

Short review and detection of *Leishmania* parasites in skin biopsies from patients coming from Echeandía, Bolívar Province, Ecuador

Breve reseña y detección de parásitos *Leishmania* en biopsias de piel de pacientes procedentes de Echeandía, provincia de Bolívar, Ecuador

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Abstract

Leishmaniasis is an important neglected tropical disease and the cause of significant morbidity in endemic countries such as Ecuador. It is transmitted by the bite of infected sand flies (Diptera Psychodidae) and has been reported from at least 22/24 provinces in the country including the foothills of the Andean mountains. Herein, we report the detection of *Leishmania* parasites, using PCR assays, in skin biopsies taken from ulcers of leishmaniasis patients from Echeandía in the province of Bolívar, Ecuador. These results suggest that most of the patients were infected with parasites from the braziliensis complex that among other species, includes *Leishmania (Viannia) panamensis* which is the most common species that causes cutaneous leishmaniasis in the Pacific coast, and *Leishmania (V.) braziliensis*, the cause of mucocutaneous lesions and significant patient disfigurement. More studies are needed to further identify the parasite species present in this area and address aspects of the parasite's life cycle and transmission dynamics. Our preliminary results showed that the R174 and R798 18 S ribosomal DNA primers used in this study are effective to amplify *Leishmania* DNA and would be a useful tool to molecularly detect New World parasites causing tegumentary leishmaniasis.

Keywords. PCR, Echeandía, leishmaniasis, skin biopsies.

Resumen

La leishmaniasis es una importante enfermedad tropical desatendida y es la causa de significativa morbilidad en países endémicos como el Ecuador. La enfermedad es transmitida por la picadura de flebotomos (Diptera:Psychodidae) infectados y ha sido reportada en al menos 22/24 provincias del Ecuador incluyendo las estribaciones de la cordillera de los Andes. En esta publicación reportamos la detección de parásitos de *Leishmania* mediante ensayos de PCR en biopsias de piel de pacientes con diagnóstico de leishmaniasis provenientes de la zona de Echeandía en la Provincia de Bolívar, Ecuador. Los resultados sugieren que la mayoría de los pacientes fueron infectados por parásitos pertenecientes al complejo braziliensis que incluye entre otras especies a *Leishmania (Viannia) panamensis*, el parásito más abundante en la costa Pacífica y causa de leishmaniasis cutánea y *Leishmania (V.) braziliensis*, la causa de lesiones mucocutáneas y significativa desfiguración del paciente. Más estudios son necesarios para identificar las especies del parásito que circulan en esta zona y abordar otros aspectos de los ciclos naturales de cada especie del parásito y la dinámica de transmisión. Los resultados preliminares muestran que los cebadores R174 and R798 del ADN 18 S ribosomal usados en este estudio son efectivos para amplificar ADN de *Leishmania* y pueden ser una herramienta útil en la detección de parásitos del Nuevo Mundo que causan leishmaniasis tegumentaria.

Palabras Clave. PCR, Echeandía, Leishmaniasis, biopsias de piel.

Introduction

Leishmaniasis is a tropical disease caused by obligate intracellular protozoan parasites of the genus *Leishmania* and transmitted in the New World by the bite of infected sand fly females of the genus *Lutzomyia* (Diptera:

Psychodidae). Cases of leishmaniasis occur mainly in the tropics and subtropics and disease burden is considered greatly underestimated; reports of the disease are only mandatory in 32 of the 88 countries affected and, according to the World Health Organization (WHO) 2010, is one of the most important neglected vector-borne dis-

eases worldwide causing significant morbidity and even mortality. It has been estimated that approximately 2 million cases occur every year with approximately 1.5 million cases of cutaneous leishmaniasis and 12 million people infected globally [1].

In the past, *Leishmania* parasites have been grouped based on the clinical manifestations into cutaneous (CL), mucocutaneous (MC), visceral (VL) and other less typical clinical forms (diffuse cutaneous and recidiva cutis), classification that has been especially useful for physicians and medical personnel. The parasites have also been grouped in a previous classification into the *Leishmania braziliensis*, *Leishmania mexicana* and the *Leishmania donovani* complexes of species. In the 1980's Lainson and Shaw proposed grouping the parasites into Suprapylaria, Peripylaria and Hypopylaria based on where the parasites were found in the vector's alimentary canal. Currently, the latest classification and the one currently accepted describes two subgenera within the genus *Leishmania*: *Viannia* and *Leishmania*. The first include species from the *braziliensis* complex whereas the second includes species from the *mexicana* complex of species.

In Ecuador, the first reported cases of the disease dated back to the beginning of the last century in 1920 [2]. Various other studies were published in local medical journals in the 1950's [3, 4] and the first and only unconfirmed report of VL was reported in 1949 [5]. This information has been listed chronologically in other publications [6] and has been reviewed elsewhere [7]. More recently, since the 1990s, several scientists have contributed to the knowledge of the parasites [8–11], the vectors [12–16], the clinical forms [17–20], prevention and treatment strategies [21–23] and other aspects of the disease in Ecuador, many studies compiled in a series of book publications [24–31]. The most recent reports referred to annotations of the presence of mucocutaneous forms at the Pacific coast [32], the presence of *Leishmania naiffi* [33], the topical treatment of cases with leishmaniasis recidiva cutis [34], and a description of patients with Chiclero's ulcer [35]. In Ecuador, at least 22/24 provinces have reported cases of the disease which is endemic in the warmer Pacific lowland where *Leishmania (V.) panamensis* is the most common parasite causing CL [36]. Nevertheless, transmission of the disease has also been well characterized in a few foci at the highlands [37, 38] and has been documented at the Amazon basin where *Le. (V.) braziliensis* is also present and the potential cause of the mucocutaneous form which is more difficult to treat and highly disfiguring [8, 39, 40].

Currently, CL is the most common clinical form in Ecuador. It produces skin ulcers with raised borders which may last for months or years if not treated and cause significant patient disfigurement due to the scars that never completely disappear [7, 41].

The novel advances in molecular techniques are an invaluable tool to address more accurately parasite and

vector taxonomic and phylogenetic issues. In the past decades, the Polymerase Chain Reaction (PCR) has been widely implemented and a variety of DNA sequences have been used to develop primers for these assays. Some of the most recent publications that use PCR in Ecuadorian studies molecularly detected *Leishmania* parasites from sand flies at the Andean region [42], at the Amazon region [43] and using molecular mass screening [44]. Due to the variety of *Leishmania* parasite species worldwide and the presence of at least 7 parasite species in Ecuador, the extensive testing of the different primer sequences using human and vector samples is crucial to evaluate their specificity and sensibility and improve parasite detection methods. For this short report, we have used two PCR assays to detect *Leishmania* infection in skin biopsies taken from patients with microscopically confirmed leishmaniasis from the area of Echeandía in the province of Bolívar, Ecuador. To our knowledge this is the first time that this 18sRNA PCR primers have been used to detect *Leishmania* parasites from Ecuador.

Materials and Methods

Human samples

Tissue samples were taken from 11 patients, with symptoms of leishmaniasis and positive microscopic diagnosis that were admitted to the Echeandía health center in the town of Echeandía, Bolívar province (Figure 1) between August 2003 and February 2004. Patient's name will not be cited in this report, but is kept as part of the medical records in the health center at Echeandía. Tissue samples were taken from the ulcer border using local anesthetic and the smear from biopsy material placed in a glass slide was stained using Giemsa. (Table 1). The rest of the tissue was stored in L6 buffer in a 1.5 ml eppendorf tube and sent to the USFQ laboratory for further analysis. Biopsies were taken primarily with a diagnosis purpose only in the cases where diagnosis based on clinical manifestations or microscopic examination of ulcer material taken routinely from scrapping the ulcer border using a scalpel was not conclusive. This explains the limited number of samples analyzed using molecular analysis. Procedures were carried out by a physician at the health center of Echeandía under the supervision of Dr. Carlos Reck strictly following safety procedures followed by the Echeandía health center.

DNA isolation and PCR Assays

For all the PCR assays, DNA was extracted from the biopsy samples using a DNeasy tissue kit (Quiagen®) following the manufacturer's instructions. For the PCRs, two different assays were used to detect Ecuadorian *Leishmania* parasites: (1) An assay based on a multiplex PCR [45] which uses one forward primer (Lu 5A) common to all *Leishmania* species and three reverse primers (LB-3C, LM 3A, LC3L) specific to species belonging to the *Leishmania braziliensis*, *Leishmania mexicana* and *Leishmania donovani* parasite complexes which amplify PCR

Preliminary results - Date: April 12/04						
Patient	Gender	Age	+Giemsa	Place	RNA mini exon	18S RNA
3	male	14	17-Jan-04	Face	braziliensis complex	+
5	male	6	15-Jan-04	Leg	-	-
6	female	10	12-Jan-04	Ankle	braziliensis complex	+
7	male	67	11-Dec-03	Arm	-	-
8	male	30	10-Dec-03	Leg	-	-
10	male	7	04-Dec-03	Leg	braziliensis complex	+
11	male	24	27-Nov-03	Leg	braziliensis complex	-
12	male	40	19-Nov-03	Face	braziliensis complex	+
13	male	42	13-Nov-03	Leg	braziliensis complex	+
15	female	1	6-Nov-03	Leg	braziliensis complex	+
16	female	20	6-Nov-03	Leg	-	+

Table 1: Information from patients with Microscopic diagnosis (Giemsa) of Leishmaniasis and results from two molecular detection PCRs (RNA mini exon PCR assay and 18S Ribosomal DNA PCR assay).

products as detailed below and (2) an assay specific to the genus *Leishmania* [46] that targets a portion of the 18 S ribosomal gene and was evaluated at the Royal Tropical Institute, KIT Medical Research (primers were kindly provided by Dr H. Schallig). In both cases, procedures described in the bibliography were modified and DNA amplification was done using PCR Ready to go Beads (Amersham Pharmacia®), a commercially available product that is easy to use where all the reagents for the PCR reaction come ready to use compacted in a solid bead inside a small eppendorf tube.

RNA mini exon gene PCR Assay

Based on a multiplex PCR assay, the RNA mini exon gene repeats were used to detect all three New World complexes of *Leishmania* parasites. The PCR primers used were as follows: the forward primer (Lu - 5A, 5'- ttt att ggt atg cga aac ttc - 3'), reverse primers (LB - 3C, 5'- cgt c/gcc gaa ccc cgt gtc - 3') specific for the braziliensis complex of *Leishmania* species amplifying a fragment of 146 - 149 bp or dimers of 375bp length as reported in the bibliography, reverse primer (LM - 3A, 5' - gca ccg cac cgg a/gcc ac - 3'), specific for the mexicana complex that produced segments of 218 - 240 bp long and reverse primer (LC - 3L, 5'- gcc cgc gc/tg tca cca ca t - 3') specific for the donovani complex that

produced fragments of 351 - 397 bp length. PCR reactions for each *Leishmania* complexes were conducted separately using the same forward primer and different reverse primers under the same thermal cyclers conditions: 95 °C for 5 minutes followed by 35 cycles of 95 °C for 30 sec, 54 °C for 45 sec, 72 °C for 30 sec with a final extension of 72 °C for 5 min.

PCR assay with 18 S ribosomal DNA primers

For the PCR reaction, 2.5 µl of DNA were amplified in a final volume of 25 µl with primers (R174, 5'- ggt tcc tt cct gat tta cg - 3') and (R798, 5'- ggc cgg taa agg ccg aat ag - 3') to yield a fragment of 600 bp length. The thermal cycling conditions used were as follows: 50 °C for 5 min, 94 °C for 10 min and 40 cycles of 94 °C for 75 sec, 60 °C for 1 min, 72 °C for 2 min with a final extension of 72 °C for 50 min.

For both assays, PCR products were analyzed by gel electrophoresis using 2% Ultra Pure or MetaPhor® agarose for the fine separation of small DNA fragments and visualized under a UV light following standard procedures. Interpretation of the patterns was based on the size and on the presence or absence of amplified DNA bands.

Results and Discussion

From the tissue biopsies analyzed for the presence of *Leishmania* parasites using PCR, only 6 (54.5%, n=11) were positive as tested with the two sets of primers used in this study as reported in Table 1. Electrophoresis from the PCR products using Ultra Pure Agarose using the RNA mini exon PCR assay showed poorly-defined bands but with clear signs of DNA amplification. MetaPhor® agarose prepared gels showed evidently more defined and clear bands of 149 bp confirming the presence of parasites from the braziliensis complex (Figure 2). Presence of additional bands of 375 bp possibly corresponded to dimers of two linked DNA fragments, that were previously reported in the bibliography [45].

Electrophoresis from samples 5, 7 and 8 revealed an unknown pattern of 4 bands using the RNA mini exon



Figure 1: Map of Ecuador showing the area of Echeandia in Bolivar province.

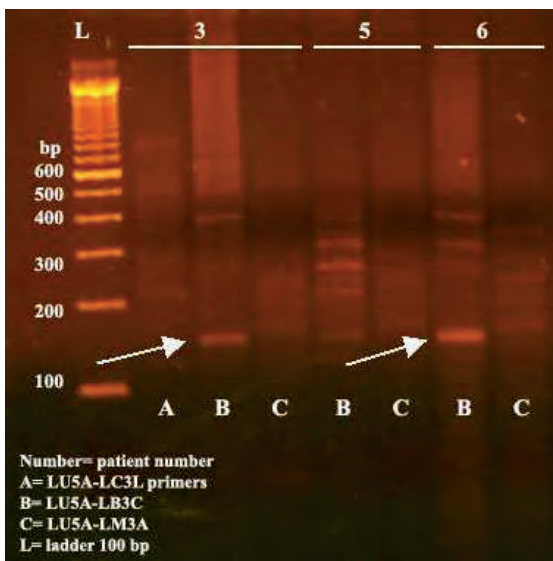


Figure 2: Detection of *Leishmania* parasites using the RNA mini exon gene PCR assay. Sample 3 and 6 showed distinct bands of 149bp and around 400 bp using LU5A-LB3C consistent with the expected band size for the *Leishmania braziliensis* complex of parasites. Sample 5 does not show any specific DNA amplification.

PCR assay (gel not shown) suggesting an unspecific amplification; no amplification was detected using the 18 S ribosomal DNA primers (Figure 3). Both PCR assays showed consistent positive/negative results in all the tissue biopsies except for samples 11 and 16. Sample 11 examined with LU5A-LB3C (*braziliensis* complex) primers showed a strong band of 149 bp and four additional bands with a similar band pattern to the one observed in samples 5, 7 and 8. However, was not recognized by the 18S ribosomal DNA primers. Sample 16 showed no amplification using the LU5A-LB-3C set of primers to the *braziliensis* complex (gel not shown) but showed a weak but distinct band of 600 bp using the 18 S ribosomal DNA primers suggesting the presence of *Leishmania* parasites (Figure 3).

The results which showed a different band pattern than the expected according to the bibliography using the RNA mini exon PCR assay may suggest that further standardization is needed and the use of beads instead of mixing the reagents individually may have affected the amplification. On the other side, it is also possible that the different band pattern might correspond to different *Leishmania* species within the *braziliensis* complex of parasites. Several past studies have reported co-infection of two parasite species [47], the presence of new parasites species (*Le. major*-like) [48] and hybrid species in Ecuador [49]; recently *Le. naiffi* was for the first time found in Ecuador [50] and *Lutzomyia tuberculata* was for the first time found constituting a new sand fly species record for the country [51], thus the immense biodiversity of parasite and vector species still need to be further investigated. According to the information reviewed, *Le. (V.) panamensis* is the only parasite species found in the Northern part of Bolívar province [7]. More studies are needed to further identify the parasite species circulating in Echeandía and to

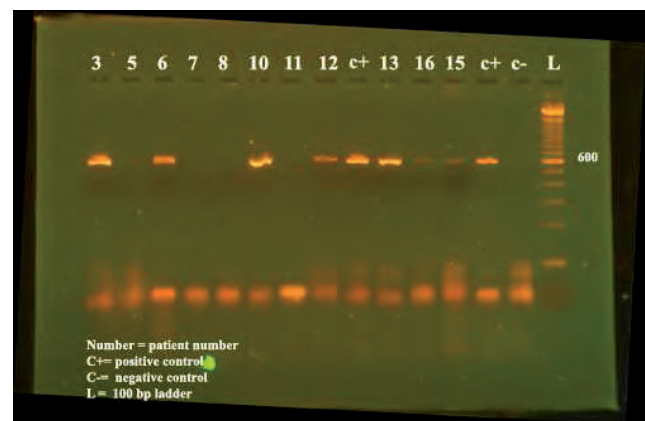


Figure 3: Detection of *Leishmania* parasites in 7 of 11 tissue biopsies as detected by PCR using 18S ribosomal DNA primers. Sample number 3, 6, 10, 12, 13, 15, 16 are positive for *Leishmania* parasites as evidenced in the gel. (Expected band size: 600 bp).

further evaluate the PCR assays used in this study. To our knowledge this is the first time that this 18S ribosomal DNA PCR assay is tested with *Leishmania* samples from Ecuador. These preliminary results suggest that R174 and R798 primers may be an effective alternative and a useful tool for the detection of *Leishmania* parasites in Ecuador and in the New World, nevertheless, other species-specific PCR methods would be necessary to identify to species the *Leishmania* parasites circulating in this region of Ecuador.

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References

- [1] WHO. 2010. "Leishmaniasis magnitude of the problem". http://www.who.int/leishmaniasis/burden/magnitude/burden_magnitude/en/print.html.
- [2] Rodríguez, J. 1974. "Lecciones de Parasitología Humana: Género Leishmania". Universidad de Guayaquil: Guayaquil.
- [3] León, L. 1957. "Leishmaniasis y Leishmaniasis". Ed. Universitaria: Quito.
- [4] Rodríguez, J.; Aviles, F. 1953. "Algunas observaciones sobre leishmaniasis cutáneo mucosa en el Ecuador". *Rev Ec Hig Med Trop*, 10:35-58.
- [5] León, L. 1952. "Leishmaniasis Visceral". *Gaceta Médica año XII No 5: Guayaquil*.

- [6] Hashiguchi, Y.; Gomez, E. 1991. "Review of leishmaniasis in Ecuador". *Bull PAHO*, 25:64–76.
- [7] Calvopiña, M.; Armijos, R.; Hashiguchi, Y. 2004. "Epidemiology of Leishmaniasis in Ecuador: Current Status of Knowledge - A Review". *Mem Inst Oswaldo Cruz*, 99 (7):663–672.
- [8] Armijos, R.; Chico, M.; Cruz, M.; Guderian, R.; Kreutzer, R.; Berman, J.; Rogers, M.; Grogl, M. 1990. "Human cutaneous leishmaniasis in Ecuador: Identification of parasites by enzyme electrophoresis". *Am J Trop Med Hyg*, 42:424–428.
- [9] Mimori, T.; Sasaki, J.; Nakata, M.; Gomez, E.; Uezato, H.; Nonaka, S.; Hashiguchi, Y.; Furuya, M.; Saya, H. 1998. "Rapid identification of *Leishmania* species from formalin-fixed biopsies by polymorphism-specific polymerase chain reaction". *Gene*, 210:179–186.
- [10] Aviles, H.; Belli, A.; Armijos, R.; Monroy, F.; Harris, E. 1999. "PCR detection and identification of *Leishmania* parasites in clinical specimens in Ecuador: A comparison with classical diagnostic methods". *J. Parasitol*, 85: 181–187.
- [11] Bañuls, A.; Jonquieres, R.; Guerrini, F.; Le Pont, F.; Barrera, C.; Espinel, I.; Guderian, R.; Echeverria, R.; Tibayrenc, M. 1999. "Genetic analysis of *Leishmania* parasites in Ecuador: Are *Leishmania (Viannia) panamensis* and *L. (V.) guyanensis* distinct taxa?". *Am J Trop Med Hyg*, 6:838–845.
- [12] Alexander, J.; Takaoka, H.; Eshita, Y.; Gomez, E.; Hashiguchi, Y. 1992. "New records of phlebotomine sand flies (Diptera: Psychodidae) from Ecuador". *Mem Inst Oswaldo Cruz*, 87:123–130.
- [13] Le Pont, F.; Leon, R.; Guerrini, F.; Gantier, J.; Mouchet, J.; Echeverria, R.; Guderian, R. 1994. "Leishmaniasis in Ecuador. 3. *Lutzomyia trapidoi*, vector of *L. panamensis*". *Ann Soc Belg Med Trop*, 74:23–28.
- [14] Le Pont, F.; Barrera, C.; Caceres, A.; Galati, E.; Jarra, O.; Riofrio, A.; Mouchet, J.; Echeverria, R.; Guderian, R. 1994. "Leishmaniasis in Ecuador. 6. Epidemiological and entomological note on the focus of leishmaniasis in Zumba". *Ann Soc Belg Med Trop*, 74:43–49.
- [15] Dujardin, J.; Le Pont, F.; Cruz, M.; Tarrieu, L.; Guderian, R.; Echeverria, R.; Tibayrenc, M. 1996. "Cryptic speciation in *Lutzomyia (Nyssomyia) trapidoi* (Diptera: Psychodidae) detected by multilocus enzyme electrophoresis". *Am J Trop Med Hyg*, 54:42–45.
- [16] Kato, H.; Uezato, H.; Katakura, K.; Calvopiña, M.; Marco, J.; Barroso, P.; Gomez, E.; Mimori, T.; Korenaga, M.; Iwata, H.; Nonaka, S.; Hashiguchi, Y. 2005. "Detection and Identification of *Leishmania* Species within Naturally Infected Sand flies in the Andean Areas of Ecuador by a Polymerase Chain Reaction". *Am. J. Trop. Med. Hyg.*, 72(1):87–93.
- [17] Calvopiña, M.; Armijos, R.; Marco, J.; Uezato, H.; Kato, H.; Gomez, E.; Korenaga, M.; Barroso, P.; Mimori, T.; Cooper, P.; Nonaka, S.; Hashiguchi, Y. 2006. "*Leishmania* isoenzyme polymorphisms in Ecuador: Relationships with geographic distribution and clinical presentation". *BMC Infectious Diseases*, 6:139.
- [18] Calvopiña, M.; Gomez, E.; Uezato, H.; Kato, H.; Nonaka, S.; Hashiguchi, Y. 2005. "Atypical Clinical Variants in New World Cutaneous Leishmaniasis: Disseminated, Erysipeloid, and Recidiva Cutis due to *Leishmania (V.) panamensis*". *Am J Trop Med Hyg*, 73(2):281–284.
- [19] Calvopiña, M.; Uezato, H.; Gomez, E.; Korenaga, M.; Nonaka, S.; Hashiguchi, Y. 2006. "Leishmaniasis recidiva cutis due to *Leishmania (Viannia) panamensis* in subtropical Ecuador: isoenzymatic characterization". *International Journal of Dermatology*, 45:116–120.
- [20] Calvopiña, M.; Gomez, E.; Sindermann, H.; Cooper, P.; Hashiguchi, Y. 2006. "Relapse of New World Diffuse Cutaneous Leishmaniasis caused by *Leishmania (Leishmania) mexicana* after Miltefosine treatment". *Am. J. Trop. Med. Hyg*, 75(6):1074–1077.
- [21] Guderian, R.; Chico, M.; Rogers, M.; Pattishall, K.; Grogl, M.; Berman, J. 1991. "Placebo controlled treatment of Ecuadorian cutaneous leishmaniasis". *Am J Trop Med Hyg*, 45:92–97.
- [22] Weigel, M.; Armijos, R. 2001. "The traditional and conventional medical treatment of cutaneous leishmaniasis in rural Ecuador". *Rev Panam Salud Publica*, 10:395–404.
- [23] Armijos, R.; Weigel, M.; Aviles, H.; Maldonado, R.; Racines, J. 1998. "Field Trial of a Vaccine against New World Cutaneous Leishmaniasis in an At-Risk Child Population: Safety, Immunogenicity, and Efficacy during the First 12 Months of Follow-Up". *The Journal of Infectious Diseases*, 177:1352–1357.
- [24] Hashiguchi, Y. 1987. "Studies on New World leishmaniasis and its transmission, with particular reference to Equateur". *Kyowa Printing Co*, 1:1–174.
- [25] Hashiguchi, Y. 1990. "Studies on New World Leishmaniasis and its Transmission with Particular Reference to Ecuador". *Kyowa Printing Co*, 2:1–238.
- [26] Hashiguchi, Y. 1992. "Studies on New World Leishmaniasis and its Transmission with Particular Reference to Ecuador". *Kyowa Printing Co*, 3:1–182.
- [27] Hashiguchi, Y. 1994. "Studies on New World Leishmaniasis and its Transmission with Particular Reference to Ecuador". *Kyowa Printing Co*, 4:1–193.
- [28] Hashiguchi, Y. 1997. "Studies on New World leishmaniasis and its transmission, with particular reference to Equateur". *Kyowa Printing Co*, 5:1–207.
- [29] Hashiguchi, Y. 2001. "Studies on New World Leishmaniasis and its Transmission with Particular Reference to Ecuador". *Kyowa Printing Co*, 6:1–218.
- [30] Hashiguchi, Y. 2004. "Studies on New World Leishmaniasis and its Transmission with Particular Reference to Ecuador, Argentina and Pakistan". *Kyowa Printing Co*, 7:1–272.

- [31] Y., H. 2007. "Studies on New World Leishmaniasis and its Transmission with Particular Reference to Ecuador, Peru, Argentina and Pakistan". *Kyowa Printing Co*, 8: 1–329.
- [32] Fernández, T.; Almeida, R. 2012. "Reporte de Lesiones Mucosas en Leishmaniosis Tegumentaria Americana en el Litoral (costa) Ecuatoriano". *Revista de Patología Tropical*, 41(3):356–366.
- [33] 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 Tropica*, 128:710–713.
- [34] Calvopiña, M.; Kato, H.; Hashiguchi, Y. 2013. "Leishmaniasis Recidiva Cutis and Its Topical Treatment in Ecuador". *Tropical Medicine and Health*, 41(3):93–94.
- [35] Calvopiña, M.; Martínez, L.; Hashiguchi, Y. 2013. "Cutaneous Leishmaniasis "Chiclero's Ulcer" in Subtropical Ecuador". *Am. J. Trop. Med. Hyg.*, 89(2):195–196.
- [36] Barrera, C.; Herrera, M.; Martínez, F.; León, R.; Richard, A.; Guderian, R.; Mouchet, R.; Echeverría, R.; Le Pont, F. 1994. "Leishmaniose en Equateur. Incidence de la Leishmaniose Tegumentaire sur la Facade Pacifique". *Ann.Soc.belge Med.Trop*, 74:1–12.
- [37] Hashiguchi, Y.; Gomez, E.; De Coronel, V.; Mimori, T.; Kawabata, M.; Furuya, M.; Nonaka, S.; Takaoka, H.; Alexander, J.; Quizhpe, A.; Grimaldi, G.; R., K.; Tesh, B. 1991. "Andean Leishmaniasis in Ecuador caused by Infection with *Leishmania Mexicana* and *L. Major-Like Parasites*". *Am. J. Trop. Med. Hyg.*, 44(2):205–217.
- [38] Gomez, E.; Katakura, K.; Matsumoto, Y.; Nonaka, S.; Mimori, T.; Eshita, Y.; Sud, R.; Sulca, F.; Vallejo, C.; Jurada, M.; Furuya, M.; Hashiguchi, Y. 1994. "Further Epidemiological Studies of Andean Leishmaniasis, with Special Reference to Huigra, Chimborazo, Ecuador. In: Studies on New World Leishmaniasis and its Transmission, with Particular Reference to Ecuador (Y. Hashiguchi, ed.)". *Research Report Series Kochi: Kyowa Printing*, 4:71–84.
- [39] Furuya, M.; Akimaru, Y.; Mimori, T.; Shiraiishi, M.; Gomez, E.; Nonaka, S.; Hashiguchi, Y. 1997. "Identification of species of Ecuadorian *Leishmania* isolates by ELISA using monoclonal antibodies". In *Studies on New World Leishmaniasis and its Transmission with Particular Reference to Ecuador. Kochi Mem Inst Oswaldo Cruz, Rio de Janeiro, Vol. 99(7), November 2004 671 Japan. Res Rep Series*, 5:11–19.
- [40] Calvopiña, M.; Guevara, A.; Armijos, R.; Gomez, E.; Mimori, T.; Cooper, P.; Hashiguchi, Y. 2001. "Clinical features of mucocutaneous leishmaniasis in the Amazonian region of Ecuador. In Studies on New World Leishmaniasis and its Transmission with Particular Reference to Ecuador". *Res Rep Series*, 6:82–89.
- [41] Herwaldt, B.; Magill, A. 2010. "Chapter 5 Leishmaniasis, cutaneous. Yellow Book". <http://wwwnc.cdc.gov/travel/yellowbook/2010/chapter-5/cutaneous-leishmaniasis.aspx>. *CDC Travelers' Health*.
- [42] Kato, H.; Uezato, H.; Katakura, K.; Calvopiña, M.; Marco, J.; Barroso, P.; Gomez, E.; Mimori, T.; Korenaga, M.; Iwata, H.; Nonaka, S.; Hashiguchi, Y. 2005. "Detection and identification of *Leishmania* species within naturally infected sandflies at the Andean areas in Ecuador by polymerase chain reaction". *Am J Trop Med Hyg.*, 72(1):87–93.
- [43] Kato, H.; Gomez, E.; Yamamoto, Y.; Calvopiña, M.; Guevara, A.; Marco, J.; Barroso, P.; Iwata, H.; Hashiguchi, Y. 2008. "Short Report: Natural Infection of *Lutzomyia tortura* with *Leishmania (Viannia) naiffi* in an Amazonian Area of Ecuador". *Am. J. Trop. Med. Hyg.*, 79(3):438–440.
- [44] Kato, H.; A., C.; Gomez, E.; Mimori, T.; Uezato, H.; Marco, J.; Barroso, P.; Iwata, H.; Hashiguchi, Y. 2008. "Short Report: Molecular Mass Screening to Incriminate Sand Fly Vectors of Andean-type Cutaneous Leishmaniasis in Ecuador and Peru". *Am. J. Trop. Med. Hyg.*, 79(5):719–721.
- [45] Harris, E.; Kropp, G.; Belli, A.; Rodriguez, B.; Agabian, N. 1998. "Single-Step Multiplex PCR Assay for Characterization of New World *Leishmania* Complexes". *J. Clin. Microbiol.*, 36(7):1989–1995.
- [46] Van der Meide, W.; Schoone, G.; Faber, W.; Zeegelaar, J.; De Vries, H.; Ozbel, Y.; Lai A Fat, R.; Coelho, L.; Kassi, M.; Schallig, H. 2005. "Quantitative nucleic acid sequence-based assay as a new molecular tool for detection and quantification of *Leishmania* parasites in skin biopsy samples". *J Clin Microbiol.*, 43(11):5560–5566.
- [47] Martínez, E.; Mollinedo, S.; Torrez, M.; Muñoz, M.; Bañuls, A.; Le Pont, F. 2002. "Co-infection by *Leishmania amazonensis* and *L. infantum/L. chagasi* in a case of diffuse cutaneous leishmaniasis in Bolivia". *Trans R Soc Trop Med Hyg.*, 96(5):529–532.
- [48] Katakura, K.; Matsumoto, Y.; Gomez, E.; Furuya, M.; Hashiguchi, Y. 1993. "Molecular karyotype characterization of *Leishmania panamensis*, *Leishmania mexicana*, and *Leishmania major-like* parasites: agents of cutaneous leishmaniasis in Ecuador". *Am J Trop Med Hyg.*, 48(5):707–715.
- [49] Bañuls, A.; Guerrini, F.; Le Pont, F.; Barrera, C.; Espinel, I.; Guderian, R.; Echeverría, R.; Tibayrenc, M. 1997. "Evidence for hybridization by multilocus enzyme electrophoresis and random amplified polymorphic DNA between *Leishmania braziliensis* and *Leishmania panamensis/guyanensis* in Ecuador". *J Eukaryot Microbiol.*, 44(5):408–411.
- [50] Kato, H.; Gomez, E.; Yamamoto, Y.; Calvopiña, M.; Guevara, A.; Marco, J.; Barroso, P.; Iwata, H.; Hashiguchi, Y. 2008. "Natural infection of *Lutzomyia tortura* with *Leishmania (Viannia) naiffi* in an Amazonian area of Ecuador". *Am J Trop Med Hyg.*, 79(3):438–440.
- [51] Arrivillaga, J.; Perez, C.; Flores, M.; Enriquez, S.; Vaca, F.; Medina, B.; Benitez, W. 2014. "Primer registro de *Lutzomyia tuberculata*". *Mangabeira, 1941 (Diptera:Psychodidae:Phlebotominae) en el Ecuador. Bol. Mal.Salud Amb.*, 54(1):103–106.