LEISHMANIASIS IN CENTRAL AND SOUTHERN TUNISIA: CURRENT GEOGRAPHICAL DISTRIBUTION OF ZYMODEMES

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Summary:
The authors report the identification of *Leishmania* strains isolated from the Centre and the South of Tunisia. 266 strains were isolated between 1998 and 2006 from human (n = 221 strains) and dogs (n = 45 strains) hosts. The isoenzymatic identification exhibits the presence of in total five zymodemes belonging to three *Leishmania* complexes: *Leishmania infantum, L. major* and *L. killicki*. All strains isolated from human and canine visceral leishmaniasis belonged to *L. infantum*. zymodeme MON-1 was the only one isolated from canine visceral leishmaniasis. However, it is predominant in human visceral leishmaniasis beside zymodeme MON-24 which was detected in two provinces of the Centre (Monastir and Kairouan) and zymodeme MON-80 isolated for the first time in Kairouan province. Three complexes are responsible for human cutaneous leishmaniasis: *L. major* MON-25 is the parasite the most frequently found in its classic foci in the Centre and the South of the country. *L. infantum* MON-24 was isolated for the first time in a small locality of Sfax (southern Tunisia) showing the appearance of a new focus of *L. infantum*. *L. killicki* was isolated in its original focus of Tataouine and in two new foci of the central part of the country (Sidi Bouzid and Kairouan).

KEY WORDS : cutaneous leishmaniasis, visceral leishmaniasis, *Leishmania major, Leishmania infantum, Leishmania killicki*, Tunisia.

Résumé : LES LEISHMANIOSES AU CENTRE ET AU SUD DE LA TUNISIE : DISTRIBUTION GÉOGRAPHIQUE ACTUELLE DES ZYMODÈMES

Les auteurs rapportent l'identification de souches de Leishmania *isolées au centre et au sud de la Tunisie. 266 souches ont été isolées entre 1998 et 2006 à partir de l'homme (n = 221 souches) et du chien (n = 45 souches). L'identification isoenzymatique de ces isolats à permis de mettre en évidence la présence de cinq zymodèmes appartenant à trois complexes leishmaniens :* Leishmania infantum, L. major *et* L. killicki. *Tous les isolats provenant de patients atteints de leishmaniose viscérale ainsi que des chiens leishmaniens appartiennent au complexe* L. infantum. *Seul le zymodème MON-1 est isolé de chiens ayant la leishmaniose viscérale. Cependant, il est prédominant dans le cas de la leishmaniose viscérale humaine à côté du zymodème MON-24 qui a été isolé dans deux gouvernorats du centre (Monastir et Kairouan) et du zymodème MON-80 isolé pour la première fois dans la région de Kairouan. Trois complexes sont responsables de la leishmaniose cutanée humaine : le complexe* L. major *zymodème MON-25 est le parasite le plus fréquemment trouvé dans les foyers classiques de la leishmaniose cutanée zoonotique du centre et du sud tunisien. Le complexe* L. infantum *MON-24 est isolé pour la première fois dans une petite délégation du gouvernorat de Sfax au sud tunisien montrant ainsi l'apparition d'un nouveau foyer de* L. infantum. *Le complexe* L. killicki *responsable de la leishmaniose cutanée anthroponotique est isolé dans son foyer original à Tataouine ainsi que dans deux nouveaux foyers au centre du pays (Sidi Bouzid et Kairouan) signalant le début d'extension de ce complexe.*

MOTS CLÉS : *leishmaniose cutanée, leishmaniose viscérale,* Leishmania major, Leishmania infantum, Leishmania killicki, *Tunisie.*

INTRODUCTION

In Tunisia, three complexes of the *Leishmania (L.)* genus are responsible for visceral and cutaneous leishmaniasis disease. Each complex is located in a focus of the country: *i*) Sporadic cutaneous leishmaniasis (SCL) and Visceral leishmaniasis (VL) due to *L. infantum* occur in the North and the Centre of Tunisia (Bel Hadj *et al.*, 2000; Bel Hadj *et al.*, 2002a); *ii*) Zoonotic cutaneous leishmaniasis (ZCL) due to *L. major* complex is distributed in the Centre and the South of the country (Zahaf *et al.*, 1986); *iii*) However, Chronic cutaneous leishmaniasis (CCL) caused by *L. killicki* complex has a limited geographical area in the micro focus of Tataouine in southeast Tunisia (Rioux *et al.*, 1986b). Human CL and human VL have been known since 1884 and 1904 respectively (Deperet & Bobinet, 1884; Laveran & Cathoire, 1904). But a precise characterization of the parasite circulating in these foci started only in 1981 (Lanotte *et al.*, 1981). Since then, *L. infantum* foci of the North of Tunisia have been intensively stu-

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died by various authors at Institut Pasteur of Tunisia and the Parasitological laboratory "Etablissement Publique de Santé" (EPS) La Rabta Tunis (Aoun *et al.*, 2000; Bel Hadj *et al.*, 2002b). However, ZCL and CCL foci of the Centre and the South have been rarely studied and no recent data have been published concerning these foci. Indeed, the latest study carried on CL in Tunisia, has included 71 strains which is the biggest number of identified CL strain until now, but only 12 studied strains originated from the Centre and the South of Tunisia (Kallel *et al.*, 2005). This little number of strains does not represent the epidemiological feature of leishmaniasis in these central and southern foci. The present report aims to complete the epidemiological studies of leishmaniasis in the remaining parts of the country and to update the result of characterization of these strains. The systematic identification of *Leishmania* strains should make possible to understand the epidemiological feature of the parasite in these endemic foci.

MATERIALS AND METHODS

Strains studied

266 strains were isolated between 1998 and 2006 in different endemic regions of the Centre (Monastir, Mahdia, Kairouan and Sidi Bouzid) and the South of the country (Sfax, Gafsa, Tataouine, Kasserine and Tozeur) (Table I). The strains were obtained from 37 cases of human visceral leishmaniasis, 184 cases of human cutaneous leishmaniasis and 45 cases of canine visceral leishmaniasis. 21 of the human VL isolates were from bone marrow and 16 from peripheral blood.

All strains obtained from human CL were isolated from skin. However, strains obtained from canine VL were isolated from popliteal lymphe nodes (n = 11 isolates), from blood (n = 9 isolates), from bone marrow (n = 9 isolates), from spleen (n = 9 isolates) and from liver (n = 7 isolates).

The collection of human strains (n = 221 isolates) has been made within the context of diagnostic investigation in laboratories hospitals in Tunisia. In fact, 67 strains came from EPS Fattouma Bourguiba Monastir, 86 from Centre d'Hygiène de Sfax, 53 from Laboratoire de Parasitologie Institut Pasteur de Tunis and 15 from EPS Farhat Hached de Sousse. However, canine strains (n = 45 isolates) were obtained from field epidemiological studies (18 isolates) and from veterinary diagnostic services (27 isolates).

Reference strains

The four reference strains of *Leishmania* used in this study were provided by the Centre National de Réfé-

	Zymodemes (number of strains)			
	Human leishmaniasis		**Canine leishmaniasis**	
Centre	**VL***	**CL****	**VL***	**Total**
Monastir	*L. infantum* MON-1 (14) *L. infantum* MON-24 (02)	*L. major* MON-25 (07)	*L. infantum* MON-1 (24)	47
Mahdia		*L. major* MON-25 (12)	–	12
Kairouan	*L. infantum* MON-1 (12) *L. infantum* MON-24 (08) *L. infantum* MON-80 (01)	*L. major* MON-25 (38) *L. infantum* MON-24 (01) *L. killicki* MON-8 (01)	*L. infantum* MON-1 (03)	64
Sousse	–	*L. major* MON-25 (06)	–	06
Sidi Bouzid	–	*L. major* MON-25 (28) *L. killicki* MON-8 (01)	–	29
South			–	
Sfax	–	*L. major* MON-25 (63) *L. infantum* MON-24 (02)	*L. infantum* MON-1 (18)	83
Gafsa	–	*L. major* MON-25 (07)	–	07
Tozeur	–	*L. major* MON-25 (03)	–	03
Tataouine	–	*L. major* MON-25 (02) *L. killicki* MON-8 (07)	–	09
Kébilli	–	*L. major* MON-25 (03)	–	03
Kasserine	–	*L. major* MON-25 (03)	–	03
Total	37	184	45	266

* VL: visceral leishmaniasis; ** CL: cutaneous leishmaniasis.

Table I. – Result of the identification of strains according to host, clinical form and focus.

rence des *Leishmania* Montpellier, France: MHOM/FR/78/LEM75, *L. infantum* MON-1, MHOM/DZ/82/LIPA59, *L. infantum* MON-24, MHOM/MA/81/LEM265, *L. major* MON-25 and MHOM/TN/LEM163, *L. killicki* MON-8.

METHODS

The strains were isolated by culture on Nicolle-Novy-MacNeal (NNN) medium and characterized by the iosenzymatic technique using starch gel electrophoresis according to Rioux *et al.*, 1990. The following 15 enzyme systems were studied: malate dehydrogenase (MDH, EC 1.1.1.37), malic enzyme (ME, EC 1.1.1.40), isocitrate dehydrogenase (ICD, EC 1.1.1.42), phosphogluconate dehydrogenase (PGD, EC 1.1.1.44), glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49), glutamate dehydrogenase (GLUD, EC 1.4.1.3), diaphorase NADH (DIA, EC 1.6.2.2), nucleoside purine phosphorylases 1 and 2 (NP1, EC 2.4.2.1. and NP2, EC 2.4.2.*), glutamate oxaloacetate transaminases 1 and 2 (GOT1 and GOT2, EC 2.6.1.1), phosphoglucomutase (PGM, EC 5.4.2.2), fumarate hydratase (FH, EC 4.2.1.2), mannose phosphate isomerase (MPI, EC 5.3.1.8) and glucose phosphate isomerase (GPI, EC 5.3.1.9).

RESULTS

During eight years, 266 strains were isolated from provinces of the Centre and the South of the country and were identified by isoenzymatic method. Three complexes and five zymodemes were present (Table I).

For the 184 strains isolated from human CL, three complexes were identified: as *L. major* zymodeme MON-25 (n = 172 strains, 93 %), *L. killicki* MON-8 (n = 09 strains, 5 %) and *L. infantum* MON-24 (n = 3 strains, 2 %). All isolates from human and canine visceral leishmaniasis belonged to *L. infantum* complex.

LEISHMANIA INFANTUM COMPLEX

Human visceral leishmaniasis

Isoenzymatic identification of 37 isolates from bone marrow or peripheral blood of patients revealed three zymodemes of *L. infantum*: zymodeme MON-1 is predominant (n = 26 strains, 70 %). The second zymodeme is the dermotropic one MON-24 (n = 10 strains, 27 %). However, *L. infantum* MON-80 is sporadic with a single strain (3 %).

All strains were isolated from patients originating from two central provinces: Monastir and Kairouan. *i)* In Monastir province, 16 human strains were characterized. Among them, 12 were from child between one and three years old. The four other were from three adult of 36, 39 and 76 years old. All strains belonged to the same complex *L. infantum*: 14 were *L. infantum* MON-1 and two were *L. infantum* MON-24. These two strains were isolated from two children coming from Ouardanine and Moknine localities. *ii)* In Kairouan province, 21 strains were obtained from children with VL. Their age ranged between one and three years old. 12 strains were identified as *L. infantum* MON-1 (57 %), eight strains were identified as *L. infantum* MON-24 (38 %) and one strain as *L. infantum* MON-80 (5 %) (Fig. 1).

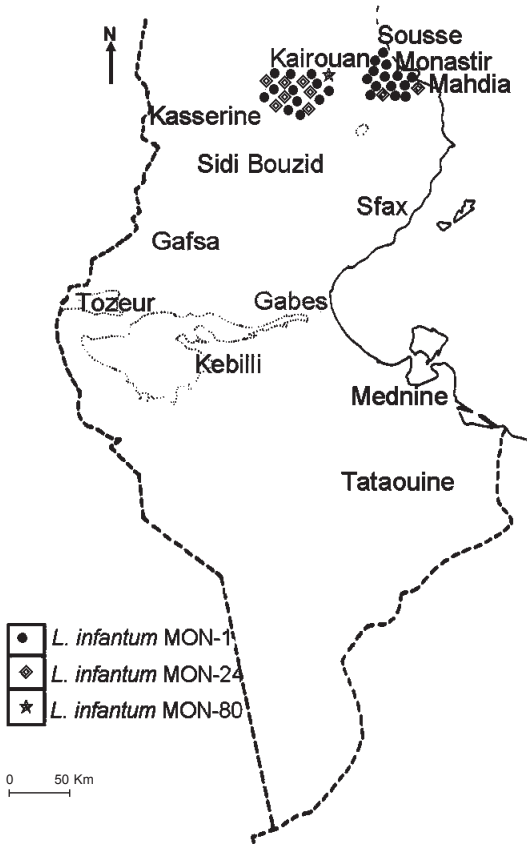

Fig. 1. – Geographical distribution of *Leishmania* strains isolated from human visceral leishmaniasis cases, according to their zymodemes.

Human cutaneous leishmaniasis

Three isolates belonging to *L. infantum* complex were obtained from patients with CL from the Centre and the South part of Tunisia (Fig. 2): out of them, one was originating from Kairouan which is a neighboring province to Seliana, the classical focus of SCL. The two others strains were surprisingly isolated from two patients originated from M'Satria district of Sfax (Southern Tunisia). This complex was never been described in this geographical arid region. All these strains were identified as *L. infantum* zymodeme MON-24. This zymodeme was classically described to be the causative agent of sporadic cutaneous and visceral leishmaniasis in Northern Tunisia.

Canine visceral leishmaniasis

45 strains of *Leishmania* were isolated from canine VL. All of them come from canine VL and no one from

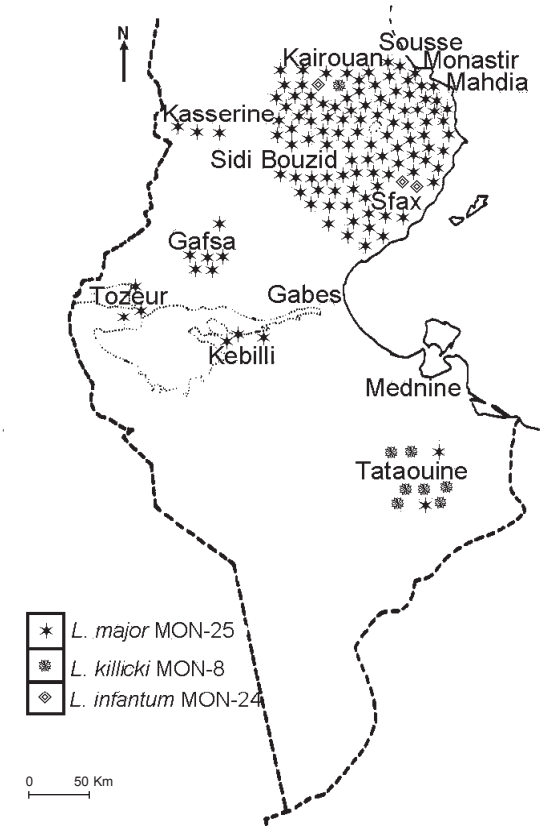

Fig. 2. – Geographical distribution of *Leishmania* strains isolated from human cutaneous leishmaniasis cases, according to their zymodemes.

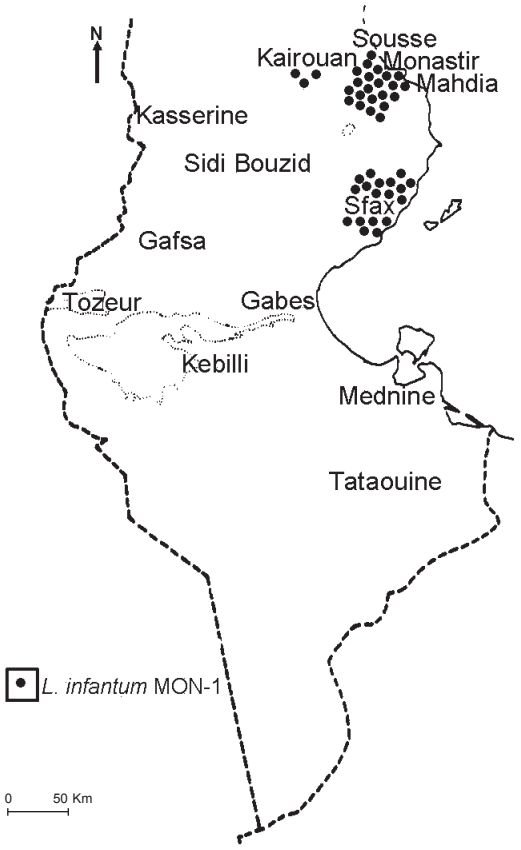

Fig. 3. – Geographical distribution of *Leishmania infantum* MON-1 strains isolated from canine visceral leishmaniasis.

canine CL. Among of these isolates, eighteen were obtained from field epidemiological investigations in Sfax province in South East Tunisia (n = 18 strains). The 27 other strains come from Monastir and Kairouan delegations in the Centre and were collected from veterinary diagnostic services. All these isolates belonged to *L. infantum* with the single zymodeme MON-1 (Fig. 3).

LEISHMANIA MAJOR COMPLEX

Human cutaneous leishmaniasis

184 strains from cutaneous leishmaniasis were studied; of which, 94 strains were collected from central Tunisia and 90 from South part of the country (Fig. 2). Out of these strains, 172 strains (93 %) belonged to *L. major* zymodeme MON-25. As expected, these strains originated from the main endemic foci of ZCL: Kairouan province (38 strains, 22 %), Sfax province (63 strains, 37 %), Sidi Bouzid province (28 strains, 16 %), Mahdia (12 strains, 7 %), Gafsa (seven strains, 4 %) and Monastir (seven strains, 4 %).

Few other isolates were collected from other regions such as Sousse (six isolates), Tataouine (two isolates), Tozeur (three isolates), Kebilli (three isolates) and Kasserine (three isolates) (n = 17 strains, 10 % together).

LEISHMANIA KILLICKI COMPLEX

Out of the 184 strains isolated from human CL, nine strains were characterized as *L. killicki* MON-8. This zymodeme was already described to be the causative agent of chronic CL which has a limited geographical area in the micro focus of Tataouine Southeast Tunisia. Two strains of this zymodeme were obtained from new foci outside Tataouine: one strain from Kairouan and one from Meknassy (delegation of Sidi bouzid). The seven others strains were isolated from patients originating and living in Tataouine province.

DISCUSSION

266 strains is the current biggest number of strains identified in Tunisia. Indeed, many studies were carried on the prevalence of cutaneous and visceral leishmaniasis in Tunisia but the characterization of the parasite was hardly ever made (Ben Salah *et al.*, 2000; Kharfi *et al.*, 2004). Previously, 65 was the largest number of identified VL strains. The majority of these studied strains originated from eight provinces of northern Tunisia (Belhadj *et al.*, 2002). For the cuta-

neous leishmaniasis, the most exclusive study has analysed 71 strains of *Leishmania*. The majority of them were isolated from the North part of the country (Kallel *et al*., 2005). So leishmaniasis disease in Centre and South of Tunisia was rarely investigated and no recent data on the characterization of the parasite were published. This prospective study updates the result of identification of *Leishmania* in the Centre and the South of Tunisia during the last eight years.

The identification of the 266 strains (from human and canine hosts and from different origins) allowed to individualize three *Leishmania* complexes responsible of CL and a single one for VL.

LEISHMANIA INFANTUM FOCI

Human visceral leishmaniasis

Tunisia has been considered as an endemic country for VL disease since the description of the first case in 1904 (Laveran & Cathoire, 1904). Since then, this form has not stopped to increase in incidence: 11 cases were registered in pediatrics department in 1970 against 16 cases in 1978 (Ben Rachid *et al*., 1983).

Then, an outbreak occurred in 1980 with 21 cases in the "Hôpital d'Enfant" of North Tunisia and in 1988 with 16 cases in the same hospital (Khaldi *et al*., 1991). The last census was reported in 1993 with 150 cases per year (Bouratbine *et al*., 1998).

Initially, VL was distributed in the North of Tunisia. It covered all humid and subhumid biolimatic zones. After a few years, transmission area of this leishmaniasis form has outstandingly extended toward the Centre and the South of Tunisia but no identification of the causative agent species was done (Ayadi *et al*., 1991; Ben Salah *et al*.2000).

37 strains of VL were isolated from patients originating from the Centre of Tunisia. About 90 % of them came from children between one and three years old. The infantile feature of the disease is the same of northern Tunisia which indicate the continuity of this focus from the North to the Centre of the country.

Three enzymatic variants of *L. infantum* complex were isolated from immunologically competent patients: *L. infantum* MON-24, *L. infantum* MON-80 and *L. infantum* MON-1. The latter was predominant (70 % of VL strains) as well as in other Mediterranean countries such as Algeria and South France (Harrat *et al*., 1996; Pratlong *et al*., 2004). The 26 strains, belonged to *L. infantum* MON-1, are distributed in two central provinces: 12 strains were from Kairouan and the 14 other were from Monastir province. Previously, this governorate was never been concerned by this leishmaniasis form and no case was reported. Since few years, sporadic cases were declared in EPS Fattouma Bourguiba Monastir and the emergence of this leishmaniasis form seems to be the consequence of the establishment of a new stable *Leishmania infantum* transmission cycle. The maintenance of this cycle could be helped by the existence of intensive agriculture in the rural areas of this province.

In spite being the main agent of VL in immunocompetent children, zymodeme MON-1 was also responsible for VL in immunosupressed patients with HIV infection in France and Spain (Pratlong *et al*., 1995). Here, we report four strains of *L. infantum* MON-1 isolated from three HIV negative adults of 36, 39 and 65 years old. Among these three patients, one was suffering from a chronic kidney disease.

Zymodeme MON-24 was previously considered to be a dermotropic zymodeme causative of sporadic CL in northern Tunisia (Aoun *et al*., 2000). In 1991, this zymodeme was isolated for the first time from a child with VL in the North (Gramiccia *et al.* 1991) than confirmed by Belhadj *et al*., in 2000.

In this report, we identify two new cases of *L. infantum* MON-24 in the Monastir province and eight other cases in Kairouan province.

Finally, the only case of *L. infantum* MON-80 was also isolated for the first time in the Kairouan Province. Until now, only four cases of *L. infantum* MON-80 were described in Tunisia (Aoun *et al*., 2001; Belhadj *et al*., 2002). All cases were reported in the North part of the country including Zaghouan province which has frontier with Kairouan province. This could confirm the geographical spread of this zymodeme to the neighboring area. This spread could be enhanced by the migration of the reservoir which is still unknown.

In total, 21 strains of VL, belonged to the three zymodemes of *L. infantum* complex, were isolated from patients originating from Kairouan province. This makes Kairouan province an interesting focus which assembles the three zymodeme of *L. infantum* behind *L. major* complex. The establishment of the transmission cycle of the two complexes would be in part the consequence of the building of the new dam last few years. This new source of water and organic materials would increase the *phlebotomus* population density the vector of *Leishmania*.

Human cutaneous leishmaniasis

In Tunisia, CL due to *L. infantum* is qualified as a sporadic disease located in the northern part of the country. Two zymodemes were reported in this form of leishmaniasis: zymodeme MON-24 was described as a predominant agent of sporadic CL in Seliana province in the North (Bel Hadj *et al*., 2002a). It is also encountered in neighboring countries such as Algeria and Morocco (Gramiccia *et al*., 1991; Harrat *et al*., 1996). However, the zymodeme MON-1 was isolated for the first time in 1999 in the North (Aoun *et al*., 2000).

Among the studied isolates, three strains were identified as *L. infantum* MON-24: one was isolated from a

patient originating from Kairouan. This province has frontier with the classical focus of this complex, Seliana. However, the two others strains were unexpectedly isolated from M'Satria district in Sfax province. This region belongs to the arid bioclimatic level where vector species of this complex could not exist. A thoroughly investigation of the history travel of the two patients shows that they had never left this region. So by reporting these two cases, we provide evidence for the presence of this zymodeme in a new focus and the geographical emergence of the MON-24 zymodeme in the south of Tunisia. This preliminary result must be continued by making other entomological and ecological studies in this new focus.

Canine visceral leishmaniasis

Conversely to the anthroponotic VL in India (Ross 1903), Mediterranean countries including Tunisia have a zoonotic type. In fact, in Tunisia, domestic dog has been incriminated in VL since the report of the first case of infected dog in 1908 (Nicolle & Comte, 1908). 45 strains of canine VL were studied. Out of them, 18 strains were obtained during epidemiological investigations carried out in the region of Sfax and 27 others were collected from veterinary service in the context of diagnosis.

Among canine VL strains, 18 were coming from Sfax region in the southern part of the country where no human VL case was isolated. The canine strains were isolated from infected dog originated from localities neighboring to endemic central foci of VL. This finding showed that VL exists in this region but the absence of data regarding human VL may be the consequence of the insufficient relationships with local practitioners in Sfax region.

All these strains were identified as *L. infantum* zymodeme MON-1. This confirms the role of domestic dog as the exclusive reservoir host of human VL due to the zymodeme MON-1 of *L. infantum* complex. This result was already reported in others Mediterranean countries as Spain (Martin-Sanchez *et al.*, 2004). However, other zymodeme of *L. infantum* complex were also isolated from dogs such as MON-77 in the Catalan focus in Spain (Gallego *et al.*, 2001) and MON-108 in Provence France (Pratlong *et al.*, 2004).

LEISHMANIA MAJOR FOCI

Leishmania major zymodeme MON-25 was described to be the causative agent of ZCL in Tunisia and in other Maghreb's countries as Morocco and Algeria (Harrat *et al.*, 1996; Rioux *et al.*, 1990).

This form of leishmaniasis is widely distributed in the central and southern part of the country covering the arid and semi arid bioclimatic zones. The first case was described in 1884; since then ZCL did not stop progressing: between 2000 and 2002, four new cases were reported in the North part of Tunisia which is a non endemic focus of this leishmaniasis form (Kallel *et al.*, 2005).

Life cycle of the causative agent *L. major* was clearly elucidated with *Phlebotomus (P.) papatasi* as the vector (Rioux *et al.*, 1986a) and *Psammomys obesus* and *Meriones shawi* as reservoir rodents (Ben Hamou *et al.*, 2006). As expected, in our study all *L. major* strains were distributed in the Centre and the South of Tunisia. The most number of strains were located in Kairouan, Sidi Bouzid and Sfax regions. All these regions belonged to the arid and per arid bioclimatic levels where rodents and *P. papatasi* are wildly spread and a stable transmission cycle is already present.

LEISHMANIA KILLICKI FOCI

Leishmania killicki was described for the first time in 1980 during an investigation in the micro focus of Tataouine south eastern Tunisia (Rioux *et al.*, 1986b). Since this date and for many years, no strains belonging to this zymodeme has been isolated out side this region.

In 2004, three strains were isolated in three new foci, Seliana, Sidi Bouzid and Gafsa showing the beginning of spread of this zymodeme to the south western and the northern part of Tunisia (Haouas *et al.*, 2005). In the same year, three other strains were isolated in Oueslatia district of Kairouan province in central Tunisia and were characterized as *L. killicki* proving this extension of the anthroponotic CL (Bouratbine *et al.*, 2005).

During our study, we identify two strains in new foci outside Tataouine province: Sidi Bouzid and Kairouan. These areas belong to different vegetation level. This finding proves one more time the extension of this focus which must be investigated by ecological study of this new area.

CONCLUSION

This report provides additional data for the studying of cutaneous and visceral leishmaniasis in the Centre and the South of Tunisia. The inclusion of such a number of strains in this study had allowed concluding to the conservation of geographical distribution of *L. major* foci, the extension of the *L. infantum* foci and the emergence of a new focus of *L. infantum* MON-24 in Sfax province.

Surely, this study brings more data for the knowledge of the spatial and temporal geographical distribution evolution of the existing *Leishmania* foci but it is not enough to elucidate the transmission cycle of each zymodeme. More investigations concerning the phle-

botomine species cartography and the study of the sand flies host preference will be very useful to response to the remains questions.

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