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New epidemiological pattern of cutaneous leishmaniasis in two pre-Saharan arid provinces, southern Morocco

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ABSTRACT

Three *Leishmania* species are responsible of cutaneous leishmaniasis (CL) in Morocco. Zoonotic CL due to *Leishmania major* and *Leishmania infantum,* the first is known as established in the eastern arid regions, whereas the latter evolves sporadically, especially in the North. While *Leishmania tropica,* classically considered anthroponotic, is endemic in the semi-arid regions and is largely distributed throughout the country. The aim of this study was to identify the *Leishmania* species causing CL in two Provinces in arid pre-Saharan region known as zoonotic CL foci, and to contribute an update to the national data concerning the distribution of *Leishmania* species in both regions. The recruitment of patients was done in six localities in Ouarzazate and Zagoura provinces in 2015 and 2016. Out of 81 samples collected, 66 were positive (81%) by ITS1-PCR amplification of *Leishmania* DNA extracted from stained smears. The highest rate of *Leishmania* infection was registered in children aged 9 years or less (71,2%). The ITS1-PCR- RFLP analysis revealed the predominance of *L. major* infecting 52 patients (79%), followed by *L. tropica* in 12 patients (18%) and *L. infantum* in 2 patients who had no history of travel outside the studied area (3%). The sequencing of the ITS1 of both *L. infantum*, showed 100% similarities with *L. infantum* strains isolated from dogs and visceral leishmaniasis patients from the south and north of Morocco. The coexistence of the 3 *Leishmania* species in the same focus, and the difficult distinction of infections associated to the different *Leishmania* species based only on clinical lesions' aspects complicate the diagnosis and then the national control strategy, as well as the therapeutic management. The epidemiological pattern of CL in the studied areas appears to have changed during the last decades, from a predominant zoonotic CL caused by *L. major* to a polymorphic disease that can be due to any of the 3 *Leishmania* species. The expansion of *L. infantum* and *L. tropica* in southern parts of Morocco, calls for in depth epidemiological investigations for a better understanding of the CL situations in Southern parts of the country and for an assessment of the climate impact and environment changes on the leishmaniasis transmission system.

1. Introduction

Leishmaniases are a complex of diseases worldwide distributed and caused by > 20 *Leishmania* species, a parasitic protozoa transmitted by the bite of infected female sand flies. The disease affects 98 endemic countries, 350 million people are considered at risk of infection, some 1.3 million new cases occur annually and 20,000–30,000 deaths are recorded yearly (WHO, 2010, 2014). The clinical symptoms of leishmaniasis vary from localized skin ulcers (cutaneous leishmaniasis) to lethal systemic disease (visceral leishmaniasis). They are included in the group of "most neglected" tropical diseases (NTD) as defined by the limited investment of resources to improve their diagnosis, treatment and control and by their strong association with poverty (den Boer et al., 2011).

Cutaneous leishmaniasis (CL) is perhaps the most neglected of all NTDs. It is caused by a number of different *Leishmania* species, producing a wide spectrum of manifestations from small skin nodules to gross mucosal tissue destruction. The endemicity of CL is high and its

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incidence has increased at an alarming rate, causing considerable morbidity of the population in the tropics and neotropics. The significance of CL has been grossly neglected because it is rarely fatal. In Morocco CL is caused by 3 species: *L. major*, *L. tropica*, and *L. infantum*. Its complex epidemiology is mainly due to species co-endemicity with different prevalence, focal distribution, transmission and cycles.

Leishmania major is the principal etiological agent of CL, responsible for the zoonotic Cutaneous Leishmaniasis (ZCL); in Morocco, ZCL has been known to exist in the vast arid pre-Saharan regions for over a century since 1914 (Rioux and Petter, 1986). *Phlebotomus Papatasi* and *Meriones shawi* are the vector and the reservoir of ZCL respectively (Rioux and Petter, 1986; Rioux et al., 2001). A total of 27,257 cases of ZCL were reported during the period of 13 years from 2000 to 2013 whence the annual incidence has increased to ~5000 cases/year for each of the subsequent last four years according to the record available from the Moroccan Ministry of Health (Ministry-of-Health-Morocco, 2014), on the other hand cases reported back to WHO are much lower (WHO, 2016) underlining the under-reporting presence for this disease which causes a real challenge in the organization of control and support strategies. Thus, the ZCL represents a serious health problem in the country. Clinical manifestations of the ZCL are particularly diverse and pleotropic, ranging from a single self-limiting lesion to multiple and disfiguring lesions that can be a social stigma, especially for women.

On the other hand, *L. tropica* is especially responsible for anthroponotic cutaneous leishmaniasis. However the possible involvement of an animal reservoir host (Domestic dog) In *L. tropica* transmission cycle was reported in Morocco (Dereure et al., 1991; Lemrani et al., 1999). CL due to *L. tropica* was initially described in the rural locality of Tanant (Azilal province, High Atlas) (Marty et al., 1989). Thereafter, a large rural CL focus was identified in Central and South Morocco (Pratlong et al., 1991) and soon after in Northern Morocco (Taza province) (Guessous-Idrissi et al., 1997). Presently, CL due to *L. tropica* has become also a major public health problem with 17,882 reported cases during the last 10 years (Ministry-of-Health-Morocco, 2014). Early in 2000, outbreaks occurred in emerging CL foci in Central and Northern Morocco, where *L. tropica* was found concurrent in established *L. major* foci (Kahime et al., 2016; Rhajaoui et al., 2007). Concerning CL due to *L. infantum*, the only dermotropic variant reported in Morocco is zymodeme MON-24. This variant was also isolated from a domestic dog (Benikhlef et al., 2004). The Ministry of Health is still considering CL due to *L. infantum* as evolving sporadically. Its distribution areas are not well defined, and it is found frequently in *L. tropica* foci (Haralambous et al., 2007). Thus the changes in CL epidemiological trends in Morocco characterized by the overlap of the geographical distribution areas of the 3 species, as well as the increasing risk of emergence and epidemics may probably be related to climate changes, ecosystems alteration due to urbanization and migrations of non-immune populations (Arroub et al., 2013; Riyad et al., 2013).

Considering the changes in the epidemiology of CL in Morocco, we chose two provinces, Ouarzazate and Zagoura, where 6695 and 6622 cases have been reported respectively the last ten years, but where no larger scale identification of *Leishmania* species have been done. So in this study, the epidemiological characteristics of CL will be examined in these two provinces by the means of molecular typing of *Leishmania* and analysis of the epidemiological data, in order to update the situation of leishmaniasis in this region, and contribute with data to the overall knowledge on the state of leishmaniasis in Morocco.

2. Materials and methods

2.1. Area of study

The study was conduct in two provinces in the region of Drâa-Tafilalet (Fig. 1): Province of Ouarzazate is in the middle of an arid plateau south of the High Atlas Mountains, at an elevation around 1100 m. The province is known for its arid climate, it is hot and dry in summer, but can be very cold in winter, with icy winds coming from the High Atlas Mountains. Precipitations are irregular during the year, with an annual mean between 115 mm and 259 mm.

Agdz Village (Province of Zagoura) located about 100 km, south Ouarzazate, at around 30°41′52″N 6°26′59″W, and at 942 m elevation. Agdz is considered to have a desert climate, with temperature ranging from 48 °C in summer to subzero temperatures in winter. The rainfall average is 106 mm.

2.2. Ethical considerations

Informed consent was obtained from all the adults who participated in the study. Consent for inclusion of young children, was obtained from parents or guardians. The study was reviewed and approved by institutional Ethical Review Committee.

2.3. Patients: recruitment and sampling

In province of Ouarzazate, sampling was done in 4 rural localities and in Ouarzazate city, whereas in Zagoura province, all patients were from the semi-rural town of Agdz.

Tissue samples were collected by dermal scraping from 81 suspected CL patients. We included in the present study, patients gathered at the Health Centre of each site during the 8-days mission we conducted in 2015 and 2016. For each patient a questionnaire was filled with all information about the patient (including code, age, sex and address, and travel history), and the lesion (including the number of lesions, localizations, onset of the disease and clinical characteristics). The stained smears for CL direct diagnosis were microscopically examined in the Health center facility and confirmed back in our laboratory. All positive patients were treated for free in the health centers in each province.

2.4. DNA extraction and PCR-RFLP analysis

The total DNA was extracted from positive stained smears, but also from negative ones taken from clinically suspected patients, using the phenol-chloroform method. Then DNA samples were purified using Bioline kit (ISOLATE II PCR & Gel kit) following the manufacturer's instructions. The DNAs were quantified by NanoDrop (Thermo Scientific), before dilution to a final concentration of 50 ng/μL when necessary.

2.5. ITS1 PCR-RFLP of leishmania species

The tissue samples obtained from CL patients were examined for the *Leishmania*-specific ribosomal internal transcribed spacer 1 region (ITS1) by PCR amplification using the primer pair L5.8S and LITSR, followed by restriction fragment length polymorphism (RFLP) analysis, as previously described (Schonian et al., 2003).

The cycling conditions were 94 °C for 2 min, followed by 32 amplification cycles, each consisting of three steps: denaturation at 94 °C for 20 s, annealing at 53 °C for 30 s and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 6 min in the thermocycler (S1000™ Thermal Cycler, Bio-Rad).

PCR products were digested with the restriction endonuclease *HaeIII* (New England Biolabs) for 2 h at 37 °C. Restriction fragments were separated by electrophoresis on a 2% agarose gel and compared with those of WHO reference strains of *L. major* (MHOM/SU/73/5ASKH), *L. tropica* (MHOM/SU/74/K27) and *L. infantum* (MHOM/TN/80/IPT1).

2.6. DNA sequencing

The final PCR products of about 300 bp were purified using the Exonuclease I/Shrimp Alkaline Phosphatase (GE Healthcare, US) then

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