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Research paper

Genetic micro-heterogeneity of *Leishmania major* in emerging foci of zoonotic cutaneous leishmaniasis in Tunisia

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ABSTRACT

Tunisia is endemic for zoonotic cutaneous leishmaniasis (ZCL), a parasitic disease caused by *Leishmania* (*L.*) *major*. ZCL displays a wide clinical polymorphism, with severe forms present more frequently in emerging foci where naive populations are dominant.
In this study, we applied the multi-locus microsatellite typing (MLMT) using ten highly informative and discriminative markers to investigate the genetic structure of 35 Tunisian *Leishmania* (*L.*) *major* isolates collected from patients living in five different foci of Central Tunisia (two old and three emerging foci). Phylogenetic reconstructions based on genetic distances showed that nine of the ten tested loci were homogeneous in all isolates with homozygous alleles, whereas one locus (71AT) had a 58/64-bp bi-allelic profile with an allele linked to emerging foci. Promastigote-stage parasites with the 58-bp allele tend to be more resistant to *in vitro* complement lysis. These results, which stress the geographical dependence of the genetic micro-heterogeneity, may improve our understanding of the ZCL epidemiology and clinical outcome.

1. Introduction

Leishmania (*L.*) are parasites transmitted by the bite of sand fly vector causing a wide spectrum of infectious diseases with large clinical polymorphism and severity ranging from cutaneous or mucocutaneous infection to diffuse cutaneous or visceral disease. In Tunisia, three different *Leishmania* species cause cutaneous leishmaniaisis. *L. major* is the most prevalent dermotropic species and cause the so-called "zoonotic cutaneous leishmaniaisis" (ZCL). *L. tropica* and *L. infantum* are typically responsible for the chronic and the sporadic cutaneous forms, respectively (Haouas et al., 2012). Diseases caused by *L. killicki* (syn. *tropica*) and *L. infantum* are largely less prevalent (100–300 cases per year for both) than ZCL, in which incidence reaches few thousands of cases each year (Aoun and Bouratbine, 2014).

ZCL displays some degree of clinical polymorphism extending from fully asymptomatic infections to benign self-limited cutaneous sore(s) or to more extensive lesion(s) causing severe disfiguration, even in immune-competent individuals (Kebaier et al., 2001).

Factors influencing the clinical features of ZCL are yet to be identified. They might include the host immune status, the intervention of vector related factors and/or parasite virulence (Bifeld and Clos, 2015).

The role of virulence in different parasite species causing various disease forms has been already investigated (Chang and McGwire, 2002; Dujardin, 2009; Smith et al., 2007). However intraspecific variations that may lead to different disease outcomes and severities are subtler when dealing with isolates belonging to the same species like *L. major*.

The experimental disease induced in BALB/c mice by different *L. major* isolates from Tunisia showed that they cluster in three groups with low, medium and high experimental pathogenicity. However, this classification did not correlate with the clinical severity of the disease, as expressed in patients from whom these isolates were obtained (Kebaier et al., 2001).

Besides the experimental pathogenicity model, several molecular tools have been used to detect genetic polymorphism between and within *Leishmania* species and strains (Le Blancq et al., 1986). They include multilocus enzyme electrophoresis (MLEE), considered as the gold standard for taxonomy and strain typing of *Leishmania*, multilocus sequence typing (MLST) or DNA-based techniques like the random amplification of polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP). MLEE electrophoretic profile comparison of

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Tunisian *L. major* strains revealed their homogeneity. Indeed all *L. major* isolates collected so far belong to MON-25 zymodeme, indicating that parasites obtained from sandflies and several rodent species were indistinguishable from those obtained from man (Le Blancq et al., 1986). In contrast, *L. infantum* belongs to three different zymodemes *i.e.*, MON-1, MON-24 and MON-80 (Haouas et al., 2012) and *L. killicki* (syn. *tropica*) belongs to MON-8 and MON-317 (Chaara et al., 2015).

The analysis of co-dominant microsatellite markers using multilocus microsatellite typing (MLMT) approach is considered one of the most discriminative DNA-based methods for population genetic studies. It is regarded as high accurate in the typing of closely related strains and has been successfully used in population genetic studies of *L. infantum*, *L. tropica*, *L. aethiopica* and *L. donovani* (for review see Krayter et al., 2015; Kuhls et al., 2007; Schwenkenbecher et al., 2006). Panels of ten to twenty microsatellite markers have been developed for detecting genetic variation in each species. Based on nucleotide sequence information, ten informative microsatellite loci have been already used to determine the heterogeneity, geographical and epidemiological distribution of *L. major* isolates collected from ten Asian and nine African countries (Al-Jawabreh et al., 2008). Among these isolates, two were originally collected in El Guettar, an old South Tunisian endemic focus, and both showed a genetic homogeneity and clustered with the subpopulation Africa-2 (Al-Jawabreh et al., 2008).

We have also recently shown by MLMT a complete genetic homogeneity between *L. major* isolates collected from various reservoirs hosts in central Tunisia (Ghawar et al., 2014).

Clinically, ZCL lesions occurring in old endemic foci are much less severe compared to those observed in emerging foci (Ben Salah, personal communication). In addition, the age distribution of patients' shifts from a skewed distribution in old foci, where only younger persons develop lesions, to a random distribution involving all age groups in newly emerging foci (Mbarki et al., 1995).

In the present study, genetic structure of 35 *L. major* isolates obtained from patients with active ZCL lesions and living in old and emerged endemic foci of central Tunisia, was analyzed by MLMT. Foci history classification was made on the basis of case notification data in the district epidemiological surveillance system.

2. Materials and methods

2.1. Ethics statement

The study protocol, consent forms and procedures were reviewed and approved by the Institut Pasteur de Tunis Ethical Review Board. All patients provided written informed consent for the collection of parasite isolates and subsequent analysis.

2.2. Study area and parasite collection

Thirty-five isolates of *L. major* were grown from patients living in villages in the governorates of Sidi Bouzid and Kairouan, central Tunisia. The study regions were described previously (Bettaieb et al., 2014) and share the same topography and climate. Mnara and Mbarkia villages (Kairouan and Sidi Bouzid governorates, respectively) are considered as old foci whereas Ksour, Dhouibet and Msadia are considered as newly emerging foci. The isolates were established between October and March (during 2008, 2009 and 2010), the period during which the cutaneous lesions appear in most individuals that have been infected by sand fly bites during the transmission season (June-October). All parasite cultures were deep frozen within the shortest time after successful growth; in order to avoid changes induced by prolonged *in vitro* cultures.

The geographical origin of each isolate is described in Table 1. DNA of the reference strain Friedlin (MHOM/IL/1980/Friedlin), for which the genome has been completely sequenced, was obtained from the Department of Parasitology, Institute of Microbiology and Hygiene, Charité University Medicine Berlin.

2.3. Parasite culture and DNA extraction

L. major promastigotes were maintained in RMPI 1640 (Sigma) medium supplemented with 10% of heat-inactivated fetal calf serum (FCS), 2 mM of L-glutamine, 100 U/ml of penicillin and 100 U/ml of streptomycin. Promastigotes were harvested at stationary phase (6–7 days old), and a pellet of 2×10^8 parasites of each isolate was conserved at −20 °C for subsequent DNA extraction. DNA purification was performed using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions.

2.4. Parasite species identification

ITS1-PCR was performed to confirm the species of all isolates included in the study, as previously described (El Tai et al., 2001; El Tai et al., 2000; Schonian et al., 2003). ITS1 sequence was amplified for all strains using LITSR/L5.8S primer pair. Final PCR mix volume was 50 μl containing 1.5 mM Mg2 +, 0.2 mM of each dNTP, 25 pmol of the forward primer (5′ CTGGATCATTTTCCGATG 3′), 25 pmol of the reverse primer (5′ TGAT ACCACTTATCGCACTT 3′), 1 U of the Taq polymerase and 20 ng of each DNA sample. PCR reaction was performed in a Biometra thermocycler (Biometra, Germany) with an initial denaturation at 95 °C for 2 min followed by 33 cycles of (i) denaturation at 95 °C for 20 s, (ii) annealing at 53 °C for 30 s and (iii) extension at 72 °C for 1 min. A final extension cycle at 72 °C for 6 min was then applied. For each experiment, DNA extracts from the three Tunisian *Leishmania* species (*L. major*, *L. tropica* and *L. infantum*) were used as controls. Double-distilled water (DdH_2O) was used as negative control.

The presence of a specific DNA amplified fragment of 300–350 bp has been visualized on 2% agarose gel. Subsequently, 17 μl of the PCR product of each amplified DNA, including the controls, were further digested with a total volume of 5 μl of a restriction mixture containing 2.5 μl of ddH2O, 1.5 μl of the NE Buffer2 10× concentrated and 1 μl (10 U) of endonuclease *Hae III*. After incubation at 37 °C, products were visualized under UV on a 2% Metaphor Agarose gel.

2.5. MLMT PCR and analysis

Microsatellite sequences were amplified in a volume of 25 μl containing 1,5 mM Mg^{2+}, 0,2 mM of each dNTP, 5 pmol of the forward primer, 5 pmol of the reverse primer, 0.5 U of the *Taq* polymerase and 10 ng of each isolate's DNA as previously described (Al-Jawabreh et al., 2008). PCR reactions were performed in a Biometra thermocycler with an initial denaturation at 95 °C for 5 min. Amplification cycles were finally followed by an extension cycle at 72 °C for 6 min. In each experiment, *L. major* MHOM/IL/1980/Friedlin DNA was used as a reference control and ddH_2O as a negative control. Presence, specificity, and quality of the PCR products were checked on a 2% agarose gel. The exact size of the PCR fragments was established by separating fluorescence labeled PCR products in a capillary sequencer and the Genemapper software (ABI). When a double peak with only one base difference was obtained due to an A-overhang created during PCR, only one peak was scored.

Sizes of the fragments generated for the ten-microsatellite markers for each isolate were estimated using Gene Mapper (v 3.2) software. The number of repeats was calculated based on the number of repeats of the corresponding marker in *L. major* MHOM/IL/1980/Friedlin strain as a reference. The repeats for each of the ten tested loci were then assembled into a multilocus microsatellite profile for each tested isolate. All MLMT experiments and analyzes were performed twice (at Institut für Mikrobiologie und Hygiene and Institut Pasteur de Tunis) and led to the same results and conclusions.