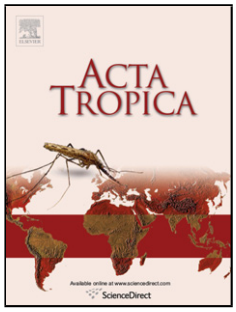

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PII: S0001-706X(17)30324-8
DOI: http://dx.doi.org/doi:10.1016/j.actatropica.2017.05.016
Reference: ACTROP 4315

To appear in: *Acta Tropica*

Received date: 17-3-2017
Revised date: 9-5-2017
Accepted date: 11-5-2017

Please cite this article as: Ait Kbaich, Mouad, Mhaidi, Idriss, Ezzahidi, Abdelkacem, Dersi, Nouredine, El Hamouchi, Adil, Riyad, Myriam, Akarid, Khadija, Lemrani, Meryem, New Epidemiological pattern of cutaneous leishmaniasis in two Pre-Saharan arid provinces, southern Morocco.Acta Tropica http://dx.doi.org/10.1016/j.actatropica.2017.05.016

New Epidemiological pattern of cutaneous leishmaniasis in two Pre-Saharan arid provinces, southern Morocco

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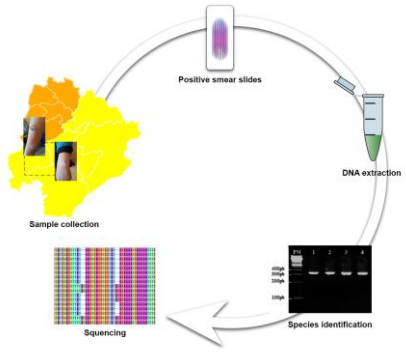
Graphical abstract



This study describes the epidemiological situation of cutaneous leishmaniasis by Leishmania molecular typing on patients in southern Morocco.

Highlights

- Epidemiological study of cutaneous leishmaniasis in Ouazazate province and Agdz town
- Analysis of clinical data of patients with CL in the region
- Molecular typing of Leishmania spp. and sequencing of L. infantum species by PCR-RFLP of ITS1
- Contribution to the epidemiological data of Morocco concerning CL
- Discussion of the change of epidemiology of CL in the region

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.

Keywords: epidemiological pattern, cutaneous leishmaniasis, arid pre-Saharan region, Morocco.

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 to 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 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, 1986). *Phlebotomus Papatasi* and *Meriones shawi* are the vector and the reservoir of ZCL respectively (Rioux, 1986, 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 ~5,000 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 6,695 and 6,622 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 1,100 meters. 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 sequenced by using BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3130 DNA automated sequencer (Applied Biosystems). Sequencing data were analyzed using Chromas v.2.6.2 software (Technelysium). Sequences were aligned and compared to entries retrieved from Genbank, using the multiple alignment program MEGA7.

2.7. Phylogenetic analysis

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The analysis involved 17 nucleotide sequences. All positions

containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7(Kumar et al., 2016).

3. Results

The clinical examination showed the existence of two lesion forms (i) exudative or ‘‘wet’’ lesion, large and complicated by superficial and secondary bacterial infections (Fig. 2a), and (ii) dry lesions, with a central crust, mainly single and located on the face (Fig. 2b).

Out of 81 clinical samples, 66 were CL positive by ITS1-PCR, of which 53 were also positive by microscopic examination. Data were analyzed with the non-parametric $\chi 2$ test. The age distribution of the CL patients ranged from 4 months to 56 years, with a mean of 10.91 years and a median of 6 years. The disease prevalence has been significantly high in the age group:

4 months - 9 year (71.21%) (P-value = 0.015) (Table 1). No statistical significance of the disease distribution has been observed between genders (p-value > 0.05).

The restriction profiles of *Leishmania* parasite amplified by PCR were identified as *L. major* in 52 clinical samples (79%) followed by *L. tropica* in 12 patients (18 %) and *L. infantum* in 2 samples (3%). *L. major* was found in 5 out of the six targeted localities while *L. tropica* and *L. infantum* were found in the semi-rural villages of Toundout and Agdz (Table 2). Clinically, 36 patients infected by *L. major* had simple lesions, whereas 16 presented multiple lesions (up to 4). For 51% patients lesions were located on the face, 46% on the extremities and 3% on both locations (Table 3). All patients presenting CL due to *L. tropica* had a single lesion except one case presenting 4 lesions on the face (Table 3).

In order to confirm the molecular identification of *L. infantum* found in this region, the amplified ITS1 products were sequenced. We also sequenced the ITS1-PCR products of 6 *L. infantum* strains isolated from 3 of visceral leishmaniasis patients (VL) and 2 dogs in northern Morocco and one strain from VL patient in the south of the country.

The Phylogenetic analysis showed that the 2 *L. infantum* sequences (KY658234, KY658235) clustered with the sequence of *L. infantum* MHOM/MA/85/LEM-684 (KY658232) strain isolated in 1985 from an indigenous VL case in Douar Ighil in southern Morocco (kindly provided by Pr. Dedet from The Laboratory of Parasitology – Mycology, Faculty of Medicine, Montpellier, France). This cluster contains also two sequences (KY658231, KY658230) of *L.infantum* strains isolated from canine reservoirs, in addition to other sequences, retrieved from GeneBank:

AJ6343551.1, AJ634339.1, and AM901451.1 from Spain, France and Sudan respectively. The sequences KY658228, KY658229 and KY658233 of *L. infantum* strains isolated from VL patients in the north showed also high similarities with our *L.infantum* dermotropic variant sequences: KY658234, KY658235. (Fig. 3.)

4. Discussion

In Morocco leishmaniasis is an important public health problem; whether zoonotic or anthroponotic, cutaneous or visceral, these affections are widely represented, from the mountains of the Rif to the per-arid palm groves of the foothills of the Anti-Atlas.

Currently, foci of ZCL are linked to palm groves, periurban and rural areas with degraded socio-economic and environmental conditions (Kahime et al., 2014). The two provinces represented in this survey were suggested as endemic area of *L. major* based only on circumstantial data: the environmental conditions, as well as the high densely presence of rodent *M. shawi*, the proven reservoir of *L. major*(Echchakery et al., 2015; Rioux et al., 1982); the prevalent presence in Ouarzazate of *P. Papatasi* the vector of *L. major* (Es-Sette et al., 2016; Rioux et al., 1986) and also *L. major* genotyping of few samples (3 patients) derived from Ouarzazate province (Rhajaoui, 2011; Rhajaoui et al., 2007). This is the first survey in which CL causative agents were identified with certainty on a large sample of cutaneous leishmaniasis human cases, since the first outbreak occurred early in the 1980s in this region. Our data show that *L. major* is the prevalent species, hence confirming the status of this region being a ZCL endemic focus. The presence of *L. tropica* in this focus is a novel finding. This species is transmitted by *P. sergenti*, widely distributed in all bioclimatic zones with preferences for semi-arid regions (Boussaa et al., 2010; Kahime et al., 2015).Here we report the presence of *L. tropica* in two semi-rural sites in the north and the south of Ouarzazate city in Toundout and Agdz respectively, (Fig.1). This finding is the first report of *L. tropica* in the South of the Atlas Mountains, with the exception of few undocumented cases of *L. tropica* in Boumalne Dades in North-East of Ouarzazate province [Ministry of health, unpublished data]. Classically *L. tropica* is found throughout the center of the country in a band stretching from the Atlantic Ocean along the length of the Atlas Mountains almost reaching the Mediterranean Sea. Thus the Atlas Mountains may constitute an ecological barrier for the spread of *L. tropica* towards the South and *L. major* towards the North (Riyad et al., 2013). Toundout town is just south of Azilal province (Fig. 2.) a known endemic CL focus of *L. tropica*, they are

separated by the Atlas Mountains (Ajaoud et al., 2015; Arroub et al., 2013). However this proximity could constitute the way for *L. tropica* spread, since people move to Azilal through the Atlas Mountains and can thus introduce the parasite, whose propagation is facilitated by the presence of the vector (Es-Sette et al., 2016). Currently, the boundaries between the different nosogeographical forms of cutaneous leishmaniasis tend to overlap and progress in Morocco; indeed several cases of CL due *L. tropica* were detected, for the first time, in an old focus of ZLC in Errachidia province (East), even-though *P. sergenti* is present in low densities (Mohamed Mahmoud el et al., 2016).The coexistence of *L. major* and *L. tropica* in the same focus complicates the control strategy, as well as therapy; moreover It is often difficult to distinguish infections of *L. major* and those of *L. tropica* based only on clinical aspect of lesions. This fact was also observed elsewhere in mixed foci of *L. major* and *L. tropica* (Al-Jawabreh et al., 2004).Thus, identification of the causative species is for great importance, as prognosis and response to treatment differ substantially between species.

We also identified *L. infantum* in two patients from Toundout and Agdz. To our knowledge, this is the first report of *L. infantum* cutaneous infection rather than visceral leishmaniasis in Pre-Saharan region. In fact *L. infantum* dermotropic variation is evolving sporadically mainly in Northern Morocco where VL is endemic (Hamdi et al., 2013; Lemrani et al., 1999). However sporadic VL cases were also reported in these two arid provinces. Indeed 6 and 15 cases were recorded during the period 2003-2013 in Ouarzazate and Zagoura provinces respectively (Ministry-of-Health-Morocco, 2014). According to the literature, the presence of *L. infantum* in arid area is not a recent event, it goes back more than 30 years in Morocco; Dereure *et al* (1986) described the first focus of zoonotic visceral leishmaniasis in South of Atlas Mountains in pre-Saharan region, where 2 cases of canine leishmaniasis and one infantile VL case were found infected by *L. infantum* MON-1(Dereure et al., 1986). The ITS1 sequence of this VL patient presents 100% similarities with the two *L. infantum* sequences described in the present study. Being the unique member of the *Larrousius* sub-genus in this area, *P. longicusp*is was the suspected vector of *L. infantum* (Dereure et al., 1986). This phlebotomine species has been represented as the second most abundant species after *P. papatasi* in Ouarzazate province according to Es-Sette *et al* (2016). Also Kahime *et al* (2015) reported the presence of *P. longicuspis* in both Ouzarzazate and Zagoura provinces. CL due to *L. infantum* was reported in many countries around the Mediterranean Basin (Aoun and Bouratbine, 2014), the spread of the disease was

suggested to be mainly through expansion of zoonotic cycle to regions where competent local vectors facilitate *L. infantum* transmission (Jacobson, 2011).

To conclude, the epidemiological pattern of CL in the studied areas appears to have changed, from a predominantly zoonotic CL caused by *L. major* to a polymorphic CL that can be due to either of the 3 *Leishmania* species. The expansion of *L. infantum* and *L. tropica* in southern parts of Morocco, calls for in depth epidemiological investigations to *shed more light* on the current and future situations of CL in Southern parts of the country. These investigations may especially focus on the vector dynamics, the identification of animal reservoirs, and the impact of climate changes on the transmission system of leishmaniasis.

Acknowledgments

We are very grateful to the local team of physicians and nurses of Ouarzazate and Zagoura province's Health Centers and the Health Delegation of the Moroccan Ministry of Health in Ouarzazate province. This work is part of the research project in the priority areas of Scientific Research and Technological Development CNRST-Type A (A / O-2013).

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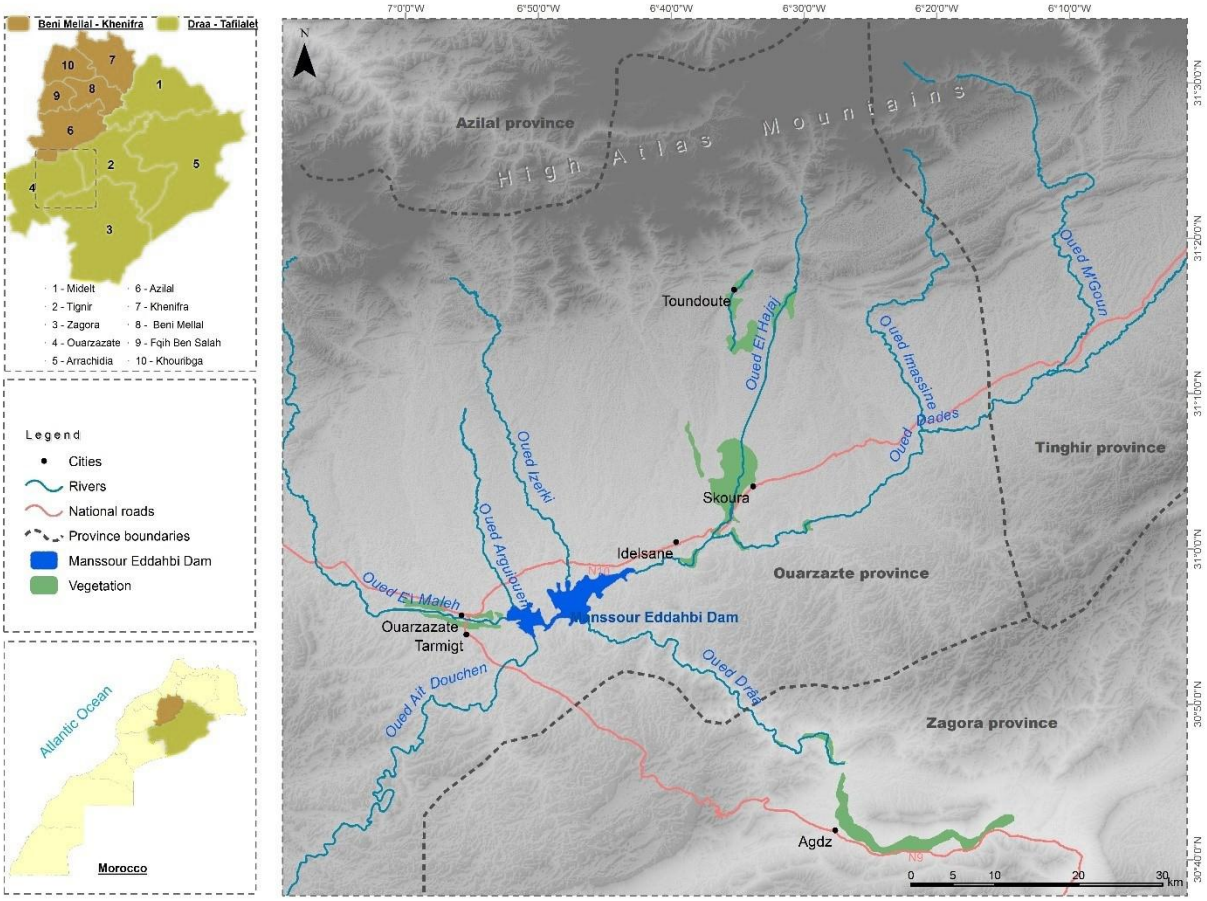

Fig. 1.

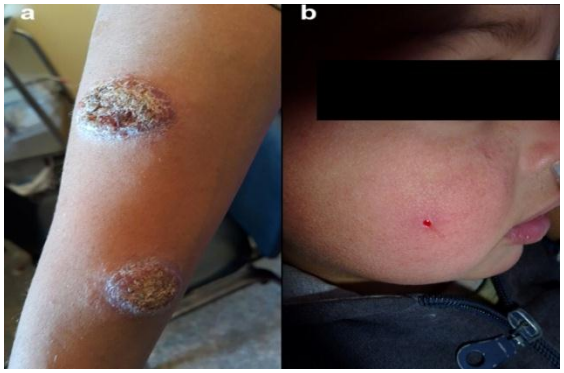

Fig. 2.

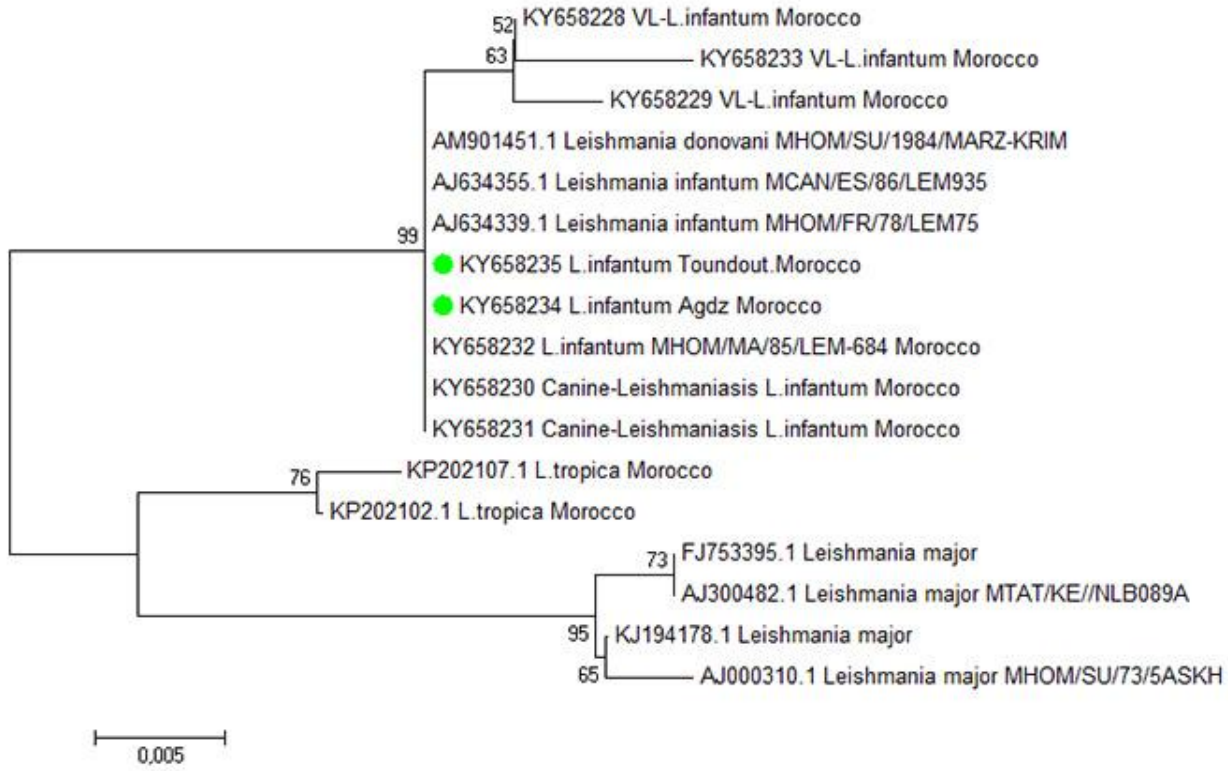

Fig. 3.

Figure titles

Fig. 1. Map showing the cities/towns where study was carried out

Fig. 2. Cutaneous leishmaniasis lesions of patients from Ouarzazate and Zagoura provinces; (a) multiple surinfected lesions on the arm; (b) single lesion on the face.

Fig. 3. Phylogenetic tree based on *Leishmania* ITS1 sequences

Table 1

Positive cutaneous leishmaniasis by age and by sex in Ouarzazate and Zagoura Provinces

Age group	+ ITS1-PCR/ Frequency %	Test $\chi 2$	+ ITS1-PCR /Gender		Test $\chi 2$
			Male	Female	
4 months - 9 years	47 (71.21%)	p-value = 0.015	39 (59.10%)	27 (40.90%)	p-value > 0.05
10 years -19 years	8 (12.12%)				
20 years and above	11 (16.67%)				
Total	66				

Table 2

ITS1-PCR-RFLP results according to localities in Ouarzazate and Zagoura provinces

Province	Locality	Samples	Positive ITS1-PCR	*Leishmania* species by PCR-RFLP		
				L. major	*L. tropica*	*L. infantum*
Ouarzazate	Ouarzazate city	15	10	10	0	0
	Tarmigte	15	11	11	0	0
	Skoura	7	5	5	0	0
	Idelsane	28	26	26	0	0
	Toundout	6	5	0	4	1
Zagoura	Agdz	10	9	1	7	1
Total	6	81	66	53	11	2

Table 3

Positive cutaneous leishmaniasis cases by Lesion number and Lesion location in Ouarzazate and Zagoura Provinces

	Lesion number		Lesion location		
Species	Single	Multiple	Face	Extremities	Both
L. major	36	17	21	30	2
L. tropica	10	1	11	0	0
L. infantum	0	2	2	0	0