ARTICLE IN PRESS

International Journal for Parasitology xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Parasitic genotypes appear to differ in leishmaniasis patients compared with asymptomatic related carriers

M. Hide [a,*], E. Marion [a], C. Pomares [b,c,d], R. Fisa [e], P. Marty [b,c,d], A.L. Bañuls [a]

[a] *Maladies Infectieuses et Vecteurs, Ecologie, Génétique, Evolution et Contrôle, MIVEGEC IRD/CNRS/UM1/UM2 (UMR 224/5290), F-34394, France*
[b] *Université de Nice Sophia Antipolis, Faculté de Médecine, Nice F-06107 Cedex 2, France*
[c] *INSERM, 1095, Centre Méditerranéen de Médecine Moléculaire, C3M, Toxines Microbiennes dans la relation hôte pathogènes, Nice F-06204 Cedex 3, France*
[d] *Centre Hospitalier Universitaire de Nice, Laboratoire de Parasitologie-Mycologie, Nice F-06202 Cedex 3, France*
[e] *Laboratory of Parasitology, Facultat de Farmàcia, Universitat de Barcelona, Avda Joan XXIII sn, 08028 Barcelona, Spain*

ARTICLE INFO

Article history:
Received 20 October 2012
Received in revised form 18 December 2012
Accepted 22 December 2012
Available online xxxx

Keywords:
Asymptomatic carriers
Cryptic infection
Reservoir
Leishmania infantum
Molecular epidemiology
Multilocus microsatellite typing (MLMT)
Southern France
Phylogenetic analysis

ABSTRACT

For numerous infectious diseases affecting humans, clinical manifestations range from asymptomatic forms to severe pathologies. The originality of this study was its focus on asymptomatic carriers of *Leishmania infantum* in southern France. The fundamental interest in these asymptomatic carriers is that they can be a reservoir of potentially pathogenic microorganisms. It remains to be established whether the parasitic genomes from asymptomatic carriers differ from those of patients. Multilocus microsatellite typing was used to investigate the genetic variation among 36 French strains of *L. infantum*. Nine *Leishmania* strains isolated from blood donors (asymptomatic carriers) were compared with 27 strains of *L. infantum* belonging to zymodemes, MON-1, -33 and -183. These strains were isolated from HIV positive or negative patients with visceral leishmaniasis, cutaneous leishmaniasis, from canine leishmaniasis or from phlebotomine sandflies. Multilocus microsatellite typing data generated using 33 loci were analyzed by a Bayesian model-based clustering algorithm and construction of a phylogenetic tree based on genetic distances. Both analyses structured the MON-1 sample into two main clusters. Furthermore, genetic analysis demonstrated that these nine asymptomatic carrier strains are divided into two clusters grouped with the MON-1 strains. One cluster with seven strains is related to, but different from, human symptomatic strains from the Alpes-Maritimes region whereas the other cluster has the two remaining strains together with canine leishmaniasis strains as well as one strain from a visceral leishmaniasis patient. Genetic diversity among asymptomatic carrier was very weak since the nine *Leishmania* strains belong to only two genotypes. Genetic differentiations were evidenced between asymptomatic carrier strains and non-asymptomatic carrier strains and especially between asymptomatic carrier and HIV+ populations, although these findings require confirmation with a larger sample size. We believe that our data explore for the first time, the genetic diversity among *L. infantum* from asymptomatic human carriers and reveal a weak polymorphism compared with *Leishmania* parasites isolated from human patients.

© 2013 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.

1. Introduction

Leishmaniases, caused by infection with the *Leishmania* parasite, are worldwide endemic diseases, with an estimated disease burden of 2,357,000 disability-adjusted life years and 59,000 deaths per year (WHO, 2002). These diseases range in severity from a healing skin ulcer to an overwhelming visceral leishmaniasis (VL). Zoonotic VL, caused by the protozoan parasite *Leishmania infantum*, is a sandfly-borne disease found in the Mediterranean area, Asia and Latin America. This species is associated with benign cutaneous leishmaniasis (CL) as well as severe VL which is fatal without treatment. In the Mediterranean Basin, dogs are the principle peridomestic reservoir of human VL. Because control programs based on the elimination of infected dogs have failed to halt or prevent epidemics of urban VL due to *L. infantum* in Brazil (Madeira Mde et al., 2004), other secondary reservoirs such as humans may be important in propagating the infection. Parasite circulation in peripheral blood has been reported in asymptomatic carriers (ACs) of leishmaniasis (persons or animals who are infected with infectious microorganisms but display no symptoms) detected either by PCR with the presence of DNA and/or by culture (Guevara et al., 1993; Le Fichoux et al., 1999; Garcia-Garcia et al., 2006; Fakhar et al., 2008; Riera et al., 2008; Scarlata et al., 2008;

* Corresponding author. Address: IRD de Montpellier, MIVEGEC, IRD/CNRS/UM1/UM2 (UMR 224/5290), F-34394, France. Tel.: +33 4 67 41 63 91; fax: +33 4 67 41 62 99.

E-mail address: mallorie.hide@ird.fr (M. Hide).

0020-7519/$36.00 © 2013 Australian Society for Parasitology Inc. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ijpara.2012.12.008

Please cite this article in press as: Hide, M., et al. Parasitic genotypes appear to differ in leishmaniasis patients compared with asymptomatic related carriers. Int. J. Parasitol. (2013), http://dx.doi.org/10.1016/j.ijpara.2012.12.008

Michel et al., 2011). Most scientific studies of infectious diseases have focused on diseased individuals, from an epidemiological or clinical point of view. However, it has been demonstrated that ACs represent a large part of the human population with infectious diseases in endemic areas (Costa et al., 2002; Riera et al., 2004). This must be considered to understand the dissemination pathways of *L. infantum* parasites and their capacity for surviving in hosts (Bañuls et al., 2011). A previous study on blood donors isolated nine *L. infantum* strains from ACs living in southern France (Le Fichoux et al., 1999). Besides these rare strains isolated from ACs, most *L. infantum* parasites are isolated from VL patients. In southern Europe, *Leishmania*/HIV co-infections have become increasingly frequent, with up to 9% of AIDS cases developing VL, particularly in France (Desjeux and Alvar, 2003). In France the incidence of VL in HIV positive (+) patients dropped from 12 in every 10,000 people per year to seven per 10,000 per year after 1996, the year in which Highly Active Anti-Retroviral therapy was introduced in this country (del Giudice et al., 2002). One of the most powerful and discriminative DNA-based methods for strain differentiation and population genetics is the analysis of highly variable, co-dominant microsatellite markers. Recently, multilocus microsatellite typing (MLMT) has been used successfully to differentiate *L. infantum* populations in the Mediterranean region of Europe. This method enabled differentiation even at the intra-zymodeme level, as shown for the predominant MON-1 zymodeme of *L. infantum* (Ochsenreither et al., 2006; Montoya et al., 2007; Kuhls et al., 2008). Here, a panel of 33 microsatellite markers was used to investigate genetic structure and gene diversity among strains of *L. infantum* isolated from healthy blood donors and to compare those with other *L. infantum* strains (isolated from sandflies, dogs or human patients) from southern France. To our knowledge this is the first genetic study on "asymptomatic" *Leishmania* strains. The results will be discussed with regard to the potential contribution of ACs in leishmaniasis transmission. The possibility of leishmaniasis transmission from an asymptomatic blood donor to a patient will also be discussed in relation to the public health risk.

2. Materials and methods

2.1. Leishmania strains

Table 1 lists the World Health Organization (WHO) code, geographical origin, clinical manifestation and Multi-Locus Enzyme Electrophoresis (MLEE) identification for the 36 local strains of *L. infantum*. All of the strains were isolated in southern France and provided by the French National Reference Center of *Leishmania* (Fig. 1). Thirty-three strains belong to zymodeme MON-1, two to MON-33 and one to MON-183 (used as an outgroup).

Table 1
Leishmania infantum strains used in this study.

Strain	WHO code	Town (department)[a]	Clinical form	HIV status	MON zymodeme[b]	STRUCTURE population[c]
LEM2355	MHOM/FR/91/LEM2355	Toulouse (31)	VL	+	183	1b
LEM356	MHOM/FR/82/LEM356	Amélie les Bains (66)	CL	–	33	1a
LEM3495	MHOM/FR/97/LPN160	Southern France	CL	–	33	1a
LEM576	IPER/FR/84/LEM576	St Clément la Rivière (34)	N/A	N/A	1	3a
LEM595	IARI/FR/84/LEM595	St Clément la Rivière (34)	N/A	N/A	1	3a
LEM3114	MCAN/FR/95/LPN122	Falicon (06)	CanL	N/A	1	3b
LEM3115	MCAN/FR/95/LPN123	St Pancrace (06)	CanL	N/A	1	3b
LEM3116	MCAN/FR/95/LPN124	Aspremont (06)	CanL	N/A	1	3b
LEM3280	MHOM/FR/96/LPN131	Monaco (98)	AC	–	1	3b
LEM3270	MHOM/FR/96/LPN134	Monaco (98)	AC	–	1	2
LEM3273	MHOM/FR/96/LPN136	Monaco (98)	AC	–	1	3b
LEM3282	MHOM/FR/96/LPN137	Monaco (98)	AC	–	1	2
LEM3283	MHOM/FR/96/LPN138	Monaco (98)	AC	–	1	2
LEM3296	MHOM/FR/96/LPN142	La Turbie (06)	AC	–	1	2
LEM3295	MHOM/FR/96/LPN143	Menton (06)	AC	–	1	2
LEM3294	MHOM/FR/96/LPN144	La Turbie (06)	AC	–	1	2
LEM3313	MHOM/IT/96/LPN145	Ventimiglia (Italy)	AC	–	1	2
LEM1098	MHOM/FR/87/LEM1098	Nebian (34)	CL	–	1	3a
LEM3496	MHOM/FR/97/LPN161	Gattieres (06)	CL	–	1	3a
LEM5459	MHOM/FR/07/LPN313	Luceram (06)	CL	–	1	2
LPN83	MHOM/FR/92/LPN83	Lavasina (2B)	CL	+	1	3a
LEM663	MHOM/FR/85/LEM663	Clermont l'Herault (34)	VL	–	1	3a
LEM716	MHOM/FR/85/LEM716	Nebian (34)	VL	–	1	3a
LEM75	MHOM/FR/78/LEM75	Béziers (34)	VL	–	1	3a
LPN105	MHOM/FR/94/LPN105	Bastia (2B)	VL	+	1	2
LPN119	MHOM/FR/95/LPN119	Cannes (06)	VL	+	1	2
LPN129	MHOM/FR/96/LPN129	Vallauris (06)	VL	+	1	3a
LPN132	MHOM/FR/96/LPN132	Mouans Sartoux (06)	VL	–	1	3b
LEM3484	MHOM/FR/97/LPN155	Nice (06)	VL	–	1	2
LEM3491	MHOM/FR/97/LPN158	Colomars (06)	VL	–	1	2
LEM3492	MHOM/FR/97/LPN159	Fayence (83)	VL	–	1	2/3a
LEM5458	MHOM/FR/07/LPN312	Grasse (06)	VL	–	1	2/3a
LEM5460	MHOM/FR/07/LPN314	Peymeinade (06)	VL	+	1	3a
LEM5535	MHOM/FR/07/LPN316	Nice (06)	VL	–	1	2
LPN92	MHOM/FR/93/LPN92	Nice (06)	VL	+	1	3a
LPN94	MHOM/FR/93/LPN94	Nice (06)	VL	+	1	2

N/A, not applicable; MHOM, human origin; IPER, *Phlebotomus perniciosus*; IARI, *Phlebotomus ariasi*; MCAN, *Canis familiaris*; VL, visceral leishmaniasis; CL, cutaneous leishmaniasis; CanL, canine visceral leishmaniasis; AC, asymptomatic carrier.

[a] Town of residence of the infected hosts. French and Monegasque departments are numbered: Alpes-Maritimes (06), Haute-Corse (2B), Haute-Garonne (31), Hérault (34), Pyrénées-Orientales (66), Var (83), Monaco (98).

[b] Zymodemes were determined with the Multi-Locus Enzyme Electrophoresis (MLEE) method performed by the World Health Organisation (WHO) reference center of Montpellier (MON), France (Rioux et al., 1990).

[c] Populations were determined according to STRUCTURE analysis ($K = 5$).

Fig. 1. Geographic origin of the 36 *Leishmania infantum* strains. (A) Southern France; (B) Nice region.

Parasites originated from either VL and CL HIV patients (eight strains), VL patients (nine strains), CL patients (five strains), canine leishmaniasis (CanL; three strains), *Phlebotomus perniciosus* (one strain), *Phlebotomus ariasi* (one strain), or healthy human blood donors (nine MON-1 strains described in (Le Fichoux et al., 1999). Promastigotes were maintained at 26 °C in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/mL of streptomycin in disposable flasks. Parasites were harvested in logarithmic phase by centrifugation (5,000g for 30 min at 4 °C) and washed twice in PBS. Parasite pellets were kept at −80 °C until DNA extraction. DNA was extracted by phenol/chloroform treatment (Sambrook et al., 1989), and DNA concentration was estimated by spectrophotometry at 260 nm.

2.2. Microsatellite genotyping

Microsatellite analysis was done using 33 microsatellite markers (Table 2). Twenty-eight microsatellite loci are already published (Rossi et al., 1994; Russell et al., 1999; el Tai et al., 2000; Jamjoom et al., 2002; Ochsenreither et al., 2006; Kuhls et al., 2007); five were developed by the authors (Table 2). The 36 strains under study were amplified according to the following conditions: every 30 μL of reaction mix was composed of 10 pmol of each primer (the forward being labeled), 100 ng of template DNA, 1 nmol of each dNTP, 3 μL of buffer 10X, and 1.5 U of Taq Polymerase (5 U/μl, Roche Diagnostics, France). Amplifications were carried out in a thermal cycler using the following conditions: 35 cycles of 94 °C for 30 s, annealing temperature of each locus (see Table 2) for 1 min, 72 °C for 1 min, and a final extension step of 72 °C for 30 min. Size analysis of the fluorescence-labeled PCR-products was carried out on an ABI Prism 3130XL genetic analyzer (Applied Biosystems, France) and data were stored and analyzed with GeneMapper analysis software (version 4.0, Applied Biosystems, France). Genescan 500 LIZ (Applied Biosystems, France) was used as internal size standard. All 36 *Leishmania* strains were genotyped at all 33 loci.

2.3. Data analysis

2.3.1. Phylogenetic analysis

Data were analyzed with Genetix version 4.05.2 (2004) and FSTat Version 2.9.3.2 software (Goudet et al., 2002), which compute estimates and test the significance of various parameters of population genetics. Genetic polymorphism was measured by the number of alleles per locus (N_a) and by Nei's unbiased genetic diversity within subsamples H_s (Nei and Chesser, 1983). F_{ST} measures the relative inbreeding in subpopulations that is attributable to the subdivision of the total population into subpopulations of limited size. F_{ST} thus also measures genetic differentiation between subpopulations. Data were heterogeneous regarding year of sampling, clinical manifestations, geographical origin, host species and patient's sex and age. To assess the possible contribution of these factors to genetic differentiation, we compared F_{ST} obtained with different sampling strategies. A neighbor-joining tree was constructed to examine the relationships between the AC population and the others by using the PHYLIP software (version 3.5c; J. Felsenstein, 1993. Department of Genetics, University of

Table 2
Characteristics of the 33 microsatellite loci used in this study for *Leishmania infantum* genotyping.

Microsatellite marker	Dye label	GenBank Accession No.	Allele size range (bp)	T_a (°C)	N_a	References
LiBTG[a]	6-FAM	nd	219–257	58	8	R. Fisa, unpublished data
LiBTA[a]	VIC	nd	226–246	58	7	R. Fisa, unpublished data
Lm4TA[a]	NED	nd	65–85	58	7	Ochsenreither et al. (2006)
LIST7026[a]	NED	AF427874	201–231	56	6	Jamjoom et al. (2002)
Li22_35[a]	VIC	AM050045	90–106	58	6	Ochsenreither et al. (2006)
Li45_24[a]	NED	AM050048	88–108	58	6	Ochsenreither et al. (2006)
TubCA[a]	6-FAM	nd	74–84	58	6	Ochsenreither et al. (2006)
LIST7039[a]	PET	AF427887	199–215	58	5	Jamjoom et al. (2002)
Li71-33	6-FAM	AM050053	104–136	57	5	Ochsenreither et al. (2006)
Rossi2[a]	VIC	X76393	140–160	57	5	Rossi et al. (1994)
LIST7021[a]	6-FAM	AF427869	228–246	54	4	Jamjoom et al. (2002)
LIST7029	6-FAM	AF427877	172–182	56	4	Jamjoom et al. (2002)
LIST7033[a]	6-FAM	AF427881	196–226	58	4	Jamjoom et al. (2002)
LIST7035[a]	PET	AF427883	188–202	56	4	Jamjoom et al. (2002)
LIST7037[a]	6-FAM	AF427885	178–194	58	4	Jamjoom et al. (2002)
Li71-7	6-FAM	AM050051	90–98	58	4	Ochsenreither et al. (2006)
Li72-20[a]	VIC	AM050057	87–95	50	4	Ochsenreither et al. (2006)
DPB1	NED	AF182167	141–147	59	3	Hide, PhD Thesis[b]
DPB2	PET	AF182167	231–235	59	3	Hide, PhD Thesis[b]
HG	6-FAM	AF170105	191–199	55	3	Hide, PhD Thesis[b]
ITS1	6-FAM	AJ000288	288–314	55	3	el Tai et al. (2000)
LIST7024	VIC	AF427872	198–224	59	3	Jamjoom et al. (2002)
LIST7025[a]	6-FAM	AF427873	171–179	56	3	Jamjoom et al. (2002)
LIST7027	PET	AF427875	177–185	59	3	Jamjoom et al. (2002)
LIST7031[a]	PET	AF427879	166–174	54	3	Jamjoom et al. (2002)
LIST7034	NED	AF427882	139–197	54	3	Jamjoom et al. (2002)
LIST7038[a]	NED	AF427886	122–130	56	3	Jamjoom et al. (2002)
Li71-5/2[a]	VIC	AM050050	104–108	54	3	Ochsenreither et al. (2006)
CS20	NED	nd	86–96	58	2	Kuhls et al., 2007
LIST7028	VIC	AF427876	140–152	58	2	Jamjoom et al. (2002)
Rossi1	6-FAM	X76394	104–110	59	2	Rossi et al. (1994)
LIST7023	PET	AF427871	153	55	1	Jamjoom et al. (2002)
LIST7030	NED	AF427878	178	59	1	Jamjoom et al. (2002)

[a] Polymorphic loci within zymodeme MON-1.
[b] Hide, M., 2004. Pathogenic variability with the *Leishmania* (*Leishmania*) *donovani* complex, agent of visceral leishmaniasis. *Comparative study of biological and genetic characters and gene expression.* Sciences/Parasitologie, Ph.D thesis, University of Montpellier II. (in french). 6-FAM, blue dye; VIC, green dye; NED, yellow dye; PET, red dye; T_a, annealing temperature (thermocycling conditions); N_a, allele number within the whole sample (genetic variation).

Washington, Seattle, USA). The neighbor-joining tree (Saitou and Nei, 1987) was constructed through calculations of Cavalli-Sforza genetic distance from allelic frequencies, and the robustness of tree topology was obtained by bootstrap resampling of loci. The tree was edited using TreeDyn software (Chevenet et al., 2006).

2.3.2. Clustering analysis

MLMT data were analyzed by a Bayesian statistics-based method implemented in STRUCTURE v.2.3.3 (2010, January) (Pritchard et al., 2000) to explore the structure of AC versus non-AC populations. STRUCTURE uses Bayesian Monte-Carlo Markov Chain (MCMC) sampling to identify the optimal number of clusters, *K*, for a given multi-locus dataset without needing to identify population subunits a priori. The parameters used were the admixture model with the length of burn-in period of 200,000 iterations, followed by 200,000 MCMC repeats after burn-in. Based on multilocus genotype data, the individuals were divided into *K* subpopulations with *K* ranging from one to 11 and 10 independent runs were performed for each value of *K*. The methods of Evanno et al. (2005) and Garnier et al. (2004) were employed to assess the optimal value of *K* (i.e. the optimal number of clusters in the dataset) corresponding to the peak in the DeltaK graph (Garnier et al., 2004; Evanno et al., 2005).

3. Results

3.1. Global genetic diversity and allelic polymorphism

Clear electropherograms for all genotypes were obtained at all 33 loci investigated, with only one or two alleles per strain at each locus. A total of 128 alleles based on 31 polymorphic microsatellite markers were identified for 36 strains of *L. infantum* (MON-1, MON-33 and MON-183) from southern France since two loci were monomorphic (LIST7023 and LIST7030). The number of alleles per locus (N_a) ranged from one to eight, the most polymorphic being LibTG (Table 2), that is, a mean value of 4.13 alleles per locus. Within MON-1, the 33 strains encompassed 50 different alleles using 18 polymorphic microsatellite markers. The number, N_a, ranged from two to six, the most polymorphic being LibTG, LibTA and Lm4TA, that is, a mean value of 3.18 alleles per locus. The observed heterozygosity (H_o) was quite weak; it ranged from 0 to 0.181 (overall 0.042) for the whole sample and varied between 0.000 and 0.104 (overall 0.018) for the MON-1 population. The mean genetic diversity, H_s, was 0.345 (0.177–0.684) for the whole sample and 0.367 (0.043–0.619) within the MON-1 population.

3.2. Phylogenetic reconstruction and clustering analysis

The neighbor-joining tree (Fig. 2A) designed from MLMT genetic distances underlined three main clusters. The MON-183 genotype was used as outgroup based on its allelic profile. The first cluster (C1) was composed of the non-MON-1 strains (two MON-33) and was separated from the MON-1 strains with a strong bootstrap value (100%). Thus, the MON-1 sample appeared monophyletic. The second cluster (C2) was composed of 16 MON-1 human strains isolated in the Alpes-Maritimes (AM), Var, and Corsica regions and sustained by a bootstrap value of 58%. This group contained two subclusters: (i) two VL strains (bootstrap value: 60%) and (ii) 14 strains (seven human patients and seven ACs) (bootstrap value: 91%). Seven of the nine strains isolated from ACs (underlined) were

ARTICLE IN PRESS

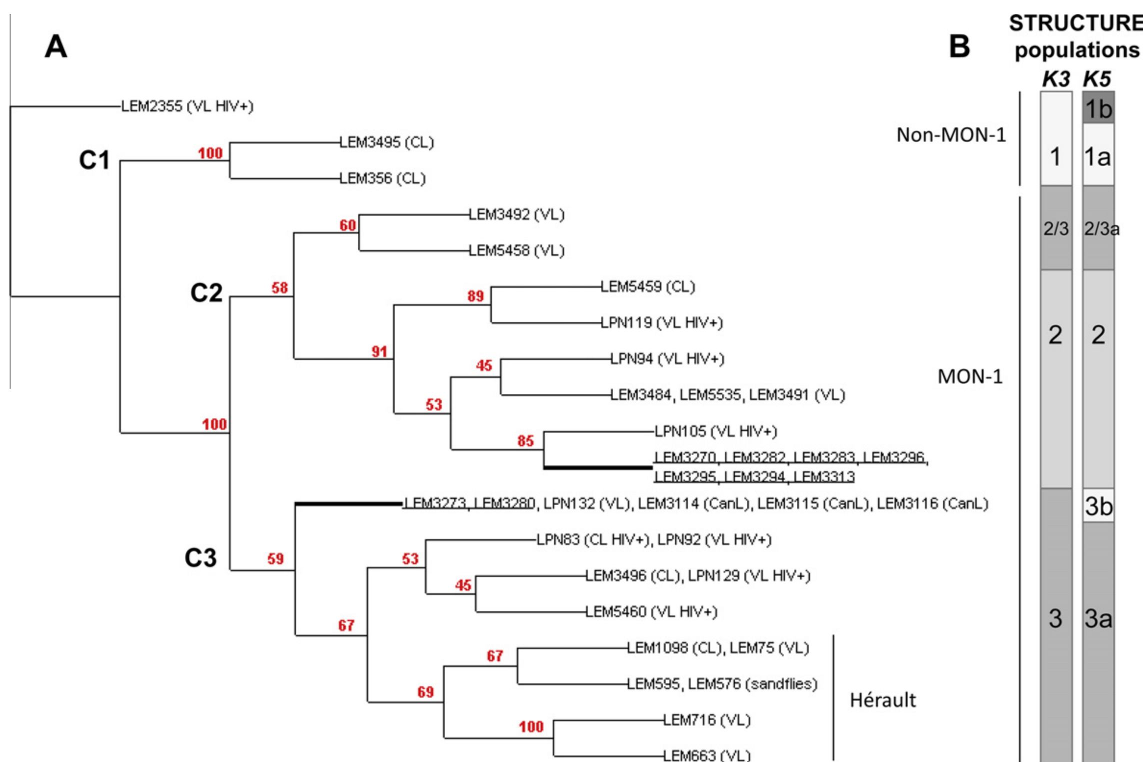

Fig. 2. Phylogenetic and clustering analysis. (A) The neighbor-joining tree inferred from the Cavalli-Sforza genetic distance results for the data from 33 microsatellite markers and 36 *Leishmania infantum* strains. The values above the branches indicate the percentage with which a given branch is supported in 100 bootstrap replications. The clusters C1–3 are indicated. *Leishmania* strains isolated from asymptomatic carriers are underlined. CL, cutaneous leishmaniasis; VL, visceral leishmaniasis; CanL, canine leishmaniasis. (B) The population structure inferred by Bayesian clustering for $K = 3$ and $K = 5$.

in the latter cluster and constituted a single genotype. The third cluster (C3), sustained by a bootstrap value of 59%, contained three subclusters: (i) the six MON-1 strains isolated from the Hérault region (bootstrap value: 69%), (ii) five MON-1 human strains from AM and Corsica regions (bootstrap value: 53%), and (iii) the three canine strains with the two remaining AC strains and a VL strain. The latter group constituted a single genotype. Interestingly, the nine AC strains were poorly polymorphic since they revealed only two genotypes; one was AC-specific (seven strains) whereas the second (two strains) was identical to the three canine strains of our sample and a VL case. In addition to the genetic distance-based approach, the model-based algorithm was used to infer the population structure of our French *L. infantum* sample (Fig. 2B). MLMT data were analyzed with STRUCTURE software to infer the population structure of (i) the whole sample (36 strains) and (ii) the MON-1 population (33 strains). According to Evanno et al. (2005) and Garnier et al. (2004), within the whole sample, the most probable K numbers are $K = 3$ and $K = 5$. In the first case, population 1 was composed of the three non-MON-1 strains (two MON-33 and one MON-183), population 2 of the MON-1 cluster C2 (14 strains), and population 3 of the MON-1 cluster C3 (17 strains) (Figs. 2B and 3). The STRUCTURE software did not allow classification of the strains LEM3492 (CL) and LEM5458 (VL) which were a mixed genotype of populations 2 and 3. For $K = 5$, population 1 (non-MON-1) was subdivided into two subpopulations (MON-183 in one part (population 1b) and MON-33 in the other part (population 1a)) and population 3 was subdivided into two subpopulations since the group (population 3b) of the three canine strains with the two remaining AC strains and a VL strain was separated from the other strains (population 3a). Within MON-1, the most probable K number is $K = 3$ with populations 2, 3a, and 3b (data not shown) (Fig. 3). Results obtained by genetic distance and clustering analysis were congruent and both dispatched the nine AC strains into two separated populations. Genetic differentiation among various populations was tested using FSTat Version 2.9.3.2 software (Goudet et al., 2002) (Table 3). With regard to geographical differentiation, strains from Hérault were differentiated from strains from the AM, Var, and Corsica regions as well as from the AM considered alone (Table 3) and were characterized by a specific homozygous allele (98 bp) for locus Li22–35. Year of isolation, sex, and age of the patient were tested but no significant results were obtained (data not shown). These parameters did not appear as significant subdividing factors. F_{ST} was significant between the human and canine populations from AM (F_{ST}: 0.3462; P value: 0.05) but we studied only three canine strains and they showed the same genotype. Thus, no conclusion could be drawn from this result, which has to be confirmed with a larger canine sample.

3.3. Comparison of AC versus non-AC strains

The nine AC strains were grouped with the MON-1 strains and were divided into two populations. For the 33 loci, the AC population revealed 42 alleles whereas the other MON-1 strains (24 strains) revealed 67 alleles. The degree of polymorphism in the AC population was much lower than among *L. infantum* MON-1 of southern France but also when considering only the samples from the Nice region. This remains true when considering only strains from 1997 or 2007 (the most abundant in our sample) which have a higher degree of polymorphism than the AC population. Seven strains presented the same genotype and belonged to population 2 as defined by STRUCTURE within cluster C2 (14 human strains). There were no specific alleles able to discriminate those among the other populations. However, three alleles were predominantly found in these seven AC strains (and were homozygous for these three loci) compared with the other MON-1 strains

Please cite this article in press as: Hide, M., et al. Parasitic genotypes appear to differ in leishmaniasis patients compared with asymptomatic related carriers. Int. J. Parasitol. (2013), http://dx.doi.org/10.1016/j.ijpara.2012.12.008

ARTICLE IN PRESS

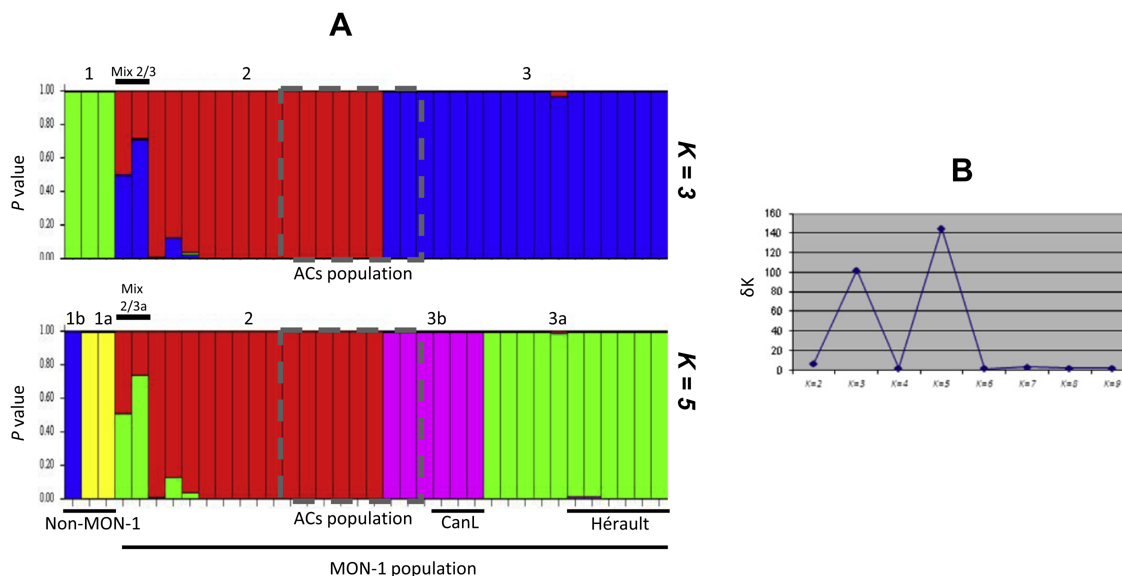

Fig. 3. Estimated population structure and substructure of *Leishmania infantum* as inferred by the STRUCTURE program. Results are based on multilocus microsatellite typing of 33 microsatellite markers obtained for the 36 *L. infantum* strains studied. (A) In the bar plots, each strain is represented by a single vertical line divided into K colors, where K is the number of populations assumed. Each color represents one population and the length of the color segment shows the strain's estimated proportion of membership in that population. Isolates are organized by membership coefficients. According to *deltaK*, the most probable numbers of populations in the data set are three and five. (B) Derived graph for *deltaK* shows a peak at $K = 3$ and $K = 5$, indicating the existence of three and five populations in the investigated strain set. CanL, Canine leishmaniasis; AC, Asymptomatic carriers.

Table 3
Differentiation measures (F_{ST}) and testing (P value) between different *Leishmania infantum* MON-1 strains according to the clinical manifestations, geographical origins and host species, and controlling for the other factors (possible only on some occasions). F_{ST} considering time, sex, and age of the patient are not significant (data not shown).

Comparison	Subsamples	F_{ST}	P value
	AC vs non-AC (All)	0.2211	0.050
	AC vs Symptomatic (humans)	0.2135	0.050
	AC vs Symptomatic (humans from AM)	0.1659	0.050
Clinical	AC vs VL	0.2097	0.030
	AC vs CL	0.3808	0.016
	AC vs HIV+	0.2876	0.016
	HIV+ vs LC/LV	−0.0070	0.400
	AC vs VL (from AM)	0.0523	0.308
	AC vs CL (from AM)	0.3303	0.083
	AC vs HIV+ (from AM)	0.3340	0.025
Geography	Hérault vs AM	0.3643	0.016
	Hérault vs AM (humans)	0.3843	0.050
	Hérault vs other	0.3668	0.050
Host	Humans vs dogs (from AM)	0.3462	0.050

AC, asymptomatic carriers; AM, Alpes-maritimes; VL, visceral leishmaniasis; CL, cutaneous leishmaniasis.

(26 strains), for which these loci were heterozygous except for LIST7026 (LIST7026 205 bp: 1 and 0.192, Lm4TA 75 bp: 1 and 0.077, LibTA 236 bp: 1 and 0.077 – allele frequencies respectively for the seven AC and other MON-1 strains). The two remaining AC (LEM3280 and LEM3273) strains from population 3b defined by STRUCTURE ($K = 5$) were grouped with the three canine strains and a VL case within cluster C3. Four homozygous loci provided specific alleles for population 3b: Li45–24 100 bp, Lm4TA 73 bp, LibTA 234 bp, and LIST7038 122 bp. There were no specific alleles able to discriminate the nine AC strains among the other populations. However, three homozygous alleles were predominantly found in the AC population compared with the other 24 MON-1 strains: Lm4TA 75 bp: 0.778 and 0.083, LibTA 236 bp: 0.778 and 0.083, and LIST7026 205 bp: 0.778 and 0.208 (allelic frequencies, respectively, for AC and other MON-1 strains). LIST7026 was homozygous for the whole MON-1 sample. Population genetics analyses using FSTat Version 2.9.3.2 software (Goudet et al., 2002) were performed, considering the whole AC sample (nine strains) as a population (Table 3). Differentiations were evidenced between the AC population and the non-AC population (24 strains) (F_{ST}: 0.2211; P value: 0.050). Because there was a genetic differentiation between the Hérault and AM samples, F_{ST} were recalculated considering only the AM sample. The AC population was differentiated from the symptomatic population (F_{ST}: 0.1659; P value: 0.050) and this was mostly due to the differentiation between the AC population and the HIV+ population (F_{ST}: 0.3340; P value: 0.025). Note that a marginally significant differentiation between the AC population and the CL population (two strains) (F_{ST}: 0.3303; P value: 0.083) was obtained but this result must be confirmed on a larger sample.

4. Discussion

The genetic structure and diversity of strains of *L. infantum* from ACs and symptomatic hosts (dogs and humans) from the south of France were for the first time investigated and compared. The MLMT approach employing 33 microsatellite markers was previously shown to be highly discriminatory for *Leishmania* typing and even for strains of *L. infantum* belonging to the zymodeme MON-1, which predominates in the Mediterranean Basin (Ochsenreither et al., 2006; Kuhls et al., 2008). The mean number of alleles per locus (N_a) was 4.13 alleles per locus. This agrees with a previous study for which the mean was 5.6 alleles per locus within a European sample of *L. infantum* and 4.6 within their European MON-1 subsample (Kuhls et al., 2008). In our sample, the level of heterozygosity was low, as demonstrated by the H_o and the H_e. This agrees with all of the previous studies, which demonstrated an overall heterozygote deficiency in all of the studied *Leishmania* spp. (Kuhls et al., 2007; Amro et al., 2009; Rougeron et al., 2009, 2011). A previous population genetics analysis showed that this deficiency could be explained by the frequent occurrence of mating

between individuals with related strains (i.e. endogamy, see (Rougeron et al., 2010)). This suggests that each *L. infantum* genotype can propagate for a long time if conditions are favorable and if they are not eliminated (by the immune system of hosts for example) during the cycle. Furthermore, the analysis revealed a geographical structuring within the sample, Hérault versus AM. This kind of structuring has already been observed when considering European countries instead of regions (Kuhls et al., 2008), as well as for another *Leishmania* spp. in Ethiopia (*Leishmania aethiopica*) (Schonian et al., 2000). Hérault and AM are only 200 miles apart but are separated by the Camargue (Rhone delta). This area could act as a barrier to gene flow by isolating Phlebotomine populations.

It is important to study *Leishmania* in healthy subjects to improve our knowledge of clinical manifestation, transmission of leishmaniasis, the pathways of parasite dissemination, and its capacity for surviving in human hosts. By using the Bayesian statistics-based clustering method, STRUCTURE, and by constructing a neighbor-joining tree based on microsatellite genetic distances, strains isolated from ACs were grouped with the zymodeme MON-1 and revealed a weak genetic polymorphism (two genotypes) compared with those isolated from symptomatic humans. Several hypotheses could explain this lack of polymorphism compared with "symptomatic" *Leishmania*. For example, (i) AC genotypes could result from a recent clonal propagation and have not yet diverged or (ii) the selective pressure to maintain this avirulent form in its human host is very high. Despite the low diversity of the AC population, we found indications for population substructures. This study included all of the *L. infantum* strains from ACs available worldwide but an effort must be made in the future to confirm these data in a larger sample. In addition, AC strains were assigned to two subpopulations. One AC population included seven AC strains and pertained to population 2 and the second, population 3b, included two AC strains, the three canine strains, and one strain from a human case of VL. There was no link between these two AC strains and the VL strain since they were cultured and studied in two different universities. This separation into two clusters cannot be explained in relation to epidemiological data since there was no correlation with isolation date, donor's sex and donor's age. The nine AC strains were isolated from blood donors between April and November 1996 from the same area (AM/Italy). Recent studies have previously used strains isolated from blood and revealed genetic diversity (A.L. Bañuls and M. Hide, personal communication). Another microsatellite study using blood, bone marrow and popliteal lymph node aspirates did not reveal specific genotypes from blood (Montoya et al., 2007). Thus, there is no culture bias due to blood that could explain the lack of diversity observed among AC parasites. Interestingly, the three canine strains from our sample belonged to population 3b. To validate this cluster, further investigation using a larger canine sample is needed, but it suggests that the "asymptomatic" human genotypes are able to propagate and to infect dogs in addition to humans. It is known that there is no structuring between CL, VL and HIV+ populations and our data confirmed this result (Kuhls et al., 2008, 2011). However, an interesting correlation was observed between *Leishmania* genotypes and clinical manifestation since genotypes from ACs are differentiated from those of symptomatic patients. This genetic differentiation is mainly due to the HIV+ subpopulation and, but much less so, to the CL subpopulation. On the one hand, in immunocompromised patients, it could be easily imagined that parasites normally not causing disease could survive the deficient immune system and genotypes normally killed by an immune competent system survive and grow in HIV+ patients. Hence, less separation between AC and HIV+ patients would be expected, however the exact opposite was observed. Regarding our results, the first hypothesis is that some genotypes could be unable to cause disease even in immunocompromised patients. On the other hand, parasites in ACs are exposed to the immune system and are under selection. They must survive attack by the immune system, even though they do not necessarily cause disease. Disease may occur in the VL patients due to some transient immune suppression that released the parasites from control by the immune system, allowing them to replicate faster and cause disease. In any case as observed in our sample, AC strains seem more similar to parasites from VL patients than HIV+ patients. Nevertheless, our sample is too small to validate these hypotheses. In this case, we wonder whether these AC strains have pathogenic properties which are able to produce clinical manifestation in humans. To explore the last hypothesis, pathogenicity of AC and symptomatic strains must be compared in vivo in immunocompromised mice. It is important to note that none of the nine donors have developed leishmaniasis since their blood donation at a Nice hospital in 1996. In addition, considering *Plasmodium falciparum*, responsible for malaria, some authors showed that, even in HIV+ patients, the percentage of clinical malaria (4%) was still lower than the percentage of malaria parasitemia (12%) (Whitworth et al., 2000). In this case, 8% of HIV+ patients remained asymptomatic for malaria infection. In any case, these AC strains must be considered as a source of material to identify parasite factors involved in the observed clinical polymorphisms.

The role of asymptomatic infection in the transmission dynamics of infectious diseases has been studied for various pathogens such as influenza or *Shigella* (Nelson et al., 1968; Hsu and Hsieh, 2008; Patrozou and Mermel, 2009). These asymptomatic infections seem to be involved in transmission dynamics. Other authors have suggested that for a given pathogen (not especially *Leishmania*), avirulent strains could have evolved to infect safe hosts whereas virulent strains would be adapted to infect hosts already infected by avirulent strains (Alizon and van Baalen, 2008). Using PCR, we found a very high AC rate in both human and canine populations, up to 70% and 40%, respectively, in southern France (Hide et al., unpublished data). The target organs of human AC strains are not known, nor whether their amastigotes are intracellular or free, or their transmission capacity. However, we know that asymptomatic infection can continue for at least 6 months (Le Fichoux et al., 1999). There are only a few studies concerning the capacity of human ACs to infect sand flies. For example, (Costa et al. 2000) showed that no sand flies acquired infection from 27 asymptomatic persons (with positive leishmanin skin tests). In a study from Brazil involving dogs, 28% of seropositive symptomatic dogs were infectious to *Lutzomyia longipalpis* sand flies, whereas only 5.4% of the asymptomatic dogs were infectious (Michalsky et al., 2007). Recently, the same results were found by Soares et al. (2011). On the contrary, some authors showed that infectivity to *P. perniciosus* sandflies was independent of the extent of symptoms in infected dogs (asymptomatics versus polysymptomatic dogs) (Molina et al., 1994). In conclusion, the high AC rate and the genetic structure of *Leishmania* from ACs must be taken into account to estimate the role of human AC populations as potential reservoirs. As we have the nine parasite samples from the AC patients, one way to answer this question would be by exploring their infectivity in *Phlebotomus* sand flies.

From the standpoint of public health, there is no *Leishmania* screening before blood transfusion, yet leishmaniases are endemic in the south of France. Transmission can occur during organ transplant (Basset et al., 2005) and a case of transfusion-transmitted VL has recently been revealed due to *Leishmania mexicana* in Colombia (Mestra et al., 2011). Other transfusion-transmitted leishmaniases have been reported and reviewed in (Dey and Singh 2006). In addition, an anthroponotic cycle has emerged among intravenous drug users, in which syringes replace the sand fly vector (Cruz et al., 2002). However, concerning blood donors, French blood products have been deleukocyted since August 1998; in 2007, the French Institute for Public Health Surveillance (InVS) considered that

Please cite this article in press as: Hide, M., et al. Parasitic genotypes appear to differ in leishmaniasis patients compared with asymptomatic related carriers. Int. J. Parasitol. (2013), http://dx.doi.org/10.1016/j.ijpara.2012.12.008

deleukocytation has vastly lowered the risk of blood donation infection with *Leishmania* (French Institute for Public Health Surveillance (InVS) – Brouard, 2007). Another study showed that leucodepletion effectively reduces parasitemia, thus minimizing the potential risk of *Leishmania* transmission through blood transfusions in endemic areas (Riera et al., 2008).

We believe that this is the first known genetic study on *Leishmania* parasites isolated from ACs (blood donors). Our data show for the first time that *Leishmania* parasites isolated from asymptomatic human carriers may differ from parasites isolated from human patients, even in a species that has very little genetic variation, such as *L. infantum*, although these findings require confirmation with a larger sample size. The AC genotypes revealed a weak polymorphism and pertained to zymodeme MON-1. The strongest genetic differentiation was observed between AC and HIV populations. This opens new research directions since these strains (especially the genotype shared by seven AC strains) can bring to light important information for understanding the outcome of leishmaniases in humans.

Acknowledgements

The authors acknowledge F. Renaud, F. Prugnolle, B. Roche, P. Delaunay and S. Alizon for helpful discussions and their assistance in analyses and interpretation of the results. We acknowledge the French National Reference Center of *Leishmania* for providing the samples. We are grateful to the Institut de Recherche pour le Développement and the Centre National de la Recherche Scientifique for their support. This study was funded by a French National Project (MIE program of CNRS). The authors acknowledge the assistance of Dr. Gary Burkhart for English manuscript corrections and to the SFR "Montpellier Environnement Biodiversité" platform from Montpellier, France. We also thank the reviewers for the improvement of the paper.

References

Alizon, S., van Baalen, M., 2008. Multiple infections, immune dynamics, and the evolution of virulence. Am. Nat. 172, E150–E168.

Amro, A., Schonian, G., Al-Sharabati, M.B., Azmi, K., Nasereddin, A., Abdeen, Z., Schnur, L.F., Baneth, G., Jaffe, C.L., Kuhls, K., 2009. Population genetics of *Leishmania infantum* in Israel and the Palestinian Authority through microsatellite analysis. Microbes Infect. 11 (4), 484–492.

Bañuls, A.L., Bastien, P., Pomares, C., Arevalo, J., Fisa, R., Hide, M., 2011. Clinical pleiomorphism in human leishmaniases, with special mention of asymptomatic infection. Clin. Microbiol. Infect. 17, 1451–1461.

Basset, D., Faraut, F., Marty, P., Dereure, J., Rosenthal, E., Mary, C., Pratlong, F., Lachaud, L., Bastien, P., Dedet, J.P., 2005. Visceral leishmaniasis in organ transplant recipients: 11 new cases and a review of the literature. Microbes Infect. 7, 1370–1375.

Chevenet, F., Brun, C., Bañuls, A.L., Jacq, B., Christen, R., 2006. TreeDyn: towards dynamic graphics and annotations for analyses of trees. BMC Bioinformatics 7, 439.

Costa, C.H., Stewart, J.M., Gomes, R.B., Garcez, L.M., Ramos, P.K., Bozza, M., Satoskar, A., Dissanayake, S., Santos, R.S., Silva, M.R., Shaw, J.J., David, J.R., Maguire, J.H., 2002. Asymptomatic human carriers of *Leishmania chagasi*. Am. J. Trop. Med. Hyg. 66, 334–337.

Costa, C.H., Gomes, R.B., Silva, M.R., Garcez, L.M., Ramos, P.K., Santos, R.S., Shaw, J.J., David, J.R., Maguire, J.H., 2000. Competence of the human host as a reservoir for Leishmania chagasi. J. Infect. Dis. 182, 997–1000.

Cruz, I., Morales, M.A., Noguer, I., Rodriguez, A., Alvar, J., 2002. *Leishmania* in discarded syringes from intravenous drug users. Lancet 359, 1124–1125.

del Giudice, P., Mary-Krause, M., Pradier, C., Grabar, S., Dellamonica, P., Marty, P., Gastaut, J.A., Costagliola, D., Rosenthal, E., 2002. Impact of highly active antiretroviral therapy on the incidence of visceral leishmaniasis in a French cohort of patients infected with human immunodeficiency virus. J. Infect. Dis. 186, 1366–1370.

Desjeux, P., Alvar, J., 2003. *Leishmania*/HIV co-infections: epidemiology in Europe. Ann. Trop. Med. Parasitol. 97 (Suppl. 1), 3–15.

Dey, A., Singh, S., 2006. Transfusion transmitted leishmaniasis: a case report and review of literature. Indian J. Med. Microbiol. 24, 165–170.

el Tai, N.O., Osman, O.F., el Fari, M., Presber, W., Schonian, G., 2000. Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of *Leishmania donovani* spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. Trans. R. Soc. Trop. Med. Hyg. 94, 575–579.

Evanno, G., Regnaut, S., Goudet, J., 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol. 14, 2611–2620.

Fakhar, M., Motazedian, M.H., Hatam, G.R., Asgari, Q., Kalantari, M., Mohebali, M., 2008. Asymptomatic human carriers of *Leishmania infantum*: possible reservoirs for Mediterranean visceral leishmaniasis in southern Iran. Ann. Trop. Med. Parasitol. 102, 577–583.

French Institute for Public Health Surveillance (InVS); Brouard, C., De Valk, H., Pilloneln J., 2007. Quantitative estimation of the risk of blood donation infection by infectious agents. In: InVS Report, pp. 1–77 (in French).

Garcia-Garcia, J.A., Martin-Sanchez, J., Gallego, M., Rivero-Roman, A., Camacho, A., Riera, C., Morillas-Marquez, F., Vergara, S., Macias, J., Pineda, J.A., 2006. Use of noninvasive markers to detect *Leishmania* infection in asymptomatic human immunodeficiency virus-infected patients. J. Clin. Microbiol. 44, 4455–4458.

Garnier, S., Alibert, P., Audiot, P., Prieur, B., Rasplus, J.Y., 2004. Isolation by distance and sharp discontinuities in gene frequencies: implications for the phylogeography of an alpine insect species, *Carabus solieri*. Mol. Ecol. 13, 1883–1897.

Goudet, J., Perrin, N., Waser, P., 2002. Tests for sex-biased dispersal using biparentally inherited genetic markers. Mol. Ecol. 11, 1103–1114.

Guevara, P., Ramirez, J.L., Rojas, E., Scorza, J.V., Gonzalez, N., Anez, N., 1993. *Leishmania braziliensis* in blood 30 years after cure. Lancet 341, 1341.

Hsu, S.B., Hsieh, Y.H., 2008. On the role of asymptomatic infection in transmission dynamics of infectious diseases. Bull. Math. Biol. 70, 134–155.

Jamjoom, M.B., Ashford, R.W., Bates, P.A., Kemp, S.J., Noyes, H.A., 2002. Towards a standard battery of microsatellite markers for the analysis of the *Leishmania donovani* complex. Ann. Trop. Med. Parasitol. 96, 265–270.

Kuhls, K., Keilonat, L., Ochsenreither, S., Schaar, M., Schweynoch, C., Presber, W., Schonian, G., 2007. Multilocus microsatellite typing (MLMT) reveals genetically isolated populations between and within the main endemic regions of visceral leishmaniasis. Microbes Infect. 9, 334–343.

Kuhls, K., Chicharro, C., Canavate, C., Cortes, S., Campino, L., Haralambous, C., Soteriadou, K., Pratlong, F., Dedet, J.P., Mauricio, I., Miles, M., Schaar, M., Ochsenreither, S., Radtke, O.A., Schonian, G., 2008. Differentiation and gene flow among European populations of *Leishmania infantum* MON-1. PLoS Negl. Trop. Dis. 2, e261.

Kuhls, K., Alam, M.Z., Cupolillo, E., Ferreira, G.E., Mauricio, I.L., Oddone, R., Feliciangeli, M.D., Wirth, T., Miles, M.A., Schonian, G., 2011. Comparative microsatellite typing of new world *Leishmania infantum* reveals low heterogeneity among populations and its recent old world origin. PLoS Negl. Trop. Dis. 5, e1155.

Le Fichoux, Y., Quaranta, J.F., Aufeuvre, J.P., Lelievre, A., Marty, P., Suffia, I., Rousseau, D., Kubar, J., 1999. Occurrence of *Leishmania infantum* parasitemia in asymptomatic blood donors living in an area of endemicity in southern France. J. Clin. Microbiol. 37, 1953–1957.

Madeira Mde, F., Schubach Ade, O., Schubach, T.M., Leal, C.A., Marzochi, M.C., 2004. Identification of *Leishmania* (*Leishmania*) chagasi isolated from healthy skin of symptomatic and asymptomatic dogs seropositive for leishmaniasis in the municipality of Rio de Janeiro, Brazil. Braz. J. Infect. Dis. 8, 440–444.

Mestra, L., Lopez, L., Robledo, S.M., Muskus, C.E., Nicholls, R.S., Velez, I.D., 2011. Transfusion-transmitted visceral leishmaniasis caused by *Leishmania* (*Leishmania*) *mexicana* in an immunocompromised patient: a case report. Transfusion 10, 1–5.

Michalsky, E.M., Rocha, M.F., da Rocha Lima, A.C., Franca-Silva, J.C., Pires, M.Q., Oliveira, F.S., Pacheco, R.S., dos Santos, S.L., Barata, R.A., Romanha, A.J., Fortes-Dias, C.L., Dias, E.S., 2007. Infectivity of seropositive dogs, showing different clinical forms of leishmaniasis, to *Lutzomyia longipalpis* phlebotomine sand flies. Vet. Parasitol. 147, 67–76.

Michel, G., Pomares, C., Ferrua, B., Marty, P., 2011. Importance of worldwide asymptomatic carriers of *Leishmania infantum* (*L. chagasi*) in human. Acta Trop. 119, 69–75.

Molina, R., Amela, C., Nieto, J., San-Andres, M., Gonzalez, F., Castillo, J.A., Lucientes, J., Alvar, J., 1994. Infectivity of dogs naturally infected with *Leishmania infantum* to colonized *Phlebotomus perniciosus*. Trans. R. Soc. Trop. Med. Hyg. 88, 491–493.

Montoya, L., Gallego, M., Gavignet, B., Piarroux, R., Rioux, J.A., Portus, M., 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. Am. J. Trop. Med. Hyg. 76, 888–895.

Nei, M., Chesser, R.K., 1983. Estimation of fixation indices and gene diversities. Ann. Hum. Genet. 47, 253–259.

Nelson, J.D., Haltalin, K.C., Hasmiesz, H.T., 1968. Endemic shigellosis: a study of fifty households. Am. J. Epidemiol. 86, 683–689.

Ochsenreither, S., Kuhls, K., Schaar, M., Presber, W., Schonian, G., 2006. Multilocus microsatellite typing as a new tool for discrimination of *Leishmania infantum* MON-1 strains. J. Clin. Microbiol. 44, 495–503.

Patrozou, E., Mermel, L.A., 2009. Does influenza transmission occur from asymptomatic infection or prior to symptom onset? Public Health Rep. 124, 193–196.

Pritchard, J.K., Stephens, M., Donnelly, P., 2000. Inference of population structure using multilocus genotype data. Genetics 155, 945–959.

Riera, C., Fisa, R., Udina, M., Gallego, M., Portus, M., 2004. Detection of *Leishmania infantum* cryptic infection in asymptomatic blood donors living in an endemic area (Eivissa, Balearic Islands, Spain) by different diagnostic methods. Trans. R. Soc. Trop. Med. Hyg. 98, 102–110.

Riera, C., Fisa, R., Lopez-Chejade, P., Serra, T., Girona, E., Jimenez, M., Muncunill, J., Sedeno, M., Mascaro, M., Udina, M., Gallego, M., Carrio, J., Forteza, A., Portus, M., 2008. Asymptomatic infection by *Leishmania infantum* in blood donors from the Balearic Islands (Spain). Transfusion 48, 1383–1389.

Rioux, J.A., Lanotte, G., Serres, E., Pratlong, F., Bastien, P., Perieres, J., 1990. Taxonomy of Leishmania. Use of isoenzymes. Suggestions for a new classification. Ann. Parasitol. Hum. Comp. 65, 111–125.

Rossi, V., Wincker, P., Ravel, C., Blaineau, C., Pages, M., Bastien, P., 1994. Structural organisation of microsatellite families in the *Leishmania* genome and polymorphisms at two (CA)n loci. Mol. Biochem. Parasitol. 65, 271–282.

Rougeron, V., De Meeus, T., Hide, M., Waleckx, E., Bermudez, H., Arevalo, J., Llanos-Cuentas, A., Dujardin, J.C., De Doncker, S., Le Ray, D., Ayala, F.J., Banuls, A.L., 2009. Extreme inbreeding in *Leishmania braziliensis*. Proc. Natl. Acad. Sci. USA 106, 10224–10229.

Rougeron, V., De Meeus, T., Kako Ouraga, S., Hide, M., Banuls, A.L., 2010. "Everything you always wanted to know about sex (but were afraid to ask)" in *Leishmania* after two decades of laboratory and field analyses. PLoS Pathog. 6, 10–15.

Rougeron, V., Banuls, A.L., Carme, B., Simon, S., Couppie, P., Nacher, M., Hide, M., De Meeus, T., 2011. Reproductive strategies and population structure in *Leishmania*: substantial amount of sex in *Leishmania Viannia guyanensis*. Mol. Ecol. 20, 3116–3127.

Russell, R., Iribar, M.P., Lambson, B., Brewster, S., Blackwell, J.M., Dye, C., Ajioka, J.W., 1999. Intra and inter-specific microsatellite variation in the *Leishmania* subgenus *Viannia*. Mol. Biochem. Parasitol. 103, 71–77.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, NY.

Scarlata, F., Vitale, F., Saporito, L., Reale, S., Vecchi, V.L., Giordano, S., Infurnari, L., Occhipinti, F., Titone, L., 2008. Asymptomatic *Leishmania infantum*/chagasi infection in blood donors of western Sicily. Trans. R. Soc. Trop. Med. Hyg. 102, 394–396.

Schonian, G., Akuffo, H., Lewin, S., Maasho, K., Nylen, S., Pratlong, F., Eisenberger, C.L., Schnur, L.F., Presber, W., 2000. Genetic variability within the species *Leishmania aethiopica* does not correlate with clinical variations of cutaneous leishmaniasis. Mol. Biochem. Parasitol. 106, 239–248.

Soares, M.R., de Mendonca, I.L., do Bonfim, J.M., Rodrigues, J.A., Werneck, G.L., Costa, C.H., 2011. Canine visceral leishmaniasis in Teresina, Brazil: relationship between clinical features and infectivity for sand flies. Acta Trop. 117, 6–9.

Whitworth, J., Morgan, D., Quigley, M., Smith, A., Mayanja, B., Eotu, H., Omoding, N., Okongo, M., Malamba, S., Ojwiya, A., 2000. Effect of HIV-1 and increasing immunosuppression on malaria parasitaemia and clinical episodes in adults in rural Uganda: a cohort study. Lancet 356, 1051–1056.

WHO, 2002. The Global Burden of disease. In: The World Health Organization Health Report, Geneva, pp. 192–197.

Please cite this article in press as: Hide, M., et al. Parasitic genotypes appear to differ in leishmaniasis patients compared with asymptomatic related carriers. Int. J. Parasitol. (2013), http://dx.doi.org/10.1016/j.ijpara.2012.12.008