


Typing of *Leishmania* isolates from vectors and leporids of the Madrid (Spain) outbreak

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Research Article

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Abstract

In 2009, a large outbreak of leishmaniasis, associated with environmental changes, was declared near Madrid (Spain), in which *Phlebotomus perniciosus* was the vector, whereas the main reservoirs were hares and rabbits. Analysis of isolates from humans, vectors and leporids from the focus identified the *Leishmania infantum* ITS-Lombardi genotype. However, multilocus enzyme electrophoresis (MLEE), the reference technique for *Leishmania* typing, and sequencing of the *hsp70* gene, a commonly used marker, were not performed. In the present study, 19 isolates from *P. perniciosus* ($n = 11$), hares ($n = 5$) and rabbits ($n = 3$) from the outbreak area, all characterized as ITS-Lombardi in previous studies, were analysed by MLEE and *hsp70* sequencing. The *hsp70* results confirmed that all the analysed strains are *L. infantum*. However, by MLEE, 4 different zymodemes of *L. infantum* were identified based on variable mobilities of the NP1 enzyme: MON-34 (NP1¹⁰⁰, $n = 11$), MON-80 (NP1¹³⁰, $n = 6$), MON-24 (NP1¹⁴⁰, $n = 1$) and MON-331 (NP1¹⁵⁰, $n = 1$). The relative frequency of these zymodemes does not correspond to their usual occurrence in Spain. Moreover, MON-34 and MON-80 were found in *P. perniciosus*, hares and rabbits for the first time. These findings continue to provide insights into the outbreak and call for further studies with a higher number of strains.

Introduction

Leishmaniasis is a parasitological disease with worldwide distribution (World Health Organization, 2010). In the Mediterranean area, the disease is autochthonous, although imported cases are also found (Berriatua *et al.*, 2021; Fernández-Arévalo *et al.*, 2022; Van der Auwera *et al.*, 2022). In Spain, the incidence reported for 2018 was 0.65 cases/100 000 habitants (Centro Nacional de Epidemiología, 2019), and in 2017 the estimated incidence rate was 0.86 for visceral leishmaniasis (VL), 1.04 for cutaneous leishmaniasis (CL) and 0.12 for mucocutaneous leishmaniasis, with an estimated underreporting of 14.7–20.2% for VL and 50.4–55.1% for CL (Humanes-Navarro *et al.*, 2021). VL and CL are the main clinical manifestations in the country, but occasionally atypical CL or mucosal presentations are also diagnosed (Aliaga *et al.*, 2003; Fernández Martínez *et al.*, 2019). Although prevalence is higher in certain regions and seasons, the disease is present in most of Spain and cases are reported throughout the year (Fernández Martínez *et al.*, 2019). The parasite responsible for autochthonous cases is *Leishmania infantum*, the principal domestic reservoir is the dog, and the main vectors are *Phlebotomus perniciosus* and *Phlebotomus ariasi* sand flies (Jiménez *et al.*, 1995; Gállego *et al.*, 2001; Martín-Sánchez *et al.*, 2004; Berriatua *et al.*, 2021). *Phlebotomus langeroni* has also been incriminated as a vector (Sáez *et al.*, 2018) and different domestic and wild animals have been suspected or confirmed to be reservoirs (Azami-Conesa *et al.*, 2021; Cardoso *et al.*, 2021; Martín-Sánchez *et al.*, 2021).

In 2009, the largest outbreak of leishmaniasis due to *L. infantum* ever detected in Europe was declared in Fuenlabrada, in the southwest Madrid region (Spain) (Arce *et al.*, 2013). At its peak (2010–2014), more than 600 human cases were reported (Dirección General de Salud Pública – Consejería de Sanidad de la Comunidad de Madrid, 2015; Fernández Martínez *et al.*, 2019) and asymptomatic carriers were also detected (Molina *et al.*, 2020). The great majority of the patients were immunocompetent adults living in the area who presented cutaneous forms of the disease (Arce *et al.*, 2013; Carrillo *et al.*, 2013). However, children and immunocompromised individuals were also affected, and VL cases and rare clinical manifestations were observed, as well as a high susceptibility of the immigrant population born in Sub-Saharan Africa (Arce *et al.*, 2013; Gomez-Barroso *et al.*, 2015; Horrillo *et al.*, 2015). Demographic and environmental changes, such as the construction of a vast green park connecting different urban zones, favoured the proliferation of *P. perniciosus*, hares (*Lepus granatensis*) and rabbits (*Oryctolagus cuniculus*), which were incriminated as reservoirs, and led to

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the outbreak (Molina *et al.*, 2012; Carrillo *et al.*, 2013; Jiménez *et al.*, 2014; González *et al.*, 2021).

Over the years, the leishmaniasis cases, reservoirs, vectors and parasites associated with the outbreak have been extensively investigated. However, few studies have characterized the parasites beyond species level. Two studies used the internal transcriber spacer (ITS) to type 3 and 4 strains isolated from hares and rabbits, respectively (Molina *et al.*, 2012; Jiménez *et al.*, 2014). All strains shared the ITS sequence of the strain MHOM/ES/87/Lombardi (AJ000295), this strain corresponding to zymodeme MON-24 of *L. infantum* (Chicharro *et al.*, 2013). Similar results were obtained for 6 DNA samples and 67 isolates from sand flies when ITS2 was tested (Jiménez *et al.*, 2013; González *et al.*, 2017). In another study, 31 human isolates related to the outbreak were characterized by ITS and *haspb* (k26) markers (Chicharro *et al.*, 2013). All strains were identified as ITS-Lombardi, but when combined with the *haspb* (k26) results, 4 different genotypes were detected, L-920 being the most prevalent. Given this background, the aim of the present study was to dig deeper into the characterization of non-human strains from the leishmaniasis outbreak using multilocus enzyme electrophoresis (MLEE), the technique most extensively used in epidemiological studies of leishmaniasis foci and the gold standard recommended by the WHO for this purpose (World Health Organization, 2010). Additionally, strains were also analysed by PCR-sequencing of the heat shock protein 70 (*hsp70*), one of the main markers used for *Leishmania* characterization (Fernández-Arévalo *et al.*, 2022; Van der Auwera *et al.*, 2022).

Materials and methods

Strains

Nineteen strains previously isolated from sand flies and leporids captured at different locations during the leishmaniasis outbreak area of Fuenlabrada (Spain) between 2011 and 2014 were selected for analysis. Eleven of them were isolated from 11 field-captured *P. perniciosus* in 2012 ($n = 4$), 2013 ($n = 4$) and 2014 ($n = 3$). The sand flies specimens had been captured in 4 collecting stations placed in institutional facilities: 3 located in the municipality of Fuenlabrada, in the border zone of the green park, (ATE-station $n = 3$, BOS-station $n = 3$, JIC-station $n = 3$) and 1 in the central area of the park in the municipality of Leganés (POL-station $n = 2$) (González *et al.*, 2017). The other 8 strains were isolated from colony specimens of *P. perniciosus* used in direct xenodiagnosis of hares and rabbits. Five of them were isolated from 3 hares living in the park captured in 2011/12 and 3 were obtained from 3 rabbits captured in the border zone in 2013 (Molina *et al.*, 2012; Jiménez *et al.*, 2014). All the isolates had been previously characterized by ITS sequencing as the Lombardi type (Molina *et al.*, 2012; Jiménez *et al.*, 2013, 2014; González *et al.*, 2017).

The strains were thawed and cultured in Schneider's insect medium supplemented with 20% fetal bovine serum and 1% sterile human urine until the exponential growth phase (Fernández-Arévalo *et al.*, 2022).

Hsp70 gene sequencing

DNA from the cultured strains was obtained by the QIAmp DNA Mini Kit (Qiagen) following the manufacturer's instructions. Amplification was done using previously described primers and cycling conditions (Van der Auwera *et al.*, 2013; Fernández-Arévalo *et al.*, 2022). PCR products were enzymatically purified using EXOSAP-IT (Affymetrix USB) and double-strand sequenced by the Sanger method at the Scientific and Technologic Centres of the University of Barcelona. The obtained sequences were edited using MEGA X software (Kumar *et al.*, 2018).

MLEE

Protein extracts were obtained from mass cultures as previously described (Piarroux *et al.*, 1994). The strains were analysed by MLEE using a panel 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] (Rioux *et al.*, 1990). The samples were run on starch gel and revealed under suitable conditions for each enzyme. Migration distances were manually measured and compared with those of the marker strains (Supplementary Table S1) to assign the corresponding electromorphs and zymodemes. Once all enzyme mobilities were determined, the strains were tested again with a second batch of protein extract to confirm the results. The isoelectric focusing technique was also used to confirm the NP1 mobility (Piarroux *et al.*, 1994).

Results

Analysis of the *hsp70* gene showed that all the analysed strains shared the same genotype, presenting the main sequence reported for *L. infantum* strains (GenBank accession numbers OQ747159–OQ747177). Neither SNPs nor heterozygous positions were detected.

The strains produced different MLEE patterns. All of them shared an electrophoretic mobility (EM) of 104 for the MDH enzyme (MDH¹⁰⁴) and an EM of 100 for the other enzymes except NP1 (EM: 100, 130, 140, 150). Thus, NP1 was the only enzyme associated with enzymatic polymorphisms in *Leishmania* strains from the outbreak. NP1¹⁰⁰ was observed in 11 out of the 19 strains, which were therefore identified as *L. infantum* zymodeme MON-34. Six strains shared NP1¹³⁰ (MON-80), whereas NP1¹⁴⁰ was observed in 1 strain (MON-24), and NP1¹⁵⁰ in the remaining strain (MON-331; also known as GR-19 and MON-24 var NP1¹⁵⁰) (Table 1).

No correlations were apparent between the zymodeme and host, year or location (Table 1, Fig. 1). The only MON-24 strain was isolated from a *P. perniciosus* specimen in 2013. MON-34 and MON-80 zymodemes were found in rabbits, hares and sand flies from all collecting stations and in all years. Finally, the zymodeme MON-331 was detected in 1 rabbit. If the strains are grouped according to the collecting station and animal reservoir of origin, 'JIC-station', 'POL-station' and 'rabbits' present the most diversity, as each strain in these groups belonged to a different zymodeme. In contrast, all 'ATE-station' strains were MON-34 (Fig. 1).

Discussion

In an unexpected outbreak in an endemic area, identification of the aetiological agent gains importance. Human leishmaniasis can be caused by about 20 of the 56 *Leishmania* species described (Akhoundi *et al.*, 2016) and in the current context of globalization and climate change, the presence of imported species cannot be ruled out. In the leishmaniasis outbreak in Fuenlabrada, although part of the immigrant population was highly affected (Arce *et al.*, 2013; Gomez-Barroso *et al.*, 2015), the causative species was promptly identified as the autochthonous *L. infantum* (Chicharro *et al.*, 2013).

When performing characterization studies within the *L. donovani* complex, and specifically for *L. infantum* strains, one of the

Table 1. Results of MLEE *Leishmania infantum* characterization related to host, location and year of isolation

Zymodeme	Original host	Location ^a	Year	WHO code		
MON-24 (n = 1)	<i>Phlebotomus perniciosus</i>	JIC station	2013	IPER/ES/2013/LEMSJIC1FF4		
MON-34 (n = 11)	<i>Phlebotomus perniciosus</i>	ATE station	2012	IPER/ES/2012/LEMATE1FL2		
			2013	IPER/ES/2013/LEMATE1FL6		
			2014	IPER/ES/2014/LEMATE1FL8		
		BOS station	2012	IPER/ES/2012/LEMBOS1FL1		
			JIC station	2012	IPER/ES/2012/LEMSJIC1FF1	
				POL station	2013	IPER/ES/2013/LEMPOL2FL21
			<i>Oryctolagus cuniculus</i>		Border zone	2013
<i>Lepus granatensis</i>	Park	2012	IPER/ES/2012/LEML34BX			
			IPER/ES/2012/LEML34DX			
			IPER/ES/2012/LEML40AX			
			IPER/ES/2012/LEML40BX			
MON-80 (n = 6)	<i>Phlebotomus perniciosus</i>	BOS station	2013	IPER/ES/2013/LEMBOS1FL10		
			2014	IPER/ES/2014/LEMBOS1FL32		
			JIC station	2014	IPER/ES/2014/LEMSJIC1FF19	
		POL station	2012	IPER/ES/2012/LEMPOL2FL7		
			<i>Oryctolagus cuniculus</i>	Border zone	2013	IPER/ES/2013/LEMC17
			<i>Lepus granatensis</i>	Park	2011	IPER/ES/2011/LEML1D18
MON-331 (n = 1)	<i>Oryctolagus cuniculus</i>	Border zone	2013	IPER/ES/2013/LEMC3		

^aJIC, 'BOS' and 'ATE' are the codes used to identify the collecting stations placed in the border zone of the park in the municipality of Fuenlabrada, and 'POL' the code the collecting station placed in the centre of the park in the municipality of Leganés.

major obstacles is the low intraspecific diversity (Maurício, 2018; Fernández-Arévalo *et al.*, 2020). Different techniques have been used for the intraspecific analysis of *L. infantum*, including MLEE, multilocus microsatellite typing, multilocus sequence typing (MLST), randomly amplified polymorphic DNA, PCR-RFLP,

and PCR-sequencing (Toledo *et al.*, 2002; Ferroglio *et al.*, 2006; Montoya *et al.*, 2007; Ait-Oudhia *et al.*, 2011; Van der Auwera and Dujardin, 2015). Depending on the technique and genetic markers used, a different resolution of genetic diversity is achieved. For instance, microsatellite typing provides extremely

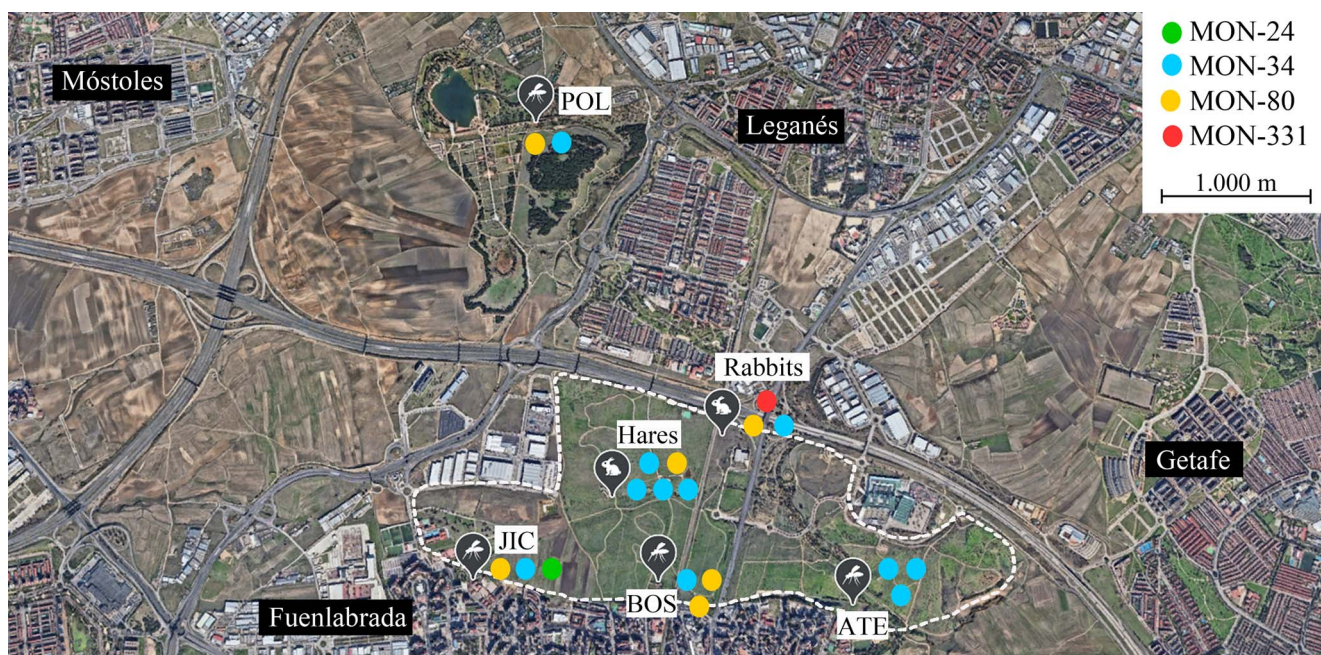


Figure 1. Distribution of *Leishmania infantum* zymodemes and hosts analysed in the leishmaniasis outbreak from Madrid region (Spain). Each circle represents 1 strain coloured according to its zymodeme. POL (placed in the municipality of Leganés), JIC, BOS and ATE (in the municipality of Fuenlabrada) are sand flies collecting stations where *L. infantum* strains were isolated from *P. perniciosus*. Rabbits were captured all around the perimeter of the Bosquesur park (marked with a dotted line) while hares were captured inside the park. The map image was taken from Google Earth (<https://earth.google.com>).

variable results, whereas single gene PCR-sequencing or PCR-RFLP techniques often show limited diversity (Van der Auwera and Dujardin, 2015). Accordingly, the 19 studied strains from the outbreak were genetically identical by both *hsp70* and ITS sequencing. The *hsp70* marker is very reliable for *Leishmania* species identification, but its resolution is too low to perform intraspecies characterization within the *Leishmania donovani* complex (Fernández-Arévalo *et al.*, 2020). The ITS region can differentiate strains below the species level, and it has been correlated with the geographical origin. Evidence for this is the distinction of the Lombardi genotype from the ITS-A genotype, which is also common in the area. However, in *L. infantum* or *L. donovani* complex strains, ITS shows a lower degree of polymorphism than in other complexes, not being sufficiently resolving for outbreak studies (Kuhls *et al.*, 2005; Fernández-Arévalo *et al.*, 2022). Sequencing results can be improved in terms of resolution and robustness by a MLST approach (Kuhls and Mauricio, 2019). However, the lack of such studies in the area, together with the absence of a consensus scheme providing sufficient resolution for *L. infantum sensu stricto* strains from the same geographical origin, hinders, in this case, an integrative understanding of the results. As a side note, it may be interesting to mention that a recent study based on complete maxicircle coding regions, which could be considered kind of MLST, detected 2 distinct *L. infantum* populations among humans in the Madrid region, in almost perfect agreement with ITS typing (Solana *et al.*, 2022).

MLEE offers an intermediate and manageable level of discrimination for these species (Fernández-Arévalo *et al.*, 2022) and it has been extensively used in epidemiological studies to assess the geographical distribution of isolates and clinical presentations, and to elucidate vectors, and reservoirs (Pratlong *et al.*, 2004; Montoya *et al.*, 2007; Ait-Oudhia *et al.*, 2011; Haouas *et al.*, 2012). However, nowadays, this methodology is falling out of use. The requirement of mass culture of the strains and its manual and laborious protocol make it a costly and time-consuming technique, limiting its use to specific situations. MLEE is not the most suitable technique for inferring taxonomy, as it relies on phenotypic traits that may not be consistent with genetics. But, when applied for *L. infantum* intraspecific typing, MLEE can provide valuable information on reservoirs and vectors, allowing comparison between foci, and providing data to fill epidemiological gaps. Here lies the interest in using MLEE to characterize the strains isolated from the vectors and reservoirs implicated in the leishmaniasis outbreak in Madrid.

The use of MLEE to type *L. infantum* has shown that strains zymodemes in humans may belong to viscerotropic, dermatropic or both (Pratlong *et al.*, 2004). In the canine reservoir, zymodeme MON-1 is predominant and few others have been isolated, giving rise to the suspicion of anthroponotic transmission cycles in some foci (Ait-Oudhia *et al.*, 2011). MON-1 is also the main zymodeme identified in isolates from other reservoir hosts, such as cats, foxes, rats, horses and even rabbits (Gállego *et al.*, 2001; Martín-Sánchez *et al.*, 2004; Campino *et al.*, 2006; Díaz-Sáez *et al.*, 2014). In contrast, greater variation has been observed in strains isolated from sand flies (Gállego *et al.*, 2001; Martín-Sánchez *et al.*, 2004). In the strains included in the present study, MLEE analysis revealed 4 different zymodemes (MON-24, MON-34, MON-80 and MON-331), which vary from each other by a single enzyme (NP1). Except for MON-331, these zymodemes are commonly found in the Madrid region, as well as in the rest of Spain and the Mediterranean basin (Jiménez *et al.*, 1995; Chicharro *et al.*, 2003, 2013; Pratlong *et al.*, 2013). Nevertheless, the frequency of the zymodemes detected here differs from what is usually found in Spain. None of the strains analysed belonged to MON-1, the predominant *L. infantum* zymodeme, which may be found in

ITS-Lombardi strains. Moreover, only a single strain was identified as MON-24, the second most frequent human *L. infantum* zymodeme including in the area of the outbreak (Jiménez *et al.*, 1995; Chicharro *et al.*, 2003, 2013), where it was isolated from a *P. perniciosus*. Other studies carried out in the Mediterranean basin have identified MON-24 in strains from cases of human VL and CL and canine leishmaniasis, as well as from sand flies (Gállego *et al.*, 2001; Martín-Sánchez *et al.*, 2004; Campino *et al.*, 2006; Haouas *et al.*, 2012). Surprisingly, the most prevalent zymodeme in our study was MON-34 (11/19 strains), followed by MON-80 (6/19 strains), both detected in hares, rabbits and sand flies captured at all the collection points. According to the literature, zymodeme MON-34 has been isolated in human CL and VL cases and in animals (dogs and racoons), but it has not been reported in sand flies until now (Jiménez *et al.*, 1995; Harrat *et al.*, 1996; Gállego *et al.*, 2001; Martín-Sánchez *et al.*, 2004; Montoya *et al.*, 2007; Pratlong *et al.*, 2013; Fernández-Arévalo *et al.*, 2022). MON-80 is less frequent in Spain, and it has been isolated mainly in humans with VL and CL throughout its distribution range (Gállego *et al.*, 2001; Chicharro *et al.*, 2003; Martín-Sánchez *et al.*, 2004; Campino *et al.*, 2006; Pratlong *et al.*, 2013). To the best of our knowledge, only 1 study in North Africa has reported MON-80 in dogs (Benikhlef *et al.*, 2009), and no previous reports describe it in sand flies, although it has been demonstrated experimentally that *P. perniciosus* could be a potential vector in the outbreak area (Remadi *et al.*, 2018). Therefore, this is the first time that MON-34 and MON-80 strains have been isolated from *P. perniciosus* and leporids.

Regarding the zymodeme MON-331, found here in a rabbit, only 1 strain has been described before (GR-19, MON-24 var NP1¹⁵⁰), in an HIV-positive human with VL (Martín-Sánchez *et al.*, 2004). Although rare, NP1¹⁵⁰ has been detected in other zymodemes of the *L. donovani* complex in isolates from Kenya, Sudan and China (Pratlong *et al.*, 2013).

Unfortunately, no human isolates from this outbreak have been typed by MLEE. Only 1 study has characterized human strains from the Fuenlabrada outbreak (Chicharro *et al.*, 2013), using ITS and *haspb* (k26) as molecular markers, also identifying 4 strain types (by *haspb* (k26)) that shared the ITS-Lombardi sequence. There is no apparent correlation between the isoenzymes and ITS sequences. Besides MON-34, MON-80 and MON-331, reported here, ITS-Lombardi has been described in MON-1, MON-24 and MON-27 strains (Chicharro *et al.*, 2013; Fernández-Arévalo *et al.*, 2022), whereas ITS sequence types other than Lombardi have been observed in MON-1, MON-24 and MON-34 strains (Kuhls *et al.*, 2005; Chicharro *et al.*, 2013; Fernández-Arévalo *et al.*, 2022). As far as we know, there is no correlation between *haspb* (k26) and MLEE either. Based on the available data, it seems that human, reservoir and vector strains from the Fuenlabrada outbreak are similar, but it is not possible to ascertain any relationship between them.

According to MLEE background records, it is likely that the human strains from the outbreak could correspond to MON-34, MON-80 and MON-24. Regarding clinical manifestations, all these zymodemes have been detected in immunocompetent and immunocompromised individuals with VL and CL (Jiménez *et al.*, 1995; Gállego *et al.*, 2001; Chicharro *et al.*, 2003; Martín-Sánchez *et al.*, 2004; Pratlong *et al.*, 2013), cases of which were also reported in the outbreak (Arce *et al.*, 2013; Carrillo *et al.*, 2013; Horrillo *et al.*, 2015). However, despite providing data of unquestionable interest, it is unlikely that MLEE would be performed in human samples due to the performance drawbacks mentioned above.

When gathering all the available information about *L. infantum* in the Fuenlabrada outbreak, various questions arise. On one hand, the typing results reflect a certain homogeneity

among the strains involved, although they belong to different zymodemes, and the strain types found are not new or exclusive to the area, which casts doubt on the emergence and transmission of a specific strain type as the detonator of the outbreak. On the other hand, the strains isolated from *P. perniciosus* in the area have shown higher virulence in the transmission of *L. infantum* to hamsters in *ex vivo* tests with experimentally infected *P. perniciosus* and in *in vivo* studies with the hamster and mouse model (Dominguez-Bernal *et al.*, 2014; Martín-Martín *et al.*, 2015; Mas *et al.*, 2021). However, the strains do not seem to cause disease in leporids (Molina *et al.*, 2012; Jiménez *et al.*, 2014), nor were they found in local dogs, and some exhibit less virulent behaviour in canine cells (Mas *et al.*, 2020). It remains uncertain if there was a selection of these strain types in the leporid reservoir or if they were simply the strain types present in the area at the onset of the outbreak, which arose from the interaction between large populations of vectors, reservoirs and humans. Furthermore, it is unknown if these strains are circulating among leporids in other endemic regions.

The lack of comparable characterization studies hampers a global understanding of the situation. To fully determine the epidemiological characteristics of the Fuenlabrada outbreak and the circulating strains in the region, more comprehensive studies with a higher number of isolates from humans, vectors and reservoirs from a range of *L. infantum* endemic regions are needed, as well as more robust and discriminative techniques.

Supplementary material. The supplementary material for this article can be found at <https://doi.org/10.1017/S0031182023001336>

Data availability statement. Sequences are available in GenBank under accession numbers OQ747159–OQ747177.

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References

- Aït-Oudhia K, Harrat Z, Benikhlef R, Dedet JP and Pratlong F (2011) Canine *Leishmania infantum* enzymatic polymorphism: a review including 1023 strains of the Mediterranean area, with special reference to Algeria. *Acta Tropica* **118**, 80–86.

- Akhoundi M, Kuhls K, Cannet A, Votýpka J, Marty P, Delaunay P and Sereno D (2016) A historical overview of the classification, evolution, and dispersion of *Leishmania* parasites and sandflies. *PLOS Neglected Tropical Diseases* **10**, e0004349.
- Aliaga L, Cobo F, Mediavilla JD, Bravo J, Osuna A, Amador JM, Martín-Sánchez J, Cordero E and Navarro JM (2003) Localized mucosal leishmaniasis due to *Leishmania (Leishmania) infantum* clinical and microbiologic findings in 31 patients. *Medicine* **82**, 147–158.
- Arce A, Estirado A, Ordobas M, Sevilla S, García N, Moratilla L, de la Fuente S, Martínez A, Pérez A, Aránguez E, Iriaso A, Sevillano O, Bernal J and Vilas F (2013) Re-emergence of leishmaniasis in Spain: community outbreak in Madrid, Spain, 2009 to 2012. *Eurosurveillance* **18**, 20546.
- Azami-Conesa I, Gómez-Muñoz MT and Martínez-Díaz RA (2021) A systematic review (1990–2021) of wild animals infected with zoonotic leishmaniasis. *Microorganisms* **9**, 1101.
- Benikhlef R, Aoun K, Bedoui K, Harrat Z and Bouratbine A (2009) First identifications of *Leishmania infantum* MON-80 in the dog in Algeria and Tunisia. *Revue de Médecine Vétérinaire* **160**, 464–466.
- Berriatua E, Maia C, Conceição C, Özbel Y, Töz S, Baneth G, Pérez-Cutillas P, Ortuño M, Muñoz C, Jumakanova Z, Pereira A, Rocha R, Monge-Maillou B, Gasimov E, van der Stede Y, Torres G and Gossner CM (2021) Leishmaniasis in the European Union and neighboring countries. *Emerging Infectious Diseases* **27**, 1723–1727.
- Campino L, Pratlong F, Abranches P, Rioux J-A, Santos-Gomes G, Alves-Pires C, Cortes S, Ramada J, Cristovão JM, Afonso MO and Dedet JP (2006) Leishmaniasis in Portugal: enzyme polymorphism of *Leishmania infantum* based on the identification of 213 strains. *Tropical Medicine and International Health* **11**, 1708–1714.
- Cardoso L, Schallig H, Persichetti MF and Pennisi MG (2021) New epidemiological aspects of animal leishmaniasis in Europe: the role of vertebrate hosts other than dogs. *Pathogens (Basel, Switzerland)* **10**, 307.
- Carrillo E, Moreno J and Cruz I (2013) What is responsible for a large and unusual outbreak of leishmaniasis in Madrid? *Trends in Parasitology* **29**, 579–580.
- Centro Nacional de Epidemiología (2019) Enfermedades de declaración obligatoria. Casos notificados por comunidades autónomas y tasas por 100.000 habitantes. España 2018. Datos definitivos (06/05/2019). Instituto de Salud Carlos III. https://www.isciii.es/QueHacemos/Servicios/VigilanciaSaludPublicaRENAVE/EnfermedadesTransmisibles/Documents/INFORMES/INFORMES_RENAVE/RENAVE_cierre_EDO_2018.pdf
- Chicharro C, Jiménez MI and Alvar J (2003) Iso-enzymatic variability of *Leishmania infantum* in Spain. *Annals of Tropical Medicine and Parasitology* **97**, S57–S64.
- Chicharro C, Llanes-Acevedo P, García E, Nieto J, Moreno J and Cruz I (2013) Molecular typing of *Leishmania infantum* isolates from a leishmaniasis outbreak in Madrid, Spain, 2009 to 2012. *Eurosurveillance* **18**, 20545.
- Díaz-Sáez V, Merino-Espinosa G, Morales-Yuste M, Corpas-López V, Pratlong F, Morillas-Márquez F and Martín-Sánchez J (2014) High rates of *Leishmania infantum* and *Trypanosoma nabiasi* infection in wild rabbits (*Oryctolagus cuniculus*) in sympatric and syntrophic conditions in an endemic canine leishmaniasis area: epidemiological consequences. *Veterinary Parasitology* **202**, 119–127.
- Dirección General de Salud Pública – Consejería de Sanidad de la Comunidad de Madrid (2015) *Leishmaniasis en la Comunidad de Madrid*. Documentos Técnicos de Salud Pública 63. <http://www.madrid.org/bvirtual/BVCM017837.pdf>
- Dominguez-Bernal G, Jiménez M, Molina R, Ordóñez-Gutiérrez L, Martínez-Rodrigo A, Mas A, Cutuli MT and Carrión J (2014) Characterisation of the *ex vivo* virulence of *Leishmania infantum* isolates from *Phlebotomus perniciosus* from an outbreak of human leishmaniasis in Madrid, Spain. *Parasites and Vectors* **7**, 499.
- Fernández-Arévalo A, El Baidouri F, Ravel C, Ballart C, Abras A, Lachaud L, Tebar S, Lami P, Pratlong F, Gállego M and Muñoz C (2020) The *Leishmania donovani* species complex: a new insight into taxonomy. *International Journal for Parasitology* **50**, 1079–1088.
- Fernández-Arévalo A, Ballart C, Muñoz-Basagoiti J, Basarte L, Lobato G, Arnau A, Abras A, Tebar S, Llovet T, Lami P, Pratlong F, Alsina M, Roe E, Puig L, Muñoz C and Gállego M (2022) 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. *Transboundary and Emerging Diseases* **69**, 1404–1418.

- Fernández Martínez B, Gómez Barroso D and Cano Portero R (2019) La leishmaniasis en España: evolución de los casos notificados a la Red Nacional de Vigilancia Epidemiológica desde 2005 a 2017 y resultados de la vigilancia de 2014 a 2017. *Boletín Epidemiológico Semanal* **27**, 15–27.
- Ferroglio E, Romano A, Trisciunglio A, Poggi M, Ghiggi E, Sacchi P and Biglino A (2006) Characterization of *Leishmania infantum* strains in blood samples from infected dogs and humans by PCR-RFLP. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **100**, 636–641.
- Gállego M, Pratlong F, Fisa R, Riera C, Rioux JA, Dedet JP and Portús M (2001) The life-cycle of *Leishmania infantum* MON-77 in the Priorat (Catalonia, Spain) involves humans, dogs and sandflies; also literature review of distribution and hosts of *L. infantum* zymodemes in the Old World. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **95**, 269–271.
- Gomez-Barroso D, Herrador Z, San Martín JV, Gherasim A, Aguado M, Romero-Maté A, Molina L, Aparicio P and Benito A (2015) Spatial distribution and cluster analysis of a leishmaniasis outbreak in the southwestern Madrid region, Spain, September 2009 to April 2013. *Eurosurveillance* **20**, 1–10.
- González E, Jiménez M, Hernández S, Martín-Martín I and Molina R (2017) Phlebotomine sand fly survey in the focus of leishmaniasis in Madrid, Spain (2012–2014): seasonal dynamics, *Leishmania infantum* infection rates and blood meal preferences. *Parasites and Vectors* **10**, 368.
- González E, Molina R, Iriso A, Ruiz S, Aldea I, Tello A, Fernández D and Jiménez M (2021) Opportunistic feeding behaviour and *Leishmania infantum* detection in *Phlebotomus perniciosus* females collected in the human leishmaniasis focus of Madrid, Spain (2012–2018). *PLoS Neglected Tropical Diseases* **15**, 1–12.
- Haouas N, Chaker E, Chargui N, Gocci M, Belhadj S, Kallel K, Aoun K, Akrouf FM, Ben Said M, Pratlong F, Dedet JP, Mezhdoud H, Lami P, Zribi M, Azaïez R and Babba H (2012) Geographical distribution updating of Tunisian leishmaniasis foci: about the isoenzymatic analysis of 694 strains. *Acta Tropica* **124**, 221–228.
- Harrat Z, Pratlong F, Belazzoug S, Dereure J, Deniau M, Rioux JA, Belkaid M and Dedet JP (1996) *Leishmania infantum* and *L. major* in Algeria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **90**, 625–629.
- Horrillo L, San Martín JV, Molina L, Madroñal E, Matía B, Castro A, García-Martínez J, Barrios A, Cabello N, Arata IG, Casas JM and Ruiz Giardin JM (2015) Atypical presentation in adults in the largest community outbreak of leishmaniasis in Europe (Fuenlabrada, Spain). *Clinical Microbiology and Infection* **21**, 269–273.
- Humanes-Navarro AM, Herrador Z, Redondo L, Cruz I and Fernández-Martínez B (2021) Estimating human leishmaniasis burden in Spain using the capture-recapture method, 2016–2017. *PLoS ONE* **16**, e0259225.
- Jiménez M, Ferrer-Dufol M, Cañavate C, Gutiérrez-Solar B, Molina R, Lagun F, López-Vélez R, Cercenado E, Daudén E, Blazquez J, de Guevara CL, Gómez J, de la Torre J, Barros C, Altes J, Serra T and Alvar J (1995) Variability of *Leishmania (Leishmania) infantum* among stocks from immunocompromised, immunocompetent patients and dogs in Spain. *FEMS Microbiology Letters* **131**, 197–204.
- Jiménez M, González E, Iriso A, Marco E, Alegret A, Fúster F and Molina R (2013) Detection of *Leishmania infantum* and identification of blood meals in *Phlebotomus perniciosus* from a focus of human leishmaniasis in Madrid, Spain. *Parasitology Research* **112**, 2453–2459.
- Jiménez M, González E, Martín-Martín I, Hernández S and Molina R (2014) Could wild rabbits (*Oryctolagus cuniculus*) be reservoirs for *Leishmania infantum* in the focus of Madrid, Spain? *Veterinary Parasitology* **202**, 296–300.
- Kuhls K and Mauricio I (2019) Phylogenetic studies. In Clos J (ed.), *Leishmania. Methods in Molecular Biology*. New York, NY: Humana Press, pp. 9–68. doi: 10.1007/978-1-4939-9210-2_2
- Kuhls K, Mauricio IL, Pratlong F, Presber W and Schönián G (2005) Analysis of ribosomal DNA internal transcribed spacer sequences of the *Leishmania donovani* complex. *Microbes and Infection* **7**, 1224–1234.
- Kumar S, Stecher G, Li M, Knyaz C and Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* **35**, 1547–1549.
- Martín-Martín I, Jiménez M, González E, Eguiluz C and Molina R (2015) Natural transmission of *Leishmania infantum* through experimentally infected *Phlebotomus perniciosus* highlights the virulence of *Leishmania* parasites circulating in the human visceral leishmaniasis outbreak in Madrid, Spain. *Veterinary Research* **46**, 138.
- Martín-Sánchez J, Gramiccia M, Di Muccio T, Ludovisi A and Morillas-Márquez F (2004) Isoenzymatic polymorphism of *Leishmania infantum* in southern Spain. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **98**, 228–232.
- Martín-Sánchez J, Torres-Medina N, Morillas-Márquez F, Corpas-López V and Díaz-Sáez V (2021) Role of wild rabbits as reservoirs of leishmaniasis in a non-epidemic Mediterranean hot spot in Spain. *Acta Tropica* **222**, 1–9.
- Mas A, Martínez-Rodrigo A, Orden JA, Viñals LM, Domínguez-Bernal G and Carrión J (2020) A further investigation of the leishmaniasis outbreak in Madrid (Spain): low-infectivity phenotype of the *Leishmania infantum* BOS1FL1 isolate to establish infection in canine cells. *Veterinary Immunology and Immunopathology* **230**, 110148.
- Mas A, Martínez-Rodrigo A, Orden JA, Molina R, Jiménez M, Jiménez MÁ, Carrión J and Domínguez-Bernal G (2021) Properties of virulence emergence of *Leishmania infantum* isolates from *Phlebotomus perniciosus* collected during the human leishmaniasis outbreak in Madrid, Spain. Hepatic histopathology and immunological parameters as virulence markers in the mouse model. *Transboundary and Emerging Diseases* **68**, 704–714.
- Mauricio IL (2018) *Leishmania* taxonomy. In Bruschi F and Gradoni L (eds), *The Leishmaniases: Old Neglected Tropical Diseases*. Cham: Springer, pp. 15–30. doi: 10.1007/978-3-319-72386-0_2
- Molina R, Jiménez MI, Cruz I, Iriso A, Martín-Martín I, Sevillano O, Melero S and Bernal J (2012) The hare (*Lepus granatensis*) as potential sylvatic reservoir of *Leishmania infantum* in Spain. *Veterinary Parasitology* **190**, 268–271.
- Molina R, Jiménez M, García-Martínez J, San Martín JV, Carrillo E, Sánchez C, Moreno J, Alves F and Alvar J (2020) Role of asymptomatic and symptomatic humans as reservoirs of visceral leishmaniasis in a Mediterranean context. *PLoS Neglected Tropical Diseases* **14**, 1–16.
- Montoya L, Gállego M, Gavignet B, Piarroux R, Rioux JA, Portús M and Fisa R (2007) Application of microsatellite genotyping to the study of a restricted *Leishmania infantum* focus: different genotype compositions in isolates from dogs and sand flies. *American Journal of Tropical Medicine and Hygiene* **76**, 888–895.
- Piarroux R, Trouvé V, Pratlong F, Martini A, Lambert M and Rioux JA (1994) The use of isoelectric focusing on polyacrylamide gel for the enzymatic analysis of 'Old World' *Leishmania* species. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **88**, 475–478.
- Pratlong F, Rioux JA, Marry P, Faraut-Gambarelli F, Dereure J, Lanotte G and Dedet JP (2004) Isoenzymatic analysis of 712 strains of *Leishmania infantum* in the south of France and relationship of enzymatic polymorphism to clinical and epidemiological features. *Journal of Clinical Microbiology* **42**, 4077–4082.
- Pratlong F, Lami P, Ravel C, Balard Y, Dereure J, Serres G, El Baidouri F and Dedet J-P (2013) Geographical distribution and epidemiological features of Old World *Leishmania infantum* and *Leishmania donovani* foci, based on the isoenzyme analysis of 2277 strains. *Parasitology* **140**, 423–434.
- Remadi L, Jiménez M, Chargui N, Haouas N, Babba H and Molina R (2018) The vector competence of *Phlebotomus perniciosus* for *Leishmania infantum* zymodemes of Tunisia. *Parasitology Research* **117**, 2499–2506.
- Rioux JA, Lanotte G, Serres E, Pratlong F, Bastien P and Perieres J (1990) Taxonomy of *Leishmania*. Use of isoenzymes. Suggestions for a new classification. *Annales de Parasitologie Humaine et Comparée* **65**, 111–125.
- Sáez VD, Morillas-Márquez F, Merino-Espinosa G, Corpas-López V, Morales-Yuste M, Pesson B, Barón-López S, Lucientes-Curdi J and Martín-Sánchez J (2018) *Phlebotomus langeroni* Nitzulescu (Diptera, Psychodidae) a new vector for *Leishmania infantum* in Europe. *Parasitology Research* **117**, 1105–1113.
- Solana JC, Chicharro C, García E, Aguado B, Moreno J and Requena JM (2022) Assembly of a large collection of maxicircle sequences and their usefulness for *Leishmania* taxonomy and strain typing. *Genes* **13**, 1070.
- Toledo A, Martín-Sánchez J, Pesson B, Sanchiz-Marín C and Morillas-Márquez F (2002) Genetic variability within the species *Leishmania infantum* by RAPD. A lack of correlation with zymodeme structure. *Molecular and Biochemical Parasitology* **119**, 257–264.
- Van der Auwera G and Dujardin JC (2015) Species typing in dermal leishmaniasis. *Clinical Microbiology Reviews* **28**, 265–294.
- Van der Auwera G, Maes I, De Doncker S, Ravel C, Cnops L, Van Esbroeck M, Van Gompel A, Clerinx J and Dujardin JC (2013) Heat-shock protein

- 70 gene sequencing for *Leishmania* species typing in European tropical infectious disease clinics. *Eurosurveillance* 18, 20543.
- Van der Auwera G, Davidsson L, Buffet P, Ruf M-T, Gramiccia M, Varani S, Chicharro C, Bart A, Harms G, Chiodini PL, Brekke H, Robert-Gangneux F, Cortes S, Verweij JJ, Scarabello A, Karlsson Söbirk S, Guéry R, van Henten S, Di Muccio T, Carra E, van Thiel P, Vandeputte M, Gaspari V and Blum J** (2022) Surveillance of leishmaniasis cases from 15 European centres, 2014 to 2019: a retrospective analysis. *Eurosurveillance* 27, 2002028.
- World Health Organization** (2010) *Control of the leishmaniasis: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22–26 March 2010*. Technical report series, 949.