

Detection and identification of *Leishmania* spp.: application of two *hsp70*-based PCR-RFLP protocols to clinical samples from the New World

Ana M. Montalvo¹ · Jorge Fraga¹ · Didier Tirado² · Gustavo Blandón² · Annia Alba¹ · Gert Van der Auwera³ · Iván Darío Vélez² · Carlos Muskus²

Received: 21 December 2016 / Accepted: 20 April 2017 / Published online: 1 June 2017
© Springer-Verlag Berlin Heidelberg 2017

Abstract Leishmaniasis is highly prevalent in New World countries, where several methods are available for detection and identification of *Leishmania* spp. Two *hsp70*-based PCR protocols (PCR-N and PCR-F) and their corresponding restriction fragment length polymorphisms (RFLP) were applied for detection and identification of *Leishmania* spp. in clinical samples recruited in Colombia, Guatemala, and Honduras. A total of 93 cases were studied. The samples were classified into positive or suspected of leishmaniasis according to parasitological criteria. Molecular amplification of two different *hsp70* gene fragments and further RFLP analysis for identification of *Leishmania* species was done. The detection in parasitologically positive samples was higher using PCR-N than PCR-F. In the total of samples studied, the main species identified were *Leishmania panamensis*, *Leishmania braziliensis*, and *Leishmania infantum* (*chagasi*). Although RFLP-N was more efficient for the identification, RFLP-F is necessary for discrimination between *L. panamensis* and *Leishmania guyanensis*, of great importance in Colombia. Unexpectedly, one sample from this country revealed an RFLP pattern corresponding to *Leishmania naiffi*. Both

molecular variants are applicable for the study of clinical samples originated in Colombia, Honduras, and Guatemala. Choosing the better tool for each setting depends on the species circulating. More studies are needed to confirm the presence of *L. naiffi* in Colombian territory.

Keywords *Leishmania* · Leishmaniasis · Species · PCR · RFLP · *hsp70*

Introduction

Human infection by *Leishmania* parasites can cause leishmaniasis, a complex of diseases presents in areas of tropical and subtropical countries. Endemic transmission occurs in 98 countries and 3 territories, where official records estimate the occurrence of 0.2 to 0.4 million cases of visceral leishmaniasis (VL) and 0.7 to 1.2 million cases of cutaneous leishmaniasis (CL) each year (Alvar et al. 2012). As several species may be pathogenic to humans, leishmaniasis can be considered a polymorphic group of diseases, in which clinical and epidemiological characteristics, response to treatment and prognosis, can differ (Pérez-Ayala et al. 2009).

The clinical presentation depends on several factors, among which the infecting species of the parasite is quite relevant (Goto and Lauletta-Lindoso 2012). In consequence, species identification has become an essential requirement for choosing the best treatment with the least side effects and late complications (de Vries et al. 2015; Hodiamont et al. 2014); in research concerning the differential response of *Leishmania* species to drugs (Fernandez et al. 2012; Obonaga et al. 2014); and in epidemiological studies involving vectors and reservoirs (Özbel et al. 2016; Córdova et al. 2011).

According to the current report on worldwide leishmaniasis incidence, a considerable number of CL cases are reported in

Electronic supplementary material The online version of this article (doi:10.1007/s00436-017-5454-6) contains supplementary material, which is available to authorized users.

✉ Ana M. Montalvo
amontalvo@ipk.sld.cu; mmontal@infomed.sld.cu

¹ Department of Parasitology, Institute of Tropical Medicine Pedro Kourí, Havana, Cuba

² PECET (Program of Study and Control of Tropical Diseases) University of Antioquia, Medellín, Colombia

³ Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium

the American region, comprising Colombia, Honduras, and Guatemala (Alvar et al. 2012). Some of these countries are also enrolled in investigations concerning the differential effect of drugs against *Leishmania* in relation to the infecting species (Reveiz et al. 2013; Obonaga et al. 2014), which makes the identification step a necessity for some laboratories.

At least six species of *Leishmania* have been reported in Colombia: *Leishmania panamensis*, *Leishmania braziliensis*, *Leishmania guyanensis*, *Leishmania mexicana*, and *Leishmania amazonensis*, causing both cutaneous and mucocutaneous diseases, and *Leishmania infantum* (*chagasi*), mainly associated with the visceral form (Corredor et al. 1990). Different protocols have been used for molecular identification of the species present in clinical isolates using several targets: the genes coding for the heat-shock protein HSP70 (*hsp70*) and cytochrome B (*cyt B*), as well as kinetoplast DNA (kDNA) (Montalvo et al. 2016; Martínez et al. 2010; Urbano et al. 2011).

In northern Guatemala, cutaneous leishmaniasis is considered endemic, being caused by *L. mexicana* and *L. braziliensis* (Zeledón et al. 1993; Herwaldt et al. 1992). Molecular detection of parasites in clinical samples was reported in this country using a real-time PCR targeting a region of the 18S rRNA gene (Wortmann et al. 2007); and more recently, a conventional PCR was applied to diagnose an imported case of Chiclero's ulcer coming from Guatemala (Blaylock and Wortmann 2012).

In Honduras, *L. braziliensis*, *L. panamensis* and *L. mexicana* have been involved as the etiological agents of the cutaneous disease, and *L. infantum* (*chagasi*) causes visceral and cutaneous lesions (Grimaldi et al. 1987; Ponce et al. 1991; Noyes et al. 1997). No recent records about the use of molecular approaches to characterize parasites from clinical samples in this country were encountered.

The gene coding for the HSP70 protein has been largely used in the last years for *Leishmania* detection and identification. Compared to the first PCR-RFLP report using this gene (García et al. 2004), three protocols were shown to have superior sensitivity and specificity (Montalvo et al. 2012, 2014; Fraga et al. 2012, 2013a, b). In the current work, the two more appropriated variants for New World species discrimination, PCR-F and PCR-N, and their respective RFLPs, are applied to the analysis of clinical samples from cutaneous leishmaniasis cases from Colombia, Honduras, and Guatemala, in order to establish their usefulness for *Leishmania* spp. typing in those settings.

Materials and methods

Clinical samples Lesion lancet scrapings or biopsies were obtained from 93 patients coming from different endemic areas of cutaneous leishmaniasis, where transmission occurs.

In some cases, lesion aspirates were obtained as well. They originated from Colombia ($n = 73$), Guatemala ($n = 10$), and Honduras ($n = 10$). Of all samples, 63 were positive for *Leishmania* according to clinical and parasitological criteria (direct smear and/or culture). The remaining 30 had clinical symptoms of CL, and lived, worked, or visited areas where transmission occurs. All samples were recruited and analyzed at the “Programa de Estudio y Control de Enfermedades Tropicales” (PECET, Universidad de Antioquia, Colombia). An approval from a review board was obtained from PECET (F-CM-0001, 26/07/2016) as well as an informed consent from all patients, allowing the use of their clinical samples in research projects related to diagnosis.

Parasitology Direct microscopic examination was done on lesion samples fixed with methanol and stained with Giemsa. Slides were analyzed with optical microscopy ($\times 100$ magnification). Lesion aspirates were taken with a tuberculin syringe and gently put in a tube containing biphasic NNN medium (Novy, Mc Neal, Nicolle) using a salt solution as the liquid phase. The tubes were incubated at 27 °C for promastigote growth. Samples were considered positive when either parasites could be detected microscopically, or could be isolated by culturing, or both. After a month, a new passage from culture was made and the primary one was considered negative.

DNA extraction DNA was isolated using the QIAamp DNA Mini Kit ($n = 70$) or the DNeasy Blood & Tissue Kit ($n = 23$), both from Qiagen (www.qiagen.com), according to manufacturer's instructions. The elution volume varied in relation to the sample size (100–150 μ L).

PCR amplification A specific *hsp70* gene fragment was amplified using the PCR-F (1286 bp) and PCR-N (593 bp) protocols described by Montalvo et al. 2012. The primers were: PCR-F (F25: GGACGCCGGCACGATKCT and R1310: CCTGGTTGTTGTTTCAGCCACTC); PCR-N (F25 and R617: CGAAGAAGTCCGATACGAGGG). The amplification conditions, for both PCR variants, were: denaturation at 95 °C for 5 min; followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 61 °C for 1 min, extension at 72 °C for 1 min (except PCR-F: 2 min); and a final extension step of 10 min at 72 °C. Five microliters of DNA obtained from each clinical sample were used as template in most cases. Negative controls were always included, along with a positive one consisting of 100 fg DNA from *L. braziliensis* strain MHOM/BR/75/M2903. Thermal cycling was performed in a MyCycler™ (Bio-Rad, www.bio-rad.com). Analysis on a 2% agarose gel was used to verify the presence and size of amplified product. The results, expressed as parasite detection sensitivity, represent the total of positive samples obtained by each PCR.

RFLP analysis For identification of *Leishmania* species, enzymatic restrictions of PCR-F and PCR-N amplified products were performed following the schemes of Montalvo et al. (2012) and Fraga et al. (2013a). The restriction enzymes used were: *HaeIII*, *RsaI*, *BsaJI*, *HindIII*, *SduI* (MBI Fermentas, St. Leon-Rot, Germany), and *BccI* (New England Biolabs, Ipswich, MA, USA). A sequential approach was followed: PCR-F was digested with *HaeIII* to separate groups of species, after which it was digested with *RsaI*, *SduI*, and *BccI* to further discriminate within these groups. For PCR-N, the first separation was done with *BsaJI*, followed by *RsaI*, *HindIII*, and *SduI*. Enzymatic digestion of strong PCR amplicons was performed in a total volume of 10 μ L, while weak amplicons were digested in 20 μ L. Each digestion contained $\times 1$ optimal buffer (recommended by the manufacturer), 7 or 17 μ L of unpurified PCR product respectively, and 2 U of enzyme. The reactions were incubated for 3 h at 37 °C (55 °C for *BsaJI*). Restriction patterns were analyzed by electrophoresis in a 3% of small fragment agarose gel (Gentaur, Brussels, Belgium), running at 3.5 V/cm for 3 h. The GeneRuler™ 100 bp DNA Ladder (MBI Fermentas, St. Leon-Rot, Germany) was used as a size reference marker. In case of weak PCR amplicons, some samples were only partially identified as the restriction algorithm could not be completed due to insufficient amounts of PCR amplified products (second or third digestion not done). The results are expressed as species identification efficiency, which represents the number of samples that could be typed or partially typed.

Results

In samples from 63 patients that were positive by parasitological methods, the detection sensitivity of PCR-N was 89%, and of PCR-F 71%. Only two cases (PE-C17 and PE-C23)

were encountered positive by PCR-F while negative by PCR-N. RFLP-N could be performed on 71% of the samples, and RFLP-F on 44%.

In all the remaining 30 suspected, but non-confirmed leishmaniasis cases, PCR-N detected *Leishmania* DNA in the samples, while PCR-F only detected 50% as positive. Species identification could be done for 57% of the samples with RFLP-N, but only for 13% with RFLP-F.

The species identified with each PCR-RFLP protocol are shown in Fig. 1, with stratification per country. A detailed listing of individual results for all samples is available in supplementary Table ESM1. When samples were typed with both protocols, a complete correspondence in the results obtained by both of them was observed. In addition, the species found agreed with those described previously in the countries of origin, with one notable exception. The particular exception was a sample from Colombia (PE-C27) showing an RFLP-N pattern corresponding to *L. naiffi* (Fig. 2).

Discussion

The higher sensitivity of PCR-N compared to PCR-F agrees with previous evaluations of the same protocols, on clinical samples from Peru, where PCR-N reached the highest sensitivity regardless of the clinical presentation or the type of clinical sample (Fraga et al. 2012). This was expected because PCR-N produces a 593 bp amplicon, half of the size of the one obtained using PCR-F (1286 bp) and which is therefore easier to amplify. Apart from this, PCR-N was also more sensitive on biopsies from Old World cutaneous leishmaniasis (Montalvo et al. 2014). However, two samples in this study (PE-C17 and PE-C23) were found positive by PCR-F and not by PCR-N. A probable explanation to this could be the amount of DNA used for the PCR-N reaction in those cases (3 μ l), instead of the 5 μ l

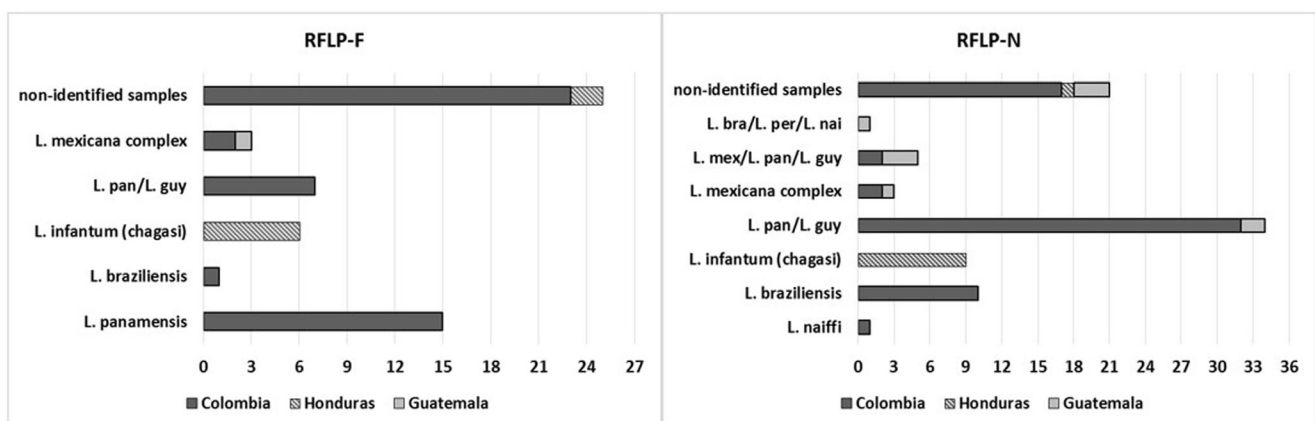


Fig. 1 *Leishmania* species identification, after RFLP-F and RFLP-N protocols. The number of species or group of species discriminated is shown according to the country of origin. A species complex (*L. mexicana* complex: *L. mexicana*, *L. amazonensis*, *L. garnhami*); individual species

(*L. infantum (chagasi)*, *L. braziliensis*, *L. panamensis*, *L. braziliensis*, *L. naiffi*); and common patterns among two or more species are shown (*L. pan*: *L. panamensis*, *L. guy*: *L. guyanensis*, *L. peruviana*, *L. nai*: *L. naiffi*, *L. braziliensis*, *L. per*: *L. peruviana*)

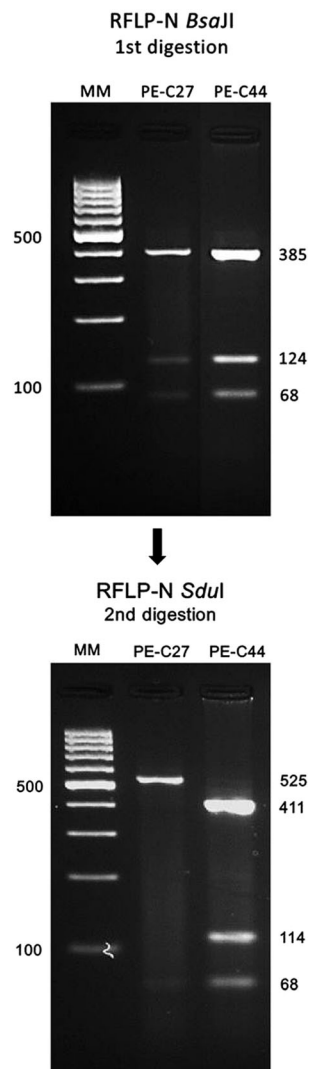


Fig. 2 Restriction patterns obtained after RFLP-N. *MM* molecular weight marker GeneRuler™ 100bp Plus bp DNA Ladder. *L. nai* RFLP-N/*SduI* pattern (525 and 68bp). *L. bra* RFLP-N/*SduI* pattern of *L. braziliensis* (411, 114, 68bp) (according to Fraga et al. 2013a, b). The RFLP-N/*SduI* pattern of sample PE-C27 is shown on the right

used in PCR-F, although in other samples this difference did not affect the amplification (PE-C 3, 6, 15, 38, 58). If those two particular cases had a considerable small amount of *Leishmania* DNA in the solution, probably the difference in the volume was crucial in the results obtained. Also, PE-C23 produced a PCR-F weak amplicon, which support this possibility.

PCR-N also detected parasite DNA in clinical samples in which no parasites from smear examination or culture could be demonstrated, probably due to a low parasite load and/or the time of evolution of the lesion. Given the previously reported 100% specificity (analytical and diagnostic) of the here used PCRs (Montalvo et al. 2012; Fraga et al. 2012), and the fact that RFLP resulted in *Leishmania*-specific patterns, we can assume that these cases are true positives. Hence, both PCRs are complementary to parasitology in identifying

leishmaniasis cases. Even though they have the additional advantage over parasitology methods of enabling species typing, they cannot replace these methods because PCRs remained negative in 10–30% of samples in which parasites were found. Nevertheless, if only parasite detection at genus level is desired, without the identification step, multicopy targets (kDNA, rDNA) would fit better for diagnosis.

When deciding between RFLP-F and RFLP-N, two aspects need be considered. On the one hand, typing efficiency is higher with RFLP-N, as a higher number of clinical samples could be typed. On the other hand, RFLP-F allows discriminating all species, whereas for instance *L. panamensis* and *L. guyanensis* (both endemic in Colombia) cannot be distinguished with RFLP-N.

In the samples infected with species from the *L. mexicana* complex, typing could not reach the species level because current *hsp70* RFLP protocols do not allow discriminating *L. mexicana* from *L. amazonensis* (syn. *L. garnhami*) (Montalvo et al. 2012). However, phylogeny of *Leishmania* species based on either HSP70 gene sequences alone (Van der Auwera et al. 2013) or in combination with HSP20 genes (Fraga et al. 2010, 2013b), supported their division into different species. Using a PCR-RFLP protocol that targets the internal transcribed spacer (ITS) of the ribosomal RNA gene array, Berzunza-Cruz et al. 2002 succeeded in differentiating *L. amazonensis* from *L. mexicana* for diagnosis.

Leishmania (V.) naiffi was previously not reported in Colombia. It was originally described as a parasite of small mammals in the Amazonian area of Brazil (Lainson and Shaw 1989). Human infection with this species resulting in CL were reported in Brazil (Naiff et al. 1991; Felinto de Brito et al. 2012) but also in French Guiana, Martinique, Guadeloupe, Surinam, and more recently, in Ecuador (Grimaldi et al. 1991; Darie et al. 1995; Pratlong et al. 2002; Fouque et al. 2007; van der Snoek et al. 2009; Kato et al. 2013). Although sequencing is needed to verify this case, the potential presence of this species in the Colombian territory (Guaviare) must be highlighted, to foresee cases of mild cutaneous infections due to *L. naiffi* in remote areas of the country.

Whereas the number of samples analyzed from Honduras was very low, 9 out of 10 were identified as *L. infantum (chagasi)*. This agrees with previous reports involving this species not only as the responsible for visceral leishmaniasis, but also causing cutaneous disease in Honduras and other Central-American countries (Grimaldi et al. 1987; Ponce et al. 1991; Noyes et al. 1997). In the case of Guatemala, only five samples were studied and four of them had patterns related to the *L. mexicana* complex and *L. braziliensis*, which have been recognized as the etiological agents of the disease (Herwaldt et al. 1992; Zeledón et al. 1993).

The here used RFLP protocols can be adapted to different settings, depending on the sensitivity required and the species that must be separated. As such, RFLP-F could be more useful for typing *Leishmania* spp. from Colombia if identification of

all circulating species is needed. On the other hand, because of its sensitivity PCR-N could be the method of choice in Guatemala and Honduras, where less species are thought to circulate.

Many studies have documented differential response to a variety of treatments in relation to the species of *Leishmania* (Llanos-Cuentas et al. 2008; Obonaga et al. 2014). A recent global analysis recommended a species-related therapy for travelers coming from endemic areas, including those from Latin America (Hodiamont et al. 2014). Taking into account that tools based on *hsp70* gene are, in parallel, among the most used and best validated typing assays (Van der Auwera and Dujardin 2015) we sustain that molecular tools applied here for typing New World *Leishmania* parasites could be very valuable for diagnostics, case management and therapeutic studies focused on differential response of treatments according to the species of *Leishmania* causing the disease.

Acknowledgements The authors appreciate the contribution of the patients for kindly donating their biological samples to assess this investigation. The assistance of Honduran and Guatemalan health services for helping to PECET'S researchers in sampling collection is acknowledged. The valuable contribution of Horacio Cadenas (PECET) is also appreciated. This work was partially funded by the third framework program of the Belgian Directorate General for Development with ITM Antwerp. The scientific support of Jean-Claude Dujardin is deeply recognized.

Authors' contributions AMM, JF, and CM conceived and designed the study. AMM, DT, GB performed the experiments. AMM, JF, AA, GVDA, and CM analyzed the data. AMM and GVDA wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M, WHO leishmaniasis control team (2012) Leishmaniasis worldwide and global estimates of its incidence. *PLoS One* 7: e35671
- Berzunza-Cruz M, Cabrera N, Crippa-Rossi M, Sosa-Cabrera T, Pérez-Monfort R, Becker I (2002) Polymorphism analysis of the internal transcribed spacer and small subunit of ribosomal RNA genes of *Leishmania mexicana*. *Parasitol Res* 88:918
- Blaylock JM, Wortmann GW (2012) A case report and literature review of “Chiclero’s ulcer”. *Travel Med Infect Dis* 10:275–278
- Córdova O, Vargas F, Hashiguchi Y, Kato H, Gómez E (2011) Identification of *Leishmania* species in patients and phlebotomines in transmission areas in a region of Peru. *Rev Peru Med Exp Salud Publica* 28:446–453
- Corredor A, Kreutzer RD, Tesh RB, Boshell J, Palau MT, Caceres E, Duque S, Pelaez D, Rodriguez G, Nichols S, Hernandez CA, Morales A, Young DG, Ferro de Carrasquilla C (1990) Distribution and etiology of leishmaniasis in Colombia. *AmJTrop Med Hyg* 42:206–214
- Darie H, Deniau M, Pratlong F, Lanotte G, Talarmin A, Millet P, Houin R, Dedet JP (1995) Cutaneous leishmaniasis of humans due to *Leishmania (Viannia) naiffi* outside Brazil. *Trans R Soc Trop Med Hyg* 89:476–477
- de Vries HCJ, Reedijk SH, Schallig HDFH (2015) Cutaneous leishmaniasis: Recent developments in diagnosis and management. *Am J Clin Dermatol* 16:99–109
- Felinto de Brito ME, Andrade MS, de Almeida EL, Medeiros AC, Werkhäuser RP, de Araújo AI, Brandao-Filho SP, Paiva-de Almeida AM, Gomes-Rodrigues GH (2012) Occupationally acquired American cutaneous leishmaniasis. *Case Rep Dermatol Med* 2012:279517. doi:10.1155/2012/279517
- Fernandez O, Diaz-Toro Y, Valderrama L, Ovalle C, Valderrama M, Castillo H, Perez M, Saravia NG (2012) Novel approach to in vitro drug susceptibility assessment of clinical strains of *Leishmania* spp. *J Clin Microbiol* 50:2207–2211
- Fouque F, Gaborit P, Issaly J, Carinci R, Gantier JC, Ravel C, Dedet JP (2007) Phlebotomine sand flies (Diptera: Psychodidae) associated with changing patterns in the transmission of the human cutaneous leishmaniasis in French Guiana. *Mem Inst Oswaldo Cruz* 102:35–40
- Fraga J, Montalvo AM, De Doncker S, Dujardin J-C, Van der Auwera G (2010) Phylogeny of *Leishmania* species based on the heat-shock protein 70 gene. *Infect Genet Evol* 10(2):238–245
- Fraga J, Veland N, Montalvo AM, Praet N, Boggild A, Valencia B, Arévalo J, Llanos-Cuentas A, Dujardin JC, Van der Auwera G (2012) Accurate and rapid species typing from cutaneous and mucocutaneous leishmaniasis lesions of the New World. *Diagn Microbiol Inf Dis* 74:142–150
- Fraga J, Montalvo AM, Maes I, Dujardin JC, Van der Auwera G (2013a) *HindIII* and *SduI* digests of heat-shock protein 70 PCR for *Leishmania* typing. *Diagn Microbiol Infect Dis* 77:245–247
- Fraga J, Montalvo AM, Van der Auwera G, Maes I, Dujardin JC, Requena JM (2013b) Evolution and species discrimination according to the *Leishmania* heat-shock protein 20 gene. *Inf Gen Evol* 18: 229–237
- García L, Kindt A, Bermúdez H, Llanos-Cuentas A, De Doncker S, Arévalo J, Quispe-Tintaya W, Dujardin JC (2004) Culture-independent species typing of neotropical *Leishmania* for clinical validation of a PCR-based assay targeting heat shock protein 70 genes. *J Clin Microbiol* 42:2294–2297
- Goto H, Lauletta-Lindoso JA (2012) Cutaneous and mucocutaneous leishmaniasis. *Infect Dis Clin N Am* 26:293–307
- Grimaldi G Jr, David JR, McMahon-Pratt D (1987) Identification and distribution of New World *Leishmania* species characterized by serodeme analysis using monoclonal antibodies. *AmJTrop Med Hyg* 36:270–287
- Grimaldi G Jr, Momen H, Naiff RD, McMahon-Pratt D, Barrett TV (1991) Characterization and classification of leishmanial parasites from humans, wild mammals, and sand flies in the Amazon region of Brazil. *AmJTrop Med Hyg* 44:645–661
- Herwaldt BL, Arana BA, Navin TR (1992) The natural history of cutaneous leishmaniasis in Guatemala. *J Infect Dis* 165:518–527
- Hodiamont CJ, Kager PA, Bart A, de Vries HJC, Van Thiel PPAM, Leenstra T, de Vries PJ, van Vugt M, Grobusch MP, van Gool T (2014) Species-directed therapy for leishmaniasis in returning travellers: a comprehensive guide. *PLoS Negl Trop Dis* 8:e2832
- Kato H, Calvopiña M, Criollo H, Hashiguchi Y (2013) First human cases of *Leishmania (Viannia) naiffi* infection in Ecuador and identification of its suspected vector species. *Acta Trop* 128:710–713
- Lainson R, Shaw JJ (1989) *Leishmania (Viannia) naiffi* sp. n., a parasite of the armadillo, *Dasyypus novemcinctus* (L.) in Amazonian Brazil. *Ann Parasitol Hum Comp* 64:3–9
- Llanos-Cuentas A, Tulliano G, Araujo Castillo R, Miranda-Verastegui C, Santamaria-Castrellon G, Ramirez L, Lazo M, De Doncker S, Boelaert M, Robays J, Dujardin JC, Arevalo J, Chappuis F (2008)

- Clinical and parasite species risk factors for pentavalent antimonial treatment failure in cutaneous leishmaniasis in Peru. *Clin Infect Dis* 46:223–231
- Martínez LP, Rebollo JA, Luna AL, Cochero S, Bejarano EE (2010) Molecular identification of the parasites causing cutaneous leishmaniasis on the Caribbean coast of Colombia. *Parasitol Res* 106: 647–652
- Montalvo AM, Fraga J, Maes I, Dujardin JC, Van der Auwera G (2012) Three new sensitive and specific heat-shock protein 70 PCRs for global *Leishmania* species identification. *Eur J Clin Microbiol Infect Dis* 31:1453–1461
- Montalvo AM, Fraga J, El Safi S, Gramiccia M, Jaffe CL, Dujardin JC, Van der Auwera G (2014) Direct *Leishmania* species typing in Old World clinical samples: evaluation of 3 sensitive methods based on the heat-shock protein 70 gene. *Diagn Microbiol Infect Dis* 80:35–39
- Montalvo AM, Fraga J, Montano I, Monzote L, Van der Auwera G, Marín M, Muskus C (2016) Identificación molecular de aislamientos clínicos de *Leishmania* spp. procedentes de Colombia con base en el gen hsp70. *Biomedica* 36:37–44
- Naiff RD, Freitas RA, Naiff MF, Arias JR, Barrett TV, Momen H, Grimaldi-Junior G (1991) Epidemiological and nosological aspects of *Leishmania naiffi* (Lainson & Shaw, 1989). *Mem Inst Oswaldo Cruz* 86:317–321
- Noyes H, Chance M, Ponce C, Ponce E, Maingon R (1997) *Leishmania chagasi*: Genotypically Similar Parasites from Honduras Cause both Visceral and Cutaneous Leishmaniasis in Humans. *Exp Parasitol* 85(3):264–273
- Obonaga R, Fernández OL, Valderrama L, Rubiano LC, Castro MM, Barrera MC et al (2014) Treatment failure and miltefosine susceptibility in dermal leishmaniasis caused by *Leishmania* subgenus *Viannia* species. *Antimicrob Agents Chemother* 58: 144–152
- Özbel Y, Karakuş M, Arserim SK, Kalkan ŞO, Töz S (2016) Molecular detection and identification of *Leishmania* spp. in naturally infected *Phlebotomus tobbi* and *Sergentomyia dentata* in a focus of human and canine leishmaniasis in western Turkey. *Acta Trop* 155:89–94
- Pérez-Ayala A, Norman F, Pérez-Molina JA, Herrero JM, Monge B, López-Vélez R (2009) Imported leishmaniasis: a heterogeneous group of diseases. *J Travel Med* 16:395–401
- Ponce C, Ponce E, Morrison A, Cruz A, Kreutzer R, McMahon-Pratt D, Neva F (1991) *Leishmania donovani chagasi*: new clinical variant of cutaneous leishmaniasis in Honduras. *Lancet* 33:67–70
- Pratlong F, Deniau M, Darie H, Eichenlaub S, Pröll S, Garrabe E, le Guyader T, Dedet JP (2002) Human cutaneous leishmaniasis caused by *Leishmania naiffi* is wide-spread in South America. *Ann Trop Med Parasitol* 96:781–785
- Revez L, Maia-Elkhoury ANS, Nicholls RS, Sierra Romero GA, Yadon ZE (2013) Interventions for American cutaneous and mucocutaneous leishmaniasis: a systematic review update. *PLoS One* 8:e61843
- Urbano J, Sánchez-Moreno M, Ovalle CE, Rosales MJ, Camargo YC, Gutiérrez-Sánchez R, Marín-Sánchez E (2011) Characterization of cutaneous isolates of *Leishmania* in Colombia by isoenzyme typing and kDNA restriction analysis. *Rev Ibero Latinoam Parasitol* 70:16–24
- Van der Auwera G, Dujardin J-C (2015) Species typing in dermal leishmaniasis. *Clin Microbiol Rev* 28:265–294
- Van der Auwera G, Maes I, De Doncker S, Ravel C, Cnops L, Van Esbroeck M, Van Gompel A, Clerinx J, Dujardin JC (2013) Heat shock protein 70 gene sequencing for *Leishmania* species typing in European tropical infectious disease clinics. *Euro Surveill* 18(30): 20543
- Van der Snoek EM, Lammers AM, Kortbeek LM, Roelfsema JH, Bart A, Jaspers CA (2009) Spontaneous cure of American cutaneous leishmaniasis due to *Leishmania naiffi* in two Dutch infantry soldiers. *Clin Exp Dermatol* 34:e889–e891
- Wortmann G, Hochberg LP, Arana BA, Rizzo NR, Arana F, Ryan FR (2007) Diagnosis of cutaneous leishmaniasis in Guatemala using a real-time polymerase chain reaction assay and the Smartcycler. *Am J Trop Med Hyg* 76:906–908
- Zeledón R, Maingon R, Ward R, Arana B, Belli A, de Carreira P, Ponce C (1993) The characterization of *Leishmania* parasites and their vectors from Central America using molecular techniques. *Arch Inst Pasteur Tunis* 70:325–329