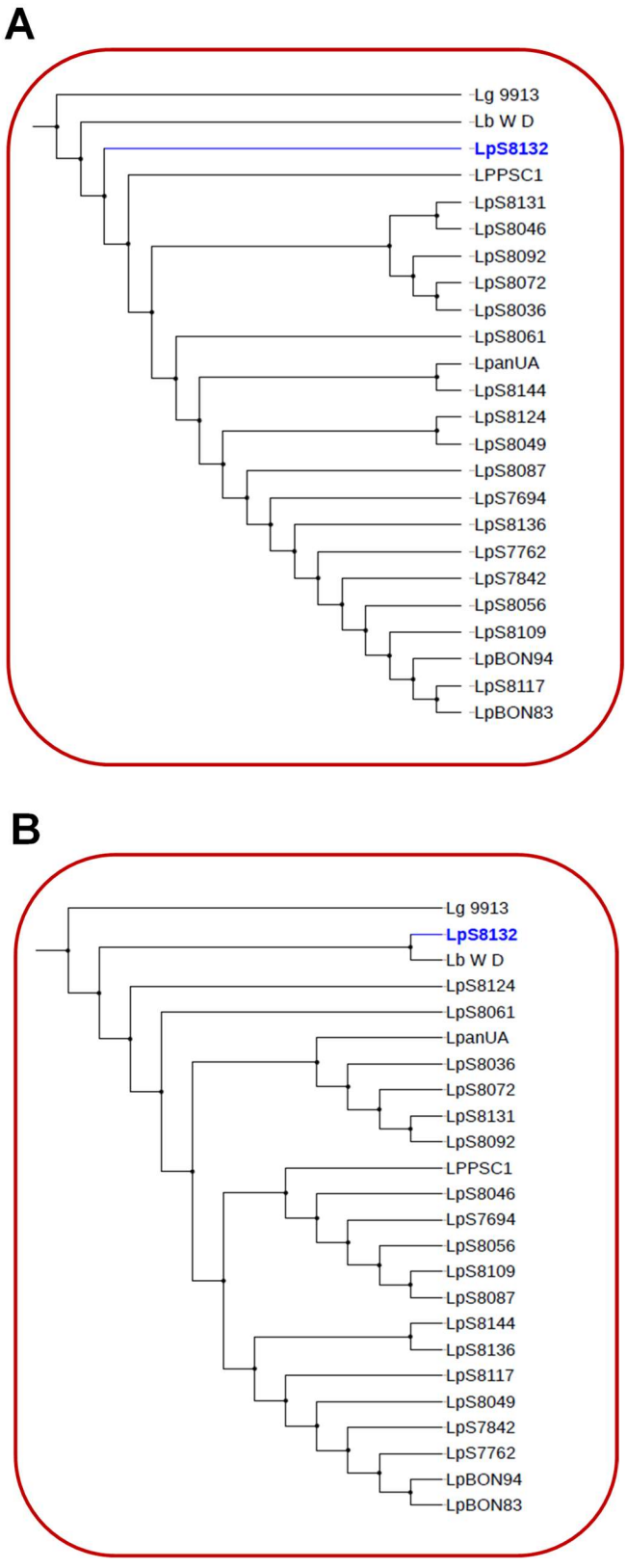

Cromosoma
Aislamiento
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35
Total
LbS7529 177 300 263 247 313 527 339 396 316 391 276 342 408 380 470 466 449 290 342 1231 381 301 399 529 469 732 561 607 768 830 898 870 983 860 1500 18611
LbS7616 170 285 245 235 267 588 355 355 433 360 368 233 359 342 379 401 450 369 358 1132 380 253 259 423 410 593 511 557 778 792 926 832 939 952 1332 17621
LbS7740 174 291 259 238 297 504 331 406 299 380 273 344 383 379 463 466 456 292 332 1172 366 287 378 537 445 718 573 615 754 828 887 835 979 888 1473 18302
LbS7864 191 278 246 271 299 510 326 412 378 391 265 376 339 326 429 471 486 284 318 1130 256 294 315 578 429 694 562 604 739 841 882 854 983 852 1539 18148
LbS7933 190 311 264 244 308 541 337 402 310 385 264 352 390 371 491 477 461 320 340 1224 386 289 394 539 460 725 576 613 817 845 888 888 1057 908 1503 18870
LbS8025 144 234 205 154 237 417 232 318 224 317 216 247 304 301 361 408 356 218 236 928 289 222 276 457 335 550 431 431 661 676 725 628 785 656 1102 14281
LbS8102 195 288 249 239 296 525 320 378 273 387 280 347 345 356 472 443 442 322 324 1188 376 299 354 483 466 655 573 566 756 804 1078 841 988 799 1421 18128

Figura 6


A
Lg 9913
Lb W D
LpS8132
LPPSC1
LpS8131
LpS8046
LpS8092
LpS8072
LpS8036
LpS8061
LpanUA
LpS8144
LpS8124
LpS8049
LpS8087
LpS7694
LpS8136
LpS7762
LpS7842
LpS8056
LpS8109
LpBON94
LpS8117
LpBON83
B
Lg 9913
LpS8132
Lb W D
LpS8124
LpS8061
LpanUA
LpS8036
LpS8072
LpS8131
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LPPSC1
LpS8046
LpS7694
LpS8056
LpS8109
LpS8087
LpS8144
LpS8136
LpS8117
LpS8049
LpS7842
LpS7762
LpBON94
LpBON83

9. CONCLUSIONES Y PERSPECTIVAS GENERALES

9.1 Conclusiones

En esta investigación se identificaron las principales especies de *Leishmania* que están circulando en una de las poblaciones más vulnerable de Colombia: Población militar, así mismo se determinó la distribución geográfica de dichas especies en todo el territorio nacional y su posible variabilidad genética intra-específica. Estos resultados junto con lo descrito previamente en la literatura describen que *Leishmania (Viannia) braziliensis* y *Leishmania (Viannia) panamensis*, son las especies más ampliamente distribuidas en población civil como en población militar colombiana.

Los análisis genómicos y transcriptómicos comparativos realizados entre cepas de *Leishmania (Viannia) braziliensis* y *Leishmania (Viannia) panamensis*, con resistencia inducida al Sb^{III} versus no inducidos, revela que así como sucede con algunas especies de *Leishmania* del viejo mundo (*L. major, L. infantum* y *L. donovani*) y algunas del nuevo mundo pertenecientes al mismo subgénero (*L. (Viannia) guyanensis*) estas especies utilizan la plasticidad de su genoma para regular la dosis genética como mecanismo de supervivencia frente a situaciones de estrés (Sb^{III}).

Por otro lado, los análisis transcriptómicos realizados en *Leishmania (Leishmania) amazonensis* revelan que al igual que otras especies, este parásito modula su perfil de expresión génica no solo para contrarrestar el efecto del antimonial si no también para modular varios procesos biológicos.

Adicionalmente, el análisis genómico realizado entre los aislamientos clínicos obtenidos de pacientes infectados con *Leishmania (Viannia) braziliensis* y *Leishmania (Viannia) panamensis*, revelo en la mayoría de aislamientos, una baja variabilidad genética intra-especie, lo cual sugiere que la manifestación clínica así como la respuesta frente al tratamiento anti-leishmanial está posiblemente asociado a otros factores diferentes al comportamiento genómico del parásito, como por ejemplo características inherentes al hospedero o al medicamento en sí.

9.2 Perspectivas Generales

- Teniendo en cuenta que el modelo macrófago-amastigote es considerado el más cercano a las condiciones fisiopatológicas de la leishmaniasis y además el método

más apropiado de tamizaje *in vitro*, se hace indispensable realizar a futuro estudios ADN-seq y ARN-seq dual que permitan analizar el comportamiento genómico y transcriptómico de forma simultánea durante dicha interacción, igualmente, realizar un análisis comparativo de dicho comportamiento molecular de amastigotes provenientes de pacientes con falla, relapso y éxito terapéutico.

- Con el propósito de seguir conociendo más acerca de la biología de *Leishmania*, y su comportamiento durante los diferentes estadios de su ciclo de vida, sería interesante ampliar el panorama de estudio e identificar el comportamiento molecular (genómico y transcriptómico) durante la interacción parásito-vector y entender cada uno de los cambios que ocurren durante el proceso de metaciclogénesis. Así mismo realizar un análisis comparativo del comportamiento genómico y transcriptómico ocurrido, cuando el parásito infecta diferentes vectores y/o hospederos mamíferos.

- Debido a la baja variación estructural observada en las especies analizadas este estudio, se hace necesario realizar un análisis genómico a gran escala en donde se involucren no solo un mayor número de aislamientos clínicos colombianos sino también aislamientos provenientes de otras partes del mundo, con el propósito de evaluar la variabilidad genética inter e intra específica en estas especies y su relación con la distribución geográfica.

- Es indispensable seguir evaluando no solo las características genómicas sino también transcriptómicas de las especies obtenidas a partir de aislamientos clínicos y evaluar la relación existente entre el comportamiento molecular y las diferentes características socio-demográficas, clínicas y terapéuticas.

- Finalmente, en vista de los resultados obtenidos en uno de los aislamientos clínicos infectados con *Leishmania (Viannia) braziliensis*, se hace necesario realizar estudios adicionales (clonación y posterior secuenciación del genoma completo) con el propósito de confirmar posibles eventos de recombinación en esta especie.

10. REFERENCIAS BIBLIOGRÁFICAS

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