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APPLICATION OF MICROSATELLITE GENOTYPING TO THE STUDY OF A RESTRICTED *LEISHMANIA INFANTUM* FOCUS: DIFFERENT GENOTYPE COMPOSITIONS IN ISOLATES FROM DOGS AND SAND FLIES

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Abstract. *Leishmania infantum* polymorphism was studied by DNA microsatellite analysis of 110 *L. infantum* stocks (94 from dogs, 15 from sand flies, and 1 from a human visceral case) from a rural leishmaniasis-endemic area (Priorat) in northeastern Spain. Three microsatellites of the eight present in three fragments (internal transcribed spacer, Lm4, and Lm2) of *L. infantum* nuclear DNA are polymorphic inside the focus, resulting in 17 genotypes. Isolates from dogs and sand flies had different allelic compositions and shared only four genotypes. Microsatellite analysis is useful for *L. infantum* genotyping and epidemiologic tracking. Its application with strains from dogs and vectors in an area endemic for leishmaniasis shows the heterogeneous distribution of *L. infantum* in hosts living in sympatric conditions.

INTRODUCTION

Leishmania infantum Nicolle, 1908 is the etiologic agent of human visceral (VL) leishmaniasis and cutaneous (CL) leishmaniasis in the European Mediterranean Basin, where dogs are considered to be the main reservoir. Although the main features of the parasite life cycle have been known for almost a century, several epidemiologic aspects of human and canine leishmaniasis are still poorly understood. Studies using multilocus enzyme electrophoresis (MLEE) in *L. infantum,* have shown polymorphism that is reflected in insect and host parasite specificity, geographic range, and virulence.[1,2] However, *L. infantum* strains from different disease-endemic areas of the Mediterranean region show distinct degrees of polymorphism. Some disease-endemic foci in southern France are characterized by low enzymatic polymorphism[1] in comparison with foci in Catalonia, Spain[1,2] or foci in southern Spain,[3] where higher polymorphism has been observed in isolates from a variety of sources (humans, dogs, rats, and sand flies).

A number of methods have proven useful in the analysis of the genetic composition of leishmanial parasite populations. Multilocus enzyme electrophoresis differentiates several zymodemes in the *L. infantum* complex.[1–6] Nevertheless, the lack of genetic uniformity detected by random amplified polymorphic DNA in *L. infantum* zymodemes in southern Spain[7] and southern France[8] has highlighted the need for other discriminatory typing methods for epidemiologic studies. Other high-resolution genetic methods, such as selective polymerase chain reaction (PCR),[9] PCR and sequencing,[10] or chromosome size polymorphism,[11] have confirmed the high polymorphism of *L. infantum* strains.

Microsatellite (MS) DNA sequences are markers used in genetic and population studies of Trypanosomatidae, such as *Trypanosoma cruzi*[12–14] and *Leishmania* spp.,[15–18] and other protozoa.[19,20] Microsatellite analyses of three sequences of the *L. infantum* genome (the internal transcribed spacer [ITS] in the rRNA genes and the Lm2 and Lm4 sequences in the nuclear genome) in strains from several areas of the Mediterranean Basin have shown a high level of polymorphism.[21] We applied MS analysis to 110 *L. infantum* stocks, mainly from dogs and sand flies, from a small disease-endemic area in northeastern Spain (Priorat region). This area is characterized by two vector species (*Phlebotomus (Larroussius) perniciosus* and *P. (L.) ariasi*),[22] with three zymodemes found in vectors (MON-1, MON-29, and MON-77) and three in dogs (MON-1, MON-28, and MON-77).[2,23] The application of a highly accurate discriminatory tool to a large number of *L. infantum* sympatric strains from dogs and vectors could provide valuable information about the parasite in this disease-endemic focus.

MATERIALS AND METHODS

Study area. The Priorat region is an area in northeastern Spain that produces grapes and hazelnuts. It has an area of 496.20 km^2 and a population of 10,000 inhabitants distributed in small rural villages. The region is mostly hilly (250–500 meters above sea level) and is crossed by the Montsant Mountains, which are 1,100 meters above sea level (Figure 1). The climate is meso-Mediterranean, with an average annual temperature of 15°C and rainfall of 500–600 mm. The transmission cycle of *L. infantum* occurs mainly in the central and southern areas of the region, which are between 200 and 500 meters above sea level (high endemic area) and have a natural vegetation predominately composed of oak (*Quercus ilex*) and pine (*Pinus halepensis*) trees. The prevalence of canine infection is about 20% in some villages, with an average prevalence of 12.2% (range = 10–14%). Lower seroprevalence rates of canine infection are found in northern part of Priorat, where *Quercus ilex ballota*, *Q. faginae*, *Q. humilis*, and *Pinus nigra salzmannii* are the dominant natural vegetation.[23,24] Although we have not recorded any cases of CL during the 20 years that we have been working in this region, the elderly community is aware of CL and some carry oriental sore scars. Seven instances of human leishmaniasis have been reported in this area from 1986 through 2003.[25]

***Leishmania infantum* stocks.** We analyzed 110 *Leishmania* stocks stored in the *Leishmania* Cryobank at Universitat de Barcelona in Spain and the International *Leishmania* Cryobank in Montpellier, France. They were collected between 1986 and 2002 in 15 municipalities in the Priorat region of Spain. Stocks were mainly isolated in those areas considered to be highly endemic for leishmaniasis (Figure 1). Ninety-four

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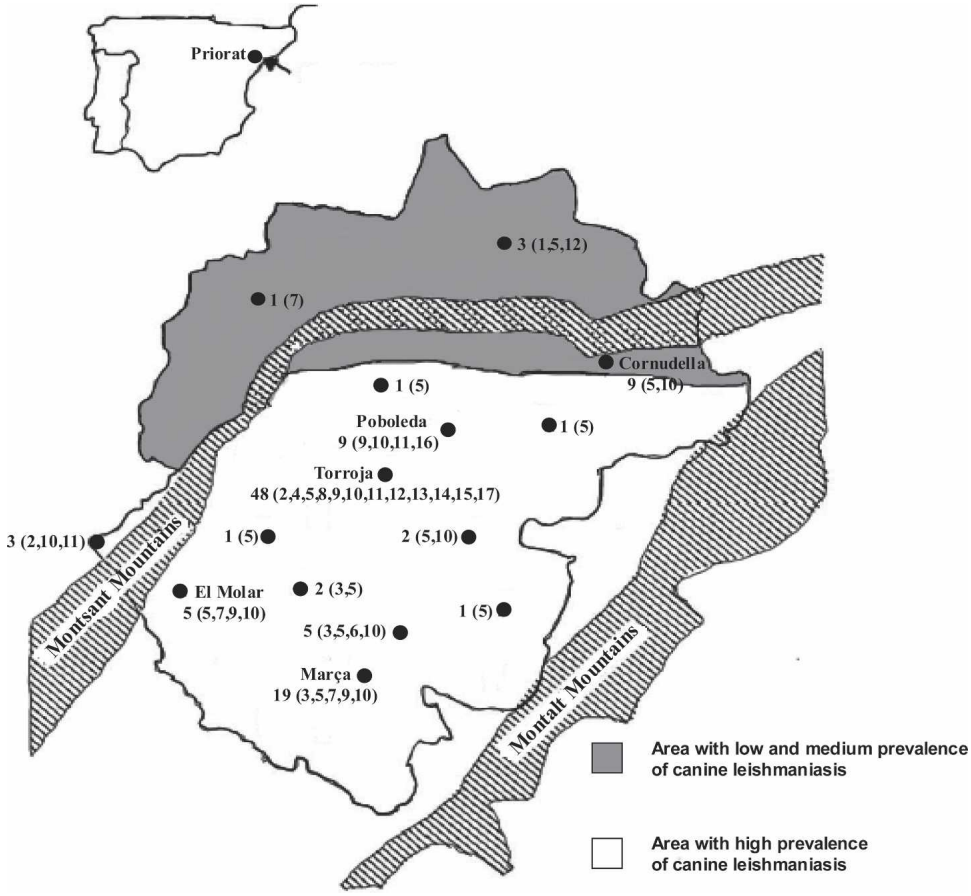

FIGURE 1. Map of the Priorat region of Spain. Dots represent municipalities, with the number of stocks isolated, and values in parentheses are multilocus genotypes observed.

stocks were isolated from dogs (34 symptomatic dogs and 60 asymptomatic dogs) as previously defined,[23] 15 from sand flies (10 from *P. ariasi* and 5 from *P. perniciosus*) from two localities (Torroja del Priorat and Poboleda),[26] and 1 from a human (a child with VL).[2] Stocks were isolated from blood (buffy coat), bone marrow, popliteal lymph node aspirates, and skin in dogs, from intestines in sand flies, and from bone marrow in the child. They were cultured in Schneider's insect medium and/or in Novy-McNeal-Nicolle's medium and incubated at 24–26°C. Promastigotes were cryopreserved in liquid nitrogen with dimethyl sulfoxide or glycerol.

Microsatellite analysis. *Isolation of DNA.* One cryovial from each stock was used for isolation of DNA. The promastigotes were quickly thawed, the pellet was washed in 500 μL of phosphate-buffered saline, suspended in 100 μL of sterile distilled water, and lysed by heating at 100°C for 20 minutes with 400 μL of a mixture of 1% Tween 20 (Sigma, St, Louis, MO), 1% Nonidet P-40 (Sigma), and 20% Chelex resin (Bio-Rad Laboratories, Hercules, CA) in sterile distilled water. The mixture was then centrifuged at 12,000 × *g* for 10 minutes at room temperature. Amplification of the supernatant by PCR was either performed immediately or after storage at –20°C.

Amplication by PCR. Eight MSs were studied: MS-AT, MS-TA (TA^1), and MS-G in the ITS region of the rRNA genes; MS-TG and the incomplete MS-TG-CG-TG in the Lm2 sequence; and MS-A, MS-T, and MS-TA (TA^2) in the Lm4 sequence. Primers and PCR conditions were as described.[21] We used primer Lm4A6 (5′-TGTGGTCTCAC-TAGCTTTCAATTC-3′) instead of Lm4A1 and primer Lm2A4 (5′-GTGAAGGGCAACACGAAAAA-3′) instead of Lm2A3. The PCRs were performed in an automated thermocycler PTC 200 DNA Engine (MJ Research, Waltham, MA) with annealing temperatures of 52°C for ITS and 63°C for Lm2 and Lm4 fragments.

DNA sequencing. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and sequenced in both directions using the BigDye® Terminator version 1.1 Cycle Sequencing Kit **(**Applied Biosystems, Foster City, CA) and the capillary electrophoresis DNA sequencer ABI Prism 3700 (Applied Biosystems) following the manufacturer's instructions. The same primers were used for sequencing reactions as for PCR amplifications.

Isoenzyme analysis. Isolates were characterized by isoenzyme electrophoresis using a panel of 13 enzymes (covering 15 enzymatic systems): malate dehydrogenase (MDH), EC 1.1.1.37; malic enzyme (ME), EC 1.1.1.40; isocitrate dehydrogenase (ICD), EC 1.1.1.42; 6-phosphogluconate dehydrogenase (6PGD), EC 1.1.1.44; glucose-6-phosphate dehydrogenase (G6PDH), EC 1.1.1.49; glutamate dehydrogenase (GLUD), EC 1.4.1.3; NADH diaphorase (DIA), EC 1.6.2.2; purine nucleoside phosphorylases 1 and 2 (NP1 and NP2), EC 2.4.2.1; glutamate-oxaloacetate transaminases 1 and 2 (GOT1 and GOT2), EC 2.6.1.1; phosphoglucomutase (PGM), EC

5.4.2.2; fumarate hydratase (FH), EC 4.2.1.2; mannose phosphate isomerase (MPI), EC 5.3.1.8; and glucose phosphate isomerase (GPI), EC 5.3.1.9.[27]

Statistical analysis. FSTAT version 2.9.3.2. was used to calculate allele frequencies at each locus.[28] Dendrograms were produced using the NTSYS program, version 1.80 (Exeter Software, Setauket, NY). Similarities were calculated using the Jaccard coefficient and clustered through the unweighted pair-group method for arithmetic averages (UPGMA) algorithm.[29]

RESULTS

Microsatellites and multilocus genotypes (MLGs). After amplification of three regions of the nuclear DNA of 110 stocks of *L. infantum* complex isolated from the Priorat focus, the sequencing study detected 17 alleles at the 8 MS examined. Only three MS were relevant by virtue of being polymorphic with 3 (MS-G), 4 (MS-TA^2), and 5 alleles (MS-TG) (Table 1). Seventeen MLGs were identified on the basis of allelic combinations and arbitrarily coded from 1 to 17 (Table 2). Half of the stocks analyzed corresponded to MLG-5 and MLG-10.

Spatial distribution of genotypes of *L. infantum*. The spatial distribution of MLGs was related to their frequency, with the most frequent MLGs (MLG-5 and MLG-10) found throughout the study area (Figure 1). In regions with low rates of seroprevalence, 13 stocks were isolated from dogs, corresponding to 5 genotypes. Eight of the nine stocks isolated from dogs over a period of 13 years (from 1989 through 2002) in one locality (Cornudella del Montsant) belonged to the same genotype (MLG-5). The remaining four stocks isolated from other places in this area of low endemicity had distinct genotypes. Clustering, such as that observed in Cornudella, was not observed in any localities in the area with high rates of seroprevalence (Table 3).

Host structuring. Allelic compositions differed between dog and sand fly isolates. The most striking difference was observed at MS-G, which was polymorphic in the 15 stocks taken from sand flies (MS-G_7 [6 of 15], MS-G_8 [1 of 15], and MS-G_9 [8 of 15]) but monomorphic in the 94 dog isolates (MS-G_7). Twelve MLGs were identified in canine stocks and 9 MLGs were identified in those from sand flies (Table 2). Forty-two canine stocks were isolated in those localities where infected sand flies were also captured (Torroja and Poboleda). The distribution of genotypes in isolates from dogs and sand flies was heterogeneous (Table 4); statistically significant differences were only observed for MLG-10 because of the low number of strains with other MLGs. The reservoir and vector shared only four genotypes and no stock from the most prevalent genotype in dogs (MLG-10) was isolated in sand flies ($P < 0.05$). In Torroja, 37 canine stocks were obtained from 1986 through 2002 and 11 sand fly stocks were obtained from 1986 through 1992 without any temporal clustering in genotypes from either host.

Most animals had no external signs of leishmaniasis when the parasite was isolated. The numbers and distribution of genotypes of stocks from asymptomatic and symptomatic animals was similar (Table 2). Consequently, no association was observed between MLGs and the virulence of the strain, which was expressed as the clinical status of the animal. However, we did not perform a statistical analysis because of the low numbers of stocks of each genotype. Five stocks were isolated from the skin of dogs in Torroja and identified as MLG-8, MLG-9, MLG-10, MLG-11, and MLG-12, all of which were also identified in stocks of visceral origin.

Correspondence of microsatellites and MLEE by clustering analysis. Microsatellite analysis of 110 *L. infantum* stocks isolated in the Priorat region identified 17 MLGs, in contrast to the 4 zymodemes identified by isoenzyme analyses of 100 of those stocks (Table 2). A dendrogram using the Jaccard index and the UPGMA algorithm enabled stocks to be clustered by microsatellites into four groups, each one featuring a different representation of zymodemes (Figure 2). Groups A and B contained stocks belonging to zymodeme MON-1 (70 stocks) and the related MON-77 (9 stocks). Group C showed a heterogeneous composition: MON-1 (3 stocks), MON-28 (6 stocks), MON-29 (4 stocks), and MON-77 (2 stocks). Group D contained isolates belonging to the two zymodemes: MON-29 (5 stocks) and MON-1 (1 stock).

TABLE 1

Allelic frequencies at eight microsatellites in the *Leishmania infantum* population studied in the Priorat region of Spain*

		All stocks‡		Dog stocks		Sand fly stocks	
Locus	Allele†	N (110)	F	N (94)	F	N (15)	F
AT	5	110	1	94	1	15	1
TA^1	5	110	1	94	1	15	1
G	7	101	0.918	94	1	6	0.400
	8	1	0.009	0	0	1	0.067
	9	8	0.073	0	0	8	0.533
TG	23	21	0.191	12	0.128	8	0.533
	24	18	0.164	14	0.149	4	0.267
	25	59	0.536	58	0.617	1	0.067
	26	1	0.009	1	0.011	0	0
	27	11	0.100	9	0.96	2	0.133
TG-CG-TG	8-5	110	1	94	1	15	1
A	10	110	1	94	1	15	1
T	9	110	1	94	1	15	1
TA^2	10	6	0.055	4	0.043	2	0.133
	11	42	0.382	40	0.426	1	0.067
	12	54	0.491	46	0.489	8	0.533
	13	8	0.073	4	0.043	4	0.267

* N = number of stocks; F = frequency.
† Number of repeats.
‡ Includes one human stock.

DISCUSSION

Few studies have been conducted to isolate and characterize sympatric strains of *L. infantum* from vertebrates and vectors in restricted foci, which explains why the epidemiologic cycle of most zymodemes is unknown.[2] In the present study, we applied microsatellite analysis to *L. infantum* stocks from dogs and sand flies from a small disease-endemic area in Catalonia in northeastern Spain characterized by two vector species (*P. perniciosus* and *P. ariasi*) and four *L. infantum* zymodemes.[2,22,23]

Studies of the molecular epidemiology of *Leishmania* species demonstrate that similar types tend to be proximal because of aggregation in space or time. The polymorphism of *L.* (*Viannia*) *braziliensis* from several areas in Brazil indicates that most genotypes are specific to certain geographic areas.[30] In the Mediterranean region, the distributions of *L. infantum* strains in southern France characterized by chromosomal

TABLE 2

Multilocus genotypes (MLGs) and zymodemes in 110 *Leishmania infantum* stocks from dogs and sand flies from the Priorat region of Spain*

	Microsatellite‡			All stocks§		Dog stocks			Sand fly stocks	
						No.				
MLG†	G	TG	TA	No.	F	A	S	Zymodemes	No.	Zymodemes
1	7	23	10	1	0.01	1	–	ND	–	–
2	7	25	10	3	0.03	1	2	MON-1	–	–
3	7	23	11	9	0.08	6	2	MON-1, MON-28, MON-77, ND	–	–
4	7	24	11	2	0.02	1	1	MON-1, ND	–	–
5	7	25	11	26	0.24	18	7	MON-1, ND	1	MON-1
6	7	26	11	1	0.01	1	–	MON-1	–	–
7	7	27	11	4	0.04	3	1	MON-1	–	–
8	7	23	12	4	0.04	1	2	MON-1, MON-77, ND	1	MON-29
9	7	24	12	10	0.09	5	3	MON-1, MON-77, ND	2	MON-1, MON-77
10	7	25	12	30	0.27	18	12	MON-1, MON-77, ND	–	–
11	7	27	12	7	0.06	4	1	MON-1	2	MON-1
12	7	24	13	4	0.04	1	3	MON-1, MON-77	–	–
13	8	23	10	1	0.01	–	–	–	1	MON-29
14	9	23	10	1	0.01	–	–	–	1	MON-29
15	9	23	12	3	0.03	–	–	–	3	MON-29
16	9	23	13	2	0.02	–	–	–	2	MON-1, MON-29
17	9	24	13	2	0.02	–	–	–	2	MON-29

* For each MLG, the no. of repetitions of each variable microsatellite, the no. of stocks belonging to each MLG, their frequency (F), and the presence of signs of leishmaniasis in the dogs from which the stock was obtained are shown. A = asymptomatic; S = symptomatic; ND = not determined.
† MLG arbitrarily coded from 1 to 17.
‡ No. of repetitions of each allele.
§ Includes one human stock (zymodeme MON-77).

size[11] or from southern Spain characterized by random amplified polymorphic DNA analysis[7] were also found to be related to their geographic origin. This geographic aggregation was also observed in the present study and became evident through the low number of polymorphic microsatellites and their low variability, particularly in comparison with the variability observed in stocks from broader areas analyzed using the same methods[21] (Montoya L and others, unpublished data).

Several MLGs were widely distributed and overrepresented (MLG-5 and MLG-10) in the Priorat region. Ten *L. infantum* MLGs found in the focus have not been described previously in other disease-endemic foci in the Mediterranean region,[21] possibly because of the low numbers of stocks from dogs and sand flies typed from other geographic areas. In all 10 cases, since these genotypes were underrepresented, they are unlikely to be related to a disease-endemic situation. Multilocus genotype polymorphism was observed across the region, particularly in localities situated in the area of high endemicity, which may be related to the ancient origin of this focus and the dispersion of infected animals. Nevertheless, the homogeneous cluster observed in dog stocks from Cornudella, a locality situated in an area with low endemicity, is quite remarkable. The repeated isolation of the same MLG in several dogs over a 13-year period in this locality could be associated with the stability of MSs[16,21] and the isolation of the microfocus of infection within the area of low transmission.[23]

Most studies on *Leishmania* have been performed on strains from the vertebrate host (humans or reservoir animals) that were compared across geographic regions. Differences in types composition in *L. infantum* populations from humans and dogs have been repeatedly found using MLEE.[2,3,31–33]

In the present study, nine stocks from sand flies were belonged to five distinct genotypes not found in dogs. This is consistent with previous MLEE results, which identified several strains such as MON-29,[22,23] which is not found in dogs in the focus, and are related to CL in immunocompetent patients in Catalonia.[34] Sand flies were captured in two neighboring villages (Torroja and Poboleda) separated by only 4 km, where 42 stocks from dogs were also isolated. A similar situation of *Leishmania* spp. strains isolated from vectors being distinct from those isolated from the vertebrate host has been found in Morocco[35,36] and in southern Spain.[3]

The polymorphism of a given *Leishmania* species in natural populations has been related to the number of sand fly vector(s) and/or animal reservoir(s) involved in the transmission cycle of the parasites.[30] The simultaneous transmission of *L. infantum* by *P. perniciosus* and *P. ariasi* in the Priorat re-

TABLE 3

Multilocus genotypes (MLGs) in *Leishmanina infantum* stocks from dogs in several localities in the Priorat region of Spain*

Location	No. ot stocks	MLGs (no. of isolates)
Torroja	37	**2**(2), **4**(2), **5**(3), **8**(3), **9**(5), **10**(18), **11**(1), **12**(3)
Marça	19	**3**(7), **5**(4), **7**(2), **9**(1), **10**(5)
Cornudella	9	**5**(8), **10**(1)
El Molar	5	**5**(1), **7**(1), **9**(2), **10**(1)
Poboleda	5	**10**(2), **11**(3)
Other	19	**1**(1), **2**(1), **3**(1), **5**(9), **6**(1), **7**(1), **10**(3), **11**(1), **12**(1)

* MLG code is in **bold.**

TABLE 4

Multilocus genotypes (MLGs) of *Leishmania infantum* stocks from dogs and sand flies in Torroja and Poboleda, Spain

	MLG code												
Stock	2	4	5	8	9	10	11	12	13	14	15	16	17
Dog (42)	2	2	3	3	5	20	4	3	0	0	0	0	0
Sand fly (15)	0	0	1	1	2	0	2	0	1	1	3	2	2

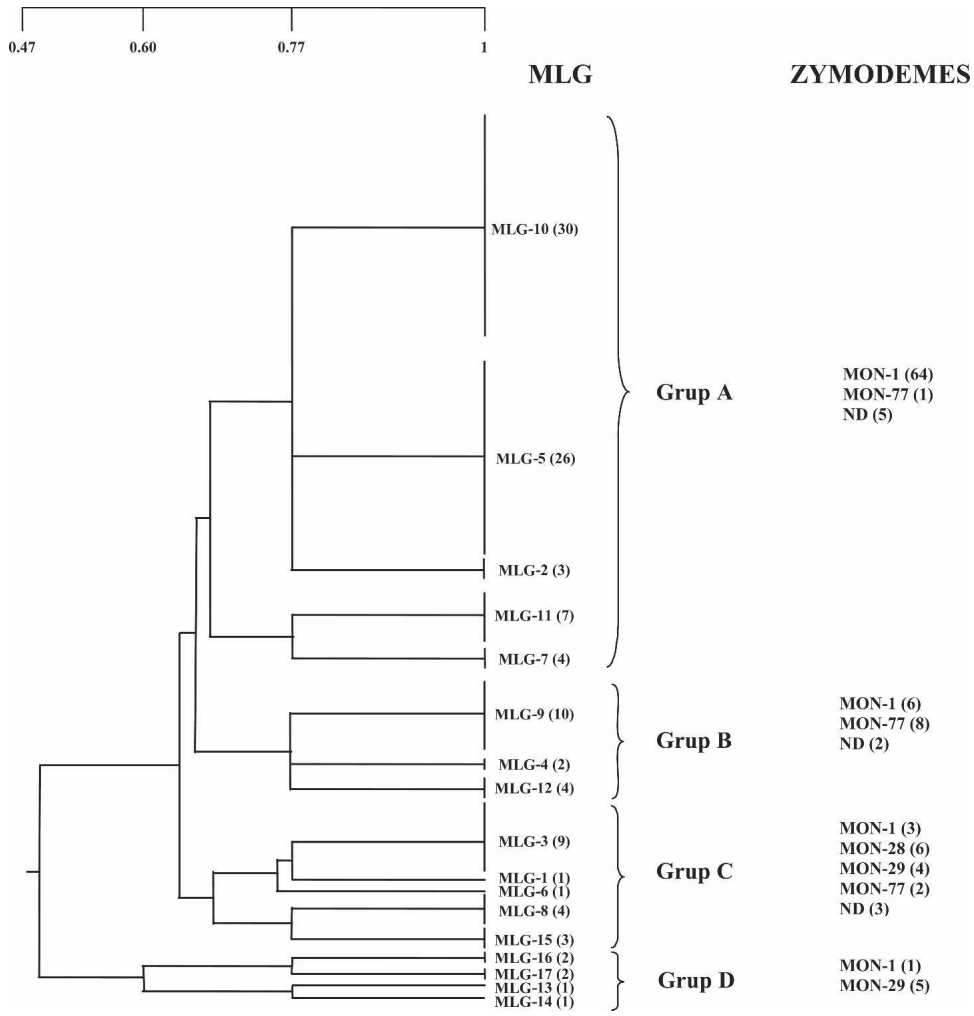

FIGURE 2. Unweighted pair-group method for arithmetic averages dendrogram generated from microsatellite analysis of 110 stocks of *Leishmania infantum* based on the Jaccard index. Results of analysis by multilocus enzyme electrophoresis (MLEE) are also indicated. Values in parentheses are the number of stocks. MLG = multilocus genotype; ND = stocks on which MLEE was not performed.

gion[22,26] is not reflected in differences in MLG distribution between the two sand fly species. Both are vectors of at least two MLG found in dogs.

With regards to their human origin, seven human cases of leishmaniasis have been reported in the Priorat area during 1986–2003,[25] but there have been no acknowledged cases in Torroja, where most of the infected sand flies were captured. The possibility of sand flies being infected by blood meals from one affected person, as might be suspected if one only considers the isoenzyme analysis (strains identified as zymodeme MON-29), can be rejected by the polymorphism detected in MLG, which indicates the existence of more than one origin. It is doubtful that several undetected human cases of CL in a village as small as Torroja were the source of these strains. Thus, the possibility of human cryptic infections, as detected in other neighboring areas such as the Balearic Islands[37] and southern France,[38] must also be considered.

There is little support for a hypothesis connecting the existence of these strains in sand flies to dermotropic strains from dogs. The five typed isolates from dog skin were all from Torroja. Stocks were obtained during a study performed on all the dogs from this village in which samples of popliteal lymph node, bone marrow, skin lesions, and healthy skin were cultured. None of the stocks analyzed matched the MLGs detected in sand flies or zymodeme MON-29.

Foxes are also considered a potential wild canine reservoir for leishmaniasis in southern Europe.[39–41] In the Priorat focus, the seroprevalence of antibodies to *Leishmania* in foxes is similar to that of dogs.[23,42] Moreover, the relatively low population of this animal and their wild habitats raise doubts as to their possible role as a reservoir of genotypes only found in vectors, especially because almost all infected sand flies were caught inside villages. The number of animal species, other than dogs, infected by *L. infantum* in the western part of the Mediterranean region is increasing and in recent years cutaneous lesions caused by this parasite have been diagnosed in cats and horses.[43–45] Infected rodents have also been repeatedly reported.[46,47] The results of parasitologic analysis (direct examination and culture) of several samples (blood, spleen, liver, bone marrow, and heart) of eight seropositive rodents captured in Torroja were negative.[23]

The possibility of original mixed infections of *L. infantum,* both in sand flies and vertebrate hosts, and the bias caused by culturing the parasite requires consideration.[48] Two *Leishma-*

nia species in the sand fly *Lutzomyia ovallesi* were reported,[49] one of which was selected in the vertebrate host. In addition, two *L. infantum* strains have been demonstrated in dogs[50] and humans,[51,52] one of which was selected in the culture. Five MLGs have been identified in 14 *L. infantum* isolates from repeated VL episodes, all from one patient coinfected with human immunodeficiency virus, which supports the existence of a mixed infection (Montoya L and others, unpublished data). We believe that strains with MLGs exclusively found in vectors have no unique origin and all the hypotheses discussed may play a role.

The dendrogram constructed from MS analyses allowed stocks to be clustered into four groups. Almost all stocks in groups A and B (8 MLG) corresponded to MON-1 (70 stocks) and those in group D (4 MLG) to MON-29 (5 stocks). Group C (5 MLG) was more heterogeneous, with stocks belonging to all four zymodemes (MON-1, MON-28, MON-29, and MON-77). Remarkably, most stocks included in groups C and D were isolated from sand flies. *Leishmania infantum* MON-1 is the most common and extensive zymodeme found[1] as a result of a great number of stocks from hosts from distinct geographic origins and pathologic situations having been studied. The application of several genetic markers to MON-1 strains demonstrated a large polymorphism within this zymodeme, and that the differences between two *L. infantum* MON-1 stocks is sometimes greater than that between different zymodemes or even between *L. infantum* and *L. donovani*.[8] It is therefore not surprising that several MON-1 stocks from the Priorat region are found in all the MLG groups in the microsatellite dendrogram (12 MLG), although with a very skewed distribution. Such a situation may also be found for other zymodemes if a large enough number of stocks are studied, as we detected in the MON-29 zymodeme (6 MLG). Our work agrees with the UPGMA phenograms established with a microsatellite analysis for *L. infantum* stocks from different geographic areas that found a large cluster mainly composed of strains belonging to zymodeme MON-1, with one MON-77 and one MON-108, and a second cluster with non-MON-1 strains.[18] It also corresponds with the idea of a high polymorphism inside the MON-1 zymodeme, when more discriminating techniques are used, even if strains from a small physical area are included in the study.

Our results confirm that MS analysis is a powerful tool for *L. infantum* strain typing and epidemiologic tracking studies. In the present study, application of MS analysis to *L. infantum* complex stocks reinforced a previous observation using MLEE analysis on the heterogeneity of strains from vectors and vertebrates in the same focus, the cause of which is unknown. However, the application of MS analysis and other highly discriminatory typing methods on cloned and sympatric strains from vectors and vertebrates (VL, CL, and cryptic infections in humans, dogs, and other reservoirs) is necessary to enhance our understanding of the population dynamics and epidemiology of Mediterranean leishmaniasis.

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