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ORIGINAL ARTICLE

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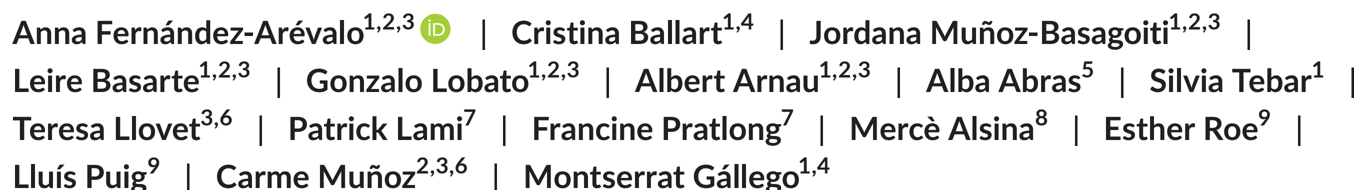
Autochthonous and imported tegumentary leishmaniasis in Catalonia (Spain): Aetiological evolution in the last four decades and usefulness of different typing approaches based on biochemical, molecular and proteomic markers



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Abstract

Leishmaniasis is a transmissible disease caused by *Leishmania* protozoa. Spain is endemic for both visceral and cutaneous leishmaniasis, the autochthonous aetiological agent being *Leishmania infantum*. Around the world, the *L. donovani* complex is associated with visceral symptoms, while any species of the *Leishmania* or *Viannia* subgenera affecting human can produce tegumentary forms. In a context of growing numbers of imported cases, associated with globalisation, the aim of this study was to analyse the aetiological evolution of human tegumentary leishmaniasis in a region of Spain (Catalonia). Fifty-six *Leishmania* strains, isolated from 1981 to 2018, were analysed using MLEE, gene sequencing (*hsp70*, *rpoIILS*, *fh* and ITS2) and MALDI-TOF. The utility of these different analytical methods was compared. The results showed an increase in leishmaniasis over the two last decades, particularly imported cases, which represented 39% of all cases studied. *Leishmania infantum*, *L. major*, *L. tropica*, *L. braziliensis*, *L. guyanensis* and *L. panamensis* were identified. The combination of molecular and enzymatic methods allowed the identification of 29 different strain types (A to AC). Strain diversity was higher in *L.* (*Viannia*), whilst the different *L. major* types were relatable with geo-temporal data. Among the autochthonous cases, type C prevailed throughout the studied period (39%). Minor types generally appeared within a short time interval. While all the techniques provided identical identification at the species complex level, MALDI-TOF and *rpoIILS* or *fh* sequencing would be the most suitable identification tools for clinical practice, and the tandem *hsp70*-ITS2 could substitute MLEE in the epidemiological field.

Carme Muñoz and Montserrat Gállego should be considered joint senior author.

[Correction added on 25 June 2021, after first online publication: The copyright line was changed.]

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KEYWORDS
aetiological evolution, characterisation methods, imported cases, Spain, tegumentary leishmaniasis

1 | INTRODUCTION

Human leishmaniasis, present in 98 countries around the world, is caused by *Leishmania* species (World Health Organization, 2020). Cutaneous leishmaniasis (CL) has a wide variety of clinical manifestations, ranging from a small dermatosis on the inoculation point, usually with spontaneous recovery, to skin lesions of variable appearance that may lead to mucocutaneous and lymphatic dissemination (Burza et al., 2018; Reithinger et al., 2007). Other manifestations include diffuse CL, disseminated CL and leishmaniasis recidivans, as well as numerous atypical forms (Meireles et al., 2017; Reithinger et al., 2007). While visceral leishmaniasis (VL) is mainly associated with *Leishmania donovani* "senso lato", human CL and its variant mucocutaneous leishmaniasis (MCL), both included in the term tegumentary leishmaniasis (TL), can be caused by nearly 20 *Leishmania* species of the subgenera *Leishmania* and *Viannia* (Van der Auwera & Dujardin, 2015; World Health Organization, 2010). The main causative agents of TL in the Old World (Europe, Africa and Asia) belong to the species complexes *L. donovani*, *L. tropica* and *L. major*, and in Latin America to *L. braziliensis*, *L. guyanensis* and *L. mexicana* (Akhoundi et al., 2017; World Health Organization, 2010). In Spain, the only autochthonous species involved in a zoonotic epidemiological cycle is *L. infantum*, included in the *L. donovani* complex (Fernández-Arévalo et al., 2020; Gállego et al., 2001; Jiménez et al., 1995; Martín-Sánchez, Gramiccia, et al., 2004). As in other foci of the Mediterranean region, *L. infantum* causes both VL and CL, including non-typical forms, and very rarely results in mucosal involvement (Aliaga et al., 2003; Alvar et al., 1997; Chicharro et al., 2003; Merino-Espinosa et al., 2018; Pratlong et al., 2013; Cortés et al., 1997). The global incidence of the disease in Spain in 2018 was 0.64 cases/100,000 habitants (Centro Nacional de Epidemiología, 2019). In the last decades, globalisation and migration have altered the classical geographical distribution of *Leishmania* species and imported cases are becoming increasingly common (Giavedoni et al., 2015; Pérez-Ayala et al., 2009). Lesion evolution and treatment effectiveness vary in each particular case, being mainly influenced by the immunological response of the patient as well as the species involved (Bañuls et al., 2011; World Health Organization, 2010). As microscopical examination does not allow discrimination between *Leishmania* species, various techniques based on biochemical, molecular or proteomic markers have been developed as identification tools (Akhoundi et al., 2017; Van der Auwera & Dujardin, 2015). Multilocus enzyme electrophoresis (MLEE), a classic technique based on the evaluation of metabolic enzyme phenotypes (Rioux et al., 1990), has been considered the gold standard for typing purposes (World Health Organization, 2010), although it is currently available only in a few laboratories. In contrast, molecular-based techniques, mainly PCR-RFLP and PCR-sequencing, are well established in laboratories worldwide (Da Silva et al., 2010; De Almeida et al., 2011; Mirahmadi et al., 2018; Van der Auwera et al., 2014) due to their relatively low cost, and because the results are easy to interpret and compare between laboratories (Van der Auwera & Dujardin, 2015). The targets of these methodologies vary considerably, the most common being ribosomal proteins, heat-shock proteins, kinetoplast regions, intergenic regions and other conserved genes (Akhoundi et al., 2017; Van der Auwera et al., 2016; Van der Auwera & Dujardin, 2015). Lately, matrix-assisted laser desorption/ionisation – time of flight mass spectrometry (MALDI-TOF MS) has also been applied for species identification, due to its speed and simplicity (Lachaud et al., 2017; Mouri et al., 2014).

The aim of this study was to evaluate the utility of different techniques and markers for the identification of *Leishmania* strains stored in the Trypanosomatid Cryobank of the Universitat de Barcelona (UB) that have caused TL in Catalonia (NE Spain), and to analyse the aetiological evolution of TL in the last four decades.

2 | METHODS

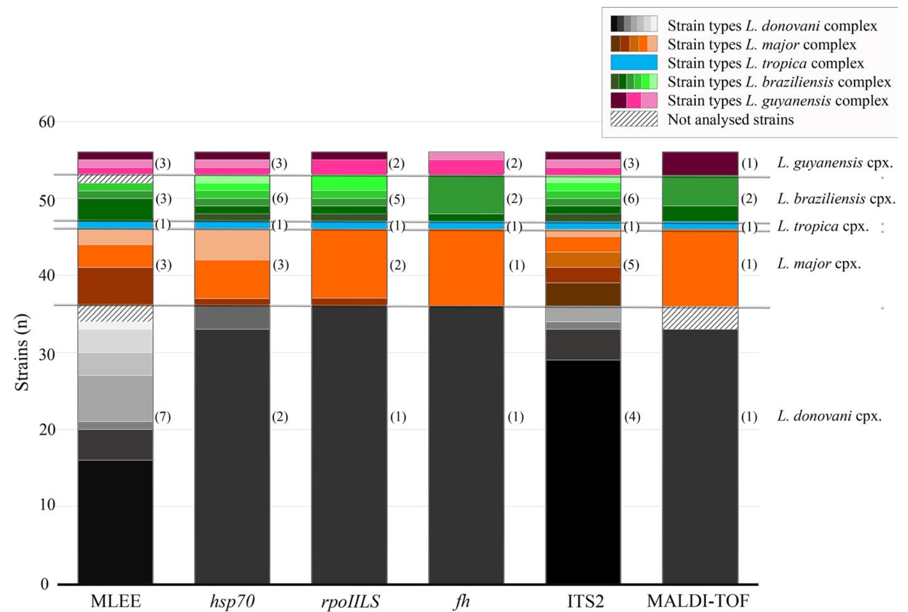
2.1 | Strains

Fifty-six strains isolated from patients with CL or MCL in Catalan hospitals from 1981 to 2018 were selected from the UB Trypanosomatid Cryobank. As all the strains had previously been identified by one of the techniques used in the current study, they were analysed with whichever methods not previously applied. All strains were thawed and cultured in Schneider's insect medium supplemented with 20% foetal bovine serum and 1% sterile human urine until the exponential growth phase, when they were analysed. All the available epidemiological and clinical information was collected.

2.2 | PCR-sequencing

DNA from the cultured strains was obtained by the Qiamp DNA Mini Kit (Qiagen) following the manufacturer's instructions. Four different regions were amplified and sequenced: heat-shock protein 70 (*hsp70*), RNA polymerase II largest subunit (*rpoIILS*), internal transcribed spacer 2 (ITS2) and fumarate hydratase (*fh*). For *hsp70*, *rpoIILS* and ITS2, primers and cycling conditions previously described by Van der Auwera et al., (2013), Ravel et al., (2006) and El Tai et al., (2001), respectively, were used. For *fh*, the forward primer described in Zemanová et al., (2007) was used for the *Leishmania* subgenus. An alternative forward primer was designed for the *Viannia* subgenus (fhnm_F, 5′-TCGTCTTCGTCTTCTGTGC-3′),

FIGURE 1 *Leishmania* complex identification by MLEE, PCR-sequencing, and MALDI-TOF. Different tonalities indicate different types within each complex. The number of types per complex are bracketed. Cpx, complex



as well as a new reverse primer for both subgenera (fhint_R, 5′-GGCAATGAAGAGGAACTCGTA-3′). Amplification conditions were an initial denaturalisation step of 5 min at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 57°C and 1 min at 72°C and a final elongation step of 10 min at 72°C. All the amplicons were enzymatically purified using EXOSAP-IT (Affymetrix USB) and double-strand sequenced at the Scientific and Technologic Centres of the UB by the Sanger method. The sequences obtained were edited using Bionumerics software (Applied Maths, version 7.6.3) and uploaded to GenBank under the accession numbers MT497923-MT497978 and MT498854-MT499021. For the analysis, sequences were aligned and compared in MEGA X software (Kumar et al., 2018). For the ITS2, each complex was analysed separately. A neighbour joining (NJ) tree was constructed from the concatenation of *hsp70*, *rpoIILS* and *fh* genes using the p-distance substitution method and a bootstrap of 1,000 replicates.

2.3 | MLEE

Protein extracts were obtained from mass cultures as previously described under the aforementioned conditions (Piarroux et al., 1994). MLEE was performed on 12 or 15 enzymes, depending on the previous identification result or the possible origin of the strains. For Old World strains, MLEE was performed on a total of 15 enzymes [malate dehydrogenase (MDH) E.C 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, NADH diaphorase (DIA) EC 1.6.2.2, nucleoside phosphorylase purine (NP) −1 and 2- EC 2.4.2.1, glutamate oxaloacetate transaminase (GOT) −1 and 2- EC 2.6.1.1, phosphoglucomutase (PGM) EC 2.7.5.1, fumarate hydratase (FH) 4.2.1.2, mannose phosphate isomerase (MPI) EC 5.3.1.8 and glucose phosphate isomerase (GPI) EC 5.3.1.9], following a previously described procedure (Rioux et al., 1990). For New World strains, MDH, ICD and GLUD enzymes were excluded (Chouicha et al., 1997). Protein extracts were run on starch gels (7 cm, 90 V) at different pH's (7.4, 8.6, 9.4) with or without coenzymes (NAD, NADP) and then revealed, using the conditions suitable for each enzyme. Migration distances were measured and compared with those from marker strains to assign the corresponding electromorph and zymodeme. The isoelectric focusing technique was occasionally used to confirm the NP_1 mobility (Piarroux et al., 1994).

2.4 | MALDI-TOF

Six replicate samples of each strain were prepared as previously described (Lachaud et al., 2017). Mass spectra were obtained with the AutoFlex II MALDI TOF/TOF instrument and the Flex Control version 3.4 software (Bruker Daltonics) on default parameters and submitted to the mass-spectral identification (MSI) application available on https://biological-mass-spectrometry-identification.com/msi/. The species result that predominated among the replicates was considered as the strain identification, and in case of a tie, the species with the best score was chosen.

3 | RESULTS

3.1 | Strain identification

The four genomic regions were sequenced in all the strains, whereas MLEE and MALDI-TOF analyses were only performed in 53 of the 56 selected strains due to culture issues. For three *L. major* strains, the ITS2 sequences had zones of poor quality; however, discriminative regions were sufficiently clear to be

TABLE 1 Enzymatic profile of the new zymodemes

Taxon	MON	ME	PGD	G6PD	DIA	NP1	NP2	GOT1	GOT2	PGM	FH	MPI	GPI	Strain WHO code
L. guyanensis	327	87	70	106	50	800	100	145	120	108.5	87	70[a]	87	MHOM/EC/2002/BCN-545
L. braziliensis	328	87	60[b]	90	50	380	112	132	125	109.6	82	70	87	MHOM/GT/2005/BCN-717
L. panamensis	329	87	45	103	50	0	100	145	90[a]	108.5	87	60	87	MHOM/PA/2014/BCN-859
L. guyanensis	330	87	45	103	50	800	100	148	118	108.5	87	60	87	MHOM/EC/2016/BCN-885

[a]New mobilities at taxon level.
[b]New mobility at genus level.

included in the analysis. All the methodologies coincided 100% in the identification at the species complex level (Figure 1), but below that, there were variations depending on the discrimination power of each technique. Thus, by consensus identification, the studied strains belonged to six different taxa: *L. infantum* ($n = 36$), *L. major* ($n = 10$), *L. tropica* ($n = 1$), *L. braziliensis* ($n = 6$), *L. guyanensis* ($n = 2$), and *L. panamensis* ($n = 1$) (Figure 1). All the complexes were represented by one species except *L. guyanensis* (*L. guyanensis* and *L. panamensis*).

Multilocus enzyme electrophoresis grouped strains in a total of 17 zymodemes, 11 of which belonged to the *Leishmania* subgenus and 6 to the *Viannia* subgenus (Figure 1). Intra-complex variability was due to different mobilities of three and up to five enzymes. Four *L.* (*Viannia*) strains (one *L. braziliensis*, two *L. guyanensis* and *L. panamensis*) presented new zymodemes. The new isoenzyme profiles were different combinations of known electrophoretic mobilities or new electromorphs for the taxa. The new zymodemes, coded from MON-327 to MON-330, are given in Table 1.

Molecular techniques showed nineteen, fifteen, eleven and seven different sequence types for ITS2, *hsp70*, *rpoIILS* and *fh*, respectively (Figure 1). Although nucleotide transitions and transversions between different complexes were observed, interspecific variation was mainly due to the presence of heterozygous alleles or, in the case of ITS2, diverse repetition of the microsatellite regions (Table 2). The latter resulted in different lengths for the ITS2 amplicons among the different complexes, but also among strains of the same taxa. Complexes of the *Leishmania* subgenus (*L. donovani*, *L. major* and *L. tropica* complexes) showed more intra-complex nucleotide differences compared to those of the *Viannia* subgenus (*L. braziliensis* and *L. guyanensis* complexes). Below the complex level, species of the subgenus *Viannia* had more inter-strain variability in nucleotide positions or regions than the species of the *Leishmania* subgenus. Consequently, in the NJ tree produced from the concatenation of the *hsp70*, *rpoIILS* and *fh* gene, greater distances were observed between the complexes of the *Leishmania* subgenus, whereas the *L.* (*Viannia*) complexes appeared more diversified (Figure 2).

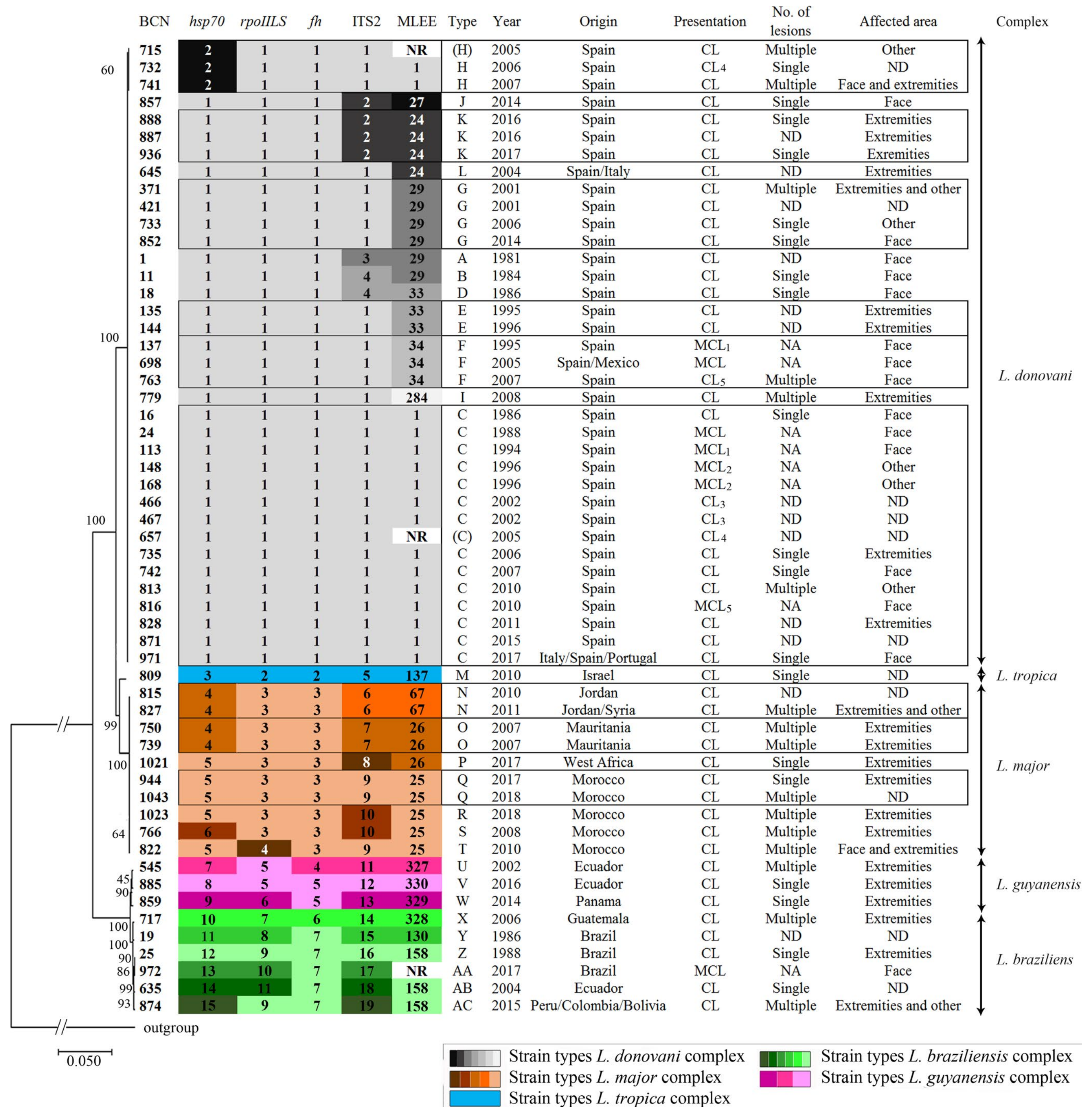
Based on MALDI-TOF, the strains were confidently associated with five complexes (Figure 1). At the species level, MALDI-TOF agreed with the consensus identification obtained by the other techniques in 87% of the strains. *L. braziliensis* and *L. guyanensis* complexes were the most controversial (Table 3, Figure 1). The MSI application did not allow any further distinction below the species level.

The discrimination power of the techniques varied depending on the complex analysed: the highest diversity in *L. donovani* was revealed by MLEE, in *L. major* by ITS2, in *L. braziliensis* by both ITS2 and *hsp70*, and in *L. guyanensis* by all three markers. When the results from molecular and enzymatic data were combined, the strains were classified into 29 types (A to AC) (Figure 2). The greatest diversity was observed in the species of the *Viannia* subgenus. Only between *L. infantum* and *L. major* strains it was possible to observe clusters sharing identical features with all the techniques used. For strains without MLEE results, the most prevalent MLEE type was assumed.

TABLE 2 Detailed intra-complex variability for *hsp70*, *rpoIILS*, *fh* and ITS2 sequences

Complex	*hsp70*			*rpoIILS*			*fh*			ITS2			
	Length	No. sequence types	Variable intra-complex positions	Length	No. sequence types	Variable intra-complex positions	Length	No. sequence types	Variable intra-complex positions	Length	No. sequence types	Variable intra-complex positions	Intracomplex variable repeated and deleted regions
L. donovani ($n = 36$)	1,245	2	504G>R	529	1	–	615	1	–	610–614	4	–	36(AT)5–6, 62(TA)4–5, 320(G)5–9
L. major ($n = 10$)	1,245	3	102C>Y, 954C>Y	529	2	9T>A	615	1	–	669–683	5	490A>G, 515T>G	106(AT)8–9, 478(TA)6–11, 605(AT)4–5
L. tropica ($n = 1$)	1,245	1	NA	529	1	NA	615	1	NA	540	1	NA	NA
L. braziliensis ($n = 6$)	1,245	6	51G>R, 264G>A, 561G>A/R, 1213T>A, 1237G>A/R	529	5	57G>K, 129G>K, 175G>K, 204A>R, 213A>R, 324G>R, 333C>Y, 375G>R, 387G>S	615	2	89G>R, 114C>Y, 414G>R	529–537	6	46A>T, 204G>C, 280G>A, 527G>A	45(TA)4–5, 94(TA)6–11, 413(CT)4–5, 452(TA)4–8, 460_463dupCATA, 503(G)4–6, 519(G)7–8
L. guyanensis ($n = 3$)	1,245	3	13C>Y, 564G>R, 1078T>G, 1017C>T, 1102A>G, 1181A>R, 1213A>G, 1236C>G	529	2	396C>A	615	2	9A>G	524–536	3	449T>C	95(TA)4–9, 221(TCCTCTC)2–3, 247_253delTCTCTTC, 254_257delTCC, 270(G)5–6, 309(G)7–9, 516(G)7–8

Note: Sequence variants were noted according to den Dunnen et al., (2016) and the International Union of Pure and Applied Chemistry (IUPAC) nomenclature. *n*: number of strains, NA: not applicable.



BCN	*hsp70*	*rpoIILS*	*fh*	ITS2	MLEE	Type	Year	Origin	Presentation	No. of lesions	Affected area	Complex
715	2	1	1	1	NR	(H)	2005	Spain	CL	Multiple	Other	
732	2	1	1	1	1	H	2006	Spain	CL$_4$	Single	ND	
741	2	1	1	1	1	H	2007	Spain	CL	Multiple	Face and extremities	
857	1	1	1	2	27	J	2014	Spain	CL	Single	Face	
888	1	1	1	2	24	K	2016	Spain	CL	Single	Extremities	
887	1	1	1	2	24	K	2016	Spain	CL	ND	Extremities	
936	1	1	1	2	24	K	2017	Spain	CL	Single	Exremities	
645	1	1	1	1	24	L	2004	Spain/Italy	CL	ND	Extremities	
371	1	1	1	1	29	G	2001	Spain	CL	Multiple	Extremities and other	
421	1	1	1	1	29	G	2001	Spain	CL	ND	ND	
733	1	1	1	1	29	G	2006	Spain	CL	Single	Other	
852	1	1	1	1	29	G	2014	Spain	CL	Single	Face	
1	1	1	1	3	29	A	1981	Spain	CL	ND	Face	
11	1	1	1	4	29	B	1984	Spain	CL	Single	Face	
18	1	1	1	4	33	D	1986	Spain	CL	Single	Face	
135	1	1	1	1	33	E	1995	Spain	CL	ND	Extremities	
144	1	1	1	1	33	E	1996	Spain	CL	ND	Extremities	
137	1	1	1	1	34	F	1995	Spain	MCL$_1$	NA	Face	
698	1	1	1	1	34	F	2005	Spain/Mexico	MCL	NA	Face	
763	1	1	1	1	34	F	2007	Spain	CL$_5$	Multiple	Face	*L. donovani*
779	1	1	1	1	284	I	2008	Spain	CL	Multiple	Extremities	
16	1	1	1	1	1	C	1986	Spain	CL	Single	Face	
24	1	1	1	1	1	C	1988	Spain	MCL	NA	Face	
113	1	1	1	1	1	C	1994	Spain	MCL$_1$	NA	Face	
148	1	1	1	1	1	C	1996	Spain	MCL$_2$	NA	Other	
168	1	1	1	1	1	C	1996	Spain	MCL$_2$	NA	Other	
466	1	1	1	1	1	C	2002	Spain	CL$_3$	ND	ND	
467	1	1	1	1	1	C	2002	Spain	CL$_3$	ND	ND	
657	1	1	1	1	NR	(C)	2005	Spain	CL$_4$	ND	ND	
735	1	1	1	1	1	C	2006	Spain	CL	Single	Extremities	
742	1	1	1	1	1	C	2007	Spain	CL	Single	Face	
813	1	1	1	1	1	C	2010	Spain	CL	Multiple	Other	
816	1	1	1	1	1	C	2010	Spain	MCL$_5$	NA	Face	
828	1	1	1	1	1	C	2011	Spain	CL	ND	Extremities	
871	1	1	1	1	1	C	2015	Spain	CL	ND	ND	
971	1	1	1	1	1	C	2017	Italy/Spain/Portugal	CL	Single	Face	
809	3	2	2	5	137	M	2010	Israel	CL	Single	ND	*L. tropica*
815	4	3	3	6	67	N	2010	Jordan	CL	ND	ND	
827	4	3	3	6	67	N	2011	Jordan/Syria	CL	Multiple	Extremities and other	
750	4	3	3	7	26	O	2007	Mauritania	CL	Multiple	Extremities	
739	4	3	3	7	26	O	2007	Mauritania	CL	Multiple	Extremities	
1021	5	3	3	8	26	P	2017	West Africa	CL	Single	Extremities	*L. major*
944	5	3	3	9	25	Q	2017	Morocco	CL	Single	Extremities	
1043	5	3	3	9	25	Q	2018	Morocco	CL	Multiple	ND	
1023	5	3	3	10	25	R	2018	Morocco	CL	Multiple	Extremities	
766	6	3	3	10	25	S	2008	Morocco	CL	Multiple	Extremities	
822	5	4	3	9	25	T	2010	Morocco	CL	Multiple	Face and extremities	
545	7	5	4	11	327	U	2002	Ecuador	CL	Multiple	Extremities	
885	8	5	5	12	330	V	2016	Ecuador	CL	Single	Extremities	*L. guyanensis*
859	9	6	5	13	329	W	2014	Panama	CL	Single	Extremities	
717	10	7	6	14	328	X	2006	Guatemala	CL	Multiple	Extremities	
19	11	8	7	15	130	Y	1986	Brazil	CL	ND	ND	
25	12	9	7	16	158	Z	1988	Brazil	CL	Single	Extremities	*L. braziliens*
972	13	10	7	17	NR	AA	2017	Brazil	MCL	NA	Face	
635	14	11	7	18	158	AB	2004	Ecuador	CL	Single	ND	
874	15	9	7	19	158	AC	2015	Peru/Colombia/Bolivia	CL	Multiple	Extremities and other	

FIGURE 2 Characterisation of all the strains and association with epidemiological and clinical data. From left to right: NJ tree constructed from concatenated sequences of *hsp70*, *rpoIILS* and *fh*; typing results (*i.e*., sequence types for the different loci and zymodemes −each one of the same species complex colour-coded in different tonalities of the same colour−and global strain type) and epidemiological and clinical data. Strains from the same type are boxed. Strains from the same patient are indicated with the same sub-index number next to the clinical presentation. NR, no results; ND, no data; NA, not applicable; CL, cutaneous leishmaniasis and MCL, mucocutaneous leishmaniasis

3.2 | Analysis of typing results considering epidemiological and clinical data of the sample

The 56 strains analysed were isolated in a 37-year period (from 1981 to 2018), 46% of them in the decade from 2001 to 2010 (Figure 2). They belonged to 51 patients aged between 8 and 76 years (average age of 43 years). Patients were 73% male and 25% female (gender was unknown for the remaining 2%) (Figure 3), and 24% were immunocompromised. Skin involvement was more abundant than mucocutaneous forms (45 versus. 5 patients and one patient having both clinical forms) (Figure 2). Although there was a high percentage of undescribed cutaneous lesions (20%, 9/46), those reported were mainly located on extremities (25 patients, 13 with lesions on upper limbs and 13 on lower limbs) or the

TABLE 3 MALDI-TOF results according to the species consensus identification

Consensus ID	MALDI-TOF ID
L. donovani complex (*L. infantum*, $n = 33$)	*L. infantum* ($n = 33$)
L. major complex (*L. major*, $n = 10$)	*L. major* ($n = 10$)
L. tropica complex (*L. tropica*, $n = 1$)	*L. killicki* ($n = 1$)
L. braziliensis complex (*L. braziliensis*, $n = 6$)	*L. braziliensis* ($n = 2$) *L. peruviana* ($n = 4$)
L. guyanensis complex (*L. guyanensis*, $n = 2$; *L. panamensis*, $n = 1$)	*L. panamensis* ($n = 3$)
Correct species ID	46 strains (87%)
Correct complex ID	6 strains (11%)

Note: Consensus ID refers to the identification obtained with MLEE and molecular techniques.
Abbreviation: ID, identification

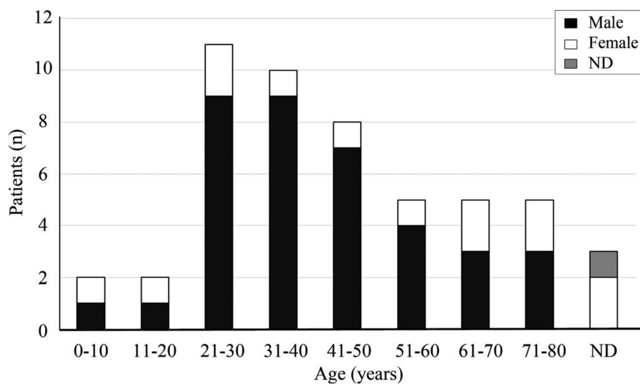


FIGURE 3 Age and sex distribution of the patients. For patients with more than one isolate, age data refer to the first isolate. ND, no data

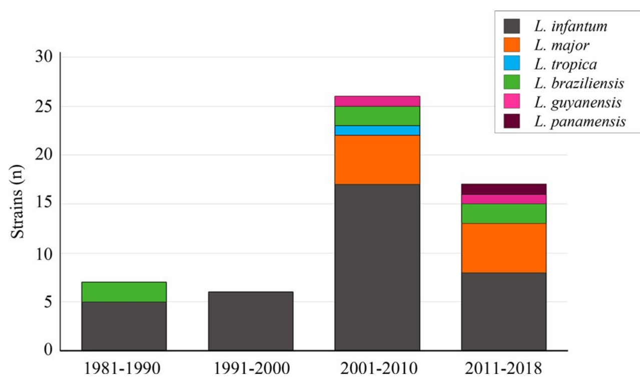


FIGURE 4 Species identification variability for each studied decade

face (11 patients); other locations were neck, lumbar area, back, buttocks, each in one patient as either single or multiple lesions (in 27 and 16 patients, respectively). Mucocutaneous lesions affected the nose (2 patients), palate, tongue and rectum (1 patient each).

Among the patients, 28 (55%) represented autochthonous cases, whereas 20 (39%) were imported, and three cases could not be identified as either. Between 2011 and 2018, imported cases were more numerous than autochthonous ones (nine versus seven, respectively, and one undetermined). The greatest taxonomic diversity was found in strains isolated in the last two decades (2001–2018) (Figure 4).

All cases considered as undoubtedly autochthonous (28 patients and 33 strains) were caused by *L. infantum* strains. Among these, the predominant patient profile was composed by males aged between 30 and 40 years, although the overall average age in the study was 48 years. Cutaneous lesions, frequently single (in at least 17 patients), were usually found on the face or extremities (9 and 10 patients, respectively). Five patients had MCL (Figure 2), and two of them had Human Immunodeficiency Virus infection (HIV+).

In five patients, four documented as immunocompromised and two previously diagnosed with VL, two different isolates of *L. infantum* were obtained (Table 4). In two cases (patients 2 and 3), the two strains were identified as identical by all the analytical techniques used. In two other cases (patients 1 and 5), the two strains were different according to MLEE, the results being MON-1 and MON-34 in both patients. In patient 4, the strains differed in at least the *hsp70* type (MLEE was not performed in one of the strains).

After combining molecular and enzymatic data, the 33 autochthonous strains were divided into 11 different types (from A to K) (Figure 5). Type C strains were the most prevalent ($n = 13$, 39%) and appeared throughout the studied period. They belonged to zymodeme MON-1 and presented the *L. infantum* consensus sequence for all the genes. Four strain types (E, F, G and I) differed from type C only in the zymodeme, MON-29 being the second most frequent. Type H differed only in the *hsp70* sequence, while five types (A, B, D, J and K) diverged in both zymodeme and sequence type. The other groups had short temporary distributions, especially when a molecular change was detected, except for types F and G that were isolated over 12 and 13 years, respectively.

Cases classified as undoubtedly imported (20 patients and strains) were caused by five different taxa (*L. major*, *L. tropica*, *L. braziliensis*, *L. guyanensis* and *L. panamensis*). Most of these patients were aged between 21 and 30 years, but the mean age was 32. Fifty percent (7 males, 3 females) were Spanish nationals who had travelled to other endemic areas, 40% were migrants (7 males and 1 female) and 10% were of unknown nationality. The only immunocompromised patient was a migrant with CL. CL lesions were single or multiple (8 and 10 cases, respectively) and affected mainly the extremities (14 cases). There was one case of MCL caused by *L. braziliensis*. As shown in Figure 2, the *L. major* strains came from North Africa and the Middle East and were usually associated with multiple lesions. The seven different profiles found among the strains differed in the geographical origin or year of isolation and were associated with MLEE and ITS2 types and to a lesser extent with the *hsp70* types. In three clusters (types N, O and Q), all the techniques gave identical characterisation results, and each was composed of two strains, which temporarily coincided in Jordan, Mauritania and

TABLE 4 Detailed information and identification results of autochthonous patients with more than one isolate (*L. infantum*)

Patient	OMS code	*hsp70*	*rpoIILS*	*fh*	ITS2	MLEE (MON)	Type	Interval	Lesion localisation	VIH
1	MHOM/ES/94/BCN-113	ST1	ST1	ST1	ST1	1	C	17 months	Palate Sublingual	pos
	MHOM/ES/95/BCN-137	ST1	ST1	ST1	ST1	34	F			
2	MHOM/ES/96/BCN-148	ST1	ST1	ST1	ST1	1	C	2 months	Rectum	pos
	MHOM/ES/96/BCN-168	ST1	ST1	ST1	ST1	1	C			
3[a]	MHOM/ES/2002/BCN-466	ST1	ST1	ST1	ST1	1	C	simultaneously	Healthy skin	pos
	MHOM/ES/2002/BCN-467	ST1	ST1	ST1	ST1	1	C		Lesion	
4[a]	MHOM/ES/2005/BCN-657	ST1	ST1	ST1	ST1	NR	(C)	17 months	ND	pos
	MHOM/ES/2006/BCN-732	ST2	ST1	ST1	ST1	1	H		ND	
5	MHOM/ES/2007/BCN-763	ST1	ST1	ST1	ST1	34	F	31 months	Face	ND
	MHOM/ES/2010/BCN-816	ST1	ST1	ST1	ST1	1	C		Nose	

Note: Bracketed type indicates that the predominant zymodeme was assumed.

Abbreviations: ND, no data; NR, not carried out; pos, positive; ST, sequence type.

[a]Patients with diagnosed VL.

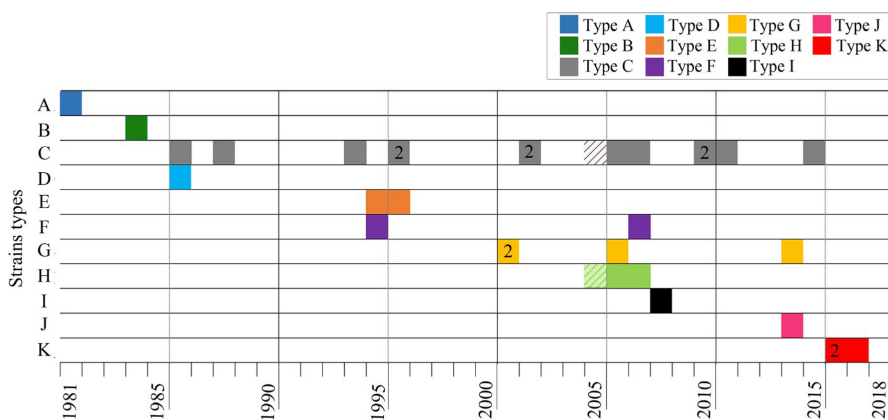


FIGURE 5 Temporal distribution of the *L. infantum* types from confirmed autochthonous cases. The number of strains is indicated when there were multiple isolates. Stripes denote the types deduced as probable due to the lack of MLEE results

Morocco, respectively. As only one *L. tropica* strain was found, acquired in Israel, a more in-depth analysis was not possible. *L. braziliensis* strains belonged to different countries of Latin America, *L. guyanensis* strains were isolated from patients originating from or visiting Ecuador, and the *L. panamensis* strain was from Panama. Each *L.* (*Viannia*) strain constituted a unique type, although some strains shared geographical origins.

4 | DISCUSSION

Since the Trypasomatid Cryobank of the UB (Spain) was created in 1983 (Gállego et al., 2003), more than 1,000 strains, belonging principally to *Leishmania* genera, have been deposited. The *Leishmania* strains were acquired mainly from patients affected by VL or are collection strains. At the end of 2018, strains isolated in hospitals in the Catalonia region from patients with TL represented only 5% of the total deposited. Despite fluctuating collaboration between the University and the hospitals over the years, the low number of TL strains is most likely to be due to under-diagnosis. As TL does not often require hospitalisation, it is frequently diagnosed and treated in primary healthcare or private dermatological centres (García-Almagro, 2005; Gil-Prieto et al., 2011; Portús et al., 2007; Suárez Rodríguez et al., 2012). Even so, in the last two decades, TL strains deposited or obtained by sample cultivation have quadrupled and tripled, respectively. This increase could be a consequence of imported cases involving other *Leishmania* species, the occurrence of rare or atypical lesions requiring differential diagnosis of CL, and the implementation of identification protocols derived from treatment differences according to the species and lesion characteristics. Although strain isolation is less sensitive than other diagnostic methods (Aronson & Joya, 2019), a similar increase in TL cases has been observed in other studies conducted in Spain and Italy (Garrido-Jareño et al., 2020; Giavedoni et al., 2015; Riera et al., 2016).

Regarding the patient profile in autochthonous cases, the average age was 48 years, which coincides with the average observed for leishmaniasis patients in other studies in Spain (Fernández Martínez et al., 2019). However, the infant population, one of the most commonly affected by leishmaniasis, was not represented. One explanation is that the hospital providing 33% of the samples does not offer paediatric health care, but another factor is that reported cases among children correspond predominantly to VL (Fernández Martínez et al., 2019; Garrido-Jareño et al., 2020). The majority of patients were male (71%), as in numerous other reports

(Fernández Martínez et al., 2019; Garrido-Jareño et al., 2020; Gil-Prieto et al., 2011; Herrador et al., 2015; Portús et al., 2007; Riera et al., 2016). Among the hypotheses proposed by researchers for this gender bias, those more applicable in our context could be the dimorphic immunological response driven by sexual hormones (Lockard et al., 2019) or the higher prevalence of *Leishmania*/HIV coinfection in males (Desjeux & Alvar, 2003; Herrador et al., 2015). It is worth mentioning that most of the patients in the study were immunocompetent, or at least no immunosuppression had been reported. In the case of immunocompromised patients (39%), who were mainly coinfected with HIV, all the strains were isolated between 1994 and 2010, and only two patients were female.

Mucocutaneous lesions are principally associated with South American *Leishmania* species, although they can also be caused by Old World species (Strazzulla et al., 2013), primarily in immunosuppressed patients (Mejia, 2018; World Health Organization, 2010). In this study, five autochthonous patients presented mucocutaneous lesions and only two of them were immunocompromised. Although mucosal involvement is rarely associated with *L. infantum*, other cases of MCL in immunocompetent patients have been described in Spain and elsewhere (Aliaga et al., 2003; Casolari et al., 2005; Freites-Martinez et al., 2015; Garrido-Jareño et al., 2020). In the current work, only one out of six MCL cases was imported, and was caused by *L. braziliensis*, the species most frequently responsible for this clinical form (Reithinger et al., 2007).

Confirmed imported cases represented 39% of the analysed strains from the last decades, which constitutes an important increase, in agreement with other studies in Spain (Giavedoni et al., 2015; Riera et al., 2016). An increasing tendency of imported leishmaniasis has been observed around the world as a result of globalisation, even affecting non-endemic countries (Bart et al., 2013; Di Muccio et al., 2015; Söbirk et al., 2018; Stark et al., 2008; Vandeputte et al., 2020). According to data from the World Tourism Organization, the number of international tourist arrivals worldwide in the studied period rose from 277 million in 1981 to 1,407 million in 2018 (World Turism Organization, 2020). Additionally, the immigrant population in Spain increased from nearly 20,000 people in the mid-1980s to over 600,000 people in 2018 (Instituto Nacional de Estadística, 2020; Romero Valiente, 2003). It is noteworthy that among samples deposited in 2011–2018, imported cases outnumbered autochthonous cases. A certain bias towards imported cases could be related to their association with more complex lesions, in contrast with autochthonous cases, with predominantly single lesions (Giavedoni et al., 2015; Merino-Espinosa et al., 2018). However, growing awareness among clinicians of the need for species identification in patients reporting travel history may also be occurring.

In imported cases, patients were younger than in autochthonous cases, with similar numbers of Spanish travellers and immigrants (10 and 8, respectively, and two patients of unknown nationality). The immigrants were often established in Spain but had recently travelled to their countries of origin. Infections were acquired to a similar extent in the Old and New World (11 and 9, respectively), unlike in other studies, which usually report a predominance of Old World cases (Bart et al., 2013; Di Muccio et al., 2015; Giavedoni et al., 2015; Pérez-Ayala et al., 2009; Vandeputte et al., 2020). Patient characteristics in terms of age, origin or destination matched those reported for the immigrant population in Spain and the profile of the Spanish traveller in several studies on imported diseases (Instituto Nacional de Estadística, 2020; Jaén-Sánchez et al., 2016; Valerio et al., 2005; Zamarrón Fuertes et al., 2010).

In three cases (1 MCL and 2 Cl) with documented travel history it was not possible to determine if the infection caused by *L. infantum* had occurred in Spain or during the stay in a foreign country (Mexico, Italy and Portugal). In South America, *L. infantum* predominantly causes VL and some cases of CL have been reported in Costa Rica, Honduras, Brazil and Venezuela (Castro et al., 2016; De Lima et al., 2009; Noyes et al., 1997; Zeledón et al., 1989). In Mexico, *L. infantum* has been reported to cause VL, but it is not associated with skin lesions (Pan American Health Organization, 2019). Without species characterisation, the case would have likely been classified as imported, based on the history of travel to Mexico and the clinical form (MCL), but the identification of *L. infantum* as the aetiological agent opens up the possibility of infection in Spain. In the other two cases, since Portugal and Italy are included in the large Mediterranean area where *L. infantum* is endemic, together with Spain (Alvar et al., 2012; Campino et al., 2006; Gramiccia et al., 2013; Pratlong et al., 2013), infection was equally possible in any of the three countries.

Similarly, in three other imported cases it was not possible to identify the country of infection, since multiple destinations with circulating species in common were reported. One patient had travelled to Jordan and Syria, both endemic for *L. major*, the species identified. However, its typing profile was identical to that of another temporally close Jordanian strain. It should be mentioned that *L. tropica* also causes CL in that area (Alvar et al., 2012; Postigo, 2010), although usually resulting in a single lesion, whereas *L. major* is more associated with multiple lesions, as suffered by this patient (Aoun & Bouratbine, 2014). Another patient, also with a *L. major* isolate but producing a single lesion, had travelled to different countries of West Africa, where this species is responsible for human CL (Kone et al., 2018). The third case was a patient who had visited Peru, Bolivia and Colombia and presented multiple CL lesions caused by *L. braziliensis*. This species is present in all three countries, where it cohabits with other species (Pan American Health Organization, 2019).

The aforementioned examples, as well as the overall taxonomic diversity found (six taxa from five complexes), indicate the growing risks of deducing the aetiological agent based only on epidemiological data, which may only be conclusive for autochthonous cases without any history of travel abroad, and support the use of species identification tools. Today, the treatment of CL varies in each patient, depending on the *Leishmania* species as well as the number, appearance and duration of the lesions, which could determine the self-healing nature of the disease, the response to certain drugs and risk of metastatic complications (Aronson & Joya, 2019; Mensa et al., 2020). From an epidemiological point of view, the imported

species identified are unlikely to represent a risk in Spain, due to the absence of reservoirs or sand fly vectors. The exception is the anthroponotic species *L. tropica*, whose vector *P. sergenti* is present elsewhere in Spain (Ballart et al., 2014; Barón et al., 2008; Gil Collado et al., 1989).

The use of high resolutive approaches or the combination of different methods can contribute other valuable information besides species identification. For example, the combination of MLEE with ITS2 allowed us to evaluate the variation of autochthonous strains through four decades. There was a prevailing strain type (type C) throughout the period (39%), which was probably equivalent to the predominant group found in other studies (Kuhls et al., 2005). Variations of type C, most of them due to different zymodemes, appeared only in narrow intervals of time, except types F and G, which had longer distribution periods. Types F and G differed from type C in being zymodemes MON-34 and MON-29, respectively, which are frequently found in CL cases in other parts of Spain (Chicharro et al., 2003; Jiménez et al., 1995; Pratlong et al., 2013). It should be noted that in the later years of the study, four strains (types J and K) showed a particular ITS2 sequence type, which differed from the consensus sequence in the number of repetitions of three microsatellite regions and which probably corresponds to the Lombardi type found by Chicharro et al., (2013) in Madrid among strains with zymodemes MON-1 and MON-24. The zymodeme for type J was MON-27, which to the best of our knowledge has only been recorded in Italy and in a Spanish man living in Argentina with an uncertain origin of infection (Martín-Sánchez, Navarro-Mari, et al., 2004; Pratlong et al., 2013). In our study, the affected patient was Spanish and without a history of travel. The zymodeme of type K strains was MON-24, which is abundant in Spain but did not appear in the current study among the autochthonous strains before 2016. Another interesting finding was a strain of MON-284 (type I). Rarely reported, this zymodeme is typically associated with HIV+ patients (Chicharro et al., 2003), as in this case. Regarding the *L. infantum* strain whose geographical origin was either Spain or Mexico, MLEE typing results (MON-34) point towards an autochthonous case. As far as we are aware, this zymodeme has not been found in America (Ribeiro Coutinho et al., 2011) and only one *L. infantum* strain from Panama has shown a profile other than MON-1 in the New World (Franssen et al., 2020). In this case, sequencing methods alone would not allow to discriminate between an autochthonous or an imported case since the predominant sequence types showed by the strain (Figure 2, BCN 698) have also been found in the New World (Kuhls et al., 2005; Van der Auwera et al., 2013).

More in-depth characterisation could also be useful to evaluate possible relapses or reinfections. In our case, five patients had two isolates. For patient 3, and probably also patient 2, both isolates were taken during the same episode and the strain type did not change. In the other three cases, 17 to 31 months elapsed between the two isolates, and in all of them there was a change in the strain type. Strains of patient 1 differed in the zymodeme, which changed from MON-1 to MON-34. However, based on the localisation of the lesion, relapse seems the most plausible scenario, and the MLEE variation could be explained by culture selection from a mixed infection (Cortés et al., 1997). An alternative hypothesis is that as long as MON-34 differs from MON-1 exclusively in the mobility of the MDH enzyme, a sequence mutation could have occurred specifically affecting enzyme mobility, as reported for other genes (Mauricio et al., 2006; Zemanová et al., 2007). Similarly, in patient 4, the presence of a heterozygous allele of the *hsp70* gene in the second isolate, together with a HIV and VL background, again suggests a relapse. As the *hsp70* sequence type was also observed in two other strains isolated within a short period of time, a mixed infection would be a reasonable explanation. A different situation was observed for patient 5. Here, the change in zymodeme, together with a higher time interval between isolates, a slight difference in the localisation of the lesion and no records of immunosuppression leads us to think of a possible reinfection.

An additional use of strain characterisation is for identification in outbreaks of disease. As we are all aware, a consequence of globalisation is that health issues are increasingly crossing borders. Among our data, we observed three *L. major* strains isolated between 2017 and 2018 that were acquired in Morocco, and with similar results for the different typing methods (two were identical and the third differed in one repetition of a microsatellite in the ITS2). In this period, an outbreak of leishmaniasis was active in Morocco (El Hamouchi et al., 2019), as also occurred in other countries at some point of the studied period (Mosleh et al., 2018; Oliveira-Neto et al., 1988; Postigo, 2010). It, therefore, seems plausible that some of the identified strains could have originated in an outbreak abroad.

Many markers and techniques have been used for *Leishmania* identification, as reviewed (Akhoundi et al., 2017; Kuhls & Mauricio, 2019; Van der Auwera & Dujardin, 2015). Nevertheless, in most studies, they have been applied to a low number of strains, to strains isolated only from the Old or New World, without covering both *Leishmania* and *Viannia* subgenera, or are limited to a single technique. In this study, we used biochemical (MLEE), molecular (sequencing) and proteomic (MALDI-TOF MS) techniques to identify 56 strains from different complexes isolated from autochthonous and imported *Leishmania* species in Catalonia. Although not always in a straightforward manner, all the techniques and markers used allowed the correct identification at the complex level, unlike the experience of another study carried out in different European laboratories (Van der Auwera et al., 2016).

Since not all identification techniques provide the same degree of discrimination, choosing the most suitable methodology depends on the specific purpose of the research. *Grosso modo*, MLEE discriminates below the species level with a high level of correspondence among zymodemes and geographical origin. Used to type nearly all the described species, MLEE constitutes the basis of current taxonomy and is considered by many as the gold standard typing method (Van der Auwera & Dujardin, 2015). However, it requires large culture volumes, is time-consuming, expensive and cumbersome, and interpretation of results is not straightforward, which has limited its use to specialized laboratories. Moreover, the phenotype observed does not always correspond to the real genotype (Mauricio et al., 2006; Zemanová et al., 2007). In contrast, MALDI-TOF is faster, easier to

use and cost-effective. However, certain difficulties could arouse in the identification below the complex level of taxonomically controversial groups and different interpretation criteria of MSI data can produce slight differences in the results. Regardless, identification at the species complex level is sufficient to manage *Leishmania*.

A third approach is sequencing, which generally takes up an intermediate position regarding discriminative power, workability and time consumption, but its pros and cons are hardly influenced by the target used (Kuhls & Mauricio, 2019). In the current study, the four analysed genetic regions permitted a confident identification of the *Leishmania* subgenus at the species level. However, if punctual sequence-type mutations were not considered, far fewer constitutive differences were observed among complexes of the *Viannia* subgenus, up to the point that the *L. braziliensis* and *L. guyanensis* complexes differed by only a single position in the *fh* fragment. Differences among taxa within the *L. guyanensis* complex were especially scarce and indistinguishable by the *fh* marker. However, since only one *L. panamensis* strain was found, it is uncertain if the observed variations in the other genes are characteristic of the taxon or polymorphisms of the strain. Below the species level, *hsp70* and ITS2 were the markers that provided additional inter-strain divergence for the entire genus. The high variability obtained among the ITS2 sequences, together with the degree of geographical and/or temporal correspondence found among the sequence types, render this molecular target suitable for epidemiological purposes beyond species identification. Nevertheless, the differences blurred the limits between the *L. braziliensis* and *L. guyanensis* complexes. The low variability observed with the *fh* fragment could be a consequence of the reduction in the fragment length made to amplify all the clinically important complexes. At least for the *L. donovani* complex, studies using the full-length gene have found greater diversity; the strains were also geographically diverse, unlike in our study (Zemanová et al., 2007). In terms of workability and time efficiency, *rpoIILS* and *fh* were the simplest targets, due to their constant and relatively short length. For the whole *hsp70* sequencing, the concatenation of two shorter fragments rendered better results than a full-length amplification, making it less straightforward. In ITS2, the abundant microsatellites hampered sequencing reads and their interpretation. In addition, the final length variability among strains did not permit the alignment of all the sequences and their addition to the NJ tree. Nevertheless, the amplicon length could preliminarily orientate towards certain complexes. Finally, even if not applied here, another factor worth considering is that some of the used regions work well with small amounts of DNA, being directly available from a clinical sample (Kuhls & Mauricio, 2019; Montalvo et al., 2017; Schönian et al., 2003); a trait inapplicable for MLEE or MALDI-TOF. This reduces costs and other culture difficulties, but drawbacks could arise related to the sensitivity of the technique.

In conclusion, both autochthonous and an increasing number of imported TL cases are diagnosed in Spain, the latter caused by human-affecting species from the New and Old World. This gives rise to a diversification of clinical manifestations, some of which may be unfamiliar to clinicians. The autochthonous cases, which have also increased, are not caused by a single strain type, which could affect the severity of the disease or the healing process. Therefore, the identification and further characterisation of the circulating strains affords valuable information for both the clinical and epidemiological management of the disease. While MLEE, sequencing and MALDI-TOF are all useful tools, they are not equally suitable for all contexts. *rpoIILS* and *fh* sequencing or MALDI-TOF (despite its culture dependence) could be key identification tools in the clinical area, where speed and simplicity are the most valuable features. Whereas *hsp70* and ITS2, due to their respective confidence and resolution, could constitute an interesting tandem to substitute MLEE in the epidemiological field.

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CONFLICT OF INTEREST

Authors declare no competing interests.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required for this original research.

DATA AVAILABILITY STATEMENT

Sequence data are available in GenBank under the accession numbers MT497923-MT497978 and MT498854-MT499021.

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