

Molecular typing of *Leishmania infantum* isolates from a leishmaniasis outbreak in Madrid, Spain, 2009 to 2012

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Leishmaniasis is endemic in south-west Europe. Recent data point to the spread and (re-)emergence of this disease in previously endemic and non-endemic European countries. A recent example is the urban community outbreak of cutaneous and visceral leishmaniasis in the south-west of Madrid autonomous community, Spain, which began on 1 July 2009. A total of 446 cases associated to this outbreak were reported up to 31 December 2012. We show molecular typing data for 73 *Leishmania infantum* isolates obtained from January 2008 to July 2012 from different areas of Madrid, including those affected by the outbreak. Seven different genotypes were identified by combining data from two targets: the ribosomal internal transcribed spacers (ITS)-1 and -2 and the *haspb* (*k26*) gene. The results contribute to a better understanding of the parasite population circulating in the region, and indicate that most of the outbreak-associated isolates (22/31) were infected by parasites with the same combined genotype. Additional data from 82 *L. infantum* isolates typed as either *MON-1* or *MON-24* by isoenzyme analysis indicate that far from concluding that the outbreak was caused by a 'new' emerging genotype, further molecular typing-based surveillance studies are required to better understand the epidemiology of leishmaniasis in the region.

Introduction

Leishmania infantum is the causative agent of autochthonous cutaneous and visceral cases of leishmaniasis in Spain, and female sandflies of the species *Phlebotomus perniciosus* and *P. ariasi* are responsible of its transmission; this depends on a zoonotic cycle, in which dogs are considered the main reservoir hosts [1]. However, reports on *Leishmania* infection in other animals from Spain, such as wild carnivores, captive macropods, rabbits and hares, are increasing in number [2-5]. Although, their role in transmission to humans has yet to be elucidated, *L. infantum* transmission from naturally infected hares to *P. perniciosus* sandflies has been recently proven [5].

In Spain, leishmaniasis is considered a hypoendemic disease (0.41 cases per 100,000 inhabitants in 2012)

[1]. However, figures for both visceral (VL) and particularly cutaneous leishmaniasis (CL) are underestimated, due to the absence of a centralised surveillance system and because leishmaniasis is not a mandatorily notifiable disease in all autonomous communities of the country [1]. After the first description of a case of acquired immune deficiency syndrome (AIDS)-associated leishmaniasis in 1985 [6], Spain faced a re-emergence of leishmaniasis related to the spread of the human immunodeficiency virus (HIV). Of the 1,911 cases of coinfection reported from south-west Europe to the World Health Organization (WHO) between 1990 and 2001, Spain accounted for 1,099 of them [7]. Fortunately, the introduction of highly active antiretroviral therapy (HAART) therapy in the late 1990s contributed to a marked decrease of coinfection cases in south-west Europe: 299 cases were reported to WHO, 130 of which were from Spain, between 2001 and 2006 [8].

In spite of the worrying *Leishmania*/HIV coinfection phenomenon, leishmaniasis seems to have been under control in south-west Europe. Nevertheless, attention has been recently drawn to the probable spread/re-emergence of leishmaniasis in Europe, including discussion of the contributing factors [9,10]. At the same time, examples appeared, such as the northward spread of human and canine leishmaniasis in Italy (in 2003) [11,12] and canine leishmaniasis in Spain (in 2011) [13], and endemic transmission of *L. infantum* to dogs in Hungary (in 2007), which until then had been regarded as free of leishmaniasis [14,15].

An urban community outbreak of CL and VL in the south-west of Madrid autonomous community (hereafter referred to as Madrid), Spain, provides a further example [16]. In Madrid, leishmaniasis surveillance has been carried out through a reporting system of mandatorily notifiable diseases since 1997, with regular records of 12 to 25 leishmaniasis cases per year [16]. However, in the last quarter of year 2010, a marked increase in the number of reported cases was noticed; subsequent investigation indicated that the outbreak had started on 1 July 2009 in the south-west of the

TABLE 1Distribution by year, Health Area and pathology of the cases of leishmaniasis^a, Madrid, Spain, 1 January 2008–31 July 2012 (n=475)

Health Area	Year					Total per Health Area	Clinical form of leishmaniasis		
	2008	2009	2010	2011	2012		CL	VL	Other ^b
A1	2	4	0	0	0	6	0	6	0
A2	2	0	3	5	0	10	3	7	0
A3	0	0	1	2	0	3	0	3	0
A4	4	10	3	5	4	26	6	18	2
A5	20	8	13	11	6	58	13	45	0
A6	1	0	0	3	2	6	3	2	1
A7	1	3	1	2	2	9	1	3	5
A8	7	8	7	4	3	29	3	23	3
A9 ^c	5	2	16	141	126	290	211	73	6
A10 ^c	3	1	2	9	10	25	10	14	1
A11	0	0	0	8	5	13	1	12	0
Total	45	36	46	190	158	475	251	206	18

CL: cutaneous leishmaniasis; VL: visceral leishmaniasis.

^a Cases diagnosed during the period stated by polymerase chain reaction at the World Health Organization Collaborating Centre for Leishmaniasis in Madrid, Spain (Instituto de Salud Carlos III).^b 'Other' comprises mucosal leishmaniasis (ML), localised lymphadenopathy (LL) (A4: 2 ML in 2009; A6: 1 ML in 2008; A7: 2 LL in 2009–2011, and 3 ML in 2011–2012; A8: 1 LL in 2009 and 2 ML in 2009–2012; A9: 5 LL in 2010–2012, and 1 ML in 2011; A10: 1 LL in 2012).^c Health Areas 9 and 10 were affected by the outbreak that began on 1 July 2009.

region. The outbreak affected four geographically close municipalities, which share wide areas in urban green parks, with a population of half a million inhabitants [16]. As reported by Arce et al. [17], a total of 446 cases of leishmaniasis associated with this outbreak were reported up to 31 December 2012, with epidemic peaks in the winter of 2010 and 2011. Of the 446 cases, 160 (35.9%) had visceral and 286 (64.1%) cutaneous forms of the disease. The median age was 44 years (range: 2 months–95 years). Risk factors associated with immunosuppression appeared only in 15.2% of the cases.

The WHO Collaborating Centre for Leishmaniasis in Madrid (Instituto de Salud Carlos III), which acts as the reference laboratory for leishmaniasis in Spain, contributed to the diagnosis of the outbreak-associated cases through molecular and serological methods, as well as parasite isolation in culture. As part of these activities, we also performed molecular typing of an assembly of 73 isolates from the outbreak area and other regions of Madrid obtained from January 2008, the year before the outbreak started, to July 2012. The results of this investigation are here presented.

Concurrently, additional data from a second assembly of 83 *L. infantum* human isolates, collected in Madrid between 1988 and 2005 and typed by multilocus enzyme electrophoresis (MLEE) as *MON-1* and *MON-24*, zymodemes responsible of most of the CL and VL cases in Spain [18], are also presented.

Methods

First assembly: isolates from the outbreak area and other regions of Madrid, 2008–2012

In the WHO Collaborating Centre for Leishmaniasis, diagnosis of leishmaniasis is made on the basis of molecular and serological methods, as well as by isolation in Novy-MacNeal-Nicolle (NNN) culture. Additionally, *Leishmania* isolates are received from different hospitals to be kept in our cryobank.

From 1 January 2008 to 31 July 2012, we diagnosed a total of 475 cases of leishmaniasis in Madrid by polymerase chain reaction (PCR) [19]. Samples of patients came from the 11 Health Areas into which the region is divided. The mean number of cases for 2008 to 2010 was 42, while 190 were diagnosed in 2011 and 158 in the first six months of 2012; this large increase was related to the outbreak of leishmaniasis in Health Areas 9 and 10, in the south-west of the region. Of the 475 cases, 251 were CL, 206 VL and 18 other forms of leishmaniasis (namely mucosal and localised lymphadenopathy). Details of the leishmaniasis cases diagnosed by PCR and their distribution by year and Health Area are shown in Table 1.

On the basis of the distribution of cases shown in Table 1 and on the availability of culture isolates successfully obtained from diagnostic samples at the WHO Collaborating Centre for Leishmaniasis or received

TABLE 2A

Molecular typing of selected *Leishmania infantum* isolates from Madrid, Spain, obtained from 1 January 2008–31 July 2012 (n=73)

Isolate WHO code ^a	Year of isolation	Health Area ^b of isolate origin	Clinical form of leishmaniasis	HIV	Age group in years	ITS type	<i>haspb</i> (<i>k26</i>) size in base pairs	Combined genotype ^c
MHOM/ES/2008/LLM-1665	2008	1	VL	NEG	>18	LOMBARDI	962	L-962
MHOM/ES/2008/LLM-1695	2008	1	VL	NEG	>18	LOMBARDI	836	L-836
MHOM/ES/2008/LLM-1643	2008	5	VL	POS	>18	A	794	A-794
MHOM/ES/2008/LLM-1657	2008	5	VL	POS	>18	LOMBARDI	920	L-920
MHOM/ES/2008/LLM-1676	2008	5	VL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2008/LLM-1667	2008	5	VL	POS	>18	A	584	A-584
MHOM/ES/2008/LLM-1644	2008	8	VL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2008/LLM-1646	2008	8	CL	POS	>18	LOMBARDI	920	L-920
MHOM/ES/2008/LLM-1653	2008	8	VL	POS	>18	A	584	A-584
MHOM/ES/2008/LLM-1681	2008	8	VL	POS	>18	A	626	A-626
MHOM/ES/2008/LLM-1648	2008	9	VL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2009/LLM-1707	2009	1	VL	POS	>18	A	626	A-626
MHOM/ES/2009/LLM-1725	2009	1	VL	NEG	>18	A	626	A-626
MHOM/ES/2009/LLM-1734	2009	1	VL	NEG	>18	A	626	A-626
MHOM/ES/2009/LLM-1750	2009	1	VL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2009/LLM-1729	2009	4	CL	NEG	>18	A	794	A-794
MHOM/ES/2009/LLM-1756	2009	4	VL	POS	>18	A	626	A-626
MHOM/ES/2009/LLM-1790	2009	5	CL	POS	>18	LOMBARDI	920	L-920
MHOM/ES/2009/LLM-1703	2009	8	VL	POS	>18	A	626	A-626
MHOM/ES/2009/LLM-1712	2009	8	VL	NEG	>18	A	626	A-626
MHOM/ES/2009/LLM-1714	2009	8	VL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2009/LLM-1896	2010	1	VL	NEG	>18	A	626	A-626
MHOM/ES/2010/LLM-1920	2010	2	VL	NEG	<5	LOMBARDI	962	L-962
MHOM/ES/2010/LLM-1873	2010	4	VL	NEG	>18	A	626	A-626
MHOM/ES/2010/LLM-1858	2010	5	VL	NEG	>18	A	626	A-626
MHOM/ES/2010/LLM-1859	2010	8	VL	NEG	<5	LOMBARDI	962/920	L-962/920
MHOM/ES/2010/LLM-1888	2010	8	VL	NEG	<5	LOMBARDI	962	L-962
MHOM/ES/2010/LLM-1854	2010	9	VL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2010/LLM-1918	2010	9	LL	NEG	>18	LOMBARDI	962	L-962
MHOM/ES/2010/LLM-1886	2010	9	VL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2010/LLM-1899	2010	9	VL	POS	>18	LOMBARDI	920	L-920
MHOM/ES/2011/LLM-2027	2011	1	VL	NEG	>18	A	626	A-626
MHOM/ES/2011/LLM-2032	2011	1	VL	NEG	>18	LOMBARDI	962/920	L-962/920
MHOM/ES/2011/LLM-2005	2011	3	VL	NEG	<5	LOMBARDI	920	L-920
MHOM/ES/2011/LLM-2051	2011	4	VL	NEG	>18	A	626	A-626
MHOM/ES/2011/LLM-1948	2011	5	CL	NEG	>18	A	626	A-626
MHOM/ES/2011/LLM-2033	2011	5	VL	NEG	<5	A	626	A-626
MHOM/ES/2011/LLM-2047	2011	5	VL	POS	>18	A	626	A-626
MHOM/ES/2011/LLM-1946	2011	7	LL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2011/LLM-2018	2011	8	VL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2011/LLM-2037	2011	9	VL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2011/LLM-1988	2011	9	VL	NEG	>18	LOMBARDI	962	L-962
MHOM/ES/2011/LLM-1964	2011	9	VL	NEG	>18	LOMBARDI	836	L-836

CL: cutaneous leishmaniasis; HIV: human immunodeficiency virus; *haspb*: hydrophilic acylated surface protein B gene; ITS: ribosomal internal transcribed spacers; LL: localised lymphadenopathy; ML: mucosal leishmaniasis; NEG: negative; POS: positive; VL: visceral leishmaniasis; WHO: World Health Organization.

^a Isolates ordered by year of isolation and Health Area.

^b Health Areas 9 and 10 were affected by the outbreak that began on 1 July 2009.

^c Combined genotypes are derived from combining the results of ITS sequence type and *haspb* (*k26*) polymerase chain reaction product size.

TABLE 2B

 Molecular typing of selected *Leishmania infantum* isolates from Madrid, Spain, obtained from 1 January 2008–31 July 2012 (n=73)

Isolate WHO code ^a	Year of isolation	Health Area ^b of isolate origin	Clinical form of leishmaniasis	HIV	Age group in years	ITS type	<i>haspb (k26)</i> size in base pairs	Combined genotype ^c
MHOM/ES/2011/LLM-1929	2011	9	VL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2011/LLM-1956	2011	9	VL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2011/LLM-1974	2011	9	CL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2011/LLM-1982	2011	9	LL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2011/LLM-1983	2011	9	CL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2011/LLM-1984	2011	9	VL	POS	>18	LOMBARDI	962/920	L-962/920
MHOM/ES/2011/LLM-1998	2011	9	ML	NEG	>18	LOMBARDI	962/920	L-962/920
MHOM/ES/2011/LLM-2001	2011	9	CL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2011/LLM-2039	2011	9	VL	NEG	>18	LOMBARDI	962/920	L-962/920
MHOM/ES/2011/LLM-2046	2011	9	CL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2011/LLM-2048	2011	9	CL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2011/LLM-1937	2011	10	VL	NEG	>18	LOMBARDI	962/920	L-962/920
MHOM/ES/2011/LLM-1954	2011	10	VL	NEG	>18	LOMBARDI	962	L-962
MHOM/ES/2011/LLM-2025	2011	11	VL	NEG	5–18	LOMBARDI	962/920	L-962/920
MHOM/ES/2012/LLM-2096	2012	1	VL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2012/LLM-2134	2012	1	VL	POS	>18	LOMBARDI	920	L-920
MHOM/ES/2012/LLM-2098	2012	5	VL	NEG	<5	A	626	A-626
MHOM/ES/2012/LLM-2118	2012	6	CL	NEG	>18	A	626	A-626
MHOM/ES/2012/LLM-2109	2012	6	VL	NEG	>18	LOMBARDI	962	L-962
MHOM/ES/2012/LLM-2139	2012	9	VL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2012/LLM-2059	2012	9	CL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2012/LLM-2063	2012	9	VL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2012/LLM-2064	2012	9	CL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2012/LLM-2072	2012	9	CL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2012/LLM-2074	2012	9	CL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2012/LLM-2076	2012	9	CL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2012/LLM-2077	2012	9	CL	NEG	>18	LOMBARDI	920	L-920
MHOM/ES/2012/LLM-2079	2012	9	VL	POS	>18	LOMBARDI	920	L-920
MHOM/ES/2012/LLM-2097	2012	9	VL	NEG	>18	LOMBARDI	962/920	L-962/920
MHOM/ES/2012/LLM-2113	2012	9	VL	NEG	>18	LOMBARDI	920	L-920

CL: cutaneous leishmaniasis; HIV: human immunodeficiency virus; *haspb*: hydrophilic acylated surface protein B gene; ITS: ribosomal internal transcribed spacers; LL: localised lymphadenopathy; ML: mucosal leishmaniasis; NEG: negative; POS: positive; VL: visceral leishmaniasis; WHO: World Health Organization.

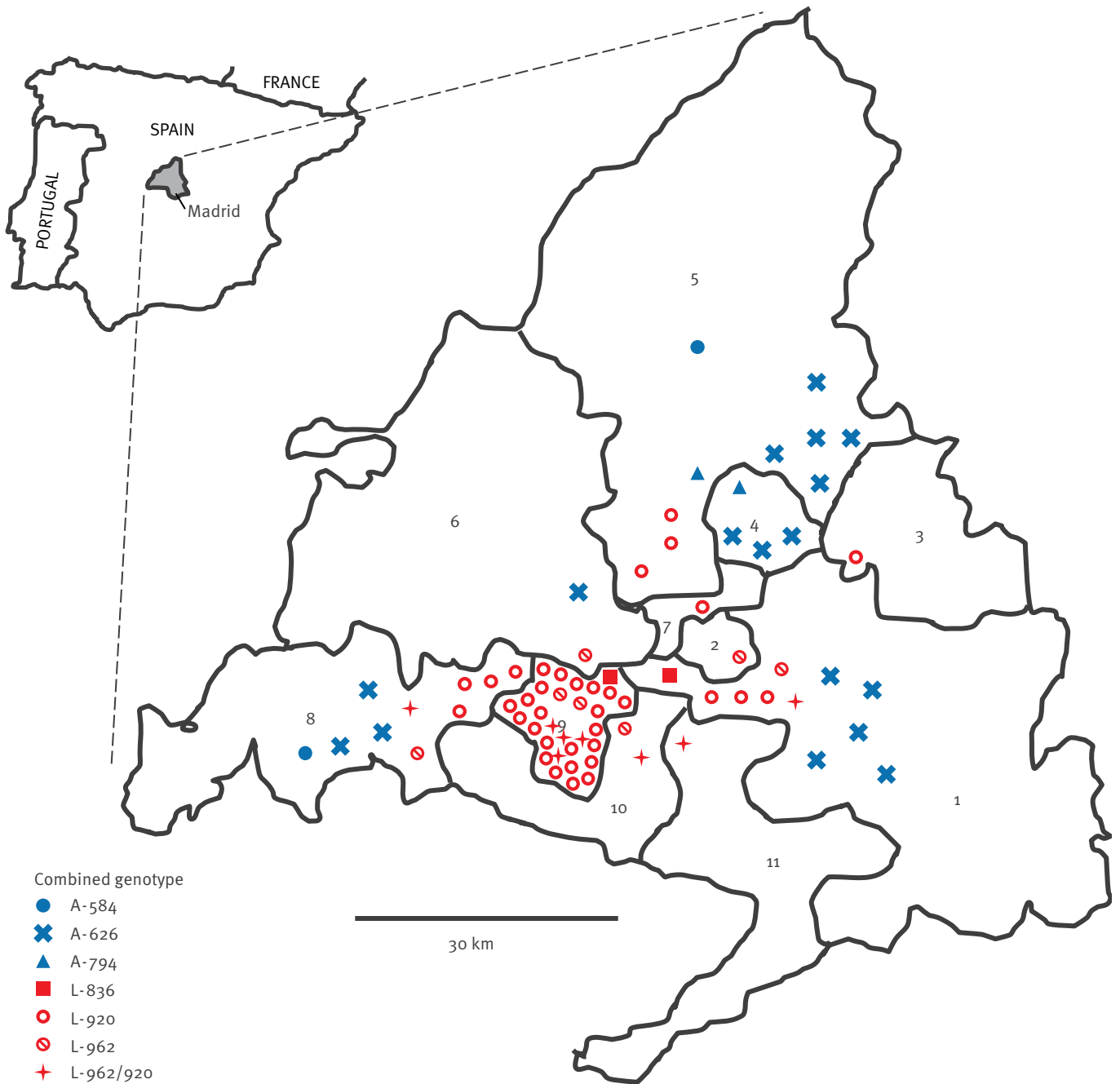
^a Isolates ordered by year of isolation and Health Area.

^b Health Areas 9 and 10 were affected by the outbreak that began on 1 July 2009.

^c Combined genotypes are derived from combining the results of ITS sequence type and *haspb (k26)* polymerase chain reaction product size.

FIGURE 1

Distribution and combined genotype of analysed *Leishmania infantum* isolates from Madrid, Spain, 1 January 2008–31 July 2012 (n=73)



The numbers 1–11 represent the 11 Health Areas in Madrid. The combined genotypes of the isolates are shown.

from referral hospitals, we prepared an assembly of *Leishmania* isolates from the cases diagnosed by the WHO Collaborating Centre during 1 January 2008 to 31 July 2012. The assembly was not solely focused on the Health Areas affected by the outbreak (Health Areas 9 and 10): in order to have a fuller picture of the context in which the outbreak occurred, isolates from all 11 Health Areas in Madrid were included. For the same reason, we also decided to include isolates obtained in 2008 (the year before the outbreak started). A total of 73 *L. infantum* isolates were included in our analysis: 16 from CL patients, 53 from VL patients, 3 from patients affected by localised lymphadenopathy and 1 from a patient with mucosal leishmaniasis. A total of 15 isolates were obtained from patients with concomitant human immunodeficiency virus (HIV) infection. Further details of the isolates included in the assembly for molecular typing concerning year of isolation, Health Area of origin, clinical form of leishmaniasis, HIV status, age and sex are shown in Table 2 and Figure 1.

Second assembly: *L. infantum* MON-1 and MON-24 isolates, 1988–2005

The intraspecies variability of *L. infantum* has long been studied by MLEE. This approach, and the subsequent identification of different zymodemes, constituted an extremely useful taxonomic tool, which has contributed much to understand the epidemiology of leishmaniasis [20,21]. Although MLEE has been used for *Leishmania* typing during the past 25 years, some drawbacks have been attributed to this methodology when compared with molecular methods [22], which are being increasingly used for epidemiological studies of visceral and cutaneous leishmaniasis and are widely available in various laboratories.

As *L. infantum* MON-1 and MON-24 zymodemes are known to be responsible for most CL and VL cases in Spain [18], we also included in this study 83 *L. infantum* isolates from Madrid that had been typed by MLEE as MON-1 (n=55) and MON-24 (n=28) at the WHO Collaborating Centre for Leishmaniasis, to have a reference at the MLEE level in our analysis. Details of these isolates are given in Table 3 and Figure 2.

DNA extraction

DNA was extracted from culture pellets cryopreserved at the WHO Collaborating Centre for Leishmaniasis using the QIAamp DNA mini kit (QIAGEN). The DNA was eluted in PCR-grade water and adjusted to a final concentration of 10 ng/µl with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). For further PCR reactions, 5 µl of each DNA sample were used.

Molecular typing

Two different targets of the *Leishmania* genome were selected for molecular typing analyses. ITS1 and ITS2 were amplified following the protocol described by Kuhls et al. [23] and the *haspb* (*k26*) gene (hydrophilic acylated surface protein B) was amplified as described by Haralambous et al. [24]. For species identification of

isolates from the first assembly, the *heat shock protein 70* (*hsp70*) gene was amplified as described by Fraga et al. [25]; species identification was not performed on the second assembly of isolates because they had been previously typed as *L. infantum* MON-1 or MON-24 by MLEE. PCR products were run on 2% agarose gels stained with ethidium bromide and visualised under ultraviolet light.

Direct sequencing of the ITS1, ITS2 and *hsp70* PCR products was performed with the corresponding forward and reverse primers; internal primers for sequencing were also used for *hsp70*, as described elsewhere [25]. Before DNA sequencing, the PCR products were excised from agarose gels and purified using the QIAquick Gel Extraction Kit (QIAGEN). The Big-Dye Terminator Cycle Sequencing Ready Reaction Kit V3.1 and the automated ABI PRISM 377 DNA sequencer (Applied Biosystems) were used. Sequences obtained were analysed and edited using the software BioEdit Sequence Alignment Editor, version 7.0.9.0 [26]. ITS1 and ITS2 sequences were compared with sequences of the ITS types described by Kuhls et al. [23], these sequences were aligned with BioEdit Sequence Alignment Editor using ClustalW multiple alignment algorithm and were manually adjusted.

For accurate estimation of the *k26* PCR product size, the bands were excised from agarose gels, purified using the QIAquick Gel Extraction Kit (QIAGEN), and analysed by capillary electrophoresis using the Agilent 2100 Bioanalyzer and the Agilent DNA 1000 kit (Agilent Technologies).

ITS genotypes were assigned to each isolate according to the sequence polymorphism of the 12 microsatellite regions in ITS1 (four sites) and ITS2 (eight sites). *k26* genotypes were assigned according to the size of the PCR product, and adjusted considering the gene size variability is due to the number of 42 nucleotide repeated motifs [24,27-29]. A combined genotype, derived from combining the results of ITS sequence type and *k26* PCR product size, was then assigned to each isolate (first assembly only).

For the second assembly, we present data on ITS genotypes only; information on the *k26* gene analysis will be published separately.

Phylogenetic analysis

Phylogenetic analysis based on the nucleotide sequences of the *Leishmania* ITS1 and ITS2 was performed based on maximum parsimony using PHYLIP (PHYLogeny Inference Package), version 3.69 [30]. To test the robustness of the internal branches generated, we performed bootstrap analysis using 10,000 replications. We included DNA sequences retrieved from GenBank (AJ634341, AJ000288, AJ000294, AJ634356, AJ634367, AJ634373, AJ000297, AJ634376) that are representative for each of the ITS types A to H, respectively, described by Kuhls et al. [23]. According to these

TABLE 3A

Selected *Leishmania infantum* MON-1 and MON-24 isolates from Madrid, Spain, 1988–2005 (n=83)

Isolate WHO code ^a	Year of isolation	Health Area of isolate origin	Clinical form of leishmaniasis	HIV	Age group in years	Zymodeme	ITS type
MHOM/ES/1988/LLM-180	1988	1	VL	NEG	>18	MON-1	A
MHOM/ES/1990/LLM-195	1990	1	VL	Unknown	Unknown	MON-1	A
MHOM/ES/1991/LLM-326	1991	4	VL	POS	Unknown	MON-1	A
MHOM/ES/1991/LLM-328	1991	4	VL	NEG	Unknown	MON-1	A
MHOM/ES/1992/LLM-339	1992	1	VL	Unknown	Unknown	MON-1	A
MHOM/ES/1992/LLM-335	1992	1	VL	POS	Unknown	MON-1	LOMBARDI
MHOM/ES/1992/LLM-315	1992	4	VL	Unknown	Unknown	MON-1	LOMBARDI
MHOM/ES/1992/LLM-323	1992	5	VL	POS	>18	MON-1	A
MHOM/ES/1992/LLM-306	1992	5	VL	POS	Unknown	MON-1	A
MHOM/ES/1993/LLM-404	1993	5	VL	POS	Unknown	MON-1	A
MHOM/ES/1994/LLM-410	1994	5	VL	POS	>18	MON-1	A
MHOM/ES/1995/LLM-442	1995	2	VL	POS	>18	MON-1	A
MHOM/ES/1995/LLM-468	1995	3	VL	Unknown	Unknown	MON-1	A
MHOM/ES/1995/LLM-450	1995	4	VL	POS	Unknown	MON-1	A
MHOM/ES/1995/LLM-464	1995	5	VL	POS	>18	MON-1	A
MHOM/ES/1995/LLM-470	1995	5	VL	POS	>18	MON-1	A
MHOM/ES/1995/LLM-482	1995	5	VL	NEG	>18	MON-1	A
MHOM/ES/1996/LLM-554	1996	4	VL	POS	Unknown	MON-1	A
MHOM/ES/1996/LLM-549	1996	5	VL	POS	>18	MON-1	A
MHOM/ES/1996/LLM-556	1996	5	VL	POS	>18	MON-1	A
MHOM/ES/1996/LLM-548	1996	5	VL	POS	Unknown	MON-1	A
MHOM/ES/1997/LLM-607	1997	4	CL	POS	>18	MON-1	A
MHOM/ES/1997/LLM-465	1997	5	VL	POS	>18	MON-1	LOMBARDI
MHOM/ES/1997/LLM-616	1997	5	VL	POS	>18	MON-1	A
MHOM/ES/1997/LLM-623	1997	5	VL	POS	Unknown	MON-1	A
MHOM/ES/1997/LLM-674	1997	5	VL	POS	>18	MON-1	A
MHOM/ES/1997/LLM-666	1997	5	CL	POS	Unknown	MON-1	A
MHOM/ES/1997/LLM-665	1997	9	VL	POS	5–18	MON-1	A
MHOM/ES/1997/LLM-690	1997	11	VL	POS	>18	MON-1	LOMBARDI
MHOM/ES/1998/LLM-739	1998	3	VL	POS	>18	MON-1	A
MHOM/ES/1999/LLM-755	1998	5	VL	POS	Unknown	MON-1	A
MHOM/ES/1998/LLM-789	1998	8	VL	NEG	>18	MON-1	A
MHOM/ES/1999/LLM-896	1999	5	VL	POS	Unknown	MON-1	LOMBARDI
MHOM/ES/1999/LLM-883	1999	8	CL	POS	>18	MON-1	A
MHOM/ES/1999/LLM-826	1999	11	VL	POS	>18	MON-1	A
MHOM/ES/2000/LLM-936	2000	4	VL	Unknown	Unknown	MON-1	LOMBARDI
MHOM/ES/2001/LLM-983	2001	5	VL	POS	>18	MON-1	A
MHOM/ES/2001/LLM-980	2001	8	VL	NEG	>18	MON-1	LOMBARDI
MHOM/ES/2001/LLM-984	2001	8	VL	NEG	>18	MON-1	LOMBARDI
MHOM/ES/2002/LLM-1220	2002	1	VL	POS	Unknown	MON-1	A
MHOM/ES/2002/LLM-1181	2002	1	VL	POS	Unknown	MON-1	A
MHOM/ES/2002/LLM-1166	2002	5	VL	POS	>18	MON-1	LOMBARDI
MHOM/ES/2003/LLM-1262	2003	1	VL	POS	Unknown	MON-1	A
MHOM/ES/2003/LLM-1304	2003	1	VL	NEG	>18	MON-1	LOMBARDI

CL: cutaneous leishmaniasis; HIV: human immunodeficiency virus; ITS: ribosomal internal transcribed spacers; NEG: negative; POS: positive; VL: visceral leishmaniasis; WHO: World Health Organization.

^a Isolates ordered by year of isolation, Health Area and zymodeme.

TABLE 3B

Selected *Leishmania infantum* MON-1 and MON-24 isolates from Madrid, Spain, 1988–2005 (n=83)

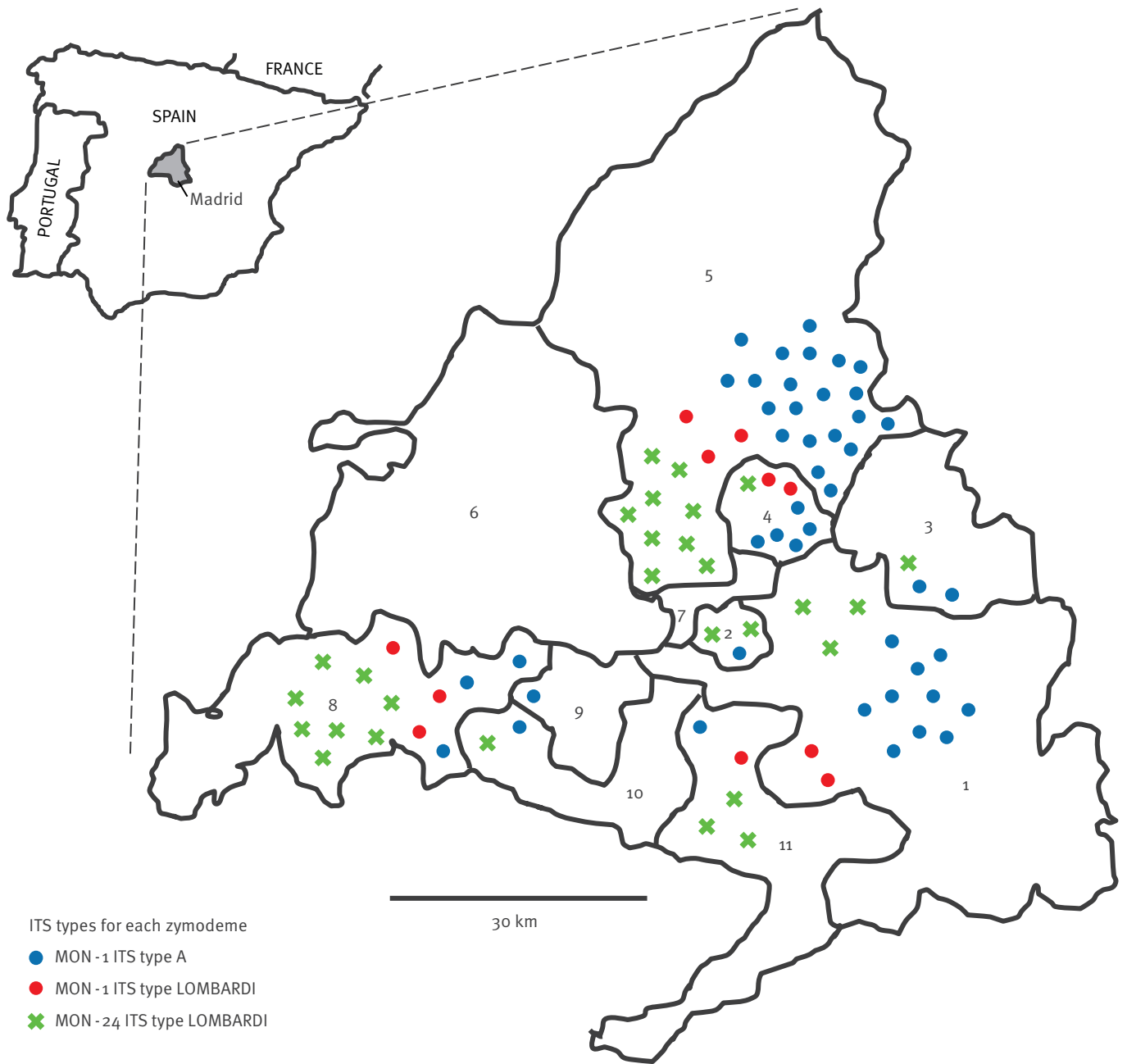
Isolate WHO code ^a	Year of isolation	Health Area of isolate origin	Clinical form of leishmaniasis	HIV	Age group in years	Zymodeme	ITS type
MHOM/ES/2003/LLM-1327	2003	5	VL	NEG	<5	MON-1	A
MHOM/ES/2003/LLM-1258	2003	8	VL	NEG	>18	MON-1	LOMBARDI
MHOM/ES/2004/LLM-1372	2004	1	VL	POS	>18	MON-1	A
MHOM/ES/2004/LLM-1377	2004	1	VL	POS	>18	MON-1	A
MHOM/ES/2004/LLM-1337	2004	5	VL	POS	Unknown	MON-1	A
MHOM/ES/2004/LLM-1461	2004	5	VL	POS	Unknown	MON-1	A
MHOM/ES/2004/LLM-1405	2004	5	VL	POS	Unknown	MON-1	A
MHOM/ES/2004/LLM-1347	2004	10	VL	POS	Unknown	MON-1	A
MHOM/ES/2005/LLM-1524	2005	1	VL	Unknown	>18	MON-1	A
MHOM/ES/2005/LLM-1523	2005	1	VL	Unknown	>18	MON-1	A
MHOM/ES/2005/LLM-1492	2005	8	VL	POS	Unknown	MON-1	A
MHOM/ES/1995/LLM-443	1995	4	VL	POS	Unknown	MON-24	LOMBARDI
MHOM/ES/1995/LLM-441	1995	5	VL	POS	Unknown	MON-24	LOMBARDI
MHOM/ES/1995/LLM-485	1995	5	VL	POS	>18	MON-24	LOMBARDI
MHOM/ES/1995/LLM-465	1995	5	VL	POS	>18	MON-24	LOMBARDI
MHOM/ES/1995/LLM-456	1995	8	VL	NEG	>18	MON-24	LOMBARDI
MHOM/ES/1996/LLM-587	1996	2	CL	POS	>18	MON-24	LOMBARDI
MHOM/ES/1996/LLM-576	1996	5	VL	POS	>18	MON-24	LOMBARDI
MHOM/ES/1996/LLM-598	1996	5	VL	POS	>18	MON-24	LOMBARDI
MHOM/ES/1996/LLM-569	1996	11	VL	POS	Unknown	MON-24	LOMBARDI
MHOM/ES/1997/LLM-713	1997	2	CL	POS	>18	MON-24	LOMBARDI
MHOM/ES/1997/LLM-711	1997	11	VL	POS	>18	MON-24	LOMBARDI
MHOM/ES/1998/LLM-779	1998	8	VL	POS	>18	MON-24	LOMBARDI
MHOM/ES/1998/LLM-730	1998	11	VL	POS	>18	MON-24	LOMBARDI
MHOM/ES/1999/LLM-845	1999	8	VL	POS	Unknown	MON-24	LOMBARDI
MHOM/ES/2000/LLM-957	2000	5	VL	Unknown	Unknown	MON-24	LOMBARDI
MHOM/ES/2001/LLM-1078	2001	1	VL	POS	Unknown	MON-24	LOMBARDI
MHOM/ES/2001/LLM-1065	2001	5	VL	POS	>18	MON-24	LOMBARDI
MHOM/ES/2001/LLM-1027	2001	8	VL	POS	Unknown	MON-24	LOMBARDI
MHOM/ES/2001/LLM-1032	2001	8	VL	POS	Unknown	MON-24	LOMBARDI
MHOM/ES/2001/LLM-982	2001	1	VL	POS	>18	MON-24	LOMBARDI
MHOM/ES/2002/LLM-1177	2002	8	CL	NEG	Unknown	MON-24	LOMBARDI
MHOM/ES/2003/LLM-1305	2003	5	VL	POS	>18	MON-24	LOMBARDI
MHOM/ES/2004/LLM-1346	2004	3	VL	POS	>18	MON-24	LOMBARDI
MHOM/ES/2004/LLM-1367	2004	8	VL	POS	Unknown	MON-24	LOMBARDI
MHOM/ES/2005/LLM-1475	2005	5	CL	NEG	<5	MON-24	LOMBARDI
MHOM/ES/2005/LLM-1526	2005	1	VL	Unknown	Unknown	MON-24	LOMBARDI
MHOM/ES/2005/LLM-1478	2005	8	VL	NEG	>18	MON-24	LOMBARDI
MHOM/ES/2005/LLM-1525	2005	10	VL	NEG	Unknown	MON-24	LOMBARDI

CL: cutaneous leishmaniasis; HIV: human immunodeficiency virus; ITS: ribosomal internal transcribed spacers; NEG: negative; POS: positive; VL: visceral leishmaniasis; WHO: World Health Organization.

^a Isolates ordered by year of isolation, Health Area and zymodeme.

FIGURE 2

Distribution and ITS type of analysed *Leishmania infantum* isolates from Madrid, Spain, 1988–2005 (n=83)

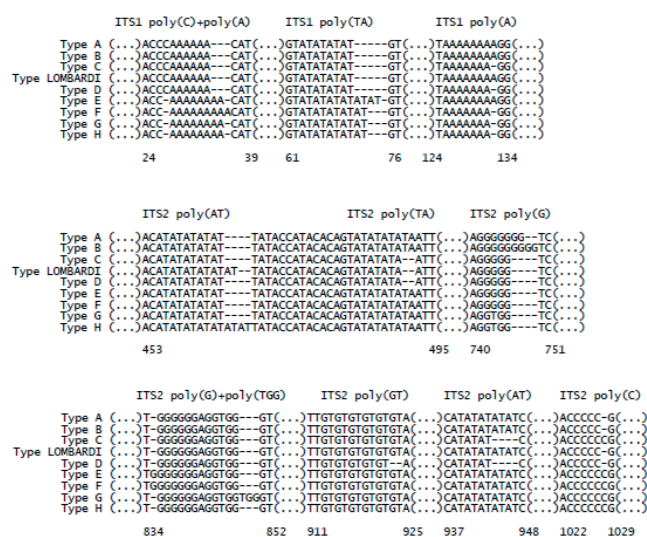


ITS: ribosomal internal transcribed spacers.

The numbers 1–11 represent the 11 Health Areas in Madrid. The ITS types for each zymodeme are shown.

FIGURE 3

Partial alignment of the eight ITS sequence types for strains of the *Leishmania donovani* complex^a and the ITS sequence type LOMBARDI identified in 52 *L. infantum* isolates from Madrid, Spain, 2008–12



ITS: ribosomal internal transcribed spacers.

Differences between the sequence types are based solely on polymorphisms of the 12 microsatellites.

^a The eight ITS types were described by Kuhls et al. [23]. Types A and B were described in *L. infantum* strains isolated in the Mediterranean basin, China and Brazil; type C in a *L. donovani* strain from China; types D, E and F in *L. donovani* strains from Ethiopia and Sudan; type G in *L. donovani* strains from Kenya and India and type H in *L. donovani* strains from India.

authors [23], ITS types A and B correspond to *L. infantum* from the Mediterranean basin, China and Brazil, ITS type C corresponds to *L. donovani* from China, ITS types D, E and F are associated with *L. donovani* from East Africa, ITS type G is found in *L. donovani* from Kenya and India, and ITS type H in *L. donovani* from India.

Results

Analysis of the 12 microsatellite sites of the ITS1 and ITS2 concatenated sequences returned two different ITS types among the 73 isolates in the first assembly (isolates from the leishmaniasis outbreak area and other regions of Madrid from 2008 to 2012). ITS type A was present in 21 of the isolates; the remaining 52 isolates were of a type that had not been described previously by Kuhls et al. [23]. The concatenated sequence of both ITS1 and ITS2 corresponding to this second ITS type was subjected to a BLASTn search [31], which returned the best score with a sequence corresponding to the *L. infantum* strain MHOM/ES/87/Lombardi (Gen Bank Accession Number AJ000295). The 12 microsatellite sites found in MHOM/ES/87/Lombardi were

identical to those observed in the second ITS type we found (hereafter called ITS-LOMBARDI). Comparison of ITS-LOMBARDI with the polymorphic microsatellite types described by Kuhls et al. for the *L. donovani* complex [23] is shown in Figure 3. ITS-LOMBARDI was common to all 31 isolates we analysed from the outbreak that affected Health Areas 9 and 10. These isolates were confirmed to be *L. infantum* by *hsp70* PCR and DNA sequencing and further comparison to the DNA sequences of strains from the *L. donovani* complex (GenBank Accession Numbers FN395027–FN395033), described elsewhere [24].

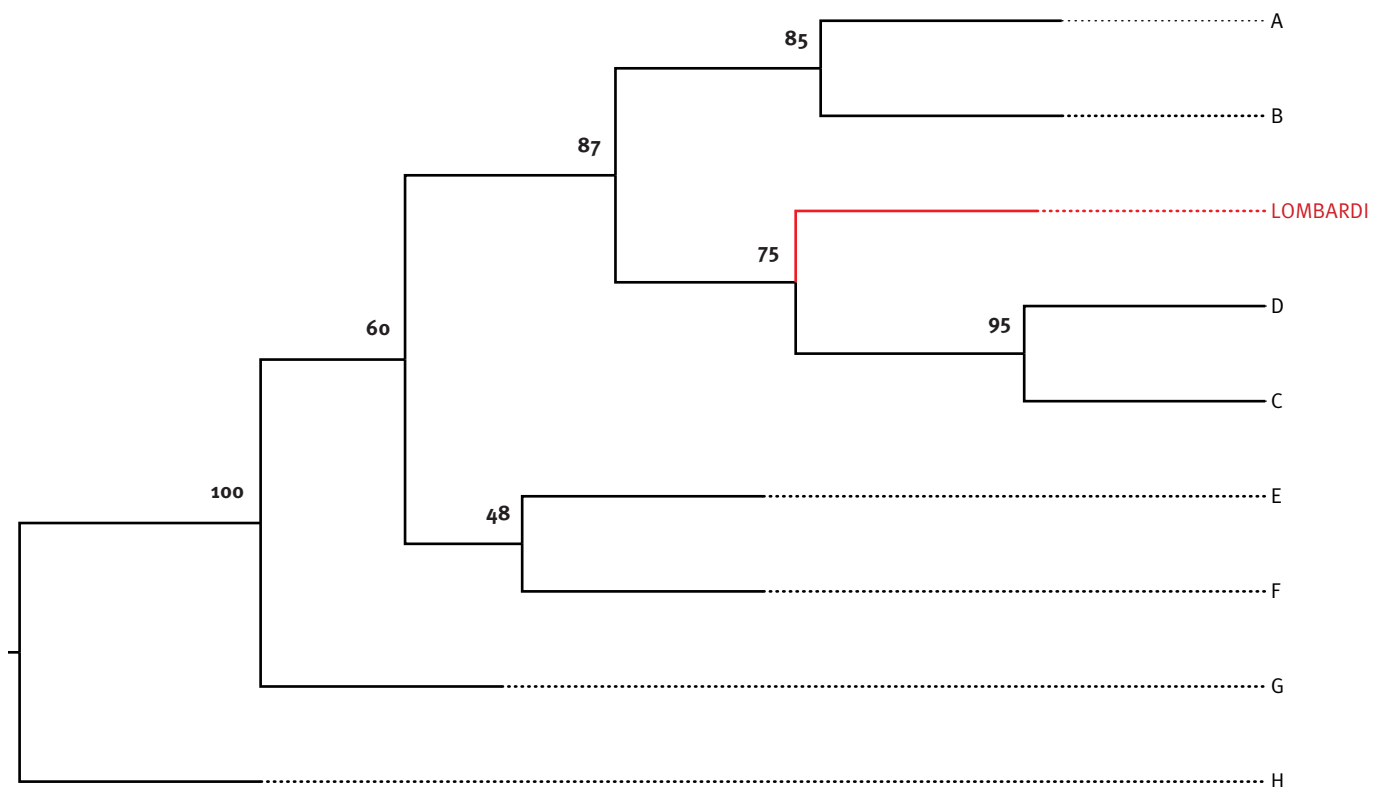
For the second assembly (*L. infantum* MON-1 and MON-24 isolates from 1988 to 2005), 44 of the 55 *L. infantum* MON-1 isolates were ITS type A, while 11 were ITS-LOMBARDI. All 28 *L. infantum* MON-24 isolates were ITS-LOMBARDI.

In both assemblies, ITS-A and ITS-LOMBARDI sequence types were found in isolates from CL and VL cases, as well as in patients who were HIV-positive and -negative. Both ITS types are widespread in Madrid, having been detected in almost all Health Areas, since 1988 (ITS-A) and since 1992 (ITS-LOMBARDI) (Tables 2 and 3). ITS-LOMBARDI was found in all the *L. infantum* MON-24 isolates studied, but is not specific to this zymodeme, given that 11 of the 55 *L. infantum* MON-1 isolates studied also had this ITS type.

Our phylogenetic analysis included ITS-LOMBARDI in a cluster – supported by a bootstrap value of 87% – with ITS types A, B, C and D. However, it is further separated from types A and B in a subsequent subcluster (Figure 4).

Capillary electrophoresis revealed seven different *k26* PCR products sizes for the 73 isolates of the first assembly studied by this method, ranging from 584 to 962 base pairs (bp). A single PCR product was obtained for 65 of the 73 isolates, while the other eight showed a double PCR product of 920 bp and 962 bp. When *k26* and ITS data were combined, again, seven genotypes were obtained, because ITS-A appears to be associated only with *k26* PCR products of 584–794 bp and ITS-LOMBARDI with larger *k26* PCR products (836–962 bp). Four combined genotypes were identified among the 32 isolates analysed from Health Areas 9 and 10 (31 of which were related to the outbreak), with L-920 (ITS-LOMBARDI in combination with a *k26* PCR product of 920 bp) the most frequent (present in 23 of the 32 isolates). In the other Health Areas (those not affected by the outbreak), the most frequent combined genotype was A-626 (present in 17/41 isolates), followed by L-920 (present in 12/41 isolates).

No particular association was found between the combined genotypes and the HIV status of the patients or clinical form of leishmaniasis (cutaneous or visceral). Of the three cases with localised lymphadenopathy (LL), two were caused by the combined genotype L-920

FIGURE 4Phylogenetic relationships of *Leishmania infantum* ITS-LOMBARDI within the *L. donovani* complex

ITS: ribosomal internal transcribed spacers.

The most parsimonious tree found by heuristic search is presented. It was inferred by parsimony analysis of the nucleotide sequences of ITS₁ and ITS₂. The numbers above the branches indicate the percentages with which a given branch is supported in 10,000 bootstrap replications. ITS types A–H are those described by Kuhls et al. [23].

and one by L-962. The case with mucosal leishmaniasis (ML) was caused by L-962/920. Two of the LL cases and the ML case occurred in Health Area 9 (Table 2).

Discussion

To the best of our knowledge, this is the first molecular typing study of the leishmaniasis outbreak in Madrid that began in July 2009 and the most comprehensive molecular typing study carried out in Spain with isolates obtained within such a short period of time (2008–12) and small geographical area (Table 2). We also provided further information on 83 *L. infantum* *MON-1* and *MON-24* isolates collected in Madrid during 1988 to 2005.

A previous molecular typing study, which included *L. infantum* isolates from Madrid and other regions in Spain (isolated between 1986 and 1993), reported only the presence of ITS types A and B for this *Leishmania* species [23]. However, in our study 71% (52/73) of the *L. infantum* isolates from the first assembly had an ITS type, ITS-LOMBARDI, which had not been previously described. This ITS type was also present in all of the

MON-24 and 20% (11/55) of the *MON-1* *L. infantum* isolates from the second assembly. It is noteworthy that this ITS type was also present in *L. infantum* *MON-24* strain MHOM/ES/87/Lombardi, which was isolated in Spain (location unknown) in 1987, and its ITS DNA sequence (AJ000295) was submitted to the GenBank in year 1998, although no report on this ITS type was published. Unfortunately, neither this ITS sequence nor any other obtained from a *MON-24* isolate were included by Kuhls et al. in their study of ITS sequence analysis of the *L. donovani* complex [23]. Our phylogenetic analysis, following a similar procedure, revealed that isolates with ITS-LOMBARDI, in spite of being *L. infantum* (as shown by MLEE and *hsp70* gene DNA sequence analyses), do not form a clear phylogenetic group with ITS types A, B (Mediterranean basin, Brazil, China) and C (China), and are not well separated from the second main group that includes all strains from East Africa and India. ITS-LOMBARDI has been circulating in Spain (region unknown) since at least 1987, based on the WHO code of the strain Lombardi (MHOM/ES/87/Lombardi). According to our data on the first and second assemblies presented here, ITS-LOMBARDI is

frequent in *L. infantum* isolates from Madrid and has been present in this region since at least 1992 (Table 3). *L. infantum* isolates obtained through xenodiagnosis from hares captured in an urban park during a study related to the Madrid outbreak described here were also typed as ITS-LOMBARDI [5] and this ITS type is also the only ITS type seen in isolates from all human cases associated with this outbreak that have been typed to date.

Another molecular typing study targeting the *haspb* (*k26*) gene included Spanish *L. infantum* isolates and revealed PCR product sizes of approximately 626 bp, 870/980 bp, 870/1200 bp and 870/1300 bp [24]. In our study *k26* PCR products sizes of 584–962 bp were found, with those of 626 and 920 bp being the most frequent, although among the outbreak-associated cases only *k26* PCR products of 836–962 bp were found (Table 2). A product size of 920 bp was the most frequent.

The epidemiological picture of the leishmaniasis outbreak affecting Health Areas 9 and 10 is consistent with focal transmission of the parasite [16]. This, together with the fact that the *L. infantum* isolates obtained from patients in these areas presented mostly the combined genotype L-920, might suggest that an emerging, less common or ‘new’ *L. infantum* strain was the causative agent of the outbreak. Care must be taken, however, before drawing this conclusion. Molecular typing-based surveillance studies of leishmaniasis are scarce in Madrid and in other parts of Spain, and probably elsewhere too. Genotype L-920 has been isolated in another five of the 11 Health Areas of Madrid and has been present in two of them (Health Areas 5 and 8) since at least 2008. Additionally, ITS-LOMBARDI has been described in an isolate from Spain obtained in 1987, and in *L. infantum* MON-1 and MON-24 isolates from Madrid since 1992 and 1995, respectively, in different Health Areas (Table 3, Figure 2). We believe that more comprehensive molecular typing-based surveillance studies should be carried out in Spain, as well as in other leishmaniasis endemic countries. Given that leishmaniasis is currently re-emerging and spreading to previously non-endemic areas [21], activities aimed at bridging research with surveillance, as suggested by Dujardin et al. [9], will contribute to a better understanding of the epidemiology of leishmaniasis and will allow control strategies to be developed.

In terms of molecular typing, further studies are needed to ascertain the magnitude and origin of the outbreak in Madrid, particularly those aiming to identify the parasite genotypes circulating among sandflies, dogs and other alternative reservoirs: the WHO Collaborating Centre for Leishmaniasis is currently working on this. The simple analysis of the *k26* PCR product size indicates that the outbreak was not caused by a single parasite strain (four combined genotypes were found). It seems more likely that the spread of a long-established transmission cycle from a nearby area, with its own

degree of parasite diversity, into an area with a population with little or no immunity against *Leishmania* could have originated this outbreak.

In spite of the wide application of molecular methods to assess *Leishmania* population structure and to help in epidemiological studies [32], there are few opportunities to validate molecular markers for leishmaniasis outbreak investigations. We believe that the results here presented will contribute to this and, together with the material collected, we would be able to validate other approaches such as multilocus sequence typing or multilocus microsatellite typing, taking advantage of this ‘experiment in nature’ – the outbreak in south-west Madrid.

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