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Leishmaniasis in the major endemic region of Plurinational State of Bolivia: Species identification, phylogeography and drug susceptibility implications☆

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ABSTRACT

The Plurinational State of Bolivia is one of the Latin American countries with the highest prevalence of leishmaniasis, highlighting the lowlands of the Department of La Paz where about 50% of the total cases were reported. The control of the disease can be seriously compromised by the intrinsic variability of the circulating species that may limit the efficacy of treatment while favoring the emergence of resistance. Fifty-five isolates of *Leishmania* from cutaneous and mucocutaneous lesions from patients living in different provinces of the Department of La Paz were tested. Molecular characterization of isolates was carried out by 3 classical markers: the rRNA internal transcribed spacer 1 (ITS-1), the heat shock protein 70 (HSP70) and the mitochondrial cytochrome b (Cyt-b). These markers were amplified by PCR and their products digested by the restriction endonuclease enzymes *Ase*I and *Hae*III followed by subsequent sequencing of Cyt-b gene and ITS-1 region for subsequent phylogenetic analysis. The combined use of these 3 markers allowed us to assign 36 isolates (65.5%) to the complex *Leishmania (Viannia) braziliensis*, 4 isolates (7, 27%) to *L. (Viannia) lainsoni*. and the remaining 15 isolates (23.7%) to a local variant of *L. (Leishmania) mexicana*. Concerning *in vitro* drug susceptibility the amastigotes from all isolates where highly sensitive to Fungizone® (mean IC_{50} between 0.23 and 0.5 μg/mL) whereas against Glucantime® the sensitivity was moderate (mean IC_{50} ranging from 50.84 μg/mL for *L. (V.) braziliensis* to 18.23 μg/mL for *L. (L.) mexicana. L. (V.) lainsoni* was not sensitive to Glucantime®. The susceptibility to miltefosine was highly variable among species isolates, being *L. (L.) mexicana* the most sensitive, followed by *L. (V.) braziliensis* and *L. (V.) lainsoni* (mean IC_{50} of 8.24 μg/mL, 17.85 μg/mL and 23.28 μg/mL, respectively).

1. Introduction

Human leishmaniasis is one of the most important neglected tropical diseases of broad global distribution. In the Americas leishmaniasis is present under a wide range of clinical manifestations, such as visceral leishmaniasis (VL) that is the most severe form of the disease, muco-cutaneous leishmaniasis (MCL) a mutilating disorder; diffuse cutaneous leishmaniasis (DCL), a long-lasting complication, and the more benign cutaneous leishmaniasis (CL) (Desjeux, 2004; David and Craft, 2009). A mosaic of phylogenetically distinct species of *Leishmania* are the responsible for all these clinical manifestations with great species-specific variation in the severity of the disease (Berzunza-Cruz et al., 2009). For this reason, the characterization of *Leishmania* parasites is critical, not only from an epidemiologic perspective, but also because the treatment choice largely depends on the levels of virulence and response to the various chemotherapeutic regimens of infecting

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species (Marfurt et al., 2003; Victoire et al., 2003).

The Plurinational State of Bolivia is one of the countries with highest incidence of leishmaniasis in Latin America, with 33 cases per 100.000 population recorded in 2006 (García et al., 2009). Data from the national programme of surveillance and control of leishmaniasis in Bolivia reflects high variation and constant increase since 1983, raising from 278 cases to 1810 cases reported in 2010. Nearly 50% of citizens affected were located in subtropical lowlands of the Department of La Paz (David et al., 1993; PNVCL-Bolivia, 2011). Besides, most cases of severe mucocutaneous leishmaniasis are reported in Bolivia, Brazil and Peru (WHO, 2010). The increases of transmission in migrants from Andean to subtropical lands and uncontrolled logging are two of the main reasons for the emergence of new highly active foci that are continually increasing in scale and extension (Alcais et al., 1997). Despite efforts to implement programs to control the leishmaniasis, the epidemiological information still do not reflect the real status of severity due to the difficult access to tropical rainforest areas where the transmission of the infection is very high.

At present, the classical multilocus enzyme electrophoresis (MLEE) continues being the gold standard for *Leishmania* species characterization in Europe and in South America (Rioux et al., 1989; Cupolillo et al., 1994). Unfortunately this method is highly time-consuming and it can only be performed in specialized laboratories. The significant advances in molecular methods have led to the development of reliable alternative tools for gene typing, all based on the polymerase chain reaction (PCR) and multiple subsets were developed such as randomly amplified polymorphic DNA (PCR-RAPD) (Tibayrenc et al., 1993; Bañuls et al., 2000; Martínez et al., 2003), multiplex PCR (Harris et al., 1998), restriction fragment length polymorphism (PCR-RFLP) (Cupolillo et al., 1995; Schönian et al., 2003; García et al., 2004). In addition the new real-time PCR assay for rapid diagnostic (Stevenson et al., 2010; Pita-Pereira et al., 2012; Jara et al., 2013), the powerful and robust developed multilocus sequence typing (MLST) (Mauricio et al., 2006; Zemanová et al., 2007; Tsukayama et al., 2009; Boite et al., 2012) and multilocus microsatellite typing (MLMT) (Kuhls et al., 2007; Kuhls et al., 2011) were also implemented. Each of them use specific coding or non-coding gene targets such as heat shock proteins (HSP70, HSP20 and UTR-HSP70), cytochrome b (Cyt-b), internal transcribed spacers (ITS-1 and ITS-2), 7SL of the ribosomal RNA, DNA microsatellite (Luyo-Acero et al., 2004; Zelazny et al., 2005; Fraga et al., 2010; De Almeida et al., 2011; Requena et al., 2012; Van der Auwera et al., 2014) and others.

In line with the recommendations from "Control strategies for visceral leishmaniasis (VL) and mucocutaneous leishmaniasis (MCL) in South America, applications of molecular epidemiology", the PCR-RFLP of ITS-1 and HSP70 genes are reliable for detection and identification of *Leishmania* species (LeishEpiNetSA, 2009).The ITS-1 region lying between the genes coding for the SSU and the 5.8S rRNA genes of the ribosomal RNA from *Leishmania* species are one of the most evolved sequences for the characterization of inter- and intra-species variation by restriction enzyme analyses or sequencing (Cupolillo et al., 1995; Dávila and Momen, 2000; Schönian et al., 2003). The HSP70 gene can be applied for direct identification of *Leishmania* species in clinical samples or cultures by a simple digestion of the PCR-product with *Hae*III enzyme (García et al., 2004; Montalvo et al., 2010). In addition, Cyt-b is one of the most useful genes for phylogenetic and taxonomic works (Escalante et al., 1998; Zhang et al., 2005; García et al., 2012).

Today there is still no effective vaccine for leishmaniasis and chemotherapy remains the most effective control measure. In Bolivia, the drugs available for the treatment are still primarily pentavalent antimony (Glucantime® and Pentostam®), although intravenous amphotericin B (Fungizone®) is used as a second line of treatment in severe or recurrence lesions and recently, oral miltefosine (MIL) has been introduced for clinical trials (Soto et al., 2008). However, none of these therapies is 100% effective and several authors reported therapy failure and variable efficacy in many scenarios (Bermudez et al., 2006; Soto et al., 2007, 2008, 2009; García et al., 2009). These failures can lead to drug resistance, lack of efficacy for American *Leishmania* species and these implications could be the most worrying problem in order to control the disease.

This work has been designed for molecular characterization and drug susceptibility assessment in autochthonous *Leishmania* isolates from patients located in the major endemic region in Bolivia. Furthermore their geographical distribution and the phylogenetic relationship among circulating species have been reconstructed using a partial sequence of ITS-1 and Cyt-b markers.

2. Material and methods

2.1. Drugs

Antimony-N-methyl-glutamine (Glucantime®, Merial), amphotericin B deoxycholate (Fungizone®, Bristol Myers Squibb) and Hexadecylphosphocholine (miltefosine, Sigma Aldrich) were used in this work.

2.2. Leishmania isolates

Fifty-five *Leishmania* isolates from patients with cutaneous and mucocutaneous lesions were kindly provided by Dr. José Santalla from the Laboratory of Parasitology of the National Institute Laboratory of Health (INLASA) of La Paz (Bolivia). Code of *Leishmania* isolates and dataset with clinical manifestations, sex, age and geographical origins of patients are listed in Table 1. Moreover, characterized *Leishmania* species isolates were used: an autochthonous isolate of *L. (L.) infantum* (MCAN/ES/92/BCN83) was gently provided by Prof. Montserrat Portús (Universidad de Barcelona, Spain), *L. (V.) braziliensis* (MHOM/BR/75/M2903), *L. (L.) amazonensis* (MHOM/Br/79/Maria) and *L. (L.) guyanensis* (141/93) were kindly donated by Drs. Alfredo Toraño and Mercedes Domínguez (Instituto de Salud Carlos III, Madrid, Spain) and *L. donovani* (MHOM/IN/80/DD8) purchased from ATCC. Two additional isolates, *L. (V.) braziliensis* (MHOM/BR/75/M2904) and *L. (L.) mexicana* (MNYC/BZ/62/M379) were a gift from Dr. César Ramírez (Universidad Javeriana, Colombia) and Dr. Lilian Yépez-Mulia (Instituto Mexicano del Seguro Social, Ciudad de México, Mexico), respectively.

2.3. In vitro culture and DNA extraction

Promastigote stages were cultured in Schneider's insect medium (Sigma®) at 26 °C supplemented with 10% heat-inactivated foetal bovine serum (HIFBS) and 100 U/mL of penicillin plus 100 µg/mL of streptomycin in 20 mL culture flask. One millilitre of cultured suspension on log growth phase per sample were centrifuged at 1500*g* and washed two times in phosphate buffer saline. The genomic DNA of the promastigotes was isolated using the *DNeasy Blood & Tissue* isolation kit (Qiagen®). DNA was recovery in mili-Q water and stored at −20 °C until use.

2.4. PCR amplification assay

A set of three standard markers previously described by other authors for *Leishmania* species identification were used. Primer LITSR (5′-CTGGATCATTTTCCGATG-3′) and L5.8S (5′-TGATACCACT TATCGCACTT-3′) (El Tai et al., 2000; Schonian et al., 2003), HSP70sen (5′-GACGGTGCCTGCCTACTTCAA-3′) and HSP70ant (5′-CCGCCCATG-CTCTGGTACATC-3′) (García et al., 2004), LCBF1 (5′GGTGTAGGTTT-TAGTTTAGG3′) and LCBR2 (5′CTACAATAAACAAATCATAATATACA-ATT3′) (Luyo-Acero et al., 2004) were purchased (Sigma-Aldrich®). *Amplitaq Gold* PCR Master Mix was purchased from Applied Biosystems®.

PCR assays were performed according to the conditions previously published by other authors with minor modifications. Briefly, PCR mix reaction was carried out in a 25 µL solution containing 12.5 µL of