The First Isoenzymatic Characterizations of the *Leishmania* Strains Responsible for Cutaneous Leishmaniasis in the Area of Annaba (Eastern Algeria)

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Abstract: The epidemiological situation of cutaneous leishmaniasis in the region of Annaba (North Eastern Algeria) is explored for the first time. During a clinical survey carried out in the Annaba Hospital between 2004 and 2008, the parasitological study of lesions has revealed 259 positive cases (43.31%). Isoenzymatic identification of 16 strains isolated showed the presence of three *Leishmania* species and four zymodemes. The hypothesis of the presence of *L. infantum* is confirmed with two zymodemes, MON-1 and MON-24. The unexpected presence of *L. major* MON-25 certifies the substantial extension of this zoonotic form to the North. The isolation of *L.killicki*, whose presence was unsuspected, reiterated the interest of eco-epidemiological analysis of households affected by the disease. Moreover, it is a new zymodeme never isolated so far, the MON-306.

Keywords: Annaba, Algeria, Cutaneaous leishmaniases, *Leishmania infantum, Leishmania killicki , Leishmania major*.

1. INTRODUCTION

The leishmaniases are known from Algeria for more than a century. Their annual incidence in the country is estimated around 30,000 reported cases in the recent years, of which 29,500 are cutaneous leishmaniasis (CL) cases [1].

Cutaneous leishmaniasis (CL) has been described in Algeria since 1860 [2]. It includes a zoonotic form, zoonotic cutaneous leishmaniasis (ZCL) , due to *Leishmania major* and distributed in arid and semi-arid areas and a sporadic form, sporadic cutaneous leishmaniasis (SCL), due to *L. infantum* and occurring in the humid and subhumid areas [1-6]. Concerning the vector, studies in Algeria confirm the presence of four *major* species, *P.perniciosus*, *P.perfiliewi*, *P.papatasi* and *P.sergenti* [7-12].

ZCL recently outspread from its usual foci and is becoming more frequent in Northern Algeria, coexisting with the visceral form, which raises questions of epidemiology. To answer these questions, epidemiological studies are required, particularly the isolation and identification of all strains circulating among humans, reservoir hosts and vectors . Recently, a third species responsible for CL, *Leishmania killicki,* has been identified in the focus of Ghardaia, Southwestern Algeria [13].

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Furthermore, foci of *L.killicki* in Tunisia [14-16] and *L.tropica* in Morocco [17-19] had been previously reported but not in Algeria, although the bio-climatic conditions and ecological epidemiology are similar. This shows the interest of establishing structures which are able to isolate all strains in endemic foci.

In spite leishmaniases were previously reported from the Annaba region, no *Leishmania* strains have been isolated and typed from this region as yet. The aim of this work is to provide a better understanding of the epidemiology of leishmaniasis in the region of Annaba, by isolation and identification of strains circulating in the area.

2. PATIENTS AND METHODS

During the period from January 2004 to December 2008, 598 patients with suspected CL were admitted in the Laboratory of Parasitology-Mycology, Hospital of Annaba, for parasitological confirmation. The patients were originating from the wilayas of Annaba (latitude 36° 54′ 00″ N and longitude 7° 46′ 00″ E), Guelma (latitude 36° 28′ 00″ N and longitude 7° 27′ 00″ E), Skikda (latitude 36° 52′ 00″ N and longitude 6° 54′ 00″ E), El Tarf (latitude 36° 46′ 02″ N and longitude 8° 18′ 50″ E), Souk Ahras (latitude 36° 17' 14.84'' N and longitude 7° 57' 14.77'' E) and Tebessa (latitude 35° 24′ 00″ N and longitude 8° 07′ 00″ E).

For each patient, an information form was carefully filled up, collecting the administrative, epidemiologic and clinical information. The samples studied were lesion scrapings col-

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lected under sterile conditions. These samples were examined after Giemsa staining and cultured on NNN medium at 25°C. Several subcultures, during one to five weeks, were often required to obtain a positive culture. The isolates obtained were sent to the *Service de Biologie Parasitaire, Institut Pasteur d'Algérie* for mass culture and cryopreservation in liquid nitrogen.

The strains were characterised by isoenzyme analysis based on starch gel electrophoresis according to Rioux *et al.*(1990) at the Centre National de Reference des *Leishmania* (CNRL), Montpellier, France. The 15 enzymes investigated were: MDH: malate dehydrogenase (EC 1.1.1.37); ME: malic enzyme (EC 1.1.1.40); ICD: isocitrate dehydrogenase (EC 1.1.1.42); PGD: phosphogluconate dehydrogenase (EC 1.1.1.44); G6PD: glucose-6-phosphate dehydrogenase (EC 1.1.1.49); GLUD: glutamate dehydrogenase (EC 1.4.1.3); DIA: NADH diaphorase (EC 1.6.2.2); NP1 and NP2: purine nucleoside phosphorylases 1 and 2 (EC 2.4.2.1 and EC 2.4.2.*); GOT1 and GOT2: glutamate-oxaloacetate transaminases 1 and 2 (EC 2.6.1.1); PGM: phosphoglucomutase (EC 5.4.2.2); FH: fumarate hydratase (EC 4.2.1.2); MPI: mannose phosphate isomerase (EC 5.3.1.8); GPI: glucose phosphate isomerase (EC 5.3.1.9).

Isoelectrofocusing was used as a complementary technique to provide greater resolving power.

The following reference strains were used: MHOM/FR/78/LEM75 (*L.infantum* zymodeme MON-1); MHOM/DZ/83/LEM417 (*L. infantum* MON-24); MHOM/TN/80/LEM163 (*L. killicki* zymodeme MON-8) and MHOM/MA/81/LEM265 (*L.major* zymodeme MON-25).

3. RESULTS

The parasitological diagnostic of the samples revealed 259 positive cases (43.31%). Cultures allowed us to isolate 22 strains of *Leishmania,* of which 16 were typed by isoenzyme electrophoresis . All patients were infected in their wilaya of origin and did not recall any notion of travel outside, so their cases should be considered as autochthonous. Positive cases were mainly from the wilaya of Annaba, with 68 cases (26.26%), followed by the province of Guelma with 63 cases (24.33%).

The isoenzymatic identification of these 16 strains showed the presence in our sampling of three *Leishmania* species and four zymodemes : 8 were *L. major* zymodeme MON-25, 7 were *L. infantum,* of which two zymodemes were identified (MON-1, 2 strains, and MON-24, 5 strains) and a single strain corresponded to a new zymodeme of *L. killicki* (Table **1**). The enzymatic profile of this strain was a new variant enzyme which the CNRL of Montpellier has assigned the code MON-306, which differs by ME from the zymodeme MON-301 isolated from Ghardaia [13](Table **2**).

The geographical distribution of zymodemes identified is illustrated in Fig. (**1**), and fully documented in Table **1**. The species isolated in the wilaya of Annaba was *L.killicki*. In the wilaya of El Tarf, *L. infantum* MON-1 was isolated from a cutaneous lesion in a patient who had been infected in the

Table 1. Clinical Data and Identification of the Strains Isolated in the Region of Annaba

PATIENT					STRAIN		
Age (Years)	**Sex**	**Organ of the lesion**	**Wilaya**	**Year**	**WHO code**	**Taxon**	**Zymodeme**
17	M	Eyelid	Annaba	2005	MHOM/DZ/2005/LPA47/05	*L.killicki*	MON-306
24	M	Cheek	El Taref	2006	MHOM/DZ/2006/LPA166/06	*L.infantum*	MON-1
08	M	Labial commissure	Skikda	2006	MHOM/DZ/2006/LPA7O/06	*L.major*	MON-25
19	M	Eyelid	Skikda	2007	MHOM/DZ/2007/LPA150/07	*L.infantum*	MON-24
30	M	Arm	Guelma	2006	MHOM/DZ/2006/LPA79/06	*L.infantum*	MON-24
25	F	Cheek	Guelma	2007	MHOM/DZ/2006/LPA57/06	*L.infantum*	MON-24
21	M	Arm	Guelma	2008	MHOM/DZ/2008/LPA04/08	*L.major*	MON-25
21	M	Hand	Guelma	2008	MHOM/DZ/2008/LPA05/08	*L.major*	MON-25
21	M	Neck	Guelma	2008	MHOM/DZ/2008/LPA15/08	*L.major*	MON-25
31	M	Hand	Souk-Ahras	2005	MHOM/DZ/2005/LPA52/05	*L.infantum*	MON-24
41	M	Arm	Souk-Ahras	2006	MHOM/DZ/2006/LPA157/06	*L.major*	MON-25
20	M	Front	Souk-Ahras	2007	MHOM/DZ/2007/LPA167/07	*L.infantum*	MON-24
40	M	Hand	Souk-Ahras	2008	MHOM/DZ/2008/LPA02/08	*L.major*	MON-25
73	M	Foot	Tebessa	2005	MHOM/DZ/2005/LPA12/05	*L.major*	MON-25
38	M	Leg	Tebessa	2007	MHOM/DZ/2005/LPA79/05	*L.infantum*	MON-1
32	M	Arm	Tebessa	2008	MHOM/DZ/2008/LPb03/08	*L.major*	MON-25

Table 2. Electromorphs of the MON-306 Zymodeme, and Comparison with the Different Zymodemes Identified within the *Leishmania Killicki* Taxon

Taxon	Zy-mode me	Enzymatic Profiles															WHO Code
		MD H	ME	ICD	PG D	G6 PD	GL UD	DIA	NP_1	NP_2	GO T_1	GO T_2	PG M	FH	MP I	GPI	
*L.killicki**	MON-8	100	100	100	93	82	110	100	300	100	127	90	100	100	110	76	MHOM/TN/80/LEM163*
L.killicki	MON-306	112	**100**	100	93	82	110	100	300	100	140	85	100	100	110	76	MHOM/DZ/2005/LPA47/05
L.killicki	MON-301	112	**93 66**	100	93	82	110	100	300	100	140	85	100	100	110	76	MHOM/DZ/2005/LIPA07

* Reference strain of *L. killicki* zymodeme MON-8.

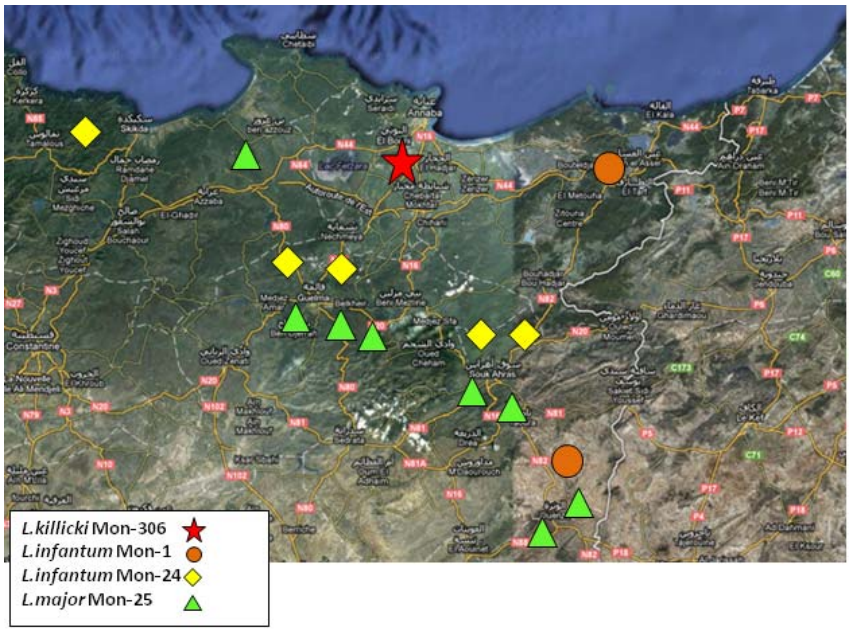

Fig. (1). Geographical distribution of strains isolated in the region Annaba.

town of Besbes. Two taxa were isolated in the wilaya of Skikda, *L. infantum* MON-24 and *L.major* MON-25.

Two species responsible for cutaneous form co-existed in the province of Guelma, *L.infantum* MON-24 and *L.major* MON-25. This supports the notion of the extension of ZCL to the North of the country. In the wilaya of Souk Ahras, both species *L. infantum* and *L. major* were simultaneously present.

The species *L. infantum* occurred in infected patients in the wilaya of Tebessa, which seems surprising considering the semi-arid character of this Sub-Saharan location. Indeed, among the three cutaneous strains identified, there is *L. infantum*. Moreover, it is the zymodeme MON-1 with a usual viscerotropic tropism (Table **1**).

4. DISCUSSION

Strain identification is a basic investigation useful for the knowledge of *Leishmania* foci and the understanding of the clinical outcome of leishmaniasis. The ecoepidemiological studies of leishmaniasis have made real progress thanks to the isoenzyme identification [20-24]. Identical clinical lesions may occur due to different species of *Leishmania,* which stresses the high interest of isoenzyme identification of the strains occurring in the area of Annaba [25]. In the present study, the region of Annaba was explored for the first time. Identification of isolates from patients infected in this area allowed us to type by isoenzyme electrophoresis 16 strains of *Leishmania* isolated from cutaneous forms.

As in other countries of North Africa, we were able to detect in the Annaba region the presence of three *Leishmania* species, namely *L. infantum*. *L.major* and *L.killicki*. In Algeria, the authors identified in previous work the two complexes *L.major* and *L. infantum,* but *L. killicki* remained unknown until its recent identification in the focus of Ghardaia (Southwestern Algeria) [13]. During a study on CL in Tunisia, Kallel *et al.* (2005) had also identified three species, *L. infantum* (MON-1 and MON-24), *L.major* (MON-25) and *L.killicki* (MON -8).

Looking closely at the strains isolated from cutaneous forms, we deduced that the two CL clinical forms, namely the SCL and ZCL, are prevalent in the area of Annaba. In fact, *L.major* responsible for the ZCL has been identified in the wilaya of Skikda, Guelma, Tebessa and Souk Ahras at the same time with *L. infantum,* responsible for SCL. Regarding El Tarf, one strain was identified, *L. infantum* responsible for the SCL. At Annaba, a new species, *L.killicki* zymodeme MON-306, was identified, responsible for the chronic cutaneous leishmaniasis (CCL).

SCL has been described in many Mediterranean countries [27-32]. It coexists geographically with canine leishmaniasis and the Mediterranean infantile visceral leishmaniasis, which are also due to zymodemes of the same taxon. In Algeria, this SCL form has also been called Northern cutaneous form, as it was first described by [33] and called by them "Clou de Mila" to distinguish it from the ZCL called "Clou de Biskra", and described in 1860 by Hamel. At that time, the causative agent, the vector and the reservoir were unknown. The answer to the first question relating to the causative agent was provided by Belazzoug *et al.*, in 1985, who isolated for the first time *L. infantum* MON-24. This classical agent of SCL remains the most common dermotropic variant zymodeme. Harrat *et al.* (1996) during a study at the Institute Pasteur of Algiers, have isolated 14 strains of *L. infantum* MON-24 from patients with SCL, compared to a single strain of *L. infantum* MON-1.

Zymodeme MON-24 was reported not only in the North African countries, Northern Tunisia [30,34] and Morocco [35], but also in some South European countries, South of France [25, 36], Italy [37], Spain [29] and Greece [31]. In Tunisia, Kallel *et al.* (2005) have isolated *L. infantum* MON-24 from cutaneous forms, together with 15 strains of *L.major* MON-25 and one of *L.killicki* MON-8. Also in Tunisia, Aoun *et al.* (2008) collected *L. infantum* MON-24 in 72.2% of cases from Northern SCL.

L. infantum MON-1 is the principal agent of Mediterranean VL [38]. However, it was isolated in cases of SCL in Algeria [3,32,39], in Tunisia [40], in France [25],in Spain [29], in Greece [31], in Italy [23] and in Portugal [41]. It has been described in Tunisia first in SCL by Aoun *et al.* (2000), with two strains against six of *L. infantum* MON-24. *L. infantum* MON-1 was also found in rare cutaneous forms in Biskra [3, 42] isolated it from dogs in Kabylie and Biskra. Identify [43] 91 strains of *L. infantum* MON-24 and 7 strains of *L. infantum* MON-1 from SCL lesions. Concerning the unexpected presence in Skikda (locality of Oued el Aneb) of the *L.major* species, typically responsible for ZCL of arid areas, it could be explained by a more important extension towards North of this form, in relation to the presence of the vector and rodent hosts and a change in eco-epidemiological conditions related to climate warming. This species we are reporting was isolated in 2006, but the patient contamination occurred in 2005, a year during which outbreaks of CL, including *L.major* ZCL, occurred in many regions of Algeria [1,6].

In our study, *L. infantum* and *L.major* were identified in Guelma and Souk Ahras wilayas which are of humid and sub-humid climates, favorable to *L. infantum* but currently not to *L.major,* which is usually prevailing in arid and semi-arid biotopes. In Tébessa also, where both species coexist, the presence of two distinct areas, the highlands to the North and the Sub-Saharan area to the South, could explain this coexistence. However, the assumption of the extension of the two species, from North to South for *L. infantum* and from South to North for *L. major,* is likely. In Tunisia, Kallel *et al.* (2005), also reported the presence of *L. infantum* MON-1 and MON-24 in Beja governorate bordering the Wilaya of El Tarf, in Jendouba governorate bordering the wilaya of Souk Ahras and in El Kef Governorate neighboring the two wilayas of Souk Ahras and Tebessa.

Concerning ZCL, it was formerly restricted to arid and semi-arid areas but an extension to the Highlands of Algeria has occurred. The species responsible, *L.major* MON-25, has spread from the old foci of Southern Algeria, namely Biskra in the East and Abadla in the West, to the neighboring regions. This species prevails in an endemic state in Northern Saharan areas and is responsible for epidemics. The only zymodeme isolated from this complex in the whole Maghreb, and particularly in Algeria, is MON-25.

In Tunisia, ZCL was also limited, for a long time, to the arid and semi-arid areas. It progressed initially towards the areas of the Tunisian Center [44] then in Béja and Jendouba (NorthTunisia) governorates bordering the two wilayas El Tarf and Souk Ahras. The authors link this increase to a migration of the rodents *Meriones* [26].

Concerning *L.killicki* species, responsible for CCL, it was isolated from a cutaneous lesion in a patient originating from the wilaya of Annaba. It is about a patient of male sex, 17 years old.The lesion localized to the eyelid was 1 cm in diameter. The strain identified proved to be *L.killicki* with a different profile from that of the Tunisian isolates which correspond to the MON-8.

L.killicki was discovered during an epidemic of cutaneous leishmaniasis in the South-East of Tunisia by Rioux *et al.*, 1986. This species was initially included within the *L.tropica* complex. It was specified as a phylogenetic complex distinct from *L.tropica* [45].

CCL due to *L.killicki* MON-8 is distributed in the Sub-Saharan arid areas of South Tunisia [14] and was found by Kallel *et al.*, 2005 in Gafsa (Center of Tunisia) probably reflecting a geographical spread. Haouas *et al.*,2005 and Aoun *et al.*, 2008 reported the presence of *L.killicki* outside its original foci (South-East) in areas of the ZCL and visceral leishmaniasis.

5. CONCLUSION

The coexistence of the *L. infantum*, *L.tropica*, *L.killicki* and *L.major* complexes in neighboring countries, Morocco and Tunisia and the recent identification of *L.killicki* in the foci of Ghardaia (Southwestern Algeria) and its present identification in the area of Annaba prove the need for further isolation and identification of strains for a better understanding of eco-epidemiological aspects in the areas affected by the disease.

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