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Detection, molecular typing and phylogenetic analysis of *Leishmania* isolated from cases of leishmaniasis among Syrian refugees in Lebanon

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ABSTRACT

Leishmania is a parasitic protozoan with more than two-dozen species causing the disease leishmaniasis. It is transmitted to humans through the bite of an infected female phlebotomine sand-fly vector. In the past two years the incidence of leishmaniasis has been drastically increasing in Lebanon. This was in parallel with the deterioration of the security in Syria forcing thousands to flee and seek shelter in poorly maintained refugee camps and collective shelters. Cutaneous leishmaniasis (CL) is now considered a public health problem, but its epidemiology has not been fully elucidated. To our knowledge, this is the first study comparing two different molecular methods for the detection and identification of *Leishmania tropica* in Lebanon.
Two molecular typing methods of 39 FFPE *Leishmania* isolates were used: the ITS1-PCR RFLP and the nested ITS1-5.8S rDNA gene amplification followed by sequencing and phylogenetic analysis. The efficiency of these two techniques in *Leishmania* identification was compared and the phylogenetic relationships among these isolates were illustrated based on the neighbor-joining (NJ) method. The results were statistically correlated with the parasitic index (PI). The DNA storage in formalin-fixed paraffin embedded (FFPE) tissues was assessed as well. The parasites identified were all *L. tropica* as determined by both techniques. ITS1-5.8S rDNA gene based typing proved to be more sensitive in the detection of parasites (positive in 69.2% of the isolates) as opposed to the ITS1-PCR RFLP method that was successful in identifying *L. tropica* in only 43.6% of the isolates. Sequencing and phylogenetic analysis revealed high levels of heterogeneity. A statistically significant correlation was observed between PI and the results of the nested ITS1-5.8S rDNA gene PCR. Genotyping at the species level is essential for monitoring the relative frequency of CL in the Mediterranean area that is correlated to three different *Leishmania* species (*Leishmania infantum*, *Leishmania major* and *L. tropica*), each characterized by distinct epidemiological features. The obtained results highlight the need to find a universally accepted diagnostic tool for *Leishmania* typing.

1. Background

Leishmania is a digenetic parasitic protozoan of the *Leishmania* genus, family Trypanosomatidae and Kinetoplastida order (Ramos et al., 2013). *Leishmania* parasites are transmitted to humans by phlebotomine female sand fly vectors (Teixeira et al.,

Abbreviations: Bp, base pair; CL, cutaneous leishmaniasis; FFPE, formalin-fixed paraffin embedded; ITS, internal transcribed spacer; *L. tropica, Leishmania tropica*; MLST, multilocus sequence typing; NJ, neighbor-joining; PCR, polymerase chain reaction; PI, parasitic index; RFLP, restriction fragment length polymorphism

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2013) and are the causative agents of leishmaniasis. At least 21 species have showed to cause disease in humans (World Health Organization, 1990). The disease ranges from localized cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) to widespread visceral leishmaniasis (VL), also known as kala-azar which is fatal if left untreated (Tsukamaya et al., 2008; Zhang et al., 2013). CL is the most common with more than 95% of the cases occurring in the Americas, the Mediterranean basin, the Near East and Central Asia especially in Afghanistan, Algeria, Brazil, Colombia, Iran and Syria. In fact, leishmaniasis is sometimes referred to as "Aleppo boil" in the medical literature (Hayani et al., 2014). Although, an estimated of 0.7 to 1.3 million new cases of CL occur worldwide each year (World Health Organization, 2014), leishmaniasis is still considered one of the world's most neglected diseases (Ramos et al., 2013).

The crisis in the Syrian Arab Republic that started in March 2011 has resulted in outbreaks of several previously overlooked diseases (World Health Organization, 2013). Since then, cases of CL in Lebanon have been drastically increasing relative to the previous years (2001–2012) (Alawieh et al., 2014; Alvar et al., 2012). From 2004 to 2008 no CL cases were reported in Lebanon compared to 22,882 cases in Syria during the same period (World Health Organization, 2013). Recently, Saroufim et al. (2014) identified *Leishmania major* in 15% and *L. tropica* in 85% out of 948 Syrian refugee patients living in Lebanon, the latter species being endemic to the Aleppo region in Syria.

Since different *Leishmania* species have been shown to cause CL and due to population travel and migration, unexpected *Leishmania* species can appear in unexpected regions (Dujardin, 2006). Also, different species show different susceptibility to drugs (Blum et al., 2004) and primary and secondary resistance (Desjeux, 2004). The occurrence of natural interspecies hybrids and sexual recombination hinders species discrimination (Tojal da Silva et al., 2006; Nolder et al., 2007; Odiwuor et al., 2011a; Ravel et al., 2006).

Several PCR-based methods have been employed for typing *Leishmania* parasites. MLEE (Multilocus Enzyme Electrophoresis) is sometimes considered the gold standard for *Leishmania* identification (Bañuls et al., 2007; Schönian et al., 2001). DNA sequencing, PCR-RFLP (Restriction Fragment Length polymorphism) (Yehia et al., 2012) and MLST (Multilocus Sequence Typing) have all been employed for this purpose (da Silva et al., 2010; Montalvo et al., 2010; Maiden et al., 1998). In terms of sensitivity and validation, the ITS1 region offers the best resolution for *Leishmania* discrimination in the Old World (Odiwuor et al., 2011b). ITS1 is the sequence in between the 18S rRNA and 5.8S rRNA genes. It contains enough conservation to serve as a PCR target but sufficient polymorphisms to facilitate species typing and identification (Roelfsema et al., 2011). However, these PCR based methods are often hindered by low sensitivity results that fail to detect amastigotes in samples that proved to be positive by microscopy. This caveat in molecular typing is often due to a low parasite load in the original sample (Shahbazi et al., 2008).

In this study, the identification and typing of *Leishmania* isolates obtained from Syrian refugees in Lebanon using two molecular typing techniques: the ITS1-PCR-RFLP and the nested ITS1-5.8S rDNA gene PCR both followed by sequencing and phylogenetic tree analysis were conducted. The obtained results were used in comparing the efficiency of these two techniques in *Leishmania* identification and assessing the evolutionary and phylogenetic relationships among these isolates. The results were correlated with the parasitic index (PI). The DNA storage in formalin-fixed paraffin embedded (FFPE) tissues was assessed as well.

2. Methods

2.1. Ethical approval

This study was approved by the American University of Beirut Institutional Review Board and the patient data used in this study was anonymized.

2.2. Sample collection and description

A total of 39 FFPE blocks were obtained from the American University of Beirut Medical Center (AUBMC) in Lebanon. FFPE blocks contained skin biopsies from patients having histologically confirmed CL (PI ≥ 2) during the years of 2013–2014. Each patient had one biopsy performed, and one FFPE block per biopsy was available. Cases included in the study were restricted to cutaneous lesions of patients who did not receive treatment prior to the biopsy. Clinical information pertaining to the lesion was also collected including: number, duration, location and dermatologic appearance. In addition, the patient's age, gender and country of residency were tabulated. Organisms collected from cultures were used as positive controls to validate the analysis. IRB approval was granted prior to the initiation of this study (PALM I.K.01).

2.3. Histopathology

Sections from each of 39 FFPE tissue blocks were stained for hematoxylin and eosin, Giemsa, Acid Fast bacilli, Gomori Methylamine Silver and Periodic Acid-Schiff. All cases were reviewed by four pathologists (JS, RK, FF and IK) and classified according to the modified Ridley's parasitic index (PI) (Table 1). The correlation between the PI, the duration of the lesion, the age of the patients and the results of the ITS1-PCR-RFLP and the nested ITS1-5.8S rDNA gene PCR was performed using the Pearson's correlation test.