

International workshop on the standardization of a real time PCR assay for the quantification of parasite load for cutaneous leishmaniasis management in the Americas

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Leishmaniasis is considered the most neglected tropical disease, according to the disability-adjusted life years (DALYs). Globally, around 12 million people are infected, and 350 million live in risk areas. The disease presents different clinical manifestations, as asymptomatic infections or the two most common forms: visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL).¹ The clinical manifestation of CL range from small-localized lesions to disseminated large ulcers all over the body. This clinical manifestation is associated with several *Leishmania* species in the New World (the Western Hemisphere), mainly *L.mexicana*, *L.amazonensis*, *L.braziliensis*, *L.guyanensis*, *L.panamensis* and *L.peruviana*, depending on the geographic region.

The diagnosis of the disease is performed by the combination of clinical, epidemiological, and parasitological tests. Parasitological diagnosis remains the gold standard and includes microscopic examination of smears or aspirates, histopathological examination of lesion biopsies, or culture of biopsy triturates or aspirates.²

Molecular parasitological approach for the diagnosis of cutaneous leishmaniasis and identification of the parasite has been in place since decades and has great potential to be applied directly in the clinical sample, avoiding the time-consuming isolation and cultivation of the parasite.

Since the beginning of the application of PCR to diagnose leishmaniasis, several methodologies were tested without consensus regarding protocols and molecular targets. It prevents data comparison, since each research group uses his own in-house protocol, even for sample preparation. Thus, the lack of standardization and validation of a consensus protocol for molecular diagnosis and parasite load estimation represents a need to conduct studies which look at the development of new drugs, epidemiological surveillance and routine clinical diagnosis.

In this context, the Communicable Disease Research Programme of the Pan American Health Organization promoted the standardization and validation of PCR for CL diagnosis and disease management across laboratories and countries. Accordingly, a project proposal was developed to validate and harmonize PCR methods during a workshop with the participation of experts from molecular biology laboratories of endemic areas working in PCR for CL. This workshop was financed by Ruta N, PAHO, and DNDi Latin America.

An international workshop with the participation of 10 experienced PCR CL laboratories from 7 Latin American Countries (Argentina, Brazil (2), Colombia (3), Costa Rica, Mexico, Panama, and Peru) was carried out in December 2016. The main objective of the workshop was to compare the performance of molecular assays for the detection and quantification of different *Leishmania* species in order to establish a standardized multiplex real time PCR protocol by the absolute quantification of the parasite load and normalization by the human DNA amount, obtained from cutaneous lesion samples.

During the activities, a silica-column based protocol for DNA extraction from skin lesion samples, containing an external quality control, was standardized. In addition, the performance of three molecular targets for *Leishmania* was compared: SSUrDNA, kDNA, and HSP70.

Preliminary results with reference strains of the most prevalent *Leishmania* species indicated a reportable range varying from 10^6 to 5 Par. Eq./mL for the SSUrDNA and kDNA targets and from 10^6 to 50 Par. Eq./mL for HSP70. For the human RNase P gene, a linearity from 10 to 10^{-3} ng/mL of human DNA was achieved in multiplex with *Leishmania* targets, indicating a wide range to be used to quantify the parasite and normalize data by human DNA. In patients' samples presenting high, moderate and low parasitism, all targets could be detected and parasite load estimated following this methodology. In contrast, during the evaluation of analytical specificity with reference samples from other tripanosomatids, such as *Trypanosoma cruzi*, *T.rangeli*, *Chitidia fasciculata*, and *Herpetomonas muscarum*, HSP70 and SSUrDNA target presented the highest specificity for the detection of *Leishmania* species.

The next step will be the standardization and clinical validation of the consensus methodology defined during this workshop. For this, the DNA extracted from ulcer samples obtained from CL patients attending health facilities in the participating countries of this initiative will be employed.

The workshop was hosted by Unidad Biología Molecular y computacional PECET - Programa de Estudio y Control de Enfermedades Tropicales - SIU-Sede de Investigación Universitaria - Universidad de Antioquia, Medellín, Colombia.

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References

1. World Health Organization. Control of the leishmaniasis. World Health OrganTech Rep Ser. 2010; (949):xii-xiii, 1-186, back cover.
2. Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, Brooker S. Cutaneous leishmaniasis. Lancet Infect Dis. 2007 Sep;7(9):581-96.