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Epidemiological surveillance of leishmaniasis in the European Union: operational and research challenges

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Leishmaniasis is complex of vector-borne diseases caused by protozoan parasites of the genus *Leishmania* transmitted by the bite of phlebotomine sandflies. A dozen nosogeographical entities – characterised by different parasite, vector and reservoir host species, geographical distribution and clinical features in humans – affect 101 countries in tropical, subtropical and temperate zones of the world [1,2]. More than 90% of 200,000–400,000 global cases of visceral leishmaniasis (VL), the most severe form, are estimated to occur annually in India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil. A less severe form, cutaneous leishmaniasis (CL), is more widely distributed, accounting for 0.7–1.2 million cases each year in countries of Latin America, Mediterranean basin, Middle East and Central Asia.

Even though many physicians and public health experts still consider leishmaniasis a tropical disease, two entities associated with several *Phlebotomus* species are endemic in southern Europe: (i) zoonotic VL and CL caused by *L. infantum* throughout the region, having dogs as reservoir host; and (ii) anthroponotic CL caused by *L. tropica*, which occurs sporadically in Greece. More recently, a third parasite species (*L. donovani*, assumed to be anthroponotic) has been recorded in Cyprus, where it causes both VL and CL [3].

VL is endemic in nine countries of the European Union (EU). The World Health Organization's Department for the Control of Neglected Tropical Diseases has estimated a total VL incidence of approximately 410–620 cases each year during 2003 to 2008 in these endemic countries, adjusted to take into account a 'mild' 1.2–1.8-fold under-reporting [2]. Recent experiences from six of the nine countries – Bulgaria, Greece, Croatia, Italy, France and Spain – are presented in this special issue.

Zoonotic CL usually occurs in the same areas endemic for VL, but there are probably many more cases than those registered (2.8–4.6-fold under-reporting has been estimated for the EU region [2]). As pointed out by Lachaud et al. for France [4], but also applicable to other EU countries endemic for CL, cutaneous lesions due to *L. infantum* are often benign and patients are seen by general practitioners or dermatologists who generally do not report these cases or notify them even when mandatory.

Despite provoking a limited number of overt clinical cases – in comparison with global leishmaniasis figures – *L. infantum* represents a latent public health threat in the EU because studies performed in several endemic foci have disclosed a high prevalence of asymptomatic parasite carriers [5]. A recent example is provided for Croatia by Šiško-Kraljević et al. [6]. Hence, immunosuppressive conditions, either due to co-morbidities (e.g. human immunodeficiency virus (HIV) infection) or therapies (e.g. organ transplantation or treatment of immunological disorders [7]) may result in the reactivation of latent infections. In this regard, it should be emphasised that dermotropic *L. infantum* genotypes – the usual agents of benign CL – may disseminate to cause severe VL in immunosuppressed individuals [8]. Such elevated prevalence of human infections could have been predicted from two strands of evidence: humans are frequently bitten by sandflies and *L. infantum* infections are widespread in dogs, a highly susceptible host [9]. In large parts of countries of southern EU, canine seroprevalence rates are estimated to be in the range of 5–30%, which means that infection rates may reach values of 40–80% [10].

Some European countries at the north of regions with natural transmission of leishmaniasis have reported large series of VL and CL imported cases, many of which have acquired the parasitic infection during holidays in southern Europe [11-14]. In several instances, a definitive diagnosis of VL proved difficult and for one case, the period before symptom onset and specific treatment was longer than a year. Delay in diagnosis or misdiagnosis can also occur in southern European countries endemic for VL, but in parts where cases occur rarely, as has been reported from a northern Italian region [15]. These observations suggest that awareness about leishmaniasis endemicity in Europe should be greatly increased among general practitioners and clinicians.

Table

Operational and research challenges concerning epidemiological surveillance of leishmaniasis in the European Union

Topic	Challenges
Surveillance by passive notification systems	Notification of leishmaniasis is not compulsory everywhere in Europe. Some endemic countries have national notification systems centralised at the Ministry of Health; others have compulsory or voluntary surveillance systems in endemic regions but not in non-endemic ones. Non-endemic countries of northern Europe rely on single (or a network of) reference centres that collect information on a voluntary basis.
	There is limited harmonisation of the existing notification systems as regards case definition, clinical presentation and patient information.
	In countries with compulsory notifiable systems, under-reporting of visceral leishmaniasis is estimated to be 1.2–1.8-fold, that of cutaneous leishmaniasis 2.8–4.6-fold [2].
Transnational information	Travellers to endemic countries are not provided with adequate information on leishmaniasis risk and physicians often do not include leishmaniasis in differential diagnosis of travel-related diseases.
	There is a lack of feedback from non-endemic countries registering leishmaniasis cases in travellers to the endemic countries visited by patients, which can hamper early identification of new or re-emerging foci.
Disease vs infection	Increasingly, there is evidence that clinical cases of leishmaniasis represent the tip of an 'infection iceberg', whose size (i.e. prevalence) is unknown in most of the endemic countries
	Determinants for human clinical susceptibility are largely unknown, apart from some co-morbidities (e.g. human immunodeficiency virus (HIV) infection) or immunocompromising conditions, i.e. through immunosuppressive therapies.
Parasite identification	Multilocus enzyme electrophoresis (MLEE), the gold standard for *Leishmania* identification, is available at reference centres of three European countries (France, Italy and Spain) [1]; however, there is risk that MLEE typing activities will be ended soon because they are expensive and laborious.
	Different levels of accuracy may be required (e.g. species level at clinical centres, genotype level for epidemiological investigations); however, common protocols for molecular *Leishmania* identification are not available yet.
Domestic vs wild reservoir hosts	Updated geographical distribution of canine leishmaniasis, representing the most efficient sentinel for leishmaniasis transmission in a territory, is not available for all endemic countries
	The epidemiological role of domestic hosts other than dogs (e.g. cats) is still unclear
	The potential role of wild mammals (rodents, lagomorphs, carnivores) as reservoir hosts of *Leishmania* requires investigation because it can change with man-made environmental changes such as witnessed by the recent outbreak in Madrid, Spain [21].
Phlebotomine vectors	Taxonomy and biology investigations on European phlebotomine species rely on a limited group of experts. Updated information on vector distribution is therefore lacking in some endemic countries and in neighbouring non-endemic ones.
	Competence of permissive sandfly species needs to be elucidated as regards potential transmission of exotic *Leishmania* species imported into Europe.
	The vectorial role of continental European species of sandflies (e.g. *Phlebotomus mascitti*) is still to be ascertained.
Control measures	The primary control measure is avoiding deaths from the most severe form of leishmaniasis (visceral). General public and health professional awareness of the disease (both leading to early diagnosis) and appropriate therapy should be the mainstay for both endemic and non-endemic countries.
	Vaccination combined with topical insecticides with sandfly anti-feeding properties should be recommended for dogs living in endemic areas or temporarily travelling from non-endemic to endemic areas.

As an endemic country comprises known areas or foci of endemicity, it is interestingly to note that in some instances, travellers became infected after visiting an area that was not considered as endemic by the health authorities of the country visited [16]. This should encourage the development of systems for appropriate transnational information following leishmaniasis diagnosis in travellers.

Deaths due to VL, although possible, are rare. The disease has a slow chronic course, so that fatal cases may be patients with individual risk factors such as severe co-morbidities or, in case of young children, malnutrition associated with late diagnosis. On the other hand, deaths due to inappropriate use of VL drugs can be even more frequent. In some European countries, antimonial drugs are still in use for some categories of patients because of the high cost of liposomal amphotericin B [17] and it is well known that overdose of pentavalent antimony in adults can cause severe cardiac failures in addition to pancreatitis.

This special issue of *Eurosurveillance*, published in two parts, is a useful instrument to review diverse aspects of leishmaniasis in Europe related to topics such as the information and surveillance systems in place in countries within the EU, the current epidemiological situation and novel aspects related to parasite identification [18,19], domestic and wild reservoir hosts [20] and vectors [9]. The main challenges associated with these topics are summarised in the Table.

In conclusion, leishmaniasis, a neglected disease, is rare in some countries of Europe, but endemic in others, having a great impact on individuals and the potential to spread further. The disease should be monitored carefully and systems for its notification should be harmonised at both national and transnational levels.

References

1. World Health Organization (WHO). Control of the leishmaniases: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22-26 March 2010. Geneva: WHO; 2010. WHO technical report series; no. 949. Available from: http://whqlibdoc.who.int/trs/WHO_TRS_949_eng.pdf
2. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS ONE 2012;7(5):e35671. http://dx.doi.org/10.1371/journal.pone.0035671 PMid:22693548 PMCid:PMC3365071
3. Antoniou M, Haralambous C, Mazeris A, Pratlong F, Dedet JP, Soteriadou K. Leishmania donovani leishmaniasis in Cyprus. Lancet Infect Dis. 2008;8(1):6-7. http://dx.doi.org/10.1016/S1473-3099(07)70297-9
4. Lachaud L, Dedet JP, Marty P, Faraut F, Buffet P, Gangneux JP, et al. Surveillance of leishmaniases in France, 1999 to 2012. Euro Surveill. 2013;18(29):pii=20534. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20534
5. Michel G, Pomares C, Ferrua B, Marty P. Importance of worldwide asymptomatic carriers of Leishmania infantum (L. chagasi) in human. Acta Trop. 2011;119(2-3):69-75. http://dx.doi.org/10.1016/j.actatropica.2011.05.012 PMid:21679680
6. Šiško-Kraljević K, Jerončić A, Mohar B, Punda-Polić V. Asymptomatic Leishmania infantum infections in humans living in endemic and non-endemic areas of Croatia, 2007 to 2009. Euro Surveill. 2013;18(29):pii=20533. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20533
7. Zanger P, Gabrysch S. Leishmaniasis in the era of tumor necrosis factor alpha antagonist therapy – a research agenda for Europe. Euro Surveill. 2013;18(30):pii=20542. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20542
8. Gradoni L, Gramiccia M. Leishmania infantum tropism: strain genotype or host immune status? Parasitol Today. 1994;10(7):264-7. http://dx.doi.org/10.1016/0169-4758(94)90142-2
9. Antoniou M, Gramiccia M, Molina R, Dvorak V, Volf P. The role of indigenous phlebotomine sandflies and mammals in the spreading of leishmaniasis agents in the Mediterranean region. Euro Surveill. 2013;18(30):pii=20540. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20540
10. Franco AO, Davies CR, Mylne A, Dedet JP, Gállego M, Ballart C, et al. Predicting the distribution of canine leishmaniasis in western Europe based on environmental variables. Parasitology. 2011;138:1878-91. http://dx.doi.org/10.1017/S003118201100148X PMid:21914251
11. Wall EC, Watson J, Armstrong M, Chiodini PL, Lockwood DN. Epidemiology of imported cutaneous leishmaniasis at the Hospital for Tropical Diseases, London, United Kingdom: use of polymerase chain reaction to identify the species. Am J Trop Med Hyg. 2012;86(1):115-8. http://dx.doi.org/10.4269/ajtmh.2012.10-0558 PMid:22232460 PMCid:PMC3247118
12. Malik AN, John L, Bruceson AD, Lockwood DN. Changing pattern of visceral leishmaniasis, United Kingdom, 1985-2004. Emerg Infect Dis. 2006;12(8):1257-9. http://dx.doi.org/10.3201/eid1708.050486 PMid:16965709 PMCid:PMC3291201
13. Weitzel T, Mühlberger N, Jelinek T, Schunk M, Ehrhardt S, Bogdan C, et al. Imported leishmaniasis in Germany 2001-2004: data of the SIMPID surveillance network. Eur J Clin Microbiol Infect Dis. 2005;24(7):471-6. http://dx.doi.org/10.1007/s10096-005-1363-1 PMid:15997368
14. Bart A, van Thiel PP, de Vries HJ, Hodiamont CJ, Van Gool T. Imported leishmaniasis in the Netherlands from 2005 to 2012: epidemiology, diagnostic techniques and sequence-based species typing from 195 patients. Euro Surveill. 2013;18(30):pii=20544. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20544
15. Varani S, Cagarelli R, Melchionda F, Attard L, Salvadori C, Finarelli AC, et al. Ongoing outbreak of visceral leishmaniasis in Bologna Province, Italy, November 2012 to May 2013. Euro Surveill. 2013;18(29):pii=20530. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20530
16. Faber WR, Hoekzema R, Bart A, Zeegelaar JE, de Vries HJ. Cutaneous leishmaniasis acquired in Jura, France. Emerg Infect Dis. 2012;18(1):183-4. http://dx.doi.org/10.3201/eid1801.110408 PMid:22257720 PMCid:PMC3310094
17. Gradoni L, Soteriadou K, Louzir H, Dakkak A, Toz SO, Jaffe C, et al. Drug regimens for visceral leishmaniasis in Mediterranean countries. Trop Med Int Health. 2008;13(10):1272-6. http://dx.doi.org/10.1111/j.1365-3156.2008.02144.x PMid:18764817
18. Van der Auwera G, Maes I, De Doncker S, Ravel C, Cnops L, Van Esbroeck M, Van Gompel A, Clerinx J, Dujardin JC. Heat-shock protein 70 gene sequencing for Leishmania species typing in European tropical infectious disease clinics. Euro Surveill. 2013;18(30):pii=20543. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20543
19. Chicharro C, Llanes-Acevedo IP, García E, Nieto J, Moreno J, Cruz I. Molecular typing of Leishmania infantum isolates from a leishmaniasis outbreak in Madrid, Spain, 2009 to 2012. Euro Surveill. 2013;18(30):pii=20545. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20545
20. Ruiz-Fons F, Ferroglio E, Gortázar C. Leishmania infantum in free-ranging hares, Spain, 2004-2010. Euro Surveill. 2013;18(30):pii=20541. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20541
21. Arce A, Estirado A, Ordobas M, Sevilla S, García N, Moratilla L, de la Fuente S, Martínez AM, Pérez AM, Aránguez E, Iriso A, Sevillano O, Bernal J, Vilas F. Re-emergence of leishmaniasis in Spain: community outbreak in Madrid, Spain, 2009 to 2012. Euro Surveill. 2013;18(30):pii=20546. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20546

Re-emergence of leishmaniasis in Spain: community outbreak in Madrid, Spain, 2009 to 2012

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Since July 2009, there has been a community outbreak of leishmaniasis in the south-west area of the Madrid autonomous community, Spain, affecting residents from four towns that are geographically close together and share extensive park areas. As of December 2012, 446 cases were reported (6 in 2009, 97 in 2010, 196 in 2011 and 147 in 2012), a mean incidence rate of 22.2 per 100,000 inhabitants during July 2009 and December 2012. The mean age was 44 years (range: 2 months to 95 years); 61.0% were male. A total of 68 (15.2%) had immunosuppressive conditions; 160 (35.9%) had visceral leishmaniasis and 286 (64.1%) cutaneous. A total of 421 (94.4%) cases were confirmed. *Leishmania infantum* was identified as the agent. Monitoring revealed high densities of the vector *Phlebotomus perniciosus*. The surveillance system for canine leishmaniasis did not detect any increase in prevalence during the period. Environmental control measures have been taken, such as improvements in sanitation and disinsection in the risk areas and control of the overpopulation of Leporidae, as xenodiagnosis studies have shown that hares play a role as active reservoirs. This is the largest reported community outbreak of leishmaniasis in Europe. The discovery of the new reservoir stands out in the multifactorial aetiology of the outbreak. Epidemiological research and environmental intervention measures are continuing.

Introduction

Human leishmaniasis is a zoonotic disease endemic in the Mediterranean basin, including Spain [1-4]. In Spain, the vector involved in the transmission of the parasite (genus *Leishmania*) is a sandfly of the *Phlebotomus* genus (primarily *P. perniciosus*), which is active between May and October and dogs are the main reservoir [3-5].

There is a formal system for reporting all compulsorily notifiable diseases, with notification protocols including case definitions. The notification process starts from physicians, primary care and hospitals, or from microbiology laboratories, which report to the Spanish and Madrid Epidemiological Surveillance Network. All cases are reviewed by an epidemiologist. In the Madrid autonomous community, leishmaniasis has been monitored through the notifiable diseases surveillance system since 1997, although state-level reporting of this disease is not compulsory [6]. The Spanish Public Health Department's approach to the disease calls for coordinated research and control actions, both epidemiological and environmental. The services in charge of environmental research are developing surveillance programmes for vectors and canine leishmaniasis in the community's animal protection centres [7].

During 2000 to 2009, between 12 and 25 leishmaniasis cases have been reported per year in the region (with an annual incidence rate of around 0.5 per 100,000 inhabitants) [6]. During the last quarter of 2010, a fivefold increase was detected in the number of cases compared with the number seen in the whole year of previous years. Subsequent research confirmed that an outbreak of leishmaniasis had been occurring since July 2009 in the south-west area of the region of Madrid [8].

The aim of this article is to describe the epidemiological characteristics of the urban community outbreak of leishmaniasis and the control measures adopted.

Methods

After detecting an unusual increase in the number of leishmaniasis cases in Madrid, the Epidemiological Surveillance Network intensified surveillance using different strategies. Coordination was strengthened through periodic meetings with the professionals involved, in both primary and secondary health care, and active case finding was conducted. A retrospective search for cases was performed using information from microbiology laboratories and hospital discharge records. Epidemiological research was intensified using a questionnaire administered by telephone, to gather information on patients' place of residence, their work environment and leisure activities. Patients

were asked about the presence of dogs, sick dogs, mosquitoes (oriented on the habitat an characteristics of the sandflies), waste and rubbish dumps, and livestock farms in these environments during last year. Questions were also asked about their travel history during the incubation period to areas that were highly endemic for the disease.

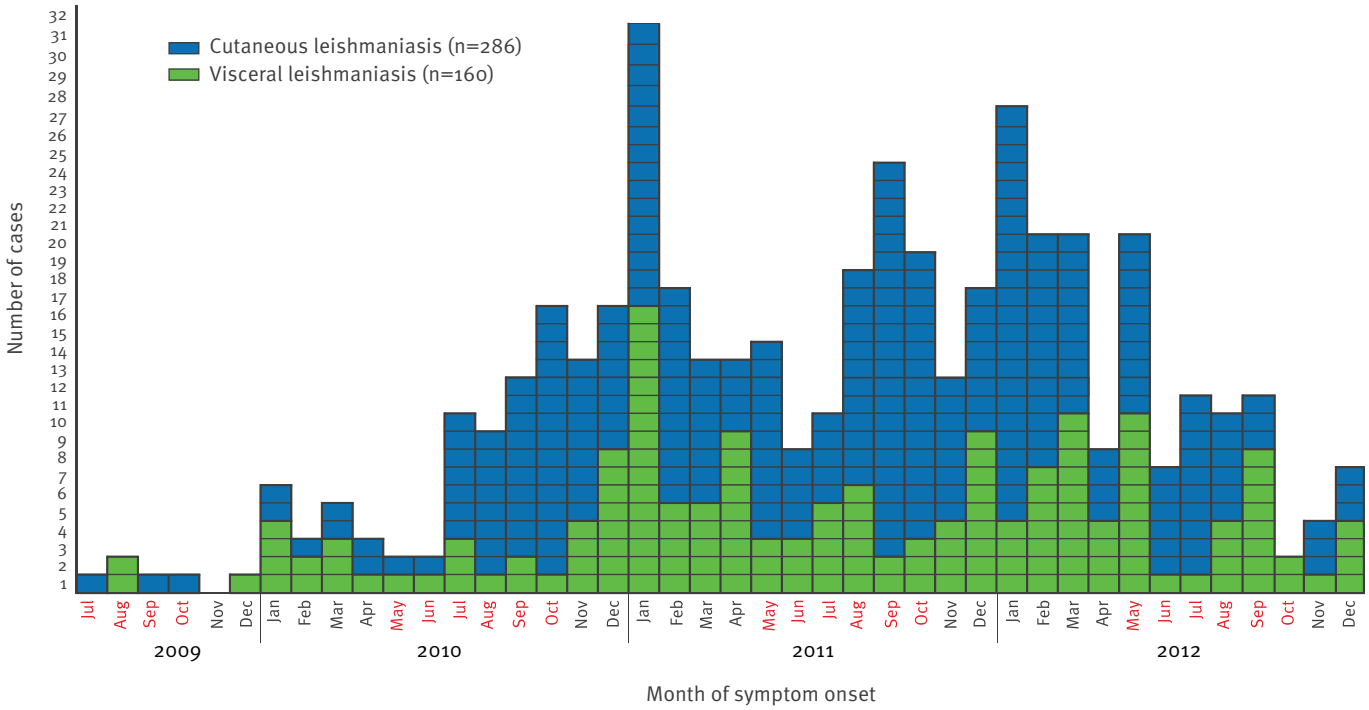
A specific case definition was established for the outbreak: a case was a person who met the clinical and laboratory criteria for leishmaniasis defined by the Epidemiological Surveillance Network, with residence in the towns located on the south-west area of the region of Madrid and with onset date of symptoms between 1 July 2009 and 31 December 2012. People affected lived in four towns – defined as the epidemic area – located geographically close together (Fuenlabrada, Leganés, Getafe and Humanes de Madrid), which share large urban parks and have a population over half a million inhabitants. It was considered that 1 July 2009 was the onset date of the outbreak because from that date, a steady increase in the number of cases was detected in the epidemic area; in the first six months of 2009, no cases were reported in this area. The Epidemiological Surveillance Network uses the case definition of leishmaniasis in the *Notification system manual of notifiable diseases* [9]. A probable case is a person that meets the clinical criteria of the case definition and may also have a positive serology (one-time positivity or titre increase of IgG). According to the manual, confirmatory diagnosis is made through demonstration of the presence of the parasite (visualisation, polymerase chain reaction (PCR) in aspirated samples or biopsy material obtained from the edges of a skin lesion (cutaneous leishmaniasis) or in a case of visceral leishmaniasis, from bone marrow, liver, spleen, lymph nodes or blood, or by the isolation of the parasite [9]. Laboratory analyses were carried out in the reference hospitals attended by each case and most cases were confirmed in the National Reference Laboratory for Leishmaniasis in Madrid (Instituto de Salud Carlos III, WHO Collaborating Centre for Leishmaniasis), where the pathogen was also classified.

We carried out a descriptive analysis of the epidemiological variables studied: sex, age, country of origin, onset date of symptoms, clinical presentation, classification of cases, diagnostic tests, intrinsic risk factors (immunosuppressive disease and/or immunosuppressive treatment), extrinsic risk factors (environmental exposure to the common vector and/or reservoir) and reporting delay. We analysed all the cases, separated according to their clinical presentation. The cases were georeferenced using the patients' place of residence.

Incidence rates for the period were calculated per town as the number of cases per 100,000 inhabitants. The population given in the continuous census for 2009

Figure 1

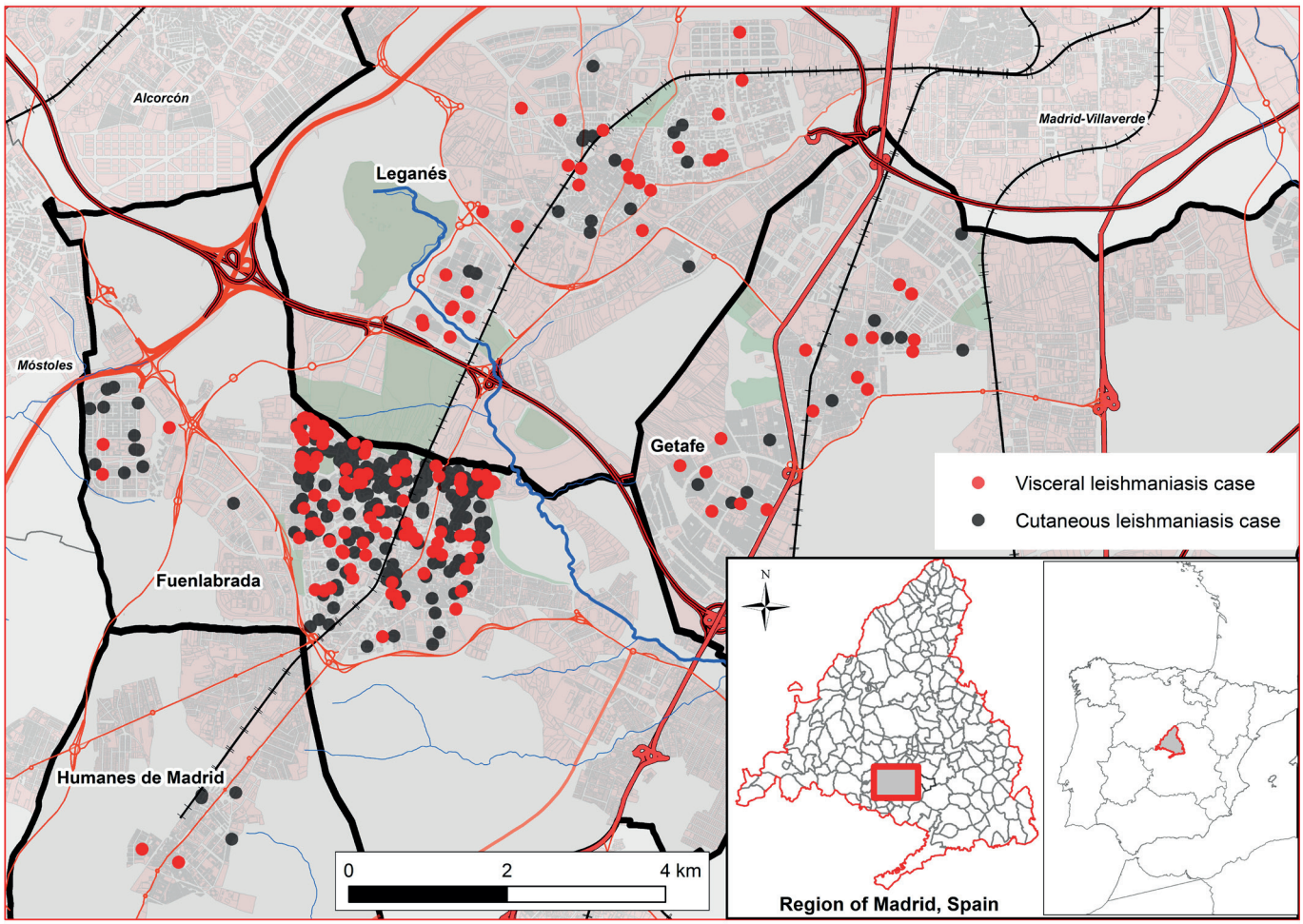
Outbreak cases of leishmaniasis by month of symptom onset and clinical presentation, region of Madrid, Spain, July 2009–December 2012 (n=446)



The months in which the vector is active (May to October) are shown in red.

Figure 2

Spatial distribution of cases by place of residence and clinical presentation, community outbreak of leishmaniasis in the region of Madrid, Spain, July 2009–December 2012 (n=446)



to 2012 published by the Institute of Statistics of the Community of Madrid [10] was used as denominator.

In environmental research, regional actions included in the canine leishmaniasis programme were adopted and specific measures were intensified in the outbreak area (monitoring of known and potential reservoirs and control measures). A sandfly surveillance system was implemented in the Madrid region in 2008 [7], involving 10 stations in various towns from May to October each year. Surveillance activities were intensified in the epidemic area, following the start of the outbreak.

Results

Epidemiological investigation

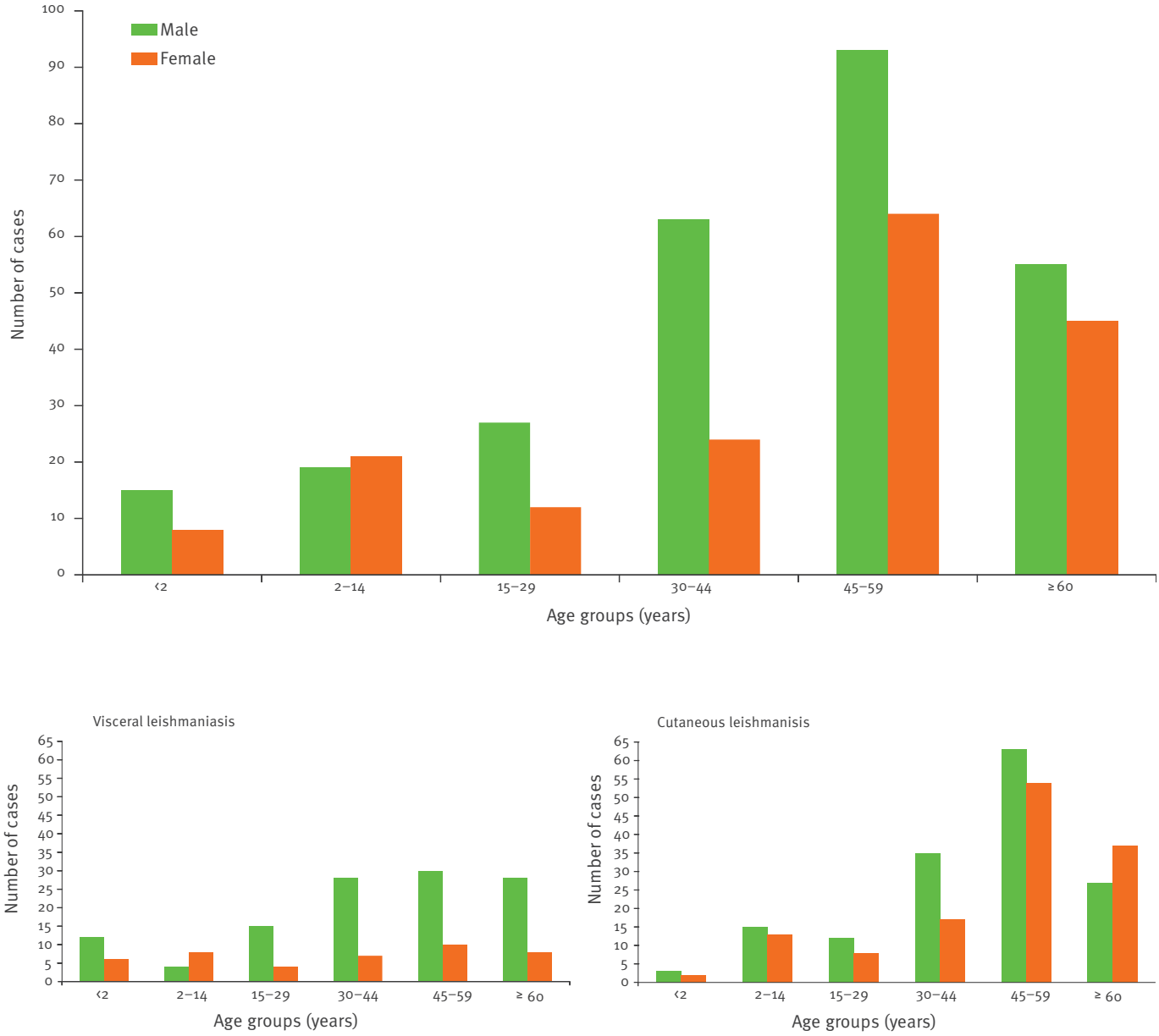
From 1 July 2009 to 31 December 2012, 542 cases of leishmaniasis were reported in the region of Madrid to the Epidemiological Surveillance Network, of which 446 (82.3%) met the outbreak case definition: 6 were identified in 2009, 97 in 2010, 196 cases in 2011 and 147 cases in 2012. The mean incidence rate in the epidemic area was 22.2 cases per 100,000 inhabitants during the period under investigation. The outbreak is under control but new cases (fewer) are being reported.

The patients lived in the following towns in the region of Madrid: Fuenlabrada (366 cases; 52.7 per 100,000 inhabitants), Leganés (48 cases; 7.3 per 100,000 inhabitants), Getafe (26 cases; 4.4 per 100,000 inhabitants) and Humanes de Madrid (6 cases; 9.2 per 100,000 inhabitants). During 2000 to 2009, between 1 and 6 cases per year were detected in these four towns, with an incidence rate below 1.0 per 100,000 inhabitants.

The clinical presentation of patients in the outbreak was 35.9% visceral leishmaniasis (160 cases; 8.0 per 100,000 inhabitants). Of these, 140 had classical disease and 20 atypical presentations (18 localised lymphadenopathic leishmaniasis and two with mucosal leishmaniasis). The remaining 64.1% had cutaneous leishmaniasis (286 cases: 14.2 per 100,000 inhabitants). The epidemic curve by date of symptom onset and clinical presentation (Figure 1) and spatial distribution

Figure 3

Distribution by sex, age group and clinical presentation, community outbreak of leishmaniasis in the region of Madrid, Spain, July 2009–December 2012 (n=446)



of cases by place of residence and clinical presentation (Figure 2) are shown.

The median reporting delay was 151 days (41 days for visceral leishmaniasis, with a minimum of 9 days and 183 days for cutaneous leishmaniasis, with a minimum of 35 days).

The distribution of cases by sex, age group and clinical presentation is shown in Figure 3. A total of 272 (61.0%) of cases were male. The mean age of all cases was 44 years (40 years for the visceral leishmaniasis cases and 46 years for the cutaneous cases), ranging from 2 months to 95 years. It is worth noting that 15 cases were infants under 1 year of age (11 with visceral leishmaniasis and 4 with cutaneous leishmaniasis) and 8 cases were aged between 12 and 23 months (7 with visceral leishmaniasis and 1 with cutaneous leishmaniasis).

The main clinical and epidemiological characteristics of the cases are shown in the Table. Some 68 (15.2%) of cases were of foreign origin: of these, 44 had visceral forms and 24 cutaneous forms. A total of 36 patients (8.1% of all cases) were born in sub-Saharan Africa (mostly from Equatorial Guinea and Nigeria), of which 32 had visceral leishmaniasis (20.0% of all the visceral leishmaniasis cases). The number of cases who were

Table

Clinical and epidemiological characteristics of leishmaniasis cases by clinical presentation, community outbreak in the region of Madrid, Spain, July 2009–December 2012 (n=446)

Characteristic	Visceral forms	Cutaneous forms	Total
	Number of cases (%)[a]	Number of cases (%)[a]	Number of cases (%)
Total	**160 (35.9)**	**286 (64.1)**	**446 (100.0)**
Sex			
Male	117 (73.1)	155 (54.2)	272 (61.0)
Female	43 (26.9)	131 (45.8)	174 (39.0)
Age in years			
<2	18 (11.2)	5 (1.7)	23 (5.2)
2–14	12 (7.5)	28 (9.8)	40 (9.0)
15–29	19 (11.9)	20 (7.0)	39 (8.7)
30–44	35 (21.9)	52 (18.2)	87 (19.5)
45–59	40 (25.0)	117 (40.9)	157 (35.2)
≥60	36 (22.5)	64 (22.4)	100 (22.4)
Country of origin			
Spain	116 (72.5)	262 (91.6)	378 (84.8)
Sub-Saharan Africa	32 (20.0)	4 (1.4)	36 (8.1)
Other countries	12 (7.5)	20 (7.0)	32 (7.2)
Year the symptoms started			
2009	3 (1.9)	3 (1.0)	6 (1.3)
2010	31 (19.4)	66 (23.1)	97 (21.8)
2011	70 (43.7)	126 (44.1)	196 (43.9)
2012	56 (35.0)	91 (31.8)	147 (33.0)
Classification			
Confirmed	137 (85.6)	284 (99.3)	421 (94.4)
Probable	23 (14.4)	2 (0.7)	25 (5.6)
Diagnosis method			
Biopsy/aspirate	126 (78.8)	283 (99.0)	409 (91.7)
Culture	13 (8.1)	23 (8.0)	36 (8.1)
Serology	100 (62.5)	0 (0.0)	100 (22.4)
Hospitalisation			
Admitted to hospital	135 (84.4)	1 (0.3)	136 (30.5)
Intrinsic risk factors			
All	50 (31.3)	18 (6.3)	68 (15.2)
Immunosuppressive treatment	25 (15.6)	13 (4.5)	38 (8.5)
HIV infection	16 (10.0)	2 (0.7)	18 (4.0)
Other immunosuppressive conditions	20 (12.5)	6 (2.1)	26 (5.8)
Alcoholism	13 (8.1)	3 (1.0)	16 (3.6)
Drug injection	1 (0.6)	1 (0.3)	2 (0.4)
Extrinsic risk factors[b]			
Contact with dogs	52 (32.5)	62 (21.7)	114 (25.6)
Contact with sick dogs	7 (4.4)	10 (3.5)	17 (3.8)
Presence of mosquitoes[c]	27 (16.9)	62 (21.7)	89 (20.0)
Waste and rubbish dumps	6 (3.8)	10 (3.5)	16 (3.6)
Walks near livestock farms	5 (3.1)	9 (3.1)	14 (3.1)
Travel history during the incubation period			
Travel to highly endemic areas	34 (21.3)	63 (22.0)	97 (21.7)

HIV: human immunodeficiency virus.

[a] Apart from the totals, the percentages shown use the number of visceral leishmaniasis cases or number of cutaneous leishmaniasis cases as appropriate.

[b] In domestic or peridomestic zones in the last year.

[c] Questions were oriented on the habitat and characteristics of sandflies. The word 'flebotomo' [sandfly] was not used, as it is not known by the general population.

born in sub-Saharan Africa was high and it should be noted that in the outbreak area, people of sub-Saharan origin represented less than 1% of the total population.

Most cases (n=421; 94.4%) were laboratory confirmed. *L.infantum* was identified as the causative agent. The remainder of the cases were probable.

Intrinsic risk factors that might decrease immunity were reported in 68 (15.2%) cases: 50 (31.3%) of cases of visceral leishmaniasis and 18 (6.3%) in the cutaneous leishmaniasis cases, with more than one immunosuppressive conditions or treatment occurring in the same patient.

Among the environmental risk factors analysed, it is noteworthy that 114 (25.6%) of cases had contact with dogs in one or more places in the domestic or peridomestic environment. A total of 56 (12.6%) cases had a dog in the home as a pet and all the animals were correctly protected against sandfly bites. A total of 17 (3.8%) cases reported having had contact with dogs that were apparently sick – without specifying the illness – which were subsequently checked to ensure that they were not affected by leishmaniasis.

Environmental research and control measures

After the increase in the number of leishmaniasis cases was detected in 2010, many different environmental actions were initiated, aimed at researching and controlling the vector and reservoir.

Monitoring of the vector

A sampling plan was developed in the epidemic area with the positioning, monitoring and analysis of both sticky and light traps for sandflies from May to October each year. In 2011, 37 stations were monitored with sticky traps (222 sampling sites) and 10,161 sandflies were studied. In 2012, 24 sampling stations (120 sampling sites) were monitored with sticky traps and 23,160 sandflies were studied, detecting a predominance of *P. perniciosus* (66.1%), the principal vector of *Leishmania* in the region. The mean density was very high, reaching 143.8 sandflies/m^2, with more than 17 sampling stations having levels above this figure (one was above 1,000 sandflies/m^2). Light traps were used in four stations, obtaining an average infection rate of 2.4% in the females collected.

The sandfly surveillance system implemented in the region of Madrid, which was intensified in the years following the start of the outbreak, showed an increase in the density of *P. perniciosus* in the epidemic area (16 sandflies/m^2 in 2008, 30 sandflies/m^2 in 2010 and 50 sandflies/m^2 in 2012) [11].

Monitoring of dogs, the main known reservoir

In 2011 and 2012, we collected information from clinical veterinarians in the epidemic area. They reported that they had not recorded any increase in the leishmaniasis detection tests performed in their clinics, where the prevalence of canine leishmaniasis was around 5%. In 2011, they performed leishmaniasis detection tests on 1,007 dogs during an anti-rabies vaccination campaign, using the rK39 blood test (BLK Fast Test, LETI), giving a prevalence of 1.0% in dogs that were household pets and 3.6% in dogs that were in dog pounds, results that were similar to those estimated in other studies performed in the region of Madrid [12,13]. Complementary analyses were also carried out on four serologically positive dogs: all four were positive by PCR for *Leishmania* and the species was analysed in 3 of them, identifying *L. infantum*.

Since 2012, these veterinarians have been piloting a sentinel system for notifying canine leishmaniasis cases. In 2012, representative sampling of 561 pet dogs during the anti-rabies vaccination campaign in the epidemic area revealed a *Leishmania* seroprevalence of 1.6%. Similarly, a sample of 502 dogs in potentially risky areas, such as dog pounds, hunting dog packs and livestock units, showed a prevalence of 2.0%.

Monitoring of other potential reservoirs, in view of the results obtained in dogs

Other potential reservoirs are being investigated, such as hares, rabbits, cats and rats. Results obtained to date indicate that 30% of the hares studied in 2011 and 2012 were infected with the parasite and in xenodiagnosis tests, evidence of the transmission of *L. infantum* from hares to sandflies has been obtained [14].

Environmental control measures

Risk areas in the epidemic towns have been identified, in which environmental sanitation steps are being carried out (removal of vegetation debris, cleaning of wasteland, removal of rubble, as well as the issuing of recommendations to individuals and companies). Burrows are being destroyed in areas where this is feasible due to the land layout. A disinsection plan has been established in risk areas, in which periodical treatments with biological insecticides and pyrethroids are carried out (in 2012, there were four treatments: every two weeks in June, one in September and one in October). In some areas where higher sandfly densities were found, intensive treatment was carried out for seven days (in September), followed by treatment once a week until the end of vector activity in October.

The collection of abandoned animals was stepped up: 406 dogs and 381 cats were collected in 2011 and 880 dogs and cats in 2012.
A control plan for the population of hares and rabbits in the environment has been set up, with around 1,000 hares having been caught to date using nets, greyhounds and falcons, and legislation has been passed for some areas in the epidemic area to declare them as temporary emergency game zones [15].

In addition to reinforcing surveillance, more information has been given to professionals from veterinary centres, dog owners and the general public. The

environmental actions have been carried out in coordination with the institutions involved (Departments of Health and the Environment, Town Councils in the area) and experts have given their advice (Instituto de Salud Carlos III Health Institute – WHO Collaborating Centre for Leishmaniasis, Veterinary Health Surveillance Centre, Veterinary Faculty and Biology Faculty of Complutense University in Madrid).

Discussion

Regular epidemiological surveillance allowed an outbreak of human leishmaniasis to be detected, which started in the second half of 2009. Up to December 2012, 446 cases were reported, representing over 80% of the cases reported in this period in the entire region of Madrid. To the best of our knowledge, this is the largest community outbreak described in Spain and in Europe. Furthermore, it occurred in an urban setting where the prevalence of leishmaniasis was previously very low, a very different case to other outbreaks described in the literature [16-22].

Under-reporting of cases becomes apparent when monitoring the disease [1,2,23], which is more noticeable in the cutaneous form. In Madrid, over the past decade of monitoring this disease, 90% of the reported cases were visceral [6], whereas in the current outbreak, they represented 36% of the cases. Visceral leishmaniasis is a serious disease that requires a specific diagnosis and treatment, normally with hospital admission, a factor that favours the notification of the disease to the surveillance network. Cutaneous leishmaniasis is a less serious disease, which can heal spontaneously, and where an aetiological diagnosis is not reached if the disease is not suspected and specific tests are not requested, such as PCR of the skin sample. Such cases are therefore generally under-represented in surveillance data. In this outbreak, given that the healthcare system in the south-west area of Madrid had been alerted, a thorough diagnosis was probably requested in patients with signs of cutaneous leishmaniasis.

The median time between the date of symptom onset and reporting to the Public Health Service was 41 days for cases of visceral leishmaniasis, as opposed to 183 days for cutaneous leishmaniasis cases. The delay arises from a number of factors that may be related to the patient (delay in seeking care) or the healthcare system (delay in diagnosis and reporting). The delay was greater for cases with cutaneous leishmaniasis due to the fact that patients take longer to request care and doctors take longer to consider the differential diagnosis of leishmaniasis and must wait for confirmation in order to be able to report the case [23].

Cases were found in all age groups. In those with visceral leishmaniasis, more men have been affected in almost all the age groups – the sex difference being particularly obvious in those over 30 years of age. In the cutaneous forms, distribution according to sex was similar. The clinical manifestations were typical for the disease (although it was remarkable that 11% of cases with visceral leishmaniasis had localised lymphadenopathic leishmaniasis as the sole clinical presentation) and the evolution was favourable after receiving the recommended treatment [2,4]. It is notable that 15 cases were infants under 1 year of age and 8 cases were aged between 12 and 23 months. It is also worth mentioning that 8% of the patients originated from sub-Saharan Africa, a percentage that rose to 20% for the visceral leishmaniasis cases.

During 2009 to 2012, there were four periods of sandfly's active life cycle, with most leishmaniasis cases occurring in the winter of 2010/11. The incubation period for the disease is variable [2]; it ranged from one week to several months and was generally longer in cases of visceral leishmaniasis, which may explain why these cases appeared more frequently during the cold months of the year. The epidemic curve allowed us to generate a hypothesis that favourable conditions for the transmission of *Leishmania* in the reservoir and/or vector began in the summer of 2009; it reached its peak in the summer of 2010 and continued in 2011. A gradual decrease in the number of cases was seen in 2012, following the introduction of control measures. Our hypothesis could be modified, depending on the evolution of the outbreak after 2012.

In most of the patients, there were no intrinsic risk factors that could alter their susceptibility to disease, although important differences were found according to the clinical form: 31% of visceral leishmaniasis cases and 6% of cutaneous leishmaniasis cases had intrinsic risk factors. In recent decades, leishmaniasis has been linked to decreased immunity and has been particularly associated with human immunodeficiency virus (HIV) infection [2-4,16]. In the outbreak described here, only 4% of all leishmaniasis cases were coinfected with HIV.

None of the cases had travelled during the incubation period to countries or areas that were highly endemic for the disease [1,2]: therefore, the infection cannot be considered imported.

In Spain, dogs are considered to be the main reservoir for *L. infantum* [1-5,11-13]; in this outbreak, only 26% of cases acknowledged contact with dogs in their domestic or peridomestic environment and the cases with dogs as pets in their homes had already applied suitable methods to protect against sandfly bites [3,24].
In order to evaluate the possible presence of vectors, we asked patients about their environment (house, neighbourhood, work, leisure pursuits and holidays). In a low percentage of cases, there were rubbish dumps, presence of mosquitoes, etc. in their peridomestic zones. We also asked patients about the areas where they walked, but no areas could be identified through which most people had passed. Therefore, our epidemiological research did not identify any of the classic environmental risk factors [2,3].

During 2011, many environmental control measures were started, aimed at monitoring and controlling the reservoir and vector: these have been intensified and optimised during 2012. Given the role that dogs classically play as the reservoir, actions initially concentrated on their study, but the surveillance system did not detect any increase in the prevalence of leishmaniasis in these animals, with level being around 5% [11-13].

Monitoring of the vector showed that *P. perniciosus* was present, a species that has been traditionally described in Spain and Madrid [25-27] and was found in high density in the epidemic area. An extension of the presence of this vector both in latitude and altitude has also been observed. Recent changes in the environment (large road-improvement works in some towns of the outbreak, warm autumns) [28,29] may have contributed to the high density.

As a high percentage of hares may be a source of infection for sandflies and may also be infected by them, these animals may be considered at least as secondary reservoirs for the infection. This would suggest the existence of a stable wild transmission cycle linked to the urban outskirts [14]. Although some of the urban parks in the areas around the four towns were recently created, there was traditionally a high rabbit and hare population in the land used for the parks. Town planning modifications over the past decade have probably modified the ecology of these Leporidae, moving from a woodland cycle to an urban one, encouraging their multiplication, as there are no predators such as birds of prey, wild boars, etc. This has also allowed their closeness to people, with whom they live alongside peacefully. The discovery of hares as reservoir has led to measures being taken aimed at controlling the hare and rabbit overpopulation [15].

Environmental aspects such as climate change, growing urbanisation, socio-economic development, etc. are causing changes in the epidemiology of infectious diseases [2,23,30,31]. Known environmental factors might have contributed to the genesis of this leishmaniasis outbreak, with the discovery of hares as secondary reservoirs being particularly significant. Epidemiological research and environmental intervention measures are continuing.

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References

1. World Health Organization (WHO). Leishmaniasis: epidemiology and access to medicines. An update based on the outcomes of WHO regional meetings, literature review and experts' opinion. Geneva: WHO. Available from: http://www.who.int/leishmaniasis/resources/leishmaniasis_epidemiology_access_to_medicine/en/
2. World Health Organization (WHO). Control of the leishmaniases: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22-26 March 2010. Geneva: WHO; 2010. WHO technical report series; no. 949. Available from: http://whqlibdoc.who.int/trs/WHO_TRS_949_eng.pdf
3. Ready PD. Leishmaniasis emergence in Europe. Euro Surveill. 2010;15(10):pii=19505. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19505 PMid:20403308
4. Marty P, Pomares C, Michel G, Delaunay P, Ferrua B, Rosenthal E. [Mediterranean visceral leishmaniasis]. Bull Acad Natl Med. 2011;195(1):181-8. French. PMid:22039711
5. Gálvez R, Descalzo MA, Miró G, Jiménez MI, Martín O, Dos Santos-Brandao F, et al. Seasonal trends and spatial relations between environmental/meteorological factors and leishmaniosis sand fly vector abundances in Central Spain. Acta Trop. 2010;115(1-2):95-102. http://dx.doi.org/10.1016/j.actatropica.2010.02.009 PMid:20171154
6. Morbilidad por enfermedades de declaración Obligatoria. Comunidad de Madrid. A-o 2010. [Report: Morbidity compulsory notifiable diseases. Community of Madrid. Year 2010]. Boletín epidemiológico de la Comunidad de Madrid. 2011;17(11). Spanish. Available from: http://www.madrid.org/cs/Satellite?blobcol=urldata&blobheader=application%2Fpdf&blobheadername1=Content-Disposition&blobheadervalue1=filename%3DNoviembre2011.pdf&blobkey=id&blobtable=MungoBlobs&blobwhere=1310910631705&ssbinary=true
7. Iriso A, Gonzalez-Mora D, Magro S, Soto MJ, Outerelo R, Sevillano O, et al. Surveillance of leishmaniasis in Madrid Region (Spain). International Conference of Emerging Vector-borne Diseases in a Changing European Environment. Montpellier, France. 10-12 May 2010. Available from: http://international-conference2010.eden-fp6project.net/var/eden_colloque/storage/fckeditor/file/Oral%20presentations_web.pdf
8. Brote comunitario de leishmaniasis en la zona suroeste de la Comunidad de Madrid. A-o 2011. [Report: Community outbreak of leishmaniasis in the southwest area of the Community of Madrid. Year 2011]. Boletín epidemiológico de la Comunidad de Madrid. 2011;17(12). Spanish. Available from: http://www.madrid.org/cs/Satellite?blobcol=urldata&blobheader=application%2Fpdf&blobheadername1=Content-Disposition&blobheadervalue1=filename%3DDiciembre2011.pdf&blobkey=id&blobtable=MungoBlobs&blobwhere=1310962031549&ssbinary=true
9. Manual de Notificación Sistema de Enfermedades de Declaración Obligatoria. Documentos Técnicos de Salud Pública no. 69. [*Notification system manual of notifiable diseases*. Public health technical papers, no. 69]. Madrid: Public Health Institute Community of Madrid; 2006. Available from: http://www.madrid.org/cs/Satellite?c=CM_Publicaciones_FA&cid=1142284809709&idConsejeria=1109266187266&idListConsj=1109265444710&idOrganismo=1142439320383&language=es&pagename=ComunidadMadrid%2FEstructura&sm=1109266101003
10. Poblaciones de referencia de la Comunidad de Madrid. Tablas. Instituto de Estadística de la Comunidad de Madrid. [Population statistics of the Community of Madrid. Basic demographic]. [Accessed 23 July 2013]. Available from: http://www.madrid.org/iestadis/fijas/estructu/demograficas/censos/ipob_ref_1.htm
11. Iriso A, Vázquez MA, Tello A, González D, Aranguez E, Soto MJ. Sistema de vigilancia de flebotomos (Diptera: Psychodidae) en la Comunidad de Madrid. [Surveillance System sandflies (Diptera: Psychodidae) in the Community of Madrid]. Rev. Salud ambient. 2013;13(Espec Congr):139. Spanish. Available from: http://ojs.easyapps.es/index.php/rsa/article/download/465/386
12. Gálvez R, Miró G, Descalzo MA, Nieto J, Dado D, Martín O, et al. Emerging trends in the seroprevalence of canine leishmanisis in the Madrid region (central Spain). Vet Parasitol. 2010;169(3-4):327-34. http://dx.doi.org/10.1016/j.vetpar.2009.11.025 PMid:20031330
13. Miró G, Montoya A, Mateo M, Alonso A, García S, García A, et al. A leishmaniasis surveillance system among stray dogs in the region of Madrid: ten years of serodiagnosis (1996-2006). Parasitol Res. 2007;101(2):253-7. http://dx.doi.org/10.1007/s00436-007-0497-8 PMid:17323100
14. Molina R, Jiménez MI, Cruz I, Iriso A, Martín-Martín I, Sevillano O, et al. The hare (Lepus granatensis) as potential sylvatic reservoir of Leishmania infantum in Spain. Vet Parasitol. 2012;190(1-2):268-71. http://dx.doi.org/10.1016/j.vetpar.2012.05.006 PMid:22677135
15. Resolución de 29 de marzo de 2012 de la Dirección General de Medio Ambiente, por la que se declara comarca de emergencia cinegética temporal los términos municipales de Alcorcón, Fuenlabrada, Getafe, Leganés y Móstoles (Boletin Oficial de la Comunidad de Madrid de 12 de abril de 2012). Spanish. Available from: http://www.madrid.org/wleg/servlet/Servidor?opcion=VerHtml&idnorma=8765&word=S&wordperfect=N&pdf=S
16. Gil-Prieto R, Walter S, Alvar J, de Miguel AG. Epidemiology of leishmaniasis in Spain based on hospitalizations records (1997-2008). Am J Trop Med Hyg. 2011;85(5):820-5. http://dx.doi.org/10.4269/ajtmh.2011.11-0310 PMid:22049034 PMCid:PMC3205626
17. Campino L, Maia C. [Epidemiology of leishmaniasis in Portugal]. Acta Med Port. 2010;23(5):859-64. Portuguese. PMid:21144327
18. Diza E, Kansouzidou A, Gerou S, Vezyri E, Metallidis S, Antoniadis A. Leishmaniases in Northern Greece: seroprevalence of the infection and incidence of the disease during the period 2001-2006. Eur J Clin Microbiol Infect Dis. 2008;27(10):997-1003. http://dx.doi.org/10.1007/s10096-008-0538-y PMid:18512088
19. Vinitsky O, Ore L, Habiballa H, Cohen-Dar M. Geographic and epidemiologic analysis of the cutaneous Leishmaniasis outbreak in northern Israel, 2000-2003. Isr Med Assoc J. 2010;12(11):652-6. PMid:21243862
20. Alvar J, Bashaye S, Argaw D, Cruz I, Aparicio P, Kassa A, et al. Kala-azar outbreak in Libo Kemkem, Ethiopia: epidemiologic and parasitologic assessment. Am J Trop Med Hyg. 2007;77(2):275-82. PMid:17690399
21. Mestre GL, Fontes CJ. [The spread of the visceral leishmaniasis epidemic in the State of Mato Grosso, 1998-2005]. Rev Soc Bras Med Trop. 2007;49(1):42-8. Portuguese. http://dx.doi.org/10.1590/S0037-86822007000100008
22. Werneck GL, Rodrigues L, Santos MV, Araújo IB, Moura LS, Lima SS, et al. The burden of Leishmania chagasi infection during an urban outbreak of visceral leishmaniasis in Brazil. Acta Trop. 2002;83(1):13-8. http://dx.doi.org/10.1016/S0001-706X(02)00058-X
23. Dujardin JC, Campino L, Ca-avate C, Dedet JP, Gradoni L, Soteriadou K, et al. Spread of vector-borne diseases and neglect of Leishmaniasis, Europe. Emerg Infect Dis. 2008;14(7):1013-8. http://dx.doi.org/10.3201/eid1407.071589 PMid:18598618 PMCid:PMC2600355
24. Palatnik-de-Sousa CB, Day MJ. One Health: the global challenge of epidemic and endemic leishmaniasis. Parasit Vectors. 2011;4:197. http://dx.doi.org/10.1186/1756-3305-4-197 PMid:21985335 PMCid:PMC3214158
25. Barón SD, Morillas Márquez F, Morales-Yuste M, Díaz-Sáez V, Irigaray C, Martín-Sánchez J. Risk maps for the presence and absence of Phlebotomus perniciosus in an endemic area of leishmaniasis in southern Spain: implications for the control of the disease. Parasitology. 2011;138(10):1234-44. http://dx.doi.org/10.1017/S0031182011000953 PMid:21854702
26. Gálvez, R, Descalzo, MA, Guerrero I, Miró G, Molina R. Mapping the current distribution and predicted spread of the leishmaniosis sand fly vector in the Madrid region (Spain) based on environmental variables and expected climate change. Vector Borne Zoonotic Dis. 2011;11(7):799-806. http://dx.doi.org/10.1089/vbz.2010.0109 PMid:21417927
27. Aransay AM, Ready PD, Morillas-Marquez F. Population differentiation of Phlebotomus perniciosus in Spain following postglacial dispersal. Heredity (Edinb). 2003:90(4)316-25. http://dx.doi.org/10.1038/sj.hdy.6800246 PMid:12692585
28. Inaugurada la nueva M-407 entre Fuenlabrada y Leganés. [Inauguration of the new M-407 between Fuenlabrada and Leganés]. EL PAÍS. 24 Mar 2011. Spanish. [Accessed 23 July 2013]. Available from: http://elpais.com/elpais/2011/03/24/actualidad/1300958250_850215.html
29. Bosque Sur. Wikipedia. [Accessed 23 July 2013]. Spanish. Available from: http://es.wikipedia.org/wiki/Bosque_Sur
30. Franco AO, Davies CR, Mylne A, Dedet JP, Gállego M, Ballart C, et al. Predicting the distribution of canine leishmaniasis in western Europe based on environmental variables. Parasitology. 2011;14:1-14.
31. Semenza JC, Menne B. Climate change and infections diseases in Europe. Lancet Infect Dis. 2009;9(6):365-75. http://dx.doi.org/10.1016/S1473-3099(09)70104-5

Imported leishmaniasis in the Netherlands from 2005 to 2012: epidemiology, diagnostic techniques and sequence-based species typing from 195 patients

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Leishmaniasis is an imported disease in the Netherlands. We report data for the period between 2005 and 2012, on clinical presentation, country where leishmaniasis was acquired, and causative species, for 195 civilian and military patients who had travelled abroad. Most patients were affected by cutaneous leishmaniasis (CL) (n=185 patients), while visceral leishmaniasis (VL) (n=8 patients) and mucocutaneous leishmaniasis (n=2 patients) were less frequently observed. All VL patients had been infected in Europe. CL was mainly acquired in Afghanistan, Surinam, Morocco and Spain. The majority of CL patients consisted of military personnel (55%, 102/185), 78 of whom had been infected during an outbreak in Afghanistan. Parasitological diagnosis was made by a combination of polymerase chain reaction (PCR), microscopy and culture. Compared to a standard of parasitological proof by any method other than the one under consideration, sensitivities of the individual methods ranged from 73% to 98%. Microscopy was least sensitive, but is fast and cheap. Mini-exon repeat PCR combines high sensitivity and specificity, and allows differentiation between species by sequencing of the PCR product. Eight different species or species complexes were identified, allowing species-specific therapy. Four patients proved infected with *Leishmania naiffi*, a hitherto rarely described cause of leishmaniasis. In comparison to previous decennia, an increase in cutaneous leishmaniasis was observed in our hospital, both in civilian and military patients who had travelled abroad. This calls for increased awareness among clinicians, availability of diagnostic tests and species-specific treatment guidelines in non-endemic countries.

Introduction

In non-endemic countries such as the Netherlands, leishmaniasis is an imported disease with increasing numbers of cases, probably due to increased travel to, migration from, and military operations in endemic regions [1-4]. Moreover, in Europe both visceral (VL) and cutaneous leishmaniasis (CL) have started a northward spread to new foci, including northern Italy, central Europe [5], and the Jura region in France [6], resulting in increasing areas where travellers can be exposed.

There are more than a dozen species of *Leishmania* parasites that can cause a wide spectrum of clinical manifestations, ranging from localised CL and disfiguring mucocutaneous leishmaniasis (MCL) to potentially lethal VL. These clinical manifestations depend on both pathogen and host genetic factors [7]. In the Netherlands, most cases of visceral leishmaniasis are acquired in the south of Europe [2,8]. In contrast, cutaneous leishmaniasis is acquired in Africa, Asia, Europe and the New World (the Americas) [4]. Travel history is often not sufficient for excluding certain species, as different species may coexist in geographical areas, and incubation times may vary widely. Also, patients may travel through several endemic areas with different species requiring different clinical management [9]. Therefore, species determination is of importance for prognosis and correct treatment.

Traditionally, diagnosis was based on microscopical examination of Giemsa stained smears, culture and histopathology of material from suspected leishmaniasis patients. Molecular methods have been introduced more recently, and are generally reported to be at least as sensitive as the combination of microscopy and culture [10]. Polymerase chain reaction (PCR)-based methods allow correct species discrimination by identification of the PCR amplicon by restriction fragment length polymorphism analysis [11] or sequencing [12].

Leishmaniasis is not a notifiable disease in the Netherlands, which hampers surveillance. The Academic Medical Center of the University of Amsterdam serves as a referral centre for leishmaniasis in our country. Therefore, our data may serve as an approximation for the leishmaniasis incidence in the Netherlands as a whole [4]. We here report the

changing epidemiology of imported leishmaniasis in 195 patients in the Netherlands in the period from 2005 to 2012. Moreover, we compared diagnostic techniques, and present the results of mini-exon repeat sequence typing of causative species.

Methods

Patients

A total of 195 patients for whom the parasitological diagnosis CL, MCL or VL was made at the Academic Medical Center in the period between June 2005 and December 2012 were included for this study. 180 patients were seen at the outpatient clinics of Dermatology or Tropical Medicine at the Academic Medical Center while 15 patients were seen in other hospitals. For the latter, data on travel were limited for this report. Demographic and clinical data of all 195 patients were aggregated in a database, including age, sex, areas visited, results of culture, impression smear, PCR and sequencing. Suspected country of acquisition

Table 1

Number of imported laboratory-confirmed leishmaniasis patients according to clinical presentation and suspected country of acquisition, Academic Medical Center, University of Amsterdam, the Netherlands, 2005–2012 (n=195)

Continent and country of acquisition	Clinical presentation Total patients[a] (military patients)		
	cutaneous	mucocutaneous	visceral
Europe	19 (0)	0 (0)	6 (0)
France	1 (0)	0 (0)	1 (0)
Italy	1 (0)	0 (0)	1 (0)
Malta	1 (0)	0 (0)	0 (0)
Portugal	1 (0)	0 (0)	0 (0)
Spain	13 (0)	0 (0)	2 (0)
Southern Europe[b]	2 (0)	0 (0)	2 (0)
Asia	98 (86)	0 (0)	0 (0)
Afghanistan	88 (86)	0 (0)	0 (0)
Iran	1 (0)	0 (0)	0 (0)
Iraq	1 (0)	0 (0)	0 (0)
Israel	3 (0)	0 (0)	0 (0)
Jordan	2 (0)	0 (0)	0 (0)
Pakistan	1 (0)	0 (0)	0 (0)
Saudi Arabia	1 (0)	0 (0)	0 (0)
Syria	1 (0)	0 (0)	0 (0)
Africa	17 (0)	0 (0)	0 (0)
Eritrea	1 (0)	0 (0)	0 (0)
Kenya	1 (0)	0 (0)	0 (0)
Morocco	15 (0)	0 (0)	0 (0)
The Americas	46 (16)	2 (0)	0 (0)
Belize	9 (9)	0 (0)	0 (0)
Bolivia	1 (0)	0 (0)	0 (0)
Brazil	4 (0)	0 (0)	0 (0)
Costa Rica	8 (0)	0 (0)	0 (0)
Peru	1 (0)	0 (0)	0 (0)
Suriname	17 (7)	1 (0)	0 (0)
Central and south America[b]	6 (0)	1 (0)	0 (0)
Multiple continents	2 (0)	0 (0)	1 (0)
East Africa/Mediterranean[b]	1 (0)	0 (0)	1 (0)
Mediterranean[b] (North Africa/Europe)	1 (0)	0 (0)	0 (0)
Not recorded	3 (0)	0 (0)	1 (0)
Total	**185 (0)**	**2 (0)**	**8 (0)**

[a] The total number of patients comprises the number of leishmaniasis patients who had travelled abroad as part of the military (which is given in parentheses) and the number of patients who had travelled abroad as civilians.

[b] These patients visited multiple countries where the causative species is endemic.

was based on travel history in combination with species typing.

Methods for confirming leishmaniasis species

Procedures for parasitological diagnosis by microscopy, culture, mini-exon repeat PCR based on the method of Marfurt et al. [13], or combinations thereof, were previously described [14]. For CL and MCL, two biopsies were taken from the edge of the lesion whereby one was used for culture, and the other for microscopy of a Giemsa stained smear and PCR. For VL, bone marrow was used for PCR, microscopy, or culture. Sequences for species determination were generated by amplification, as detailed earlier, with primer Rme2 as one of the two primers[13], followed by single strand sequencing with primer Rmeseq (5'-ACA GAA ACT GAT ACT TAT ATA GCG TTA GTT-3'). Sequence analysis and comparison was performed using the CodonCode software (CodonCode Corporation, Dedham, MA), using consensus sequences for different species as references. References were composed of previously published sequences [11,15] from GenBank, sequences derived from reference strains, and iteratively added patient sequences. Discrimination between the genotypically highly similar *Leishmania braziliensis* and *L. peruviana*, and between *L. infantum* and *L. donovani*, is not feasible for the mini-exon. The clinical relevance of such distinction is limited, as preferred treatment in the Netherlands is identical for both species, and only depends on the clinical presentation. Of note, discrimination between these species is impossible or difficult by other targets as well [16,17], and therefore their taxonomic status has been a continuing matter of debate [18]. We therefore refer to these species as *L. braziliensis/peruviana* and *L. donovani/infantum*, respectively. Other species that can be can be discriminated include *L. major, L. tropica, L. aethiopica, L. mexicana, L. amazonensis, L. guyanensis, L. panamensis, L. lainsoni,* and *L. naiffi*.

Sensitivity of diagnosis techniques

Sensitivity of different diagnostic techniques was calculated relative to parasitological evidence of leishmaniasis by at least one other method than the technique under consideration, assuming 100% specificity of culture, microscopy and PCR each. This is warranted, as stringent measures were used to avoid contamination in PCR [19], and typing by sequence analysis should reveal possible contamination for both PCR and culture.

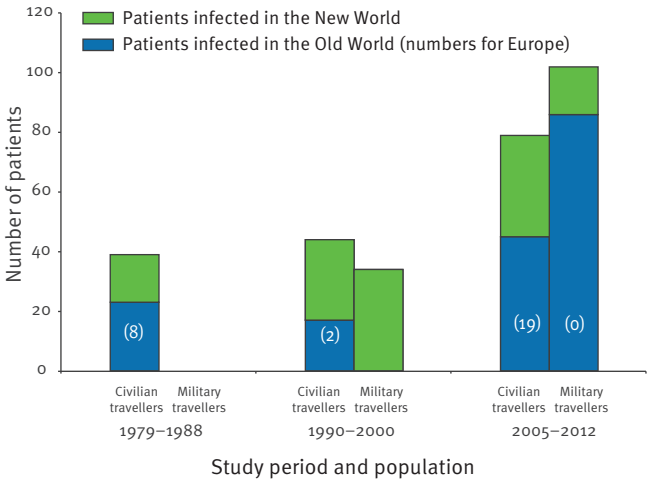
Results

Patients

From June 2005 to December 2012, leishmaniasis was diagnosed and laboratory confirmed in 195 patients. The endemic countries visited and clinical forms of leishmaniasis are listed in Table 1. The vast majority of patients (95%, 185/195) presented with CL. VL (n=8 patients) and MCL (n=2 patients) were only rarely encountered. Patients consisted of 102 military personnel who had travelled as part of their duties, and 93 civilian patients who had been travelling abroad (which comprise tourists, business travellers and travellers originating from an endemic country visiting family and friends). Median age for military patients, who all had CL, was 24 years (range: 19–50). Median age for civilian patients with MCL was 46 years (range: 2–78), and for travellers with VL 55 years (range: 2–62). The male to female ratio in the total 93 civilian patients was 1.53:1.

Figure 1

Distribution over three time periods of imported laboratory-confirmed cutaneous leishmaniasis patients, according to military or civilian status, and geographical area of infection, Academic Medical Center, University of Amsterdam, the Netherlands, 1999–2012 (n=302)



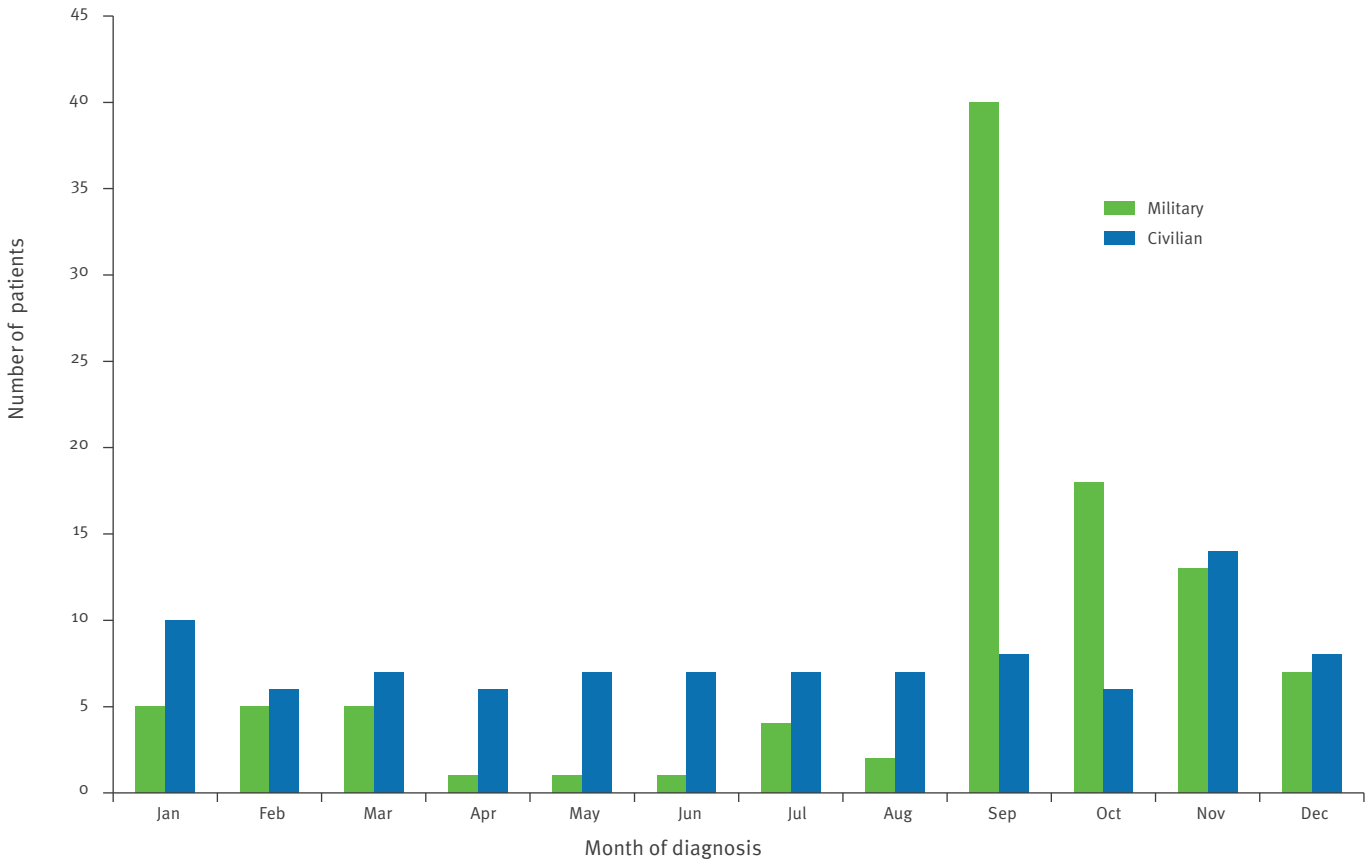
New World refers to the Americas. Old World comprises Africa, Asia and Europe.

A previous study described an increasing incidence of imported CL in our population in the period from 1990 to 2000 (78 cases) as compared to between 1979 and 1989 (39 cases) [4]. For the current, shorter, study period between 2005 and 2012, the number of detected CL patients was 185 (Figure 1), including 78 military personnel, who had acquired CL in an outbreak in north Afghanistan [20-22]. Even if the latter are not considered, the 107 remaining patients in the current study represent more patients than in previous periods.

Between 1990 and 2000, most patients acquired CL in the New World (78%, 61/78). In the present study, more than twice as many patients acquired CL in the Old World (Europe, Asia and Africa) (n=136) as in the New World (n=46). The number of patients who acquired CL in the Old World was still higher (n=58) when the military patients who had been deployed to north Afghanistan (n=78) were put aside [20-22].

Figure 2

Distribution of laboratory-confirmed leishmaniasis imported patients, according to military and civilian status, by month of diagnosis, Academic Medical Center, University of Amsterdam, the Netherlands, 2005–2012 (n=195)



More than half of the total CL patients (102/185) in the present study were military personnel. This constitutes an increase in military personnel with CL compared to the previous study periods (with a total of 34 cases in 1990–2000 and none in 1979–1988). Most military patients got infected in the Old World in Afghanistan (n=86), whereas infections in the New World were acquired in Belize (n=9) and Suriname (n=7). An increase in imported infected civilian CL patients (n=83), which comprise tourists and business travellers as well as those originating from an endemic country visiting family and friends, was also observed compared to previous studies (44 in 1990–2000 and 39 in 1979–1988), as shown in Figure 1. Most civilian patients who were infected in the New World acquired CL in Suriname (10 of 30), but by different species than the military patients. Most civilian patients infected in the Old World contracted CL in various countries in Europe (19 of 48), as was also the case for VL (Table 1).

As shown in Figure 2, the distribution of patients per month is different for military patients when compared to civilian patients. Military patients usually present as groups after duty abroad. In contrast, imported civilian patients present throughout the year with only a relatively small increase towards the end of the year.

Diagnostic methods for cutaneous leishmaniasis and sequence-based typing

The sensitivity of PCR, microscopy and culture, and combinations thereof for diagnosis of CL (including MCL), are listed in Table 2.

Sequencing of the mini-exon repeat PCR product obtained from either direct biopsy material or from cultured parasites, allowed identification of the causative species in patients affected with VL, CL or MCL by comparison to consensus sequences. Altogether the species responsible for the disease was identified in 186 of the 195 patients.

Leishmania species distribution according to geographical region of acquisition

Eight different species or species groups were detected, three in the Old World and six in the New World (Figure 3).

In patients infected in the Old World, *L. major, L. tropica* and L. *donovani/infantum* were detected. The high number of L. major patients was predominantly found among the Dutch soldiers deployed to Afghanistan. Patients infected in Europe were exclusively infected with *L. infantum/donovani*. In the New World, *L.*

Table 2

Sensitivity of different (combinations) methods for diagnosis of (muco)cutaneous leishmaniasis, Academic Medical Center, University of Amsterdam, the Netherlands, 2005–2012 (n=187)

Diagnostic methods	Positive	Negative	ND[a]	Sensitivity
PCR[b]	183	4	0	98%
Microscopy	127	47	13	73%
Culture	138	29	20	83%
Microscopy and/or culture[c]	151	15	21	91%
Microscopy and/or PCR[c]	172	2	13	99%
Culture and/or PCR[c]	164	2	21	99%
Microscopy, culture and/or PCR[c]	166	0	21	100%

ND: not determined; PCR: polymerase chain reaction.

[a] These samples represent either requests from other hospitals or patients for which no biopsy was taken for culture due to the small size of the lesion.

[b] Mini-exon repeat PCR based on the method of Marfurt et al [13].

[c] Patients for whom not all methods were performed were included in the group labelled as ND.

guyanensis was most prevalent (Figure 3), and mainly found among patients that visited Suriname (Table 1).

Leishmania species distribution among civilian and military travellers

The species distribution differed markedly between military and civilian patients, with *L. tropica*, *L. panamensis* and *L. donovani/infantum* exclusively found in civilian patients, and *L. naiffi* only in military patients (Table 3). This difference reflects the endemic countries visited, as military patients acquired leishmaniasis in Afghanistan (n=86), Belize (n=9) and Suriname (n=7). For the latter country, infection with *L. naiffi* has been related to different epidemiological circumstances during military manoeuvres [14].

Discussion

Visceral and cutaneous leishmaniases are imported diseases in the Netherlands. The cases of VL are mostly imported from countries in southern Europe, as confirmed in our study where all VL patients were civilians who had been infected there. Also among the 83 civilian CL patients, 19 (23%) of the CL infections were acquired in Europe, with 13 in Spain. This is noteworthy, as misdiagnosis, due to the misconception that leishmaniasis is a tropical disease, has occurred in the Netherlands for cases acquired in southern Europe [8]. CL for civilians and military combined was mainly acquired in Afghanistan, Suriname, Morocco and Spain (Table 1). Our data show an increase in patients diagnosed with CL in our hospital between 2005 and 2012 compared to the periods from 1979 to 1988 and 1990 to 2000 [4] (Figure 1). An analysis of nationwide pathological records from 1996 to 2007 found an increase during that period as well [2]. Comparison of the pathological data available only until 2007 [2] to our data shows that our patient population represented 56% (39/70) of the CL cases in 2006 and 45% (14/31) in 2007 in the

Figure 3

Distribution of *Leishmania* species derived from leishmaniasis patients according to geographical region of infection, Academic Medical Center, University of Amsterdam, the Netherlands, 2005–2012 (n=183)

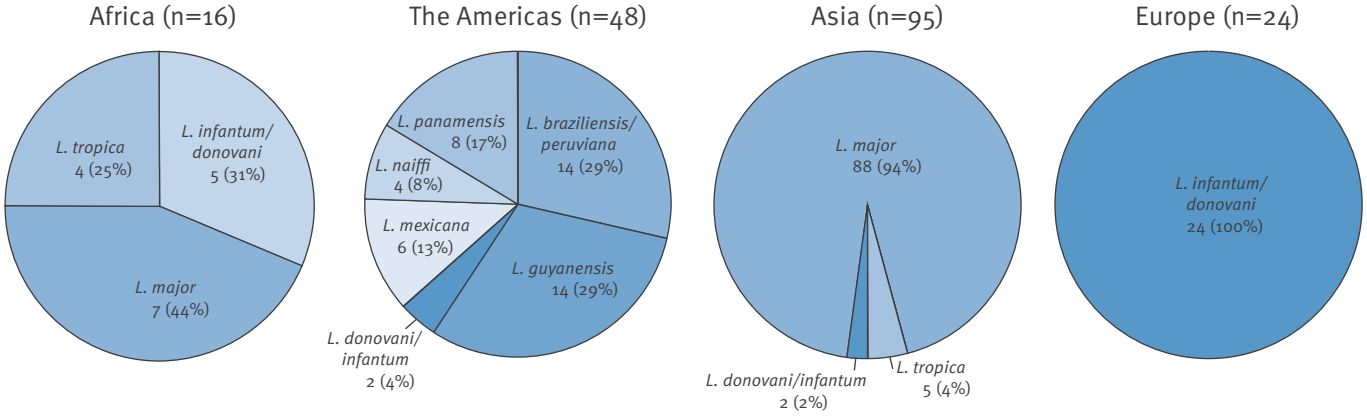


Table 3

Causative *Leishmania* species identified in military and civilian populations, Academic Medical Center, University of Amsterdam, the Netherlands, 2005–2012 (n=195)

Species	(Muco)cutaneous		Visceral	Total
	military	civilian	civilian	
L. braziliensis/peruviana	5	9[a]	0	**14**
L. donovani/infantum	0	29	8	**37**
L. guyanensis	3	11[a]	0	**14**
L. major	77[b]	14	0	**91**
L. mexicana	4	2	0	**6**
L. naiffi	4	0	0	**4**
L. panamensis	0	8	0	**8**
L. tropica	0	12	0	**12**
ND[c]	9	0	0	**9**
Total	**102**	**85**	**8**	**195**

[a] Of these patients, one patient presented with mucocutaneous disease due to *L. braziliensis* and one due to *L. guyanensis*.

[b] These patients belonged to troops deployed to north Afghanistan, and were part of a larger outbreak described elsewhere [20-22].

[c] Of these patients, two were positive by microscopy only. Seven patients obtained prior treatment; polymerase chain reaction was weakly positive but yielded insufficient product for sequence analysis in these seven patients.

Netherlands. Therefore, our observations with respect to epidemiology and causative species are probably valid for most patients in the Netherlands.

Part of the increase in cases is due to increased exposure, due to larger numbers of military personnel sent to endemic countries. An increase in imported leishmaniasis is a common problem in non-endemic countries that send troops abroad, both for training and active duty [23-25]. Military patients usually present as groups after duty abroad, and awareness in a unit is high after initial cases are identified. As a result, diagnosis of leishmaniasis patients among military are more clustered in time (Figure 2).

The number of infected civilian travellers increased also as compared to previous years [4] (Figure 1), and patients presented throughout the year (Figure 2). This more evenly spread distribution probably reflects a combination of travel throughout the year, variation in incubation times, health seeking behaviour and variation in delay before referral for diagnosis. Only a relatively small increase towards the end of the year was noted, which is probably the consequence of increased travel during summer.

Apart from increased exposure and possible changes in health seeking behaviour for CL in immigrant communities [26], improved diagnostic methods, and awareness among clinicians may also have contributed to the increased number of leishmaniasis patients detected. During the study period, PCR was a routine diagnostic procedure for leishmaniasis, in contrast to the previous study periods [4]. In the preceding years, both specificity and sensitivity have benefited from improved measures to avoid contamination of PCR [19] and higher quality of reagents and equipment for PCR and DNA extraction. In the present study, sensitivity of PCR was higher (98%, Table 2) as compared to previous years (89%) [4]. Though PCR alone has a high sensitivity, both microscopy and culture have added value (Table 2). Apart from increasing overall sensitivity, microscopy can be used as point of care test, and results are available within one hour at low cost. Culture allows expansion of strain collections for research purposes, e.g. for quality control programmes and comparison of different typing methods as advocated by the LeishMan consortium [27].

Follow-up of CL is based on clinical evaluation. Only if therapy failure is suspected, are laboratory diagnostics performed. Whole parasites as demonstrated by microscopy, culture, or the detection of *Leishmania* RNA [28] are considered a sign of relapse. Detection of parasite DNA by PCR is no definitive proof of relapse, since this can also be present in scars of successfully treated patients [29,30].

For accurate treatment and precise prognosis of CL, characterisation of the causative *Leishmania* species is often needed, e.g. pentamidine is effective for treatment of *L. guyanensis* but less efficient against disease caused by *L. braziliensis* [4]. This was accomplished by sequence analysis in the vast majority (95.1%) of our patients, detecting eight different species or species groups (Figure 3). The species distribution was different between military and civilan patients (Table 3), probably as a result of different endemic countries

visited and different epidemiological circumstances encountered [14].

The relevance of typing is best illustrated in our population for the CL and MCL patients that were infected in Suriname (Table 1). Because of its historic ties with the Netherlands, Suriname is a popular destination for Dutch tourists, persons visiting friends and relatives, and has been used for jungle training by the Dutch military. Traditionally, *L. guyanensis* was regarded as the causative species of CL from Suriname. Recently, *L. amazonensis*, *L. lainsoni*, *L. naiffi*, and *L. braziliensis* have been reported for the first time in Suriname as well [31,32]. Awareness that any of these species can be present in patients returning from Suriname is important, as the differences between these species influence clinical management.

In conclusion, the number of imported leishmaniasis patients in our hospital, and probably the Netherlands as a whole, continues to increase. This increase affects both civilian and military patients. Although most patients in this study were infected with CL, however it is noteworthy that all patients with VL had acquired their infection in European endemic countries. CL was also acquired in Europe for approximately 20% of civilian patients. Among all imported cases, eight different *Leishmania* species or species groups were identified. Improved diagnostic procedures, including sequence-based typing, allow species-specific treatment.

References

1. Antinori S, Gianelli E, Calattini S, Longhi E, Gramiccia M, Corbellino M. Cutaneous leishmaniasis: an increasing threat for travellers. Clin Microbiol Infect. 2005;11(5):343-6. http://dx.doi.org/10.1111/j.1469-0691.2004.01046.x PMid:15819858
2. Herremans T, Pinelli E, Casparie M, Nozari N, Roelfsema J, Kortbeek L. Increase of imported leishmaniasis in the Netherlands: a twelve year overview (1996-2007). Int Health. 2010;2(1):42-6. http://dx.doi.org/10.1016/j.inhe.2009.12.005
3. Schwartz E, Hatz C, Blum J. New world cutaneous leishmaniasis in travellers. Lancet Infect.Dis. 2006;6:342-349 http://dx.doi.org/10.1016/S1473-3099(06)70492-3
4. Zeegelaar JE, Steketee WH, van Thiel PP, Wetsteyn JC, Kager PA, Faber WR. Changing pattern of imported cutaneous leishmaniasis in the Netherlands. Clin.Exp.Dermatol. 2005; 30(1):1-5. http://dx.doi.org/10.1111/j.1365-2230.2004.01677.x PMid:15663490
5. Aspock H, Gerersdorfer T, Formayer H, Walochnik J. Sandflies and sandfly-borne infections of humans in Central Europe in the light of climate change. Wien.Klin.Wochenschr. 2008;120(19-20 Suppl 4):24-9.
6. Faber WR, Hoekzema R, Bart A, Zeegelaar JE, de Vries HJ. Cutaneous leishmaniasis acquired in Jura, France. Emerg Infect Dis. 2012;18(1):183-4. http://dx.doi.org/10.3201/eid1801.110408 PMid:22257720 PMCid:PMC3310094
7. Schriefer A, Wilson ME, Carvalho EM. Recent developments leading toward a paradigm switch in the diagnostic and therapeutic approach to human leishmaniasis. Curr Opin Infect Dis. 2008;21(5):483-8. http://dx.doi.org/10.1097/QCO.0b013e32830d0ee8 PMid:18725797 PMCid:PMC2593452
8. van Vliet MJ, Veeken H, Hart W, Tamminga RY. [Clinical reasoning and decision-making in practice. A young boy with fever, pancytopenia and an enlarged spleen]. Ned Tijdschr Geneeskd. 2006;150(30):1662-8. Dutch. PMid:16922351
9. Blum J, Desjeux P, Schwartz E, Beck B, Hatz C. Treatment of cutaneous leishmaniasis among travellers. J Antimicrob Chemother. 2004;53(2):158-66. http://dx.doi.org/10.1093/jac/dkh058 PMid:14729756
10. Faber WR, Oskam L, van GT, Kroon NC, Knegt-Junk KJ, Hofwegen H, et al. Value of diagnostic techniques for cutaneous leishmaniasis. J Am Acad Dermatol. 2003;49(1):70-4. http://dx.doi.org/10.1067/mjd.2003.492 PMid:12833011
11. Marfurt J, Niederwieser I, Makia ND, Beck HP, Felger I. Diagnostic genotyping of Old and New World Leishmania species by PCR-RFLP. Diagn Microbiol Infect Dis. 2003; 46(2):115-24. http://dx.doi.org/10.1016/S0732-8893(03)00040-3
12. Requena JM, Chicharro C, Garcia L, Parrado R, Puerta CJ, Canavate C. Sequence analysis of the 3'-untranslated region of HSP70 (type I) genes in the genus Leishmania: its usefulness as a molecular marker for species identification. Parasit Vectors. 2012;5:87. http://dx.doi.org/10.1186/1756-3305-5-87 PMid:22541251 PMCid:PMC3425316
13. Marfurt J, Nasereddin A, Niederwieser I, Jaffe CL, Beck HP, Felger I. Identification and differentiation of Leishmania species in clinical samples by PCR amplification of the miniexon sequence and subsequent restriction fragment length polymorphism analysis. J Clin Microbiol. 2003;41(7):3147-53. http://dx.doi.org/10.1128/JCM.41.7.3147-3153.2003 PMid:12843055 PMCid:PMC165364
14. van Thiel PP, Gool Tv, Kager PA, Bart A. First cases of cutaneous leishmaniasis caused by Leishmania (Viannia) *naiffi* infection in Surinam. Am J Trop Med Hyg. 2010;82(4):588-90 http://dx.doi.org/10.4269/ajtmh.2010.09-0360 PMid:20348504 PMCid:PMC2844559
15. Fernandes O, Murthy VK, Kurath U, Degrave WM, Campbell DA. Mini-exon gene variation in human pathogenic Leishmania species. Mol Biochem Parasitol. 1994;66(2):261-71. http://dx.doi.org/10.1016/0166-6851(94)90153-8
16. Lukes J, Mauricio IL, Schonian G, Dujardin JC, Soteriadou K, Dedet JP, et al. Evolutionary and geographical history of the Leishmania donovani complex with a revision of current taxonomy. Proc Natl Acad Sci U S A. 2007;104(22):9375-80. http://dx.doi.org/10.1073/pnas.0703678104 PMid:17517634 PMCid:PMC1890502
17. Fraga J, Montalvo AM, De Doncker S, Dujardin JC, Van der Auwera G. Phylogeny of Leishmania species based on the heat-shock protein 70 gene. Infect Genet Evol. 2010;10(2):238-45. http://dx.doi.org/10.1016/j.meegid.2009.11.007 PMid:19913110
18. Banuls AL, Hide M, Prugnolle F. Leishmania and the leishmaniases: a parasite genetic update and advances in taxonomy, epidemiology and pathogenicity in humans. Adv Parasitol. 2007;64:1-109. http://dx.doi.org/10.1016/S0065-308X(06)64001-3
19. Aslanzadeh J. Preventing PCR amplification carryover contamination in a clinical laboratory. Ann Clin Lab Sci. 2004;34(4):389-96. PMid:15648778
20. van Thiel PP, van Gool T, Faber WR, Leenstra T, Kager PA, Bart A. Variation in clinical presentation and genotype of causative Leishmania major strain in cutaneous leishmaniasis in north and south Afghanistan. Am J Trop Med Hyg. 2011;85(1):60-3. http://dx.doi.org/10.4269/ajtmh.2011.10-0531 PMid:21734125 PMCid:PMC3122344
21. van Thiel PP, Leenstra T, de Vries HJ, van der Sluis A, van GT, Krull AC, et al. Cutaneous leishmaniasis (Leishmania major infection) in Dutch troops deployed in northern Afghanistan: epidemiology, clinical aspects, and treatment. Am J Trop Med Hyg. 2010;83(6):1295-300. http://dx.doi.org/10.4269/ajtmh.2010.10-0143 PMid:21118937 PMCid:PMC2990047
22. van Thiel PP, Leenstra T, Kager PA, de Vries HJ, van VM, van der Meide WF, et al. Miltefosine treatment of Leishmania major infection: an observational study involving Dutch military personnel returning from northern Afghanistan. Clin Infect Dis. 2010;50(1):80-3. http://dx.doi.org/10.1086/648726 PMid:19951107
23. Bailey MS. Cutaneous leishmaniasis in British troops following jungle training in Belize. Travel Med Infect Dis. 2011;9(5):253-4. http://dx.doi.org/10.1016/j.tmaid.2011.07.001 PMid:21856236
24. Berger F, Romary P, Brachet D, Rapp C, Imbert P, Garrabe E, et al. [Outbreak of cutaneous leishmaniasis in military population coming back from French Guiana]. Rev Epidemiol Sante

Publique. 2006;54(3):213-21. French.
http://dx.doi.org/10.1016/S0398-7620(06)76717-7

25. Elston DM, Miller SD. Leishmaniasis acquired in the Iraqi Theater of Operations: lessons learned. Cutis. 2004;74(4):253-5.
PMid:15551719

26. Mohammedamin RS, van der Wouden JC, Koning S, Schellevis FG, van Suijlekom-Smit LW, Koes BW. Reported incidence and treatment of dermatophytosis in children in general practice: a comparison between 1987 and 2001. Mycopathologia. 2007;164(6):271-8.
http://dx.doi.org/10.1007/s11046-007-9062-3
PMid:17891509 PMCid:PMC2780650

27. European Network for Tropical Medicine and Travel Health (TropNet). LeishMan - Surveillance of imported leishmaniasis with regard to diagnostic and therapeutic procedures. Basel: TropNet. [Accessed: 2012]. Available from: http://www.tropnet.net/index.php?id=103

28. van der Meide WF, Schoone GJ, Faber WR, Zeegelaar JE, de Vries HJ, Ozbel Y, et al. Quantitative nucleic acid sequence-based assay as a new molecular tool for detection and quantification of Leishmania parasites in skin biopsy samples. J Clin Microbiol. 2005;43(11):5560-6.
http://dx.doi.org/10.1128/JCM.43.11.5560-5566.2005
PMid:16272487 PMCid:PMC1287793

29. Mendonca MG, de Brito ME, Rodrigues EH, Bandeira V, Jardim ML, Abath FG. Persistence of leishmania parasites in scars after clinical cure of American cutaneous leishmaniasis: is there a sterile cure? J Infect Dis. 2004;189(6):1018-23.
http://dx.doi.org/10.1086/382135
PMid:14999605

30. Schubach A, Haddad F, Oliveira-Neto MP, Degrave W, Pirmez C, Grimaldi G Jr, et al. Detection of Leishmania DNA by polymerase chain reaction in scars of treated human patients. J Infect Dis. 1998;178(3):911-4.
http://dx.doi.org/10.1086/515355
PMid:9728572

31. Hu RV, Kent AD, Adams ER, van der Veer C, Sabajo LO, Mans DR, et al. First case of cutaneous leishmaniasis caused by Leishmania (Viannia) braziliensis in Suriname. Am J Trop Med Hyg. 2012;86(5):825-7.
http://dx.doi.org/10.4269/ajtmh.2012.11-0728
PMid:22556081 PMCid:PMC3335687

32. van der Meide WF, Jensema AJ, Akrum RA, Sabajo LO, Lai A Fat RF, Lambregts L. Epidemiology of cutaneous leishmaniasis in Suriname: a study performed in 2006. Am J Trop Med Hyg. 2008;79(2):192-7.
PMid:18689623

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 underestimates, 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 been 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 1

Distribution 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	*haspb (k26)* 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	*haspb (k26)* 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.

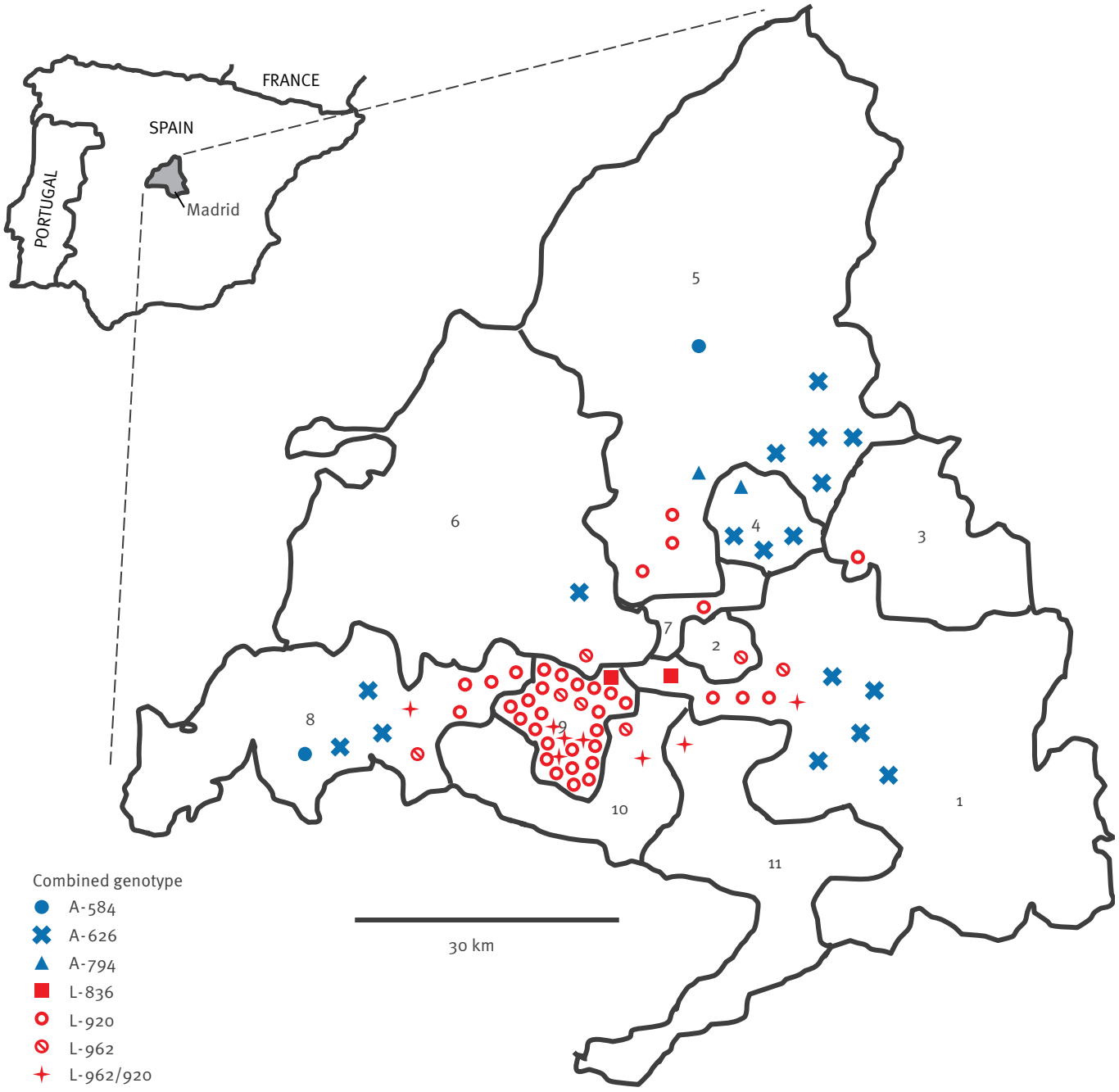
[a] Isolates ordered by year of isolation and Health Area.

[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 leishmaniases 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 of10 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)

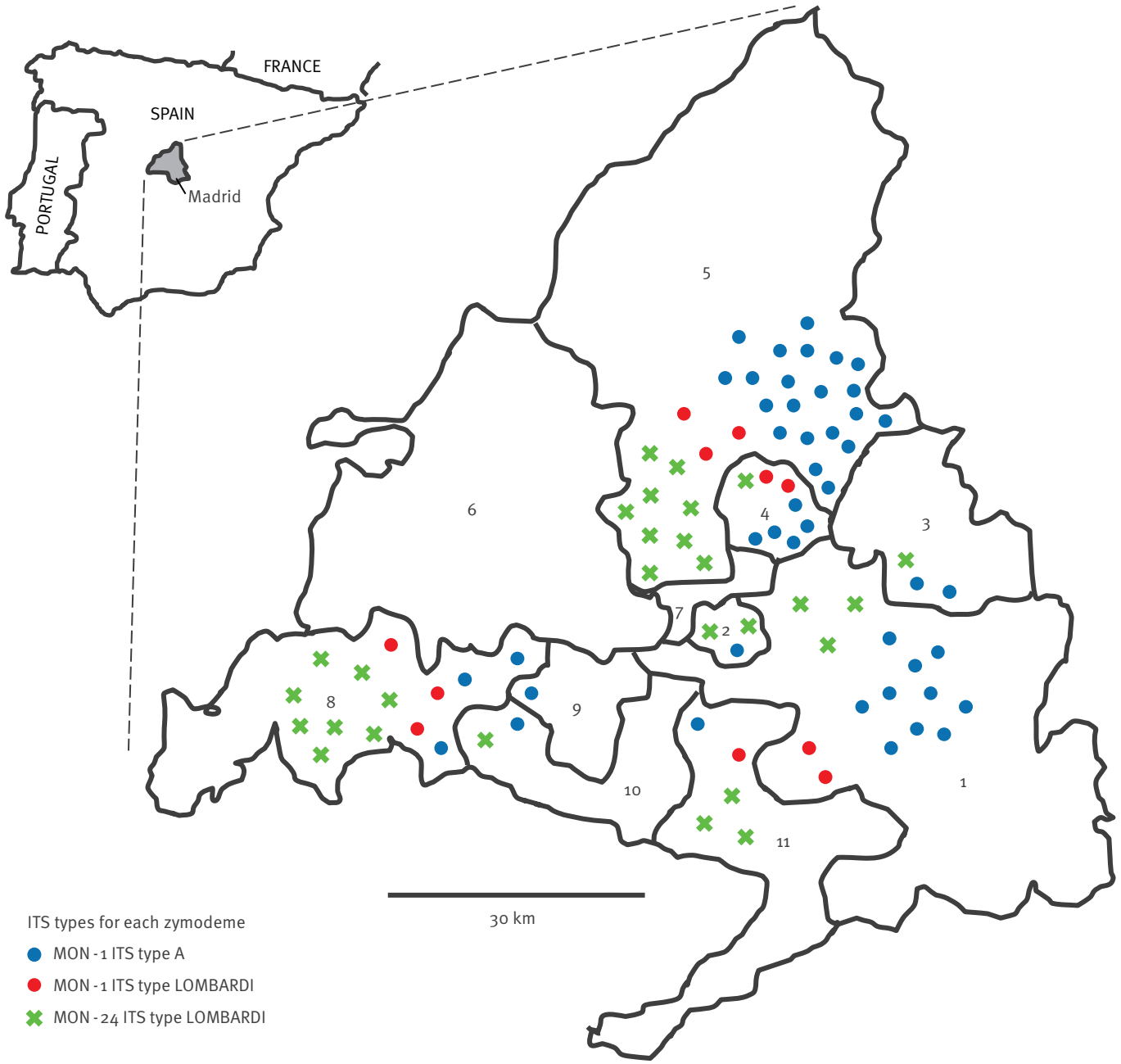
Isolate WHO code[a]	Year of isolation	Health Area of isolate origin	Clinical form of leishmaniasis	HIV	Age group in years	Zymodeme	ITS type
MHOM/ES/2003/LLM-1327	2003	5	VL	NEG	‹5	MON-1	A
MHOM/ES/2003/LLM-1258	2003	8	VL	NEG	›18	MON-1	LOMBARDI
MHOM/ES/2004/LLM-1372	2004	1	VL	POS	›18	MON-1	A
MHOM/ES/2004/LLM-1377	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

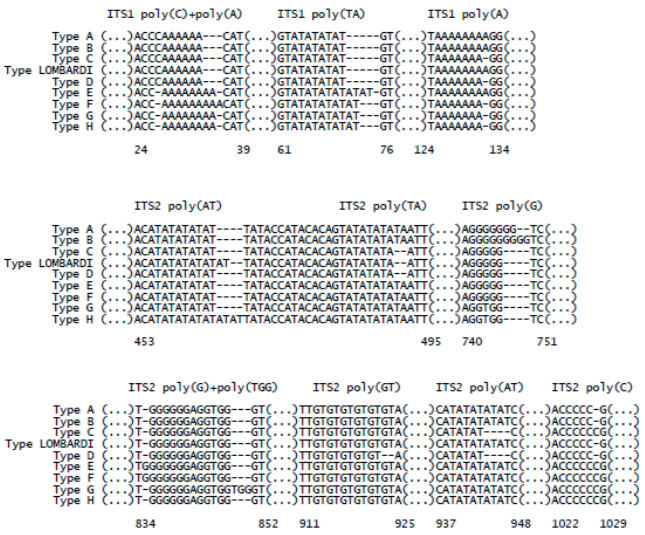
Distribution and ITS type of analysed *Leishmania infantum* isolates from Madrid, Spain, 1988–2005 (n=83)



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 being 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.

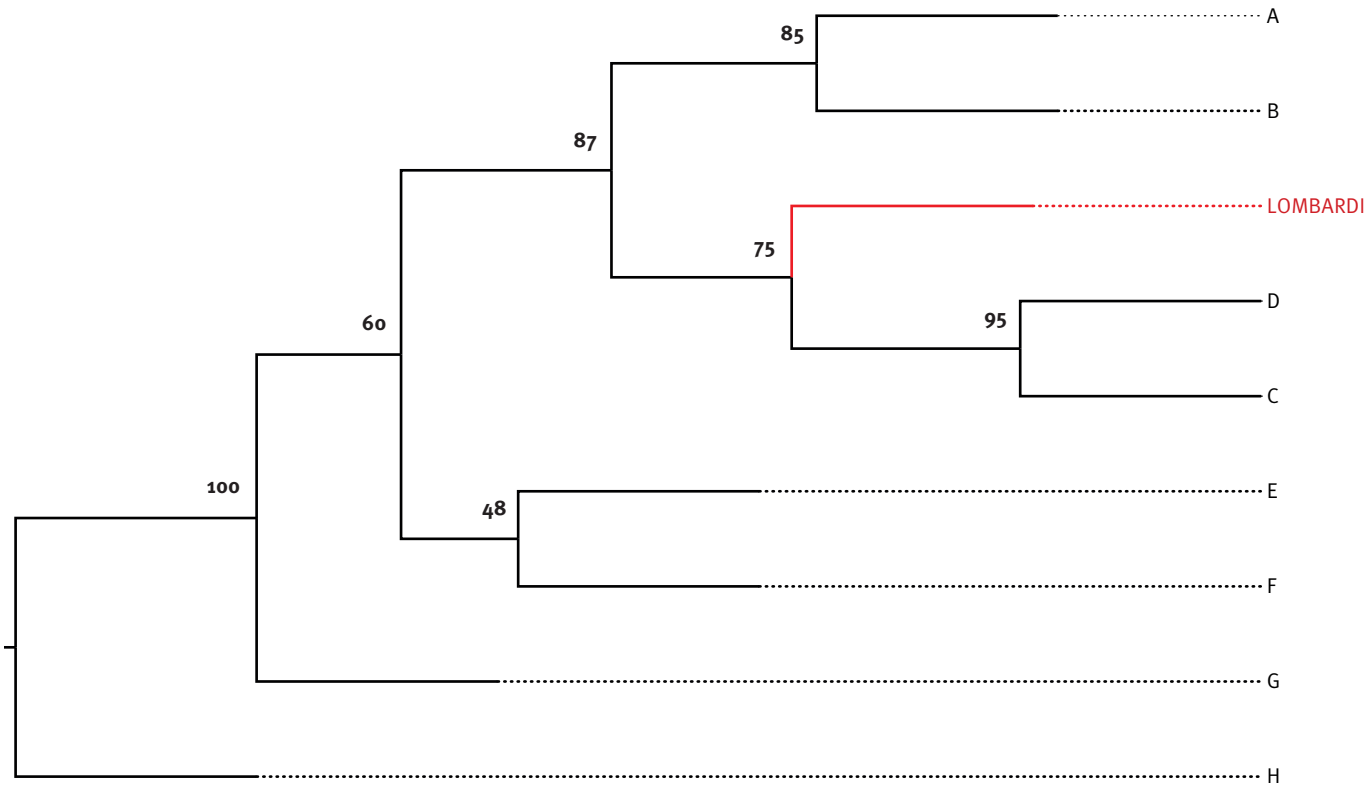
Our phylogenetic analysis included ITS-LOMBARDI in a cluster – supported by a bootstrap value of 87% – with ITS types A, B, C and D. However, it is further separated from types A and B in a subsequent subcluster (Figure 4).

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 leishamaniasis (cutaneous or visceral). Of the three cases with localised lymphadenopathy (LL), two were caused by the combined genotype L-920

Figure 4

Phylogenetic 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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References

1. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS One. 2012;7(5):e35671. http://dx.doi.org/10.1371/journal.pone.0035671 PMid:22693548 PMCid:PMC3365071
2. Chitimia L, Mu-oz-García CI, Sánchez-Velasco D, Lizana V, Del Río L, Murcia L, et al. Cryptic Leishmaniosis by Leishmania infantum, a feature of canines only? A study of natural infection in wild rabbits, humans and dogs in southeastern Spain. Vet Parasitol. 2011;181(1):12-6. http://dx.doi.org/10.1016/j.vetpar.2011.04.016 PMid:21592669
3. Ramírez GA, Pe-afiel-Verdú C, Altimira J, García-González B, Vilafranca M. Naturally acquired visceral leishmaniosis in a captive Bennett's wallaby (Macropus rufogriseus rufogriseus). Vet Pathol. 2013;50(1):188-90. http://dx.doi.org/10.1177/0300985812446155 PMid:22692623
4. Sobrino R, Ferroglio E, Oleaga A, Romano A, Millan J, Revilla M, et al. Characterization of widespread canine leishmaniasis among wild carnivores from Spain. Vet Parasitol. 2008;155(3-4):198-203. http://dx.doi.org/10.1016/j.vetpar.2008.05.009 http://dx.doi.org/10.1016/j.vetpar.2008.05.003 PMid:18579311
5. Molina R, Jiménez MI, Cruz I, Iriso A, Martín-Martín I, Sevillano O, et al. The hare (Lepus granatensis) as potential sylvatic reservoir of Leishmania infantum in Spain. Vet Parasitol. 2012;190(1-2):268-71. http://dx.doi.org/10.1016/j.vetpar.2012.05.006 PMid:22677135
6. de la Loma A, Alvar J, Martinez Galiano E, Blázquez J, Alcalá Mu-oz A, Nájera R. Leishmaniasis or AIDS? Trans R Soc Trop Med Hyg. 1985;79(3):421-2. http://dx.doi.org/10.1016/0035-9203(85)90400-6
7. Desjeux P, Alvar J. Leishmania/HIV co-infections: epidemiology in Europe. Ann Trop Med Parasitol. 2003;97 Suppl 1:3-15. http://dx.doi.org/10.1179/000349803225002499 PMid:14678629
8. Alvar J, Aparicio P, Aseffa A, Den Boer M, Ca-avate C, Dedet JP, et al. The relationship between leishmaniasis and AIDS: the second 10 years. Clin Microbiol Rev. 2008;21(2):334-59. http://dx.doi.org/10.1128/CMR.00061-07 PMid:18400800 PMCid:PMC2292576
9. Dujardin JC, Campino L, Ca-avate C, Dedet JP, Gradoni L, Soteriadou K, et al. Spread of vector-borne diseases and neglect of Leishmaniasis, Europe. Emerg Infect Dis. 2008;14(7):1013-8. http://dx.doi.org/10.3201/eid1407.071589 PMid:18598618 PMCid:PMC2600355
10. Ready PD. Leishmaniasis emergence in Europe. Euro Surveill. 2010;15(10):pii=19505. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19505 PMid:20403308
11. Maroli M, Rossi L, Baldelli R, Capelli G, Ferroglio E, Genchi C, et al. The northward spread of leishmaniasis in Italy: evidence from retrospective and ongoing studies on the canine reservoir and phlebotomine vectors. Trop Med Int Health. 2008;13(2):256-64. http://dx.doi.org/10.1111/j.1365-3156.2007.01998.x PMid:18304273
12. Biglino A, Bolla C, Concialdi E, Trisciuoglio A, Romano A, Ferroglio E. Asymptomatic Leishmania infantum infection in an area of northwestern Italy (Piedmont region) where such infections are traditionally nonendemic. J Clin Microbiol. 2010;48(1):131-6. http://dx.doi.org/10.1128/JCM.00416-09 PMid:19923480 PMCid:PMC2812267
13. Miró G, Checa R, Montoya A, Hernández L, Dado D, Gálvez R. Current situation of Leishmania infantum infection in shelter dogs in northern Spain. Parasit Vectors. 2012;5:60. http://dx.doi.org/10.1186/1756-3305-5-60 PMid:22452948 PMCid:PMC3348073
14. Farkas R, Tánczos B, Bongiorno G, Maroli M, Dereure J, Ready PD. First surveys to investigate the presence of canine leishmaniasis and its phlebotomine vectors in Hungary. Vector Borne Zoonotic Dis. 2011;11(7):823-34. http://dx.doi.org/10.1089/vbz.2010.0186 PMid:21254904
15. Tánczos B, Balogh N, Király L, Biksi I, Szeredi L, Gyurkovsky M, et al. First record of autochthonous canine leishmaniasis in Hungary. Vector Borne Zoonotic Dis. 2012;12(7):588-94. http://dx.doi.org/10.1089/vbz.2011.0906 PMid:22607079 PMCid:PMC3398396
16. Servicio de Epidemiología de la Comunidad de Madrid. Brote comunitario de leishmaniasis en la zona suroeste de la Comunidad de Madrid, 2009-2012. [Community outbreak of leishmaniasis in the southwest region of Madrid Autonomous Community]. Boletín Epidemiológico de la Comunidad de Madrid. 2011;17(12):25-32. Spanish. Available from: http://www.madrid.org/cs/Satellite?blobcol=urldata&blobheader=application%2Fpdf&blobheadername1=Content-Disposition&blobheadervalue1=filename%3DDiciembre2011.pdf&blobkey=id&blobtable=MungoBlobs&blobwhere=1310962031549&ssbinary=true
17. Arce A, Estirado A, Ordobas M, Sevilla S, García N, Moratilla L, et al. Re-emergence of leishmaniasis in Spain: community outbreak in Madrid, Spain, 2009 to 2012. Euro Surveill. 2013;18(30):pii=20546. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20546
18. Chicharro C, Jiménez MI, Alvar J. Iso-enzymatic variability of Leishmania infantum in Spain. Ann Trop Med Parasitol. 2003;97 Suppl 1:57-64. http://dx.doi.org/10.1179/000349803225002534 PMid:14678633
19. Cruz I, Chicharro C, Nieto J, Bailo B, Ca-avate C, Figueras MC, et al. Comparison of new diagnostic tools for management of pediatric Mediterranean visceral leishmaniasis. J Clin Microbiol. 2006;44(7):2343-7. http://dx.doi.org/10.1128/JCM.02297-05 PMid:16825347 PMCid:PMC1489479
20. Pratlong F, Rioux JA, Marty P, Faraut-Gambarelli F, Dereure J, Lanotte G, et al. Isoenzymatic analysis of 712 strains of Leishmania infantum in the south of France and relationship of enzymatic polymorphism to clinical and epidemiological features. J Clin Microbiol. 2004;42(9):4077-82. http://dx.doi.org/10.1128/JCM.42.9.4077-4082.2004 PMid:15364993 PMCid:PMC516332
21. World Health Organization (WHO). Control of the leishmaniases: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22-26 March 2010. Geneva: WHO; 2010. WHO technical report series; no. 949. Available from: http://whqlibdoc.who.int/trs/WHO_TRS_949_eng.pdf
22. Schönian G, Kuhls K, Mauricio IL. Molecular approaches for a better understanding of the epidemiology and population genetics of Leishmania. Parasitology. 2011;138(4):405-25. http://dx.doi.org/10.1017/S0031182010001538 PMid:21078222
23. Kuhls K, Mauricio IL, Pratlong F, Presber W, Schönian G. Analysis of ribosomal DNA internal transcribed spacer sequences of the *Leishmania donovani* complex. Microbes Infect. 2005;7(11-12):1224-34. http://dx.doi.org/10.1016/j.micinf.2005.04.009 PMid:16002315
24. Haralambous C, Antoniou M, Pratlong F, Dedet JP, Soteriadou K. Development of a molecular assay specific for the *Leishmania donovani* complex that discriminates L. donovani/Leishmania infantum zymodemes: a useful tool for typing *MON-1*. Diagn Microbiol Infect Dis. 2008;60(1):33-42. http://dx.doi.org/10.1016/j.diagmicrobio.2007.07.019 PMid:17889482
25. Fraga J, Montalvo AM, De Doncker S, Dujardin JC, Van der Auwera G. Phylogeny of Leishmania species based on the heat-shock protein 70 gene. Infect Genet Evol. 2010;10(2):238-45. http://dx.doi.org/10.1016/j.meegid.2009.11.007 PMid:19913110
26. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. 1999;41:95-8.
27. Depledge DP, Maclean LM, Hodgkinson MR, Smith BA, Jackson AP, Ma S, et al. Leishmania-specific surface antigens show sub-genus sequence variation and immune recognition. PLoS Negl Trop Dis. 2010;4(9):e829. http://dx.doi.org/10.1371/journal.pntd.0000829 PMid:20927190 PMCid:PMC2946902
28. Zackay A, Nasereddin A, Takele Y, Tadesse D, Hailu W, Hurissa Z, et al. Polymorphism in the HASPB repeat region of East African *Leishmania donovani* strains. PLoS Negl Trop Dis. 2013;7(1):e2031. http://dx.doi.org/10.1371/journal.pntd.0002031 PMid:23358849 PMCid:PMC3554577
29. Bhattacharyya T, Boelaert M, Miles MA. Comparison of visceral leishmaniasis diagnostic antigens in African and Asian *Leishmania donovani* reveals extensive diversity and region-specific polymorphisms. PLos Negl Trop Dis. 2013;7(2):e2057. http://dx.doi.org/10.1371/journal.pntd.0002057 PMid:23469296 PMCid:PMC3585016
30. Felsenstein J. PHYLIP-Phylogeny Inference Package (version 3.2). Cladistics.1989; 5: 164-6.
31. BLAST. Basic local alignment search tool. [Accessed 15 Mar 2012]. Available from: http://blast.ncbi.nlm.nih.gov/
32. Schönian G, Mauricio I, Gramiccia M, Ca-avate C, Boelaert M, Dujardin JC. Leishmaniases in the Mediterranean in the era of molecular epidemiology. Trends Parasitol. 2008;24(3):135-42. http://dx.doi.org/10.1016/j.pt.2007.12.006 PMid:18262469

Leishmania infantum in free-ranging hares, Spain, 2004-2010

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Iberian hares (*Lepus granatensis*) were recently deemed responsible for an outbreak of human leishmaniasis affecting metropolitan Madrid, Spain. However, the reservoir potential of hares in Europe is poorly known. We report a retrospective survey on *Leishmania infantum*, the causal agent of zoonotic endemic leishmaniasis in the Mediterranean basin, infection status of Iberian, European (*Le. europaeus*) and Broom (*Le. castroviejoi*) hares in Spain. Spleen samples from 94 hares were tested by polymerase chain reaction. Sequencing and restriction fragment length polymorphism (RFLP) assays were performed on positive samples and RFLP patterns compared with those of strains reported in the scientific literature. DNA prevalence in hare spleen samples was 43.6% (95% confidence interval: 33.6-53.6). In all six regions studied at least one positive sample was found. RFLP revealed existence of specific hare strains of *L. infantum* differing from those reported in wild carnivores in Spain. The widespread presence of *L. infantum* in the most abundant Spanish hare species and the recent evidence of the ability of naturally infected hares to transmit the pathogen to *Phlebotomus perniciosus*, its main vector in the western Mediterranean, suggest that hares may have an unexpected role in the epidemiology of *L. infantum* in Spain.

Introduction

Diseases at the wildlife-livestock-human interface are an increasing concern for public health, animal health and animal conservation authorities worldwide [1]. Also, wildlife-associated infectious diseases are at the top of human emerging diseases [2]. Basic epidemiologic knowledge would constitute the foundation for targeted prevention and control measures of wildlife-associated diseases, but knowledge is scarce for many of the currently emerging threats; Leishmaniasis in Europe is a good example. Endemic Mediterranean leishmaniasis is a disease of animals and humans caused by *Leishmania infantum*, a protozoan causing both visceral and cutaneous zoonotic leishmaniasis in the Mediterranean basin. *L. infantum* has recently spread northward from Mediterranean to temperate climates in Europe (e.g. Hungary and northern Italy), apparently linked to climate change [3] but perhaps also linked to increased movements of infected hosts, mostly dogs, from endemic Mediterranean areas [4]. Thus, leishmaniasis caused by *L. infantum* can be considered as a potentially emerging threat for central and northern European countries [4].

Dogs are deemed as major reservoirs of *L. infantum* since they efficiently replicate the protozoan parasite and are preferred hosts for vector phlebotomine sandflies [4]. Wild carnivores such as the wolf (*Canis lupus*), the red fox (*Vulpes vulpes*), the Egyptian mongoose (*Herpestes ichneumon*), the genet (*Geneta geneta*), the pine marten (*Martes martes*) or the Iberian lynx (*Lynx pardinus*) have also been implicated in the maintenance of *L. infantum* [5,6]. Recently, naturally infected Iberian hares (*Lepus granatensis*) were found to efficiently allow infection of *Phlebotomus perniciosus* sandflies with *L. infantum* [7]. Iberian hares were deemed as the main reservoirs of a leishmaniasis outbreak causing more than 260 human cases in the southwestern metropolitan area of Madrid since 2009 [7,8].

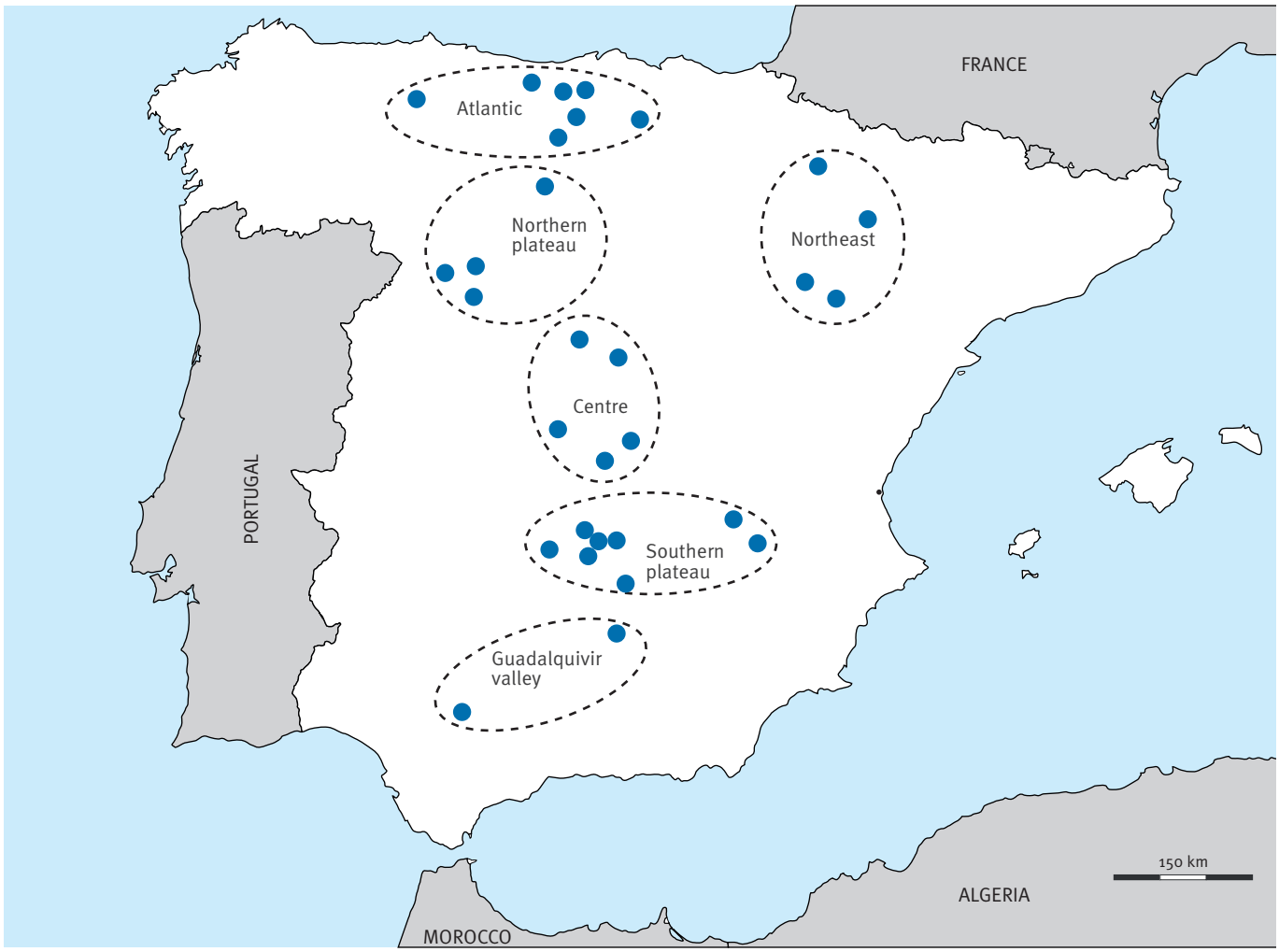
Three different hare species inhabit the Iberian Peninsula: (i) the Iberian hare that is present in vast areas of the Iberian Peninsula, (ii) the European brown hare (*Le. europaeus*) that lives in northern and northeastern Spain, and (iii) the Broom hare (*Le. castroviejoi*) that can be found in the Cantabrian Mountains [9]. Since basic information on the relationship between *L. infantum* and Iberian hare species is only anecdotal [7] and *Le. granatensis* populations are abundant and stable [10, unpublished data], we designed a retrospective survey on samples collected from Spanish hares through wildlife disease surveillance programs.

Methods

Spleen samples from a subset of hares (n=94) collected during necropsies performed over carcasses of animals found dead or harvested by hunters in Spain from 2004 to 2010 were used in this study. Sample collection was opportunistic since they were obtained

Figure

Location of hare sampling points by geographic region, Spain, 2004-2010



through Spanish wildlife disease surveillance programs. Hare tissues were preserved frozen at -20ºC until analysed.

According to their origin, samples were allocated to six geographic regions (listed here from north to south): Atlantic, Northern plateau, Northeast, Centre, Southern plateau and Guadalquivir river valley (Figure).

Total genomic DNA from spleen samples was extracted using a commercial kit (GenomeElute, Sigma-Aldrich, St. Louis, MO) following the manufacturer's protocol. A previously described polymerase chain reaction (PCR) targeting a 145 bp fragment present on the high copy of kDNA minicircles of *L. infantum* was performed on spleen samples [11]. PCR products were elicited by PCR fragment size estimation in comparison with two molecular weight standards: PCR 100 pb Low Ladder and pBR 322 HaeIII Digest (Sigma–Aldrich, St. Louis, MO) after electrophoresis in 2% agarose gel. Amplicons from 23 positive hares were sequenced to confirm *L. infantum* infection. Moreover, a restriction fragment length polymorphism (RFLP) assay was performed on all positive samples to compare amplicon patterns with previous studies from peninsular Spain [5]. For the RFLP assay, 15 µl of PCR product were digested with restriction enzymes BsiY I and Mlun NI as previously reported [12].

Basic comparison of prevalence values between sexes, species and geographic regions was performed by means of chi-squared tests. Statistical uncertainty was assessed by calculating the 95% confidence interval (CI) for each of the proportions according to the expression 95% CI. = $1.96[p(1 - p)/n]^{1/2}$ (where "p" is the proportion in its unitary value and "n" is the sample size) and expressed in percentage.

Table 1

Prevalence of *Leishmania infantum* infection in hares by geographic region and species, Spain, 2004-2010 (n=94)

Geographic region	Hare species	Number of samples	Positive	Prevalence in percent (95% CI)
Atlantic	*Le. europaeus*	14	9	64.3 (39.2-89.4)
	Le. castroviejoi	2	0	0.0 (n.a.)
Northern plateau	*Le. granatensis*	5	1	20.0 (0.0-55.1)
Northeast	*Le. europaeus*	2	0	0.0 (n.a.)
	Le. granatensis	5	3	60.0 (17.1-100.0)
Centre	*Le. granatensis*	10	6	60.0 (29.6-90.3)
Southern plateau	*Le. granatensis*	54	21	38.8 (21.8-51.8)
Guadalquivir river valley	*Le. granatensis*	2	1	50.0 (0.0-100.0)
Total		**94**	**41**	**43.6 (33.6-53.6)**

CI: confidence interval; Le: *Lepus*; n.a.: not applicable.

IBM SPSS 19.0 Statistical Package software (IBM Corporation, New York, USA) was employed for statistical analyses.

Results

Spleen samples analysed belonged to *Le. granatensis* (n=76; 24 males, 29 females and 23 unsexed), *Le. europaeus* (n=16; 5 males, 8 females and 3 unsexed) and *Le. castroviejoi* (n=2; both females).

The collected hare species were from the (i) Atlantic region:14 *Le. europaeus* and two *Le. castroviejoi* ; (ii) Northern plateau region: five *Le. granatensis*; (iii) the Northeastern region: five *Le. granatensis* and two *Le. europaeus*; (iv) Centre region: 10 *Le. granatensis*; (v) Southern plateau region: 54 *Le. granatensis*; and (vi) Guadalquivir river valley region: two *Le. granatensis*.

Overall, 41 out of 94 Spanish hares (43.6%; 95% CI: 33.6 to 53.6) were positive for the presence of *L. infantum* DNA. At least one positive hare was found in each of the six geographic regions surveyed (Table 1). Both Iberian and European hares tested positive for presence of *L. infantum* DNA by PCR.

No statistically significant differences in prevalence were observed between sexes: males (n=29) 44.8% (95% CI: 26.8 to 62.8) and females (n=39) 46.2% (95% CI: 30.2 to 62.2); species: *Le. granatensis* 42.1% (95% CI: 31.1 to 53.1), *Le. europaeus* 56.3% (95% CI: 32.3 to 80.3) and *Le. castroviejoi* 0%; and regions (Table 1). Interestingly, the highest prevalence value was observed in hares from Central and Atlantic regions.

Sequencing was successful from nine hares and homology with *L. infantum* kinetoplast DNA ranged from 94% to 99%.

Twenty-two RFLP patterns were obtained from 32 hares (see Table 2). Thirteen RFLP patterns were found in thirteen individuals; six from the Atlantic region, six from Southern plateau region and one from Centre region. Eight patterns were each present in two different hares; two of the patterns were exclusively present in hares from Southern plateau region and one was only present in hares from Centre region, while five of these eight patterns were present in hares from different geographic regions – two in hares from Atlantic and Southern plateau regions respectively, one in hares from Northern plateau and Southern plateau regions, one in hares from Centre and Southern plateau regions and one in hares from Atlantic and Centre regions. Finally, one of the 22 patterns was present in three different hares: two from Southern plateau and one from Northeast region.

Nine different patterns were found in the nine positive hares (all *Le. europaeus*) from Atlantic region (Table 2). Five hares from Centre region (all *Le. granatensis*) presented four different patterns. Sixteen hares from Southern plateau (all *Le. granatensis*) presented 13 different patterns. RFPL patterns identified in one hare from Northeast region and one hare from Northern plateau were also present in other regions.

No similarities were found between hare RFLP patterns and those previously found in wild carnivores from continental Spain [5].

Discussion

This study shows that *L. infantum* is present in two of the three Spanish hare species and that specific 'hare strains' of *L. infantum* circulate in Spain. However, the low number of samples from *Le. castroviejoi* – an endangered species – prevented determining if they are exposed to *L. infantum*. We selected testing the presence of *L. infantum* DNA in spleen samples instead of detecting antibody presence because we aimed to measure the occurrence of effective infections rather than detecting exposure. The effect of possible local

Table 2

Allocation to geographic region of the 22 *Leishmania infantum* restriction fragment length polymorphism patterns identified from hares, Spain 2004-2010 (n=32)

Hare number	RFLP pattern number	Species	Geographic region
1	1	*Le. europaeus*	Atlantic
2	2	*Le. europaeus*	Atlantic
3	3	*Le. granatensis*	Southern plateau
4	4	*Le. granatensis*	Southern plateau
5	4	*Le. granatensis*	Southern plateau
6	5	*Le. europaeus*	Atlantic
7	6	*Le. europaeus*	Atlantic
8	6	*Le. granatensis*	Southern plateau
9	7	*Le. europaeus*	Atlantic
10	8	*Le. granatensis*	Northern plateau
11	8	*Le. granatensis*	Southern plateau
12	9	*Le. granatensis*	Southern plateau
13	10	*Le. granatensis*	Centre
14	11	*Le. granatensis*	Southern plateau
15	12	*Le. europaeus*	Atlantic
16	13	*Le. granatensis*	Southern plateau
17	13	*Le. granatensis*	Centre
18	14	*Le. europaeus*	Atlantic
19	14	*Le. granatensis*	Centre
20	15	*Le. granatensis*	Southern plateau
21	15	*Le. granatensis*	Southern plateau
22	16	*Le. granatensis*	Southern plateau
23	17	*Le. granatensis*	Southern plateau
24	18	*Le. granatensis*	Centre
25	18	*Le. granatensis*	Centre
26	19	*Le. granatensis*	Southern plateau
27	19	*Le. granatensis*	Northeast
28	19	*Le. granatensis*	Southern plateau
29	20	*Le. granatensis*	Southern plateau
30	21	*Le. granatensis*	Southern plateau
31	21	*Le. europaeus*	Atlantic
32	22	*Le. europaeus*	Atlantic

Le: *Lepus*; RFLP: restriction fragment length polymorphism.

temporal trends in *L. infantum* prevalence caused by changes in vector and host population dynamics could have had an effect on prevalence rates found in this study.

In spite of sample size limitations in this study, overall *L. infantum* DNA prevalence in *Le. granatensis* and *Le. europaeus* seems to be above the 30% (lower limits of estimated confidence intervals). The finding of positive hares in each of the six geographic regions surveyed suggests that *L. infantum* is widely spread in Spanish hare populations. These findings together with the recent evidence of the ability of *Ph. perniciosus* to get infected through feeding on *Le. granatensis* [7], evidences the reservoir potential of hares for *L. infantum*. Infection by *L. infantum* in Iberian hares seems not to cause clinical disease [7] and thus *L. infantum* may not be of direct concern for hare conservation purposes. However, since *L. infantum* is an important pathogen for humans and other mammals, animal health and conservation authorities in Spain should be aware of the indirect consequences on conservation and wildlife management caused by their potential role as *L. infantum* reservoir.

Both Iberian and European hares are widely distributed in Spain, and their impact in the epidemiology of Mediterranean leishmaniasis deserves further research. Our findings suggest that hares have the potential to modulate the ecology of *L. infantum* in the near future as already evidenced in the outskirts of Madrid. The European hare inhabits vast areas of central Europe [13], constituting a potential European reservoir for *L. infantum*. This should be carefully considered when modeling the spread of *L. infantum* in those areas. Translocation of European and Iberian hares for hunting purposes between European countries is frequent e.g. between Spain and France [14,15]. This increases the chance of introducing *L. infantum* to new areas or other European countries via infected hares. It could also explain the great diversity of RFLP patterns found in European hares from the Atlantic region in Spain, where introduction of animals from French farms is common [15]. Moreover, translocation of hares at the national scale is frequent, which could explain the finding of similar PCR-RFLP patterns from hares surveyed in different geographic regions. Otherwise, our findings would indicate the existence of a widespread pattern of *L. infantum* strains in hares across Spain.

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References

1. Kuiken T, Ryser-Degiorgis MP, Ferroglio E, Gortázar C. Establishing a European network for wildlife health surveillance. Rev Sci Tech. 2011;30:755-61. PMid:22435188.
2. Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, et al. Global trends in emerging infectious diseases. Nature. 2008;451(7181):990-3. http://dx.doi.org/10.1038/nature06536. PMid:18288193.
3. Ferroglio E, Maroli M, Gastaldo S, Mignone M, Rossi L. Canine leishmaniosis in Italy. Emerg Infect Dis 2005;11(10):1618-20.

http://dx.doi.org/10.3201/eid1110.040966 PMid:16318709. PMCid:PMC3366729.

4. Ready PD. Leishmaniasis emergence in Europe. Euro Surveill. 2010;15(10):pii=19505. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19505. PMid:20403308.
5. Sobrino R, Ferroglio E, Oleaga A, Romano A, Millán J, Revilla M, et al. Characterization of widespread canine leishmaniasis among wild carnivores from Spain. Vet Parasitol. 2008;155(3-4):198-203. http://dx.doi.org/10.1016/j.vetpar.2008.05.003. PMid:18579311.
6. Millán J, Zanet S, Gomis M, Trisciuoglio A, Negre N, Ferroglio E. An Investigation into alternative reservoirs of canine Leishmaniasis on the endemic Island of Mallorca (Spain). Transbound Emerg Dis. 2011;58(4):352-7. http://dx.doi.org/10.1111/j.1865-1682.2011.01212.x. PMid:21733133.
7. Molina R, Jiménez MI, Cruza I, Iriso A, Martín-Martín I, Sevillano O, et al. The hare (Lepus granatensis) as potential sylvatic reservoir of Leishmania infantum in Spain. Vet Parasitol. 2012;190(1-2):268-71. http://dx.doi.org/10.1016/j.vetpar.2012.05.006. PMid:22677135.
8. Arce A, Estirado A, Ordobas M, Sevilla S, García N, Moratilla L, et al. Re-emergence of leishmaniasis in Spain: community outbreak in Madrid, Spain, 2009 to 2012. Euro Surveill. 2013;18(30):pii=20546. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20546
9. Palomo LJ, Gisbert J, Blanco JC. Atlas y libro rojo de los mamíferos terrestres de Espa-a. [Atlas and red book of terrestrial mamals in Spain]. 1st ed. Madrid: Dirección General para la Biodiversidad-SECEM-SECEMU; 2007. Spanish.
10. Gortázar C, Millán J, Acevedo P, Escudero MA, Marco J, Fernández de Luco D. A large-scale survey of Brown hare Lepus europaeus and Iberian hare L. granatensis populations at the limit of their ranges. Wildl Biol. 2007;13(3):244-50. http://dx.doi.org/10.2981/0909-6396(2007)13[244:ALSOBH]2.0.CO;2
11. Lachaud L, Marchergui-Hammami S, Chabbert E, Dereure J, Dedet JP, Bastien P. Comparison of six PCR methods using peripheral blood for detection of canine visceral leishmaniasis. J Clin Microbiol. 2002;40(1):210–5. http://dx.doi.org/10.1128/JCM.40.1.210-215.2002. PMid:11773118. PMCid:PMC120090.
12. Ferroglio E, Romano A, Trisciuoglio A, Poggi M, Ghiggi E, Sacchi P, Biglino A. Characterization of Leishmania infantum strains in blood samples from infected dogs and humans by PCR-RFLP. Trans R Soc Trop Med Hyg. 2006; 100(7):636-41. http://dx.doi.org/10.1016/j.trstmh.2005.09.008. PMid:16343571
13. Mitchell-Jones AJ, Amori G, Bogdanowicz W, Kryŝtufek B, Reijnders PJH, Spitzenberger F, et al. The Atlas of European mammals. 1st ed. London: Academic Press. 1999.
14. Bordes F, Langand J, Feliu C, Morand S. Helminth communities of an introduced hare (Lepus granatensis) and a native hare (Lepus europaeus) in southern France. J Wildl Dis. 2007;43(4):747–51. PMid:17984274.
15. Alzaga V, Tizzani P, Acevedo P, Ruiz-Fons F, Vicente J, Gortázar C. Deviance partitioning of host factors affecting parasitization in the European brown hare (Lepus europaeus). Naturwissens. 2009;96(10):1157–68. http://dx.doi.org/10.1007/s00114-009-0577-y. PMid:19565211.

Heat-shock protein 70 gene sequencing for *Leishmania* species typing in European tropical infectious disease clinics

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We describe *Leishmania* species determination on clinical samples on the basis of partial sequencing of the heat-shock protein 70 gene (*hsp70*), without the need for parasite isolation. The method is especially suited for use in non-endemic infectious disease clinics dealing with relatively few cases on an annual basis, for which no fast high throughput diagnostic tests are needed. We show that the results obtained from this gene are in nearly perfect agreement with those from multilocus enzyme electrophoresis, which is still considered by many clinicians and the World Health Organization (WHO) as the gold standard in *Leishmania* species typing. Currently, 203 sequences are available that cover the entire *hsp70* gene region analysed here, originating from a total of 41 leishmaniasis endemic countries, and representing 15 species and sub-species causing human disease. We also provide a detailed laboratory protocol that includes a step-by-step procedure of the typing methodology, to facilitate implementation in diagnostic laboratories.

Introduction

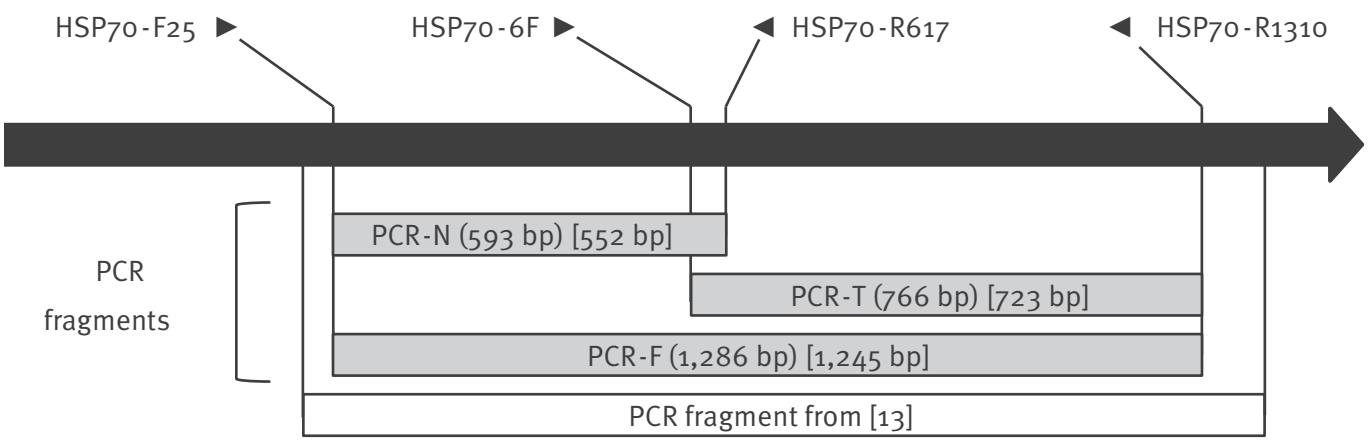
As a result of current human mobility, European infectious disease clinics are occasionally confronted with leishmaniasis patients who got infected in an area endemic for *Leishmania* outside their own country. Typically it concerns tourists, expatriates, military staff, migrants, and relatives visiting friends or family. In many of these centres, the number of such cases seen annually is limited, and investing in the validation of high-throughput methods for discriminating the medically relevant species is therefore too costly. Nevertheless, especially in the case of tegumentary leishmaniasis, knowledge of the aetiological agent is highly relevant, as the disease prognosis and treatment choice depend on it [1-8]. However, one cannot always rely on the known epidemiology in the suspected region of infection. Firstly, because such information is often inaccurate or outdated, and secondly, because the geographic area where the patient got infected may not be known exactly if they resided in different endemic areas or countries. Moreover, even if the exact location of infection and epidemiology are known, different species may circulate sympatrically in a given region. Hence, there is a need for easily applicable, straightforward and standardised species discrimination methods that must above all be accurate, rather than allowing to handle many samples in a high-throughput fashion.

Over the past few years, we have been investing in the use of the heat-shock protein 70 gene (*hsp70*) for discrimination of medically important *Leishmania* species worldwide [9-12]. Initially developed for species discrimination in the New World subgenus *L. (Viannia)* by restriction fragment length polymorphism (RFLP) analysis [13], we have upgraded the specificity and sensitivity of the *hsp70* PCR amplification strategy to suit all *Leishmania* species [12]. In this paper we report on the power of *Leishmania* species typing on the basis of *hsp70* sequences rather than RFLP. The approach is directed specifically towards diagnosis in clinical laboratories dealing with relatively few cases on an annual basis, such as our Institute of Tropical Medicine in Antwerp where an average of 15 patients are diagnosed each year. The method described here was developed in the framework of a European consortium of tropical infectious disease clinics called 'LeishMan' (www.leishman.eu), embedded in the European network for tropical medicine and travel health TropNet (www.tropnet.net). LeishMan aims at characterising *Leishmania* parasites using a standardised molecular assay that can be applied for clinical samples, without the need for parasite culture.

Several other single-locus assays have been used for sequence-based species discrimination, such as the mini-exon, the 7SL-RNA, and the ribosomal DNA-ITS1

Figure 1

Position of PCR primers and products used for sequencing on the *hsp70* coding region of *Leishmania major* strain MHOM/IL/81/Friedlin



GenBank accession number FR796424.

The size of the PCR products is indicated between round brackets. The size of the sequenced fragments between the PCR primers is indicated between square brackets.

Black arrow in 5' to 3' direction. ▶ primer extending in the sense direction of the gene; ◀ primer extending in the antisense direction of the gene. The region in the white box is the PCR fragment reported in [13].

[14-17]. We found that *hsp70* has some advantages over these (data not shown): it is easily comparable across all *Leishmania* species worldwide as there is no size variation in the gene [9], it discriminates all relevant species in both subgenera *L.* (*Leishmania*) and L. *(Viannia)*, and PCRs have been optimised for direct amplification from clinical samples [10,12]. The gene is arranged as a tandem repeat unit, with almost no sequence variation between the coding sequences of the different copies [18,19]. In this paper we assess the concordance of *Leishmania* species typing with *hsp70* sequences on the one hand, and results obtained from other genetic targets and multilocus enzyme electrophoresis (MLEE) on the other hand.

Methods

Hsp70 amplification and sequencing

Leishmania hsp70 sequences from 64 cultures and 36 rDNA-PCR-confirmed [20,21] clinical samples were determined on the basis of a single PCR amplicon, i.e. PCR-F in Figure 1. Ca. 50 of these cultures were obtained from the Centre National de Référence des *Leishmania* (Montpellier, France). Among the clinical samples, 27 were from cutaneous lesions (mostly biopsies), one from a mucocutaneous lesion, two from visceral leishmaniasis patients, and six from an unknown clinical background. In the rare occasions where direct amplification of PCR-F failed from the clinical sample DNA extract, or when an insufficient amount of amplicon was obtained for sequencing, two shorter PCRs were used that together cover the same fragment: PCR-N and PCR-T (Figure 1). These can be run directly on the sample DNA, or alternatively as hemi-nested PCRs using the PCR-F amplicon as first round PCR. A detailed protocol is available from www.itg.be/LeishmaniaHSP70.

All PCRs were performed in 25 µl 1x standard PCR buffer (Qiagen, Hilden, Germany), supplemented by 1 mM $MgCl^2$ and 1x Qiagen Q-solution. Each reaction used 200 µM of each dNTP, 0.8 µM of each PCR primer (Table), and 1U of HotStarTaq Plus DNA polymerase (Qiagen). Up to 2.5 µl of template were used. Cycling conditions were as follows: 5 min at 95 °C denaturation; 35 cycles of 40 sec at 94 °C, 1 min at 61 °C, 2 min at 72 °C; and finally 10 min at 72 °C. For PCR-N and PCR-T, the elongation step was shortened to 1 min at 72 °C.

PCR products were analysed on a 2% agarose gel to check for sufficient and specific amplification, based on the expected product sizes outlined in Figure 1. The fragments were sequenced with primers internal in the PCR fragment (see protocol on www.itg.be/LeishmaniaHSP70). In some strains, a second nucleotide was detected below the main trace signal at some sequence positions. In such cases, IUPAC ambiguity codes [22] were introduced in the sequence whenever the secondary nucleotide showed at least 20% of the intensity of the main peak in each sequence read covering the respective position. The sequences from reference strains were submitted to the European Nucleotide Archive (www.ebi.ac.uk/ena).

Sequence analysis and typing

For the analysis presented in this paper, we compiled

Table

PCR primers used for amplification of the partial *hsp70* coding region

Primer name	PCR	Sequence (5'-3')	Length	Orientation	Annealing start (5' of primer)[a]	Annealing end (3' of primer)[a]
HSP70-F25	PCR-F/N	GGACGCCGGCACGATTKCT	19	Sense	480	498
HSP70-6F	PCR-T	GTGCACGACGTGGTGCTGGTG	21	Sense	1,000	1,020
HSP70-R617	PCR-N	CGAAGAAGTCCGATACGAGGGA	22	Antisense	1,072	1,051
HSP70-R1310	PCR-F/T	CCTGGTTGTTGTTCAGCCACTC	22	Antisense	1,765	1,744

[a] Annealing position in GenBank entry FR796424 (*hsp70* of *L. major* Friedlin strain).

Primers are listed in order of annealing in the coding sequence of the gene, from 5'-3' terminus.

107 available sequences covering the 1,245 bp *hsp70* PCR-F fragment (Figure 1) from GenBank (www.ncbi.nlm.nih.gov/genbank, accessed on 25 June 2013). These were aligned with the 100 sequences determined in this study. From the total of 207 sequences, 84 were typed by MLEE, and 54 on the basis of genes other than *hsp70*. Many MLEE-typed isolates were analysed with genetic methods as well. For 12 sequences, we relied on the species identification as listed in GenBank, where the typing method is not specified. Finally, no typing data were available for the remaining 57 sequences, which included those determined for diagnosis. For the sequences described in the paper by Zhang et al. [23], we did not rely on the GenBank identification, as this was in conflict with data in the paper itself. Aligning was done manually, which was straightforward as no size variation was detected in 205 sequences, while two sequences showed a deletion of three nucleotides, corresponding to one amino acid.

Species delineation was based upon the clustering of aligned sequences in a comparative dendrogram, which was constructed with the freely available software package MEGA5 [24]. Dendrograms were built from the variable sites in the alignment using the neighbour-joining method, with pairwise gap deletion and 2,000 bootstrap replicates. As our aim was to find the most discriminative analysis method rather than to study evolution, we based our dendrograms on p distances, and not on other models such as the popular Kimura 2-parameter method for calculating corrected distances. More details are available from the protocol on www.itg.be/LeishmaniaHSP70.

Results

The final alignment contained 207 sequences from 42 *Leishmania*-endemic countries, representing 18 species of which 15 are causing human disease (Figure 2 and supplementary dendrogram available at www.itg.be/LeishmaniaHSP70). As further detailed in the Discussion, four GenBank entries contained sequences that did not correspond to the indicated isolate, reducing the number of trustworthy sequences to 203 from 41 countries.

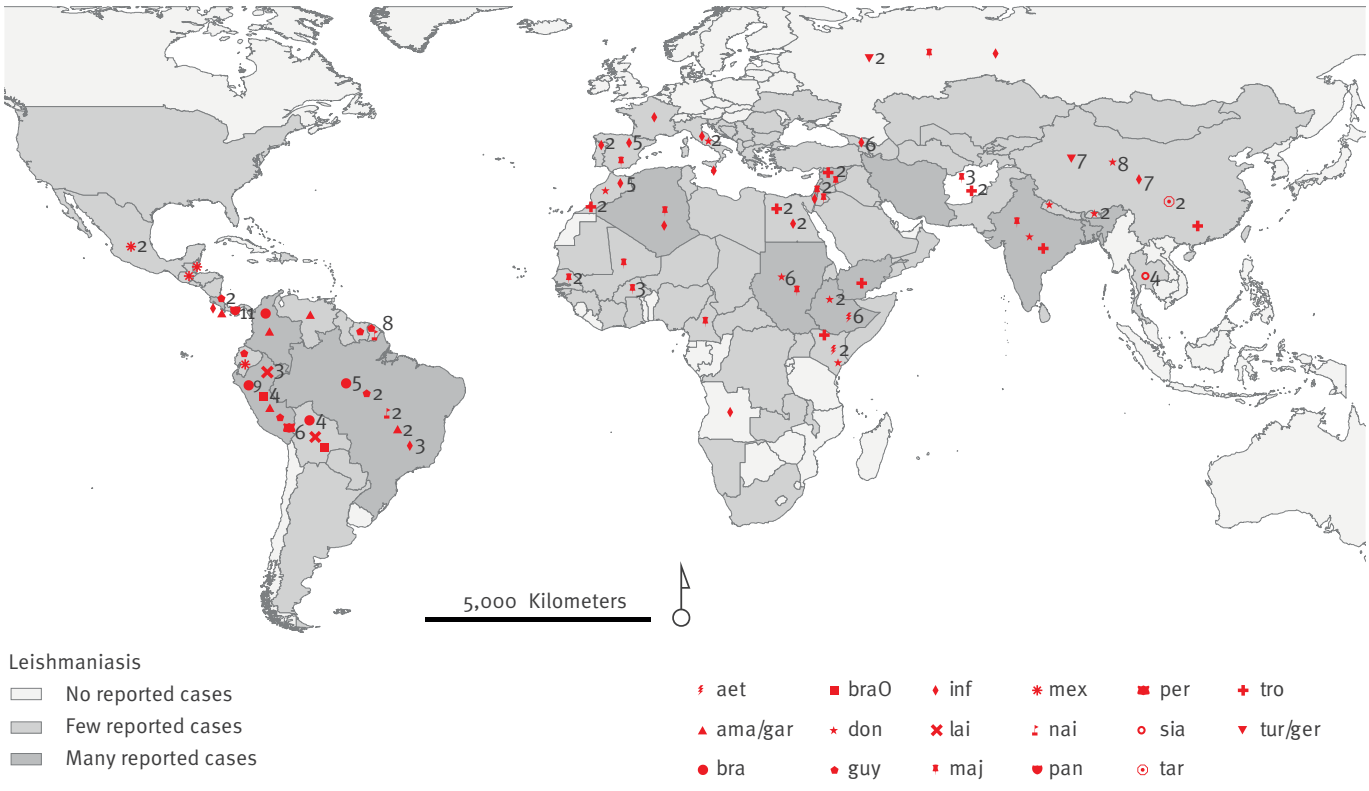
The clustering of a representative selection of the *hsp70* sequences is depicted in Figure 3. A dendrogram of the complete set of available *hsp70* sequences can be found at www.itg.be/LeishmaniaHSP70. The medically relevant clusters indicated in bold in these figures could be easily discriminated, and were supported by bootstrap values between 89 and 99% (Figure 3). These generally coincided with recognised species complexes. Within these complexes, a further distinction was possible, as indicated by the dotted lines. These subdivisions had a lower bootstrap support, between 53 and 71% (Figure 3).

Almost all *hsp70* clusters showed a perfect agreement with MLEE-based classifications (isolates identified with 'M. species') and typing results from genetic loci different from *hsp70* (identified with 'G. species'). There were nevertheless a few exceptions, which are indicated with * and ** following the taxon designation. Of the 81 isolates typed on the basis of MLEE and from which a trustworthy *hsp70* sequence was reported, 76 (94%) grouped in the respective *hsp70* cluster. Of the 54 isolates typed on the basis of non-*hsp70* genetic loci, 50 (93%) grouped in the respective *hsp70* cluster. A few isolates did not group with any known species clade, notably IMON/CN/90/KXG-Y, MHOM/--/94/CRE58, MHOM/PE/--/CU00181, MHOM/PE/95/LQ-8, and MCAN/IR/96/LON-49.

The *L. braziliensis* isolates separated into two clearly distinct clusters, named *L. braziliensis* outlier and *L. braziliensis* complex, which also contained *L. peruviana*. Even though these two clusters are sister taxa, the bootstrap support was weak (53%). In some dendrograms, the two clusters did not form sister clades, and the outliers rather grouped with *L. naiffi* (results not shown). One strain, MHOM/PE/--/CU00181, was intermediate between both *L. braziliensis* clusters.

Figure 2

Geographic origin of *Leishmania hsp70* sequences analysed in this study (n=190)



Of 203 trustworthy sequences, this figure includes the 190 with known origin of infection and species.

The shaded areas are considered endemic for Leishmania, the darkly shaded areas carry the heaviest burden of visceral and/or cutaneous leishmaniasis according to [46]. Strains are assigned at country level, the position of the symbols within a country has no meaning. The former Soviet Union is considered as one country; Costa Rica and Panama are joined because of their small size. If one symbol represents several strains, the number is given on the right, otherwise it represents only one strain.

Species: aet: *L. aethiopica*; ama: *L. amazonensis*; arc: *L. archibaldi*; bra: *L. braziliensis*; braO: *L. braziliensis* outlier; bra-braO: hybrid; cha: *L. chagasi*; don: *L. donovani*; gar: *L. garnhami*; ger: *L. gerbilli*; guy: *L. guyanensis*; inf: *L. infantum*; lai: *L. lainsoni*; maj: *L. major;* mex: *L. mexicana*; nai: *L. naiffi*; pan: *L. panamensis*; per: *L. peruviana*; sia: *L. siamensis*; tar: *L. tarentolae*; tro: *L. tropica*; tur: *L. turanica*.

Using the here presented *hsp70* clustering system, we have so far been able to determine the infecting species in 33 clinical samples presented for diagnosis in our institute, and three from military personnel on mission in Afghanistan. These were from 14 different countries and represented eight *Leishmania* species (Figure 3 and supplementary dendrogram). The majority (n=27) were from cutaneous lesions (mostly biopsies), one from a mucocutaneous lesion, and two from visceral leishmaniasis patients. From six samples the clinical presentation was not known.

Discussion

In general there is good agreement between typing results on the basis of *hsp70* and those based on other genes and MLEE, with the following exceptions: (i) *L. chagasi* isolates could not be distinguished from *L. infantum*, which agrees with previous studies showing that both are in fact one species, whereby *L. chagasi* is synonym of South-American *L. infantum* [25]. (ii) *L. archibaldi* grouped with *L. donovani*, in line with the current notion that it is not a separate species [26]. (iii) Two *L. infantum* isolates, MHOM/CN/94/KXG-LIU and MHOM/CN/93/KXG-XU, were found clustering with *L. donovani*. According to the authors who published these sequences, however, the species identification is disputable and depends on the technique applied [23]. (iv) The MLEE identified *L. donovani* isolate MHOM/SU/84/MARZ-KRIM clustered with *L. infantum*. On the basis of at least eight other genes, this strain was indeed identified as *L. infantum* (data not shown). (v) Two *L. guyanensis* strains, MHOM/CO/83/REST417 and MHOM/EC/90/UI.031 were found in the *L. panamensis* cluster. Although it has been argued that *L. panamensis* is merely a geographically confined subcluster of *L. guyanensis* rather than a distinct species [9,27-29], both strains merit a more profound genetic analysis to evaluate their *hsp70* classification. (vi) The *L. braziliensis* isolate MHOM/PE/2001/LH2140 clustered with *L. peruviana*, even though its sequence is different from those of the other *L. peruviana* strains. A genome-wide amplified fragment length polymorphism

Figure 3

Dendrogram of selected *Leishmania hsp70* sequences analysed in this study, including for each indicated cluster the most divergent sequences (n=91)

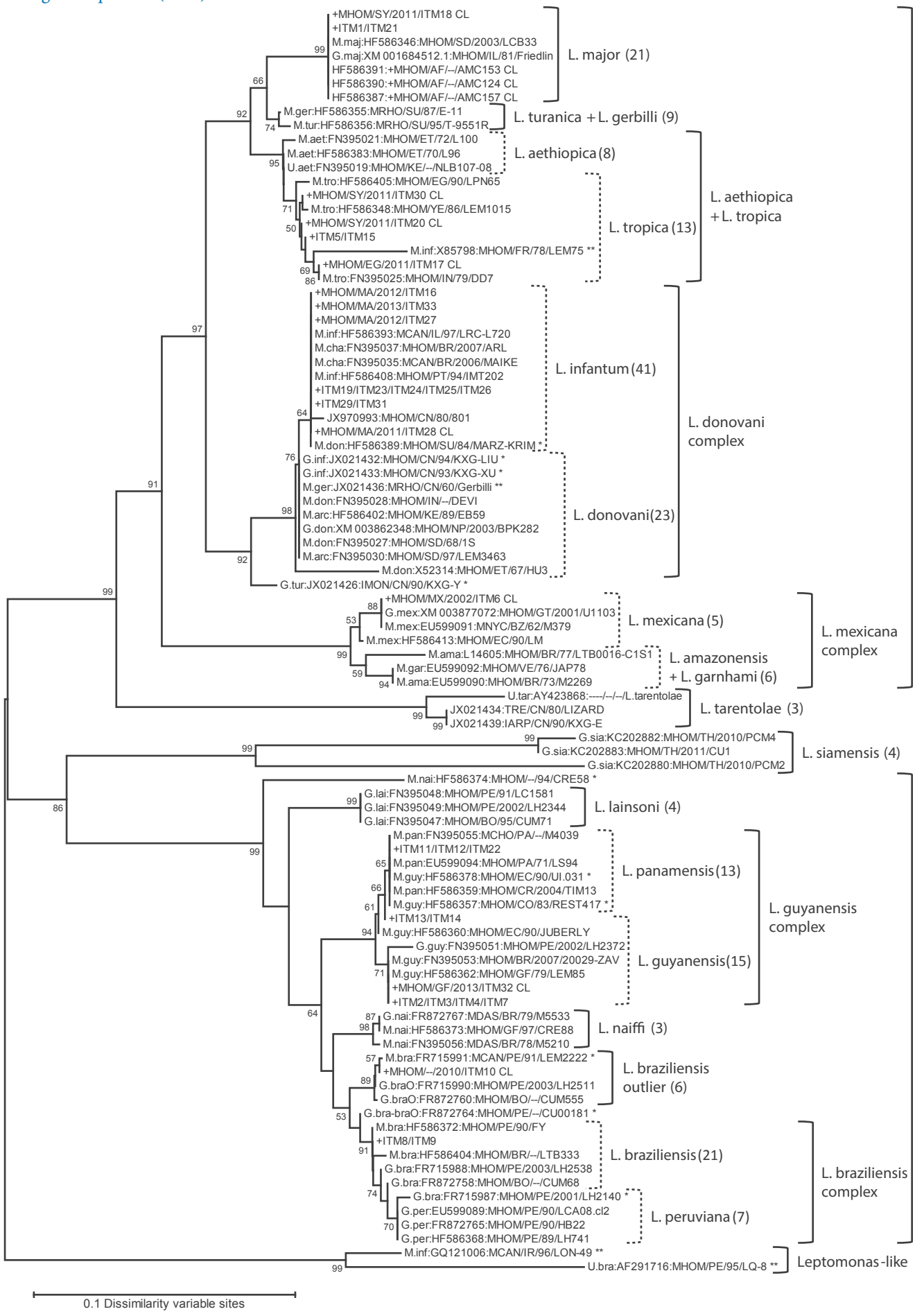


Figure 3 notes

Dendrogram of selected *Leishmania hsp70* sequences analysed in this study, including for each indicated cluster the most divergent sequences (n=91)

Each taxon is identified as follows:

(i) Identification method if available: G: genetic analysis other than *hsp70*; M: multilocus enzyme electrophoresis; U: unknown typing method.

(ii) Species based on this identification method: aet: *L. aethiopica*; ama: *L. amazonensis*; arc: *L. archibaldi*; bra: *L. braziliensis*; braO: *L. braziliensis* outlier; bra-braO: hybrid; cha: *L. chagasi*; don: *L. donovani*; gar: *L. garnhami*; ger: *L. gerbilli*; guy: *L. guyanensis*; inf: *L. infantum*; lai: *L. lainsoni*; maj: *L. major*; mex: *L. mexicana*; nai: *L. naiffi*; pan: *L. panamensis*; per: *L. peruviana*; sia: *L. siamensis*; tar: *L. tarentolae*; tro: *L. tropica*; tur: *L. turanica*.

(iii) EBI/GenBank accession number if available.

(iv) World Health Organization (WHO) code: Missing data are indicated by .

(v) Clinical samples diagnosed in this study are indicated with + in front of the taxon name, and those from the Institute of Tropical Medicine Antwerp are identified by ITM without WHO code, whereby identical sequences are presented as one taxon. CL following the WHO code indicates that the sample was taken from a cutaneous lesion.

(vi) Strains indicated with * cluster differently compared with other methods, those indicated with ** do not represent the strain as reported in GenBank.

The dissimilarity scale is presented at the bottom. Bootstrap values higher than 50% from a 2,000 replicate analysis are shown in percentages at the internodes. Clusters strongly supported are indicated in bold, those less supported are indicated by dotted lines. For each recognised cluster, the number of strains in the total of 207 available sequences is given between brackets.

(AFLP) analysis clearly identified this strain as *L. braziliensis* [19]. (vii) The MLEE-typed *L. braziliensis* isolate MCAN/PE/91/LEM2222 clustered with the *L. braziliensis* outliers, confirming results from several other genes (data not shown). This is in line with the fact that MLEE does not separate both *L. braziliensis* groups. (viii) *L. braziliensis* isolate MHOM/PE/--/CU00181 clustered intermediate between *L. braziliensis* and *L. braziliensis* outliers, which agrees with AFLP data [19]. (ix) Two sequences were found in an incorrect species cluster: MRHO/CN/60/Gerbilli and MHOM/FR/78/LEM75. According to GenBank data, JX021436 is the sequence from the WHO *L. gerbilli* reference strain MRHO/CN/60/Gerbilli, but it clustered among *L. donovani*, apart from the other *L. gerbilli* sequences. As the strain was not included in the publication describing related GenBank entries [23], there are no independent data to confirm the identity of the sequence. Moreover, several species designations in this set of GenBank entries (especially those listed as *L. donovani*) do not match those in the corresponding paper. MHOM/FR/78/LEM75 is a type strain of *L. infantum*, but it strongly grouped with *L. tropica*. Given that all 17 other *L. infantum* strains clustered correctly with *hsp70*, it is reasonable to assume that the sequence in GenBank is erroneous. (x) Finally, four isolates, MHOM/PE/95/LQ-8, MCAN/IR/96/LON-49, MHOM/--/94/CRE58, and IMON/CN/90/KXG-Y, did not group with any of the designated species complexes, the reason for which is unclear. The sequences reported for MHOM/PE/95/LQ-8 and MCAN/IR/96/LON-49 were found related to the *Leptomonas* sp. sequence described in [30], and hence these do not match with the *Leishmania* isolates listed in GenBank (results not shown). MHOM/--/94/CRE58 and IMON/CN/90/KXG-Y were typed as *L. naiffi* and *L. turanica*, respectively, using several genes, and it is unclear why they did not group with their respective species.

Taken all evidence together, of the 135 trustworthy sequences for which either MLEE or independent genetic species identification was done, 130 (96.3%) grouped with the correct species in the *hsp70* sequence dendrogram; two (1.5%) did not group with any species; and three (2.2%) were assigned to the correct species complex, but the wrong species. Since we started routine species typing on the basis of *hsp70* sequences, we could type 33 clinical samples that were sent to our clinic for diagnosis, along with three samples sent to us by other institutes (accessions HF586387, HF586390, HF586391). In the same period, amplification failed from two samples with an extremely low parasite load. We provide a detailed protocol and sequence reference set on the website www.itg.be/LeishmaniaHSP70, which outlines a step-by-step guideline of the PCRs, sequencing, and interpretation. We acknowledge that implementing sequence analysis in a routine diagnostic laboratory may be difficult in some settings and that the entire analysis may take a few days. Nevertheless, in our hands the method proved highly convenient, and in view of the few samples diagnosed per year, more cost-effective than validating a high-throughput system with a simple readout. Alternatively, sequencing could provide a clear identification in case other assays fail .

The more disputable species designations are *L. infantum*, *L. panamensis*, and *L. peruviana*, as all these were moderately bootstrap-supported subgroups of the highly robust *L. donovani*, *L. guyanensis*, and *L. braziliensis* complexes, respectively, as previously documented [9,19,26-29,31-35]. In case of doubt, the complex level should be reported rather than the exact species. From a clinical point of view, discriminating *L. infantum* from *L. donovani* is not highly relevant, since both species can cause visceral leishmaniasis and treatment is the same [26,36]. Also the discrimination

between *L. guyanensis* and *L. panamensis* is not a priority in clinical practice [36]. Separating *L. braziliensis* from *L. peruviana* is considered more relevant, because *L. braziliensis* potentially causes mucocutaneous complications, while *L. peruviana* generally does not [34]. As no markers are currently available that discriminate strains that do from those that do not cause mucocutaneous leishmaniasis, identification at the species level is the only option. Both MLEE and genetic analyses have revealed that *L. peruviana* is a subcluster in the *L. braziliensis* complex, but discrimination is impaired by the fact that many parasites of this complex seem to have a composite genotype carrying signatures of both species [19,32-35]. The situation is further complicated by the fact that occasionally, *L. peruviana* can cause mucocutaneous disease [34]. Isolates belonging to the *L. braziliensis* outlier group have been isolated from mucous lesions as well (data not shown), but whether these were primary or secondary infections is not known. Two other tightly linked species in the *hsp70* dendrogram are *L. tropica* and *L. aethiopica*. Although the *L. tropica* isolates cover the entire endemic region, from Morocco to eastern Africa, the Middle-East, and India, they form a clearly separated recognisable group. The same applies to separating *L. mexicana* from *L. amazonensis*, even though the latter could not be distinguished from *L. garnhami*.

One may wonder why some of the above species in the recognised larger complexes seem less clearly defined by sequencing than by single-nucleotide polymorphism (SNP) assays such as species-specific PCRs or RFLP analysis. The reason is that these assays use a point mutation in the genome of the parasite, which is either present or absent, thereby allowing a binary discrimination. When using sequences, much more information is provided from many polymorphisms and is sometimes contradictory. Typing based on sequencing can therefore be more difficult, but it is more reliable as it uses more data. An accidental mutation may lead to erroneous conclusions in a SNP-based assay, while this is less likely when analysing entire sequences.

The current complete set of trustworthy sequences that can be used for typing amounts to 203, representing 15 species of human medical importance, and originating from 41 endemic countries. This reference set is updated continuously for further improvement of the geographic and genetic coverage, to ensure an adequate representation of the existing inter- and intraspecies variability. Some species are over-represented from some regions (such as in Peru), but that does not interfere with the typing outcome. It is of crucial importance to base species typing upon sequences that have been quality-checked. In practice, BLAST searches are often used for identification purposes, on the basis of *hsp70* sequences found in public databases such as GenBank. We have found several instances where the species designation reported in these databases was incorrect. For example, in entries JX021425 up to JX021443 and JX970993 up to JX970996, several erroneous *L. donovani* sequences are reported, even disagreeing with the species assignment as listed in the related publication [23]. Two entries were here shown related to *Leptomonas* rather than *Leishmania* (Figure 3 and supplementaty dendrogram). Two entries were determined from the *L. infantum* type strain MHOM/FR/78/LEM75: Y08020 and X85798. Both sequences clearly grouped with *L. tropica*, unlike all genuine *L. infantum* sequences in our analysis. This illustrates that one should be extremely careful when using sequences that have not been quality-controlled for species typing by comparison with other sequences from the same species, as this could result in incorrect typing outcomes, with potential adverse consequences for the patient.

As with all other assays based on the analysis of a single genomic locus, it is assumed that the relationship between the sequences mirror the relationship between the parasites. A first requirement to meet this objective is to avoid the use of paralogous sequences. This poses no problem in the case of *hsp70* because, although this gene is part of a gene family [18], the primers used in our protocol specifically amplify only one of the family members. Another problem is presented by the occasional inter-species hybrids that have been reported [19,34,37-40], and that are not necessarily evidenced in all genes. Nevertheless, such hybrids do not necessarily go undetected when looking at single genomic loci. For instance, isolate MHOM/PE/2006/CU00181, by AFLP clearly identified as a hybrid between *L. braziliensis* and *L. braziliensis* outliers [19], also holds an intermediate position in *hsp70* sequences (Figure 3 and supplementary dendrogram). On the contrary, MHOM/PE/2003/LH2538, also shown to be a hybrid between these two clusters, grouped with *L. braziliensis*. As this isolate derived a much smaller proportion of its genome from the *L. braziliensis* outliers, such classification is however not problematic. In natural *L. donovani–L. aethiopica* hybrids, both species alleles were present in all genes investigated, including *hsp70* [38]. In an *L. infantum–L. major* hybrid, both genomes were present [39], hence enabling to type the parasite based on a single gene assay. Theoretically, the chance of detecting inter-species recombinants increases as more loci are analysed, such as in multilocus-microsatellite, -sequence and -enzyme electrophoresis assays, but this also raises the cost and time of species typing. Given all currently available evidence on the potential of *hsp70* to detect reported inter-species hybrids, and as such hybrids are rare, we consider this a negligible setback of using the *hsp70* single-locus assay for routine species typing. Nevertheless, additional more variable genes may be able to perform better in discriminating within the complexes, but this would probably require a separate approach for each complex or subgenus [32,41,42].

Ultimately, the use of single-locus sequencing for species discrimination could be substituted by whole-genome sequencing [43]. With this method becoming

cheaper, it may soon be the standard in clinical studies. Comparison of whole-genome information could reveal clinically relevant intra-species differences, and has the highest chance of detecting recombination events. Such typing methodology could even make abstraction of the classical concept of typing at the taxonomic levels of species and species complexes [44]. It could open up a whole new era of relating strains on the basis of a selection of genes relevant for disease progression and treatment options, rather than based upon species definitions that may at times not correlate with clinical outcome. Nevertheless, whole-genome sequencing seems at present miles away from being implemented in everyday clinical practice, not only because of the complexity of data analysis, but also because it is complicated by the presence of human DNA contamination in clinical samples and related ethical issues. In the meantime there is a need for standardised methods to identify *Leishmania* strains. We advocate that *hsp70* has this potential: the gene is easily amplified, it can be analysed by sequencing in high-resource settings, and by simpler methods such as RFLP in limited-resource endemic areas [12,45]. If a global database of *hsp70* sequences from endemic regions were to be established, new sequences found in imported leishmaniasis could immediately be related to documented parasites, with clinical information on the patients from whom they were isolated. Such analysis could even be independent of currently used species boundaries, and provide adequate links based on genetic similarity irrespective of the species.

Conclusion

We present in this paper a complete validation and globally applicable standardised protocol for the use of *hsp70* sequences in *Leishmania* typing. As this validation includes a detailed comparison with other species identification methods currently used in various laboratories, we feel that implementation of the here presented typing strategy in a clinical diagnostic laboratory should be straight-forward, and could entail the validation of only the sequencing process itself rather than the actual species assignment, in view of this report. We intend to further promote our strategy, to identify additional strains with linked clinical information, and to establish a global database of circulating *Leishmania* parasites.

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References

1. Bailey MS, Lockwood DN. Cutaneous leishmaniasis. Clin Dermatol. 2007;25(2):203-11. http://dx.doi.org/10.1016/j.clindermatol.2006.05.008 PMid:17350500
2. Bailey MS, Green AD, Ellis CJ, O'Dempsey TJ, Beeching NJ, Lockwood DN, et al. Clinical guidelines for the management of cutaneous leishmaniasis in British military personnel. J R Army Med Corps. 2005;151(2):73-80. http://dx.doi.org/10.1136/jramc-151-02-03 PMid:16097110
3. Schwartz E, Hatz C, Blum J. New world cutaneous leishmaniasis in travellers. Lancet Infect Dis. 2006;6(6):342-9. http://dx.doi.org/10.1016/S1473-3099(06)70492-3
4. Blum JA, Hatz CF. Treatment of cutaneous leishmaniasis in travelers 2009. J Travel Med. 2009;16(2):123-31. http://dx.doi.org/10.1111/j.1708-8305.2008.00286.x PMid:19335813
5. Mitropoulos P, Konidas P, Durkin-Konidas M. New World cutaneous leishmaniasis: updated review of current and future diagnosis and treatment. J Am Acad Dermatol. 2010;63(2):309-22. http://dx.doi.org/10.1016/j.jaad.2009.06.088 PMid:20303613
6. Goto H, Lindoso JA. Current diagnosis and treatment of cutaneous and mucocutaneous leishmaniasis. Expert Rev Anti Infect Ther. 2010;8(4):419-33. http://dx.doi.org/10.1586/eri.10.19 PMid:20377337
7. Buffet PA, Rosenthal E, Gangneux JP, Lightburne E, Couppie P, Morizot G, et al. Therapy of leishmaniasis in France: consensus on proposed guidelines. Presse Med. 2011;40(2):173-84. http://dx.doi.org/10.1016/j.lpm.2010.09.023 PMid:21106333
8. Boecken G, Sunderkotter C, Bogdan C, Weitzel T, Fischer M, Muller A, et al. Diagnosis and therapy of cutaneous and mucocutaneous leishmaniasis in Germany. J Dtsch Dermatol Ges. 2011;9 Suppl 8:1-51. http://dx.doi.org/10.1111/j.1610-0379.2011.07820.x PMid:22050890
9. Fraga J, Montalvo AM, De Doncker S, Dujardin JC, Van der Auwera G. Phylogeny of Leishmania species based on the heat-shock protein 70 gene. Infect Genet Evol. 2010;10(2):238-45. http://dx.doi.org/10.1016/j.meegid.2009.11.007 PMid:19913110
10. Fraga J, Veland N, Montalvo AM, Praet N, Boggild AK, Valencia BM, et al. Accurate and rapid species typing from cutaneous and mucocutaneous leishmaniasis lesions of the New World. Diagn Microbiol Infect Dis. 2012;74(2):142-50. http://dx.doi.org/10.1016/j.diagmicrobio.2012.06.010 PMid:22819605
11. Montalvo AM, Fraga J, Monzote L, Montano I, De Doncker S, Dujardin JC, et al. Heat-shock protein 70 PCR-RFLP: a universal simple tool for Leishmania species discrimination in the New and Old World. Parasitology. 2010;137(8):1159-68 http://dx.doi.org/10.1017/S0031182010000089 PMid:20441679
12. Montalvo AM, Fraga J, Maes I, Dujardin JC, Van der Auwera G. Three new sensitive and specific heat-shock protein 70 PCRs for global Leishmania species identification. Eur J Clin Microbiol Infect Dis. 2012;31(7):1453-61. http://dx.doi.org/10.1007/s10096-011-1463-z PMid:22083340
13. Garcia L, Kindt A, Bermudez H, Llanos-Cuentas A, De Doncker S, Arévalo J, et al. Culture-independent species typing of neotropical Leishmania for clinical validation of a PCR-based assay targeting heat shock protein 70 genes. J Clin Microbiol. 2004;42(5):2294-7. http://dx.doi.org/10.1128/JCM.42.5.2294-2297.2004 PMid:15131217 PMCid:PMC404633
14. Marfurt J, Nasereddin A, Niederwieser I, Jaffe CL, Beck HP, Felger I. Identification and differentiation of Leishmania species in clinical samples by PCR amplification of the miniexon sequence and subsequent restriction fragment length polymorphism analysis. J Clin Microbiol. 2003;41(7):3147-53. http://dx.doi.org/10.1128/JCM.41.7.3147-3153.2003 PMid:12843055 PMCid:PMC165364
15. Schönian G, Nasereddin A, Dinse N, Schweynoch C, Schallig HD, Presber W, et al. PCR diagnosis and characterization of Leishmania in local and imported clinical samples. Diagn Microbiol Infect Dis. 2003;47(1):349-58 http://dx.doi.org/10.1016/S0732-8893(03)00093-2
16. Stevenson LG, Fedorko DP, Zelazny AM. An enhanced method for the identification of Leishmania spp. using real-time polymerase chain reaction and sequence analysis of the 7SL

RNA gene region. Diagn Microbiol Infect Dis. 2010;66(4):432-5. http://dx.doi.org/10.1016/j.diagmicrobio.2009.11.005 PMid:20226334 PMCid:PMC2856081

17. Roelfsema JH, Nozari N, Herremans T, Kortbeek LM, Pinelli E. Evaluation and improvement of two PCR targets in molecular typing of clinical samples of Leishmania patients. Exp Parasitol. 2011;127(1):36-41. http://dx.doi.org/10.1016/j.exppara.2010.06.024 PMid:20599989
18. Folgueira C, Ca-avate C, Chicharro C, Requena JM. Genomic organization and expression of the HSP70 locus in New and Old World Leishmania species. Parasitology. 2007;134(Pt 3):369-77. http://dx.doi.org/10.1017/S0031182006001570 PMid:17054823
19. Odiwuor S, Veland N, Maes I, Arévalo J, Dujardin JC, Van der Auwera G. Evolution of the Leishmania braziliensis species complex from amplified fragment length polymorphisms, and clinical implications. Infect Genet Evol. 2012;12(8):1994-2002. http://dx.doi.org/10.1016/j.meegid.2012.03.028 PMid:22516226
20. Deborggraeve S, Laurent T, Espinosa D, Van der Auwera G, Mbuchi M, Wasunna M, et al. A simplified and standardized polymerase chain reaction format for the diagnosis of leishmaniasis. J Infect Dis. 2008;198(10):1565-72. http://dx.doi.org/10.1086/592509 PMid:18816188
21. Odiwuor S, Muia A, Magiri C, Maes I, Kirigi G, Dujardin JC, et al. Identification of Leishmania tropica from micro-foci of cutaneous leishmaniasis in the Kenyan Rift Valley. Pathog Glob Health. 2012;106(3):159-65. http://dx.doi.org/10.1179/2047773212Y.0000000015 PMid:23265373
22. DNA Baser Sequence Assembler. Nucleotide ambiguity code. Pitesti: Heracle BioSoft S.R.L. Available from: http://www.dnabaser.com/articles/IUPAC%20ambiguity%20codes.html
23. Zhang CY, Lu XJ, Du XQ, Jian J, Shu L, Ma Y. Phylogenetic and evolutionary analysis of Chinese Leishmania isolates based on multilocus sequence typing. PLoS One. 2013;8(4):e63124. http://dx.doi.org/10.1371/journal.pone.0063124 PMid:23646184 PMCid:PMC3639960
24. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28(10):2731-9. http://dx.doi.org/10.1093/molbev/msr121 PMid:21546353 PMCid:PMC3203626
25. Mauricio IL, Stothard JR, Miles MA. The strange case of Leishmania chagasi. Parasitol Today. 2000;16(5):188-9. http://dx.doi.org/10.1016/S0169-4758(00)01637-9
26. Lukeš J, Mauricio IL, Schönian G, Dujardin JC, Soteriadou K, Dedet JP, et al. Evolutionary and geographical history of the Leishmania donovani complex with a revision of current taxonomy. Proc Natl Acad Sci USA. 2007;104(22):9375-80. http://dx.doi.org/10.1073/pnas.0703678104 PMid:17517634 PMCid:PMC1890502
27. Ba-uls AL, Hide M, Tibayrenc M. Evolutionary genetics and molecular diagnosis of Leishmania species. Trans R Soc Trop Med Hyg. 2002;96 Suppl 1:S9-13. http://dx.doi.org/10.1016/S0035-9203(02)90045-3
28. Bañuls AL, Jonquieres R, Guerrini F, Le Pont F, Barrera C, Espinel I, et al. Genetic analysis of Leishmania parasites in Ecuador: are Leishmania (Viannia) panamensis and Leishmania (V.) guyanensis distinct taxa? Am J Trop Med Hyg. 1999;61(5):838-45.
29. Cupolillo E, Grimaldi G Jr, Momen H. A general classification of New World Leishmania using numerical zymotaxonomy. Am J Trop Med Hyg. 1994;50(3):296-311. PMid:8147488
30. Srivastava P, Prajapati VK, Vanaerschot M, Van der Auwera G, Dujardin JC, Sundar S. Detection of *Leptomonas* sp. parasites in clinical isolates of Kala-azar patients from India. Infect Genet Evol. 2010;10(7):1145-50. http://dx.doi.org/10.1016/j.meegid.2010.07.009 PMid:20633704 PMCid:PMC2933273
31. Odiwuor S, Vuylsteke M, De Doncker S, Maes I, Mbuchi M, Dujardin JC, et al. Leishmania AFLP: paving the way towards improved molecular assays and markers of diversity. Infect Genet Evol. 2011;11(5):960-7. http://dx.doi.org/10.1016/j.meegid.2011.03.008 PMid:21439405
32. Oddone R, Schweynoch C, Schönian G, de Sousa CS, Cupolillo E, Espinosa D, et al. Development of a multilocus microsatellite typing approach for discriminating strains of Leishmania (Viannia) species. J Clin Microbiol. 2009;47(9):2818-25. http://dx.doi.org/10.1128/JCM.00645-09 PMid:19587302 PMCid:PMC2738093
33. Veland N, Boggild AK, Valencia C, Valencia BM, Llanos-Cuentas A, Van der Auwera G, et al. Leishmania (Viannia) species identification on clinical samples from cutaneous leishmaniasis patients in Peru: assessment of a molecular stepwise approach. J Clin Microbiol. 2012;50(2):495-8. http://dx.doi.org/10.1128/JCM.05061-11 PMid:22116151 PMCid:PMC3264178
34. Nolder D, Roncal N, Davies CR, Llanos-Cuentas A, Miles MA. Multiple hybrid genotypes of Leishmania (Viannia) in a focus of mucocutaneous leishmaniasis. Am J Trop Med Hyg. 2007;76(3):573-8. PMid:17360886
35. Bañuls AL, Dujardin JC, Guerrini F, De Doncker S, Jacquet D, Arevalo J, et al. Is Leishmania (Viannia) peruviana a distinct species? A MLEE/RAPD evolutionary genetics answer. J Eukaryot Microbiol. 2000;47(3):197-207. http://dx.doi.org/10.1111/j.1550-7408.2000.tb00039.x
36. World Health Organization (WHO). Control of the leishmaniasis: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22-26 March 2010. Geneva, Switzerland: WHO; 2010. Available from: http://whqlibdoc.who.int/trs/WHO_TRS_949_eng.pdf
37. Belli AA, Miles MA, Kelly JM. A putative Leishmania panamensis/Leishmania braziliensis hybrid is a causative agent of human cutaneous leishmaniasis in Nicaragua. Parasitology. 1994;109(Pt 4):435-42. http://dx.doi.org/10.1017/S0031182000080689 PMid:7800411
38. Odiwuor S, De Doncker S, Maes I, Dujardin JC, Van der Auwera G. Natural Leishmania donovani/Leishmania aethiopica hybrids identified from Ethiopia. Infect Genet Evol. 2011;11(8):2113-8. http://dx.doi.org/10.1016/j.meegid.2011.04.026 PMid:21558020
39. Ravel C, Cortes S, Pratlong F, Morio F, Dedet JP, Campino L. First report of genetic hybrids between two very divergent Leishmania species: Leishmania infantum and Leishmania major. Int J Parasitol. 2006;36(13):1383-8. http://dx.doi.org/10.1016/j.ijpara.2006.06.019 PMid:16930606
40. Delgado O, Cupolillo E, Bonfante-Garrido R, Silva S, Belfort E, Grimaldi JG, et al. Cutaneous leishmaniasis in Venezuela caused by infection with a new hybrid between Leishmania (Viannia) braziliensis and L. (V.) guyanensis. Mem Inst Oswaldo Cruz. 1997;92(5):581-2. http://dx.doi.org/10.1590/S0074-02761997000500002 PMid:9566221
41. Kuhls K, Keilonat L, Ochsenreither S, Schaar M, Schweynoch C, Presber W, et al. Multilocus microsatellite typing (MLMT) reveals genetically isolated populations between and within the main endemic regions of visceral leishmaniasis. Microbes Infect. 2007;9(3):334-43. http://dx.doi.org/10.1016/j.micinf.2006.12.009 PMid:17307010
42. Zemanová E, Jirku M, Mauricio IL, Horák A, Miles MA, Lukeš J. The Leishmania donovani complex: genotypes of five metabolic enzymes (ICD, ME, MPI, G6PDH, and FH), new targets for multilocus sequence typing. Int J Parasitol. 2007;37(2):149-60. http://dx.doi.org/10.1016/j.ijpara.2006.08.008 PMid:17027989
43. Downing T, Imamura H, Decuypere S, Clark TG, Coombs GH, Cotton JA, et al. Whole genome sequencing of multiple Leishmania donovani clinical isolates provides insights into population structure and mechanisms of drug resistance. Genome Res. 2011;21(12):2143-56. http://dx.doi.org/10.1101/gr.123430.111 PMid:22038251 PMCid:PMC3227103
44. Van der Auwera G, Fraga J, Montalvo AM, Dujardin JC. Leishmania taxonomy up for promotion? Trends Parasitol. 2011;27(2):49-50. http://dx.doi.org/10.1016/j.pt.2010.11.007 PMid:21147036
45. da Silva LA, dos Santos de Sousa C, da Graça GC, Porrozzi R, Cupolillo E. Sequence analysis and PCR-RFLP profiling of the hsp70 gene as a valuable tool for identifying Leishmania species associated with human leishmaniasis in Brazil. Infect Genet Evol. 2010;10(1):77-83 http://dx.doi.org/10.1016/j.meegid.2009.11.001 PMid:19913112
46. Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis Worldwide and Global Estimates of Its Incidence. PLoS One. 2012;7(5):e35671. http://dx.doi.org/10.1371/journal.pone.0035671 PMid:22693548 PMCid:PMC3365071

Leishmaniasis in the era of tumor necrosis factor alpha antagonist therapy – a research agenda for Europe

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A number of published case reports suggest an association of tumor necrosis factor (TNF) alpha antagonist use and manifest leishmaniasis. Despite increasing popularity of antagonising TNF alpha for the treatment of autoimmune disorders, systematic research on the risk of opportunistic leishmaniasis in patients receiving these drugs is lacking. This perspective identifies areas of uncertainty regarding the safety profile of TNF alpha antagonist drugs and their clinical use in patients at risk of leishmaniasis. Then, we reflect on how current pharmacovigilance activities in Europe could be enhanced to help reduce these uncertainties. Our aim is to stimulate a debate about this important drug safety issue with potential consequences for patients receiving TNF alpha antagonists living in or travelling to areas endemic for leishmaniasis.

Introduction

Since their introduction about a decade ago, tumour necrosis factor (TNF) alpha antagonist drugs have greatly improved the clinical management of autoimmune disorders and are now widely used in rheumatology. The flipside of the coin is an increase in infectious disease risk in patients treated with these drugs. Both reactivation of latent infections and increased susceptibility to new infections have been observed in patients receiving TNF alpha antagonist therapy. Depending on their mode of action, the TNF alpha antagonist drugs most widely used can be distinguished in two major groups: monoclonal antibodies, such as infliximab and adalimumab, and the receptor construct etanercept [1].

Leishmaniasis is endemic in large parts of southern Europe [2-5] where studies suggest a focal prevalence of latent infection of up to 53% in the adult population [5]. Two species, *Leishmania infantum* and *L. tropica* are transmitted around the Mediterranean basin by the bite of sandfly species (order Diptera, family Psychodidae, subfamily Phlebotominae) [4]. The spectrum of manifest infection ranges from a well localised, self-healing cutaneous papule or ulceration to rapidly fatal visceral disease. Host immunity is known to be a key determinant of the clinical manifestation and outcome. Immuno¬suppression, in particular conditions that alter the type 1 helper T-cell-mediated immune response of which TNF alpha is an important component, is generally regarded as a risk factor for manifest leishmaniasis and has been identified as a major contributor to its re-emergence in Europe [4]. A number of published case reports suggest an association of TNF alpha antagonist use and manifest leishmaniasis [6, 7]. Despite this, no systematic research on opportunistic leishmaniasis in patients receiving TNF alpha antagonists has been conducted.

Based on a brief summary of published information on opportunistic leishmaniasis and use of TNF alpha antagonists, this perspective identifies areas of uncertainty regarding their safety profile and their clinical use in patients at risk of leishmaniasis. We then reflect on how current pharmacovigilance activities in Europe could be enhanced to help reduce these uncertainties. Our aim is to stimulate a debate about this important drug safety issue with potential consequences for patients receiving TNF alpha antagonists living in or travelling to areas endemic for leishmaniasis.

Current knowledge on tumor necrosis factor alpha antagonist therapy and leishmaniasis

Published research on therapy with TNF alpha antagonists and leishmaniasis is scarce and limited to case reports and case series. We recently reviewed the literature [6] and identified 19 descriptions of patients with leishmaniasis while receiving TNF antagonists, published between 2004 and 2011. Nearly all of the identified cases (18/19) occurred in Europe, and more than two thirds were published in 2010-11. The vast majority were reported from endemic regions of southern Europe (14/18) whereas fewer occurred after travel to and migration from endemic areas (4/18). The reported time period from initiation of treatment to onset of leishmaniasis ranged from 0.5 to 48 months, consistent with both reactivation of latent infection and increased susceptibility to new infection.

Only one reported case was treated with etanercept while all others received either infliximab or

Box 1

Aims and objectives of a coordinated research effort on opportunistic leishmaniasis in patients treated with tumor necrosis factor alpha antagonists in Europe

Aims

- Estimate the impact of TNF alpha use on the occurrence of leishmaniasis in Europe.
- Reduce leishmaniasis risk by tailoring TNF alpha antagonist therapy to individual risk profile.
- Optimise anti-parasitic and anti-rheumatic therapy in patients with opportunistic leishmaniasis.

Objectives

- Define the absolute risk of leishmaniasis in patients treated with TNF alpha antagonists. Define the relative risk of leishmaniasis in patients treated with TNF alpha antagonists
  - with respect to classic immunosuppressive regimens; and
  - with respect to type of TNF alpha antagonist used.
- Define the proportionate contribution of new infection and reactivation towards the burden of leishmaniasis in patients treated with TNF alpha antagonists
  - overall; and
  - by type of TNF alpha antagonist used.
- Define the role of screening (serology, intradermal leishmanin) in patients treated with TNF alpha antagonists for clinical decision making by determining their predictive values
  - with regard to the overall risk of leishmaniasis; and
  - depending on the type of TNF alpha antagonist used.
- Identify groups at risk of and factors associated with developing leishmaniasis during TNF alpha antagonist therapy namely
  - environmental/behavioural (e.g. companion animals, travel, region of residence) factors;
  - host factors (e.g. co-morbidity); and
  - others (e.g. immunosuppressive co-medication).
- Optimise therapy of leishmaniasis as complication of TNF alpha therapy through either
  - interruption or continuation of TNF alpha antagonist; and
  - systemic or local treatment of cutaneous leishmaniasis.
- Explore whether TNF alpha antagonists can be restarted after cure of leishmaniasis and if
  - there is a need for potential modifications of this therapy;
  - treatment can be continued with identical or alternative TNF alpha antagonist;
  - immunosuppressive co-medication needs to be modified; and
  - anti-parasitic maintenance therapy is necessary to prevent relapses (secondary prevention).

TNF: tumor necrosis factor.

adalimumab. This was surprising, since prescription data from the countries where the cases occurred showed that each of the three drugs was prescribed about equally often. These findings suggest that opportunistic leishmaniasis is more likely to occur in patients receiving TNF alpha monoclonal antibodies than in patients treated with etanercept. This interpretation is supported by similar findings for tuberculosis [8, 9] and by studies in mice [10] and in vitro [11].

Interestingly, published case reports describe various approaches including discontinuation as well as continuation of TNF alpha antagonist therapy during and after anti-parasitic treatment while using the same or a different type of drug for ongoing TNF alpha antagonisation [6]. Their number, however, is too small and the data and observation period reported too heterogeneous to decide whether one or the other approach is associated with an increased risk of recurrent leishmaniasis.

Although it provided us with a hypothesis on the possible differences of leishmaniasis risk by type of TNF alpha antagonist used, our analysis of case reports is limited by potential publication bias and confounding underlying the observed associations. Moreover, the number of published cases is likely to represent only a small fraction of patients treated with TNF alpha antagonists with opportunistic leishmaniasis, leaving the magnitude of this drug safety issue unclear. Hence more systematic research is needed to improve our

understanding of opportunistic leishmaniasis in these patients.

Unsolved questions

The main areas of uncertainty pertaining to opportunistic leishmaniasis in patients receiving TNF alpha antagonist therapy, to be addressed by future research, are outlined below and summarised in Box 1.

There is a clear need to estimate the potential impact of TNF alpha antagonist use on the incidence of leishmaniasis. This information is required to clarify to what extent there is a need to further investigate and corroborate this association and to justify funding of research activities that aim at elucidating risk factors for leishmaniasis among those treated with TNF alpha antagonists.

Any study on risk factors should attempt to answer whether there truly is a difference in risk of leishmaniasis depending on the type of TNF alpha antagonist used. Once supported by evidence from prospective studies, physicians (mainly clinical rheumatologists) could directly translate this knowledge into practice by choosing the TNF alpha antagonist with the lowest risk for opportunistic leishmaniasis for patients living in or travelling to endemic areas. Moreover, the role of behavioural and host-associated risk factors for clinically manifest leishmaniasis under TNF alpha antagonist therapy should be evaluated as knowledge gained could be relevant in clinical decision-making or counselling of patients.

Once a risk difference for the various types of TNF alpha antagonists has been established, it will be important to clarify if this is due to reactivation of latent leishmaniasis or increased susceptibility to new infection.

It needs to be determined whether screening for latent infection before initiation of TNF alpha antagonist therapy can play a role in preventing opportunistic leishmaniasis. Studies evaluating the predictive values of serologic and intradermal leishmanin testing have great potential to inform clinical decision-making and will equally contribute to our understanding of latent versus newly acquired infection for the onset of opportunistic leishmaniasis in patients receiving anti-TNF alpha therapy.

Apart from defining risk factors, there is a need for clinical research to improve medical care for patients suffering from opportunistic leishmaniasis. In particular, better evidence on whether TNF alpha antagonists can be continued in patients with clinically active leishmaniasis, or after its cure, is of high clinical relevance since many of these patients depend on TNF alpha antagonists to adequately control the underlying autoimmune disease. Besides, future research has to address whether there is a need for secondary prevention of opportunistic leishmaniasis in patients that require sustained antagonisation of TNF alpha. For instance, type and duration as well as indicators for the initiation of anti-parasitic maintenance therapy need to be established. Of note, anti-TNF alpha therapy has been continued or re-initiated in several cases of opportunistic leishmaniasis without subsequent relapse [6, 12], indicating that a first episode does not justify long-term anti-parasitic treatment.

Finally, we have to learn more about the risk of generalised infection secondary to localised cutaneous leishmaniasis in patients receiving TNF alpha antagonists. Published case reports indicate that most clinicians fear this complication and opt for systemic anti-parasitic therapy. This, however, has to be balanced against increased toxicity when compared to local treatment which could be an option in a setting where close monitoring of the patient is ensured.

Research challenges concerning opportunistic leishmaniasis and tumor necrosis factor alpha antagonist therapy

Traditionally, spontaneous notification of adverse events to national pharmacovigilance systems has been used to define a drug's safety profile with regard to rare events and long-term effects that may have remained undetected during pre-licensure clinical trials. This approach, however, is subject to significant underreporting, does not allow analysing the number of reported events relative to the number of subjects treated, and cannot provide an estimate of the baseline risk.

In response to these shortcomings and with the support of the pharmaceutical industry, national rheumatology societies in several European countries have initiated national drug registers as post-marketing surveillance tools [13]. Many of these were put in place simultaneously to the licensing of the first TNF alpha antagonists about a decade ago. Their methodologies were recently reviewed in detail by Zink et al. [13]. In brief, selected care providers in rheumatology enrol patients receiving TNF alpha antagonists or other biotherapies into epidemiological cohort studies or into registers, thus overcoming some of the limitations of traditional pharmacovigilance activities.

Although a huge improvement compared to the traditional reporting systems, it has been called into question whether national drug registers cover a sufficient patient base to detect rare adverse events. Zink et al. estimate that events with an incidence equal or below one in 1,000 patient-years may not be adequately detected by this approach [13]. With regard to leishmaniasis, we observed that the French register only detected half of leishmaniasis cases reported in publications from France over a defined time period [6], implying that underreporting may not be adequately addressed by the national registers. Besides, none of these registers had detected sufficient numbers of cases to allow analysing whether leishmaniasis risk

Table

Components of a coordinated research effort on opportunistic leishmaniasis in Europe

Component	Function/role
Coordinating board hosted by European institution including representatives from existing European network structures in clinical parasitology/ tropical medicine, dermatology, rheumatology, infectious diseases and supported by public health experts	• Design of post-authorisation safety studies (cohort studies) and observational clinical studies • Ensure uniform methodology and data collection • Enhance national drug registers in countries endemic for leishmaniasis (where necessary) • Host online platform for case registration by non-endemic countries (i.e. travellers, migrants) • Increase awareness among policy-makers and professional societies
National drug registers hosted by professional societies in rheumatology	• Implementation of cohort studies • Increase awareness/ case reporting among clinicians
Institutions specialised in diagnosis and therapy of leishmaniasis, represented through existing European network structures in clinical parasitology/ tropical medicine, dermatology, infectious diseases	• Implementation of clinical studies • Detection of cases outside endemic countries • Increase awareness / case reporting among clinicians
Pharmaceutical industry	• Data on drug sales with sufficient detail allowing geographically high resolution in leishmaniasis endemic areas • Funding

varies by type of TNF alpha antagonist. As pointed out before, this was possible by combining published information from different European countries. Based hereon, it becomes obvious that fragmentation of data at national level hampers a better description of the safety profile of TNF alpha antagonists with regard to rare opportunistic infections. Therefore, evaluation of data across registers and at European level has been suggested to increase the ability to detect such events [13]. Although this approach has potential to contribute to our understanding of opportunistic leishmaniasis, a range of challenges remain.

Firstly, transmission of leishmaniasis occurs geographically in confined foci since it is bound to the presence of the phlebotomine vector. This is reflected in the large variation of sero-prevalence reported in regions around the Mediterranean basin [2, 3, 5]. Hence, to further advance our knowledge on opportunistic leishmaniasis in patients receiving biotherapies, it will be crucial that national drug registers adequately cover the population in areas where transmission occurs. The variation in transmission risk also implies that estimates of the absolute risk of leishmaniasis in TNF alpha antagonist users calculated from register data will vary according to the proportion of subjects live in endemic regions enrolled in the register.

Secondly, European national drug registers on the safety of TNF alpha antagonist drugs and other biotherapies use different methodology and do not record data in a uniform manner. To facilitate collaboration between national drug registers and to allow supranational data analyses, harmonisation of European registers is a prerequisite. A uniform reporting scheme has so far only been adopted by Great Britain, Sweden, and Germany, but not by France and Spain, countries where leishmaniasis is endemic [13].

Thirdly, it is unclear, whether all countries with leishmaniasis endemic regions have an active national drug register or cohort. For instance, from a number of countries in the Balkan region, there are no peer-reviewed publications available on PubMed reporting on such activities.

The Table outlines potential components of a coordinated research effort on opportunistic leishmaniasis in Europe and Box 2 the resulting strengths.

Conclusions

Although the initiation of national registers with support of the pharmaceutical industry was a major step forward in better defining the safety profile of TNF alpha antagonist drugs and other biotherapies, there are shortcomings of this system with regard to opportunistic leishmaniasis. Crucial for improved case detection and reduced underreporting will be a closer link between existing national drug registers and institutions specialised in the diagnosis and therapy of leishmaniasis, e.g. tropical medicine institutes, dermatology departments, etc. This will likely enable the detection of a considerable number of additional cases of opportunistic leishmaniasis that are either directly seen at these institutions or were suspected elsewhere but laboratory confirmed there. Using existing network structures in Europe such as TropNet (www.tropnet.net) and EuroTravNet (www.istm.org/eurotravnet/

Box 2

Strengths of a coordinated research effort at European level on opportunistic leishmaniasis in patients treated with tumor necrosis factor alpha antagonists

- Impact – a coordinated research effort will allow estimating the public health impact of tumor necrosis factor alpha (TNF) alpha antagonists on leishmaniasis in Europe.
- Statistical power – a coordinated research effort will increase statistical power through improved case detection and more comprehensive coverage of populations in endemic regions.
- Defragmentation – a coordinated research effort will ensure that data available is not restricted to single countries.
- Standardisation – a coordinated research effort will improve database quality for supra-national analysis through uniform methodology, reporting scheme, and common denominators (e.g. data on drug sales).
- Flexibility – a coordinated research effort will allow rapid incorporation of newly emerging biotherapies and rare infections other than leishmaniasis.

main.html) could facilitate the creation of such links. An extended network of clinical specialists in rheumatology, infectious diseases, tropical medicine, and dermatology will be necessary to answer the research questions pertaining to the clinical management of patients with opportunistic leishmaniasis set out in this paper.

Prospective data from a well-defined population at risk of acquiring or being latently infected with leishmania will be needed to obtain meaningful estimates of the incidence of clinical manifest infection that can be attributed to TNF alpha antagonist use and to evaluate the clinical role of screening for latent infection. This can only be achieved through focused post-marketing safety studies that make use of large cohorts in areas endemic for leishmaniasis. To ensure uniform methodology and data collection, such activity should be designed and coordinated at European level before being implemented by the existing national registers. In European countries endemic for leishmaniasis but without a well-functioning register or cohort, this may require enhancing or newly creating such structures. Taken together, these activities will need adequate funding and most importantly, substantial collaboration across disciplines including input from epidemiologists and public health experts.

Conflict of interest

None declared.

References

1. Scallon B, Cai A, Solowski N, Rosenberg A, Song XY, Shealy D, et al. Binding and functional comparisons of two types of tumor necrosis factor antagonists. J Pharmacol Exp Ther. 2002;301(2):418-26. http://dx.doi.org/10.1124/jpet.301.2.418
2. Diza E, Kansouzidou A, Gerou S, Vezyri E, Metallidis S, Antoniadis A. Leishmaniases in northern greece: Seroprevalence of the infection and incidence of the disease during the period 2001-2006. Eur J Clin Microbiol Infect Dis. 2008;27(10):997-1003. http://dx.doi.org/10.1007/s10096-008-0538-y PMid:18512088
3. Federico G, Damiano F, Caldarola G, Fantini C, Fiocchi V, Ortona L. A seroepidemiological survey on leishmania infantum infection. Eur J Epidemiol. 1991;7(4):380-3. http://dx.doi.org/10.1007/BF00145003
4. Ready PD. Leishmaniasis emergence in europe. Euro Surveill. 2010;15(10):19505. (10):pii=19505. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19505
5. Moral L, Rubio EM, Moya M. A leishmanin skin test survey in the human population of l'alacanti region (spain): Implications for the epidemiology of leishmania infantum infection in southern europe. Trans R Soc Trop Med Hyg. 2002;96(2):129-32. http://dx.doi.org/10.1016/S0035-9203(02)90278-6
6. Zanger P, Kötter I, Kremsner PG, Gabrysch S. Tumor necrosis factor alpha antagonist drugs and leishmaniasis in europe. Clin Microbiol Infect. 2012;18(7):670-6. http://dx.doi.org/10.1111/j.1469-0691.2011.03674.x PMid:21985586
7. Xynos ID, Tektonidou MG, Pikazis D, Sipsas NV. Leishmaniasis, autoimmune rheumatic disease, and anti-tumor necrosis factor therapy, europe. Emerg Infect Dis. 2009;15(6):956-9. http://dx.doi.org/10.3201/eid1506.090101 PMid:19523302 PMCid:PMC2727307
8. Wallis RS, Broder MS, Wong JY, Hanson ME, Beenhouwer DO. Granulomatous infectious diseases associated with tumor necrosis factor antagonists. Clin Infect Dis. 2004;38(9):1261-5. http://dx.doi.org/10.1086/383317 PMid:15127338
9. Tubach F, Salmon D, Ravaud P, Allanore Y, Goupille P, Bréban M, et al. Risk of tuberculosis is higher with anti-tumor necrosis factor monoclonal antibody therapy than with soluble tumor necrosis factor receptor therapy: The three-year prospective french research axed on tolerance of biotherapies registry. Arthritis Rheum. 2009;60(7):1884-94. http://dx.doi.org/10.1002/art.24632 PMid:19565495 PMCid:PMC2921546
10. Allenbach C, Launois P, Mueller C, Tacchini-Cottier F. An essential role for transmembrane tnf in the resolution of the inflammatory lesion induced by leishmania major infection. Eur J Immunol. 2008;38(3):720-31. http://dx.doi.org/10.1002/eji.200737662 PMid:18266271
11. Lohmann-Matthes ML, Luttig B, Hockertz S. Involvement of membrane-associated tnf in the killing of leishmania donovani parasites by macrophages. Behring Inst Mitt. 1991;88:125-32. PMid:2049029
12. Zanger P, Kötter I, Raible A, Gelanew T, Schonian G, Kremsner PG. Successful treatment of cutaneous leishmaniasis caused by leishmania aethiopica with liposomal amphothericin b in an immunocompromised traveler returning from eritrea. Am J Trop Med Hyg. 2011;84(5):692-4. http://dx.doi.org/10.4269/ajtmh.2011.10-0712 PMid:21540377 PMCid:PMC3083735
13. Zink A, Askling J, Dixon WG, Klareskog L, Silman AJ, Symmons DP. European biologicals registers: Methodology, selected results and perspectives. Ann Rheum Dis. 2009;68(8):1240-6. http://dx.doi.org/10.1136/ard.2008.091926 PMid:18647854

The role of indigenous phlebotomine sandflies and mammals in the spreading of leishmaniasis agents in the Mediterranean region

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An updated view of the establishment and spread of the leishmaniases in Europe is presented, mostly with respect to newly emerging and re-emerging foci and the incrimination of neglected as well as new reservoir hosts. At the same time, a concept of specific versus permissive vectors reassesses the potential role of various sandfly species in *Leishmania* transmission and considers the risk of introduction of exotic *Leishmania* species in Europe. The leishmaniases are dynamic diseases and the circumstances of transmission are continually changing in relation to environmental, demographic and human behavioural factors. Changes in the habitat of the natural hosts and vectors, immunosuppressive conditions (like infection with human immunodeficiency virus (HIV) or organ transplantation-associated therapies in humans) and the consequences of war, all contribute to the transformation of the epidemiology of leishmaniasis. Such changes should be considered when studying the spread of the disease throughout Europe for targeted control measures to safeguard public health.

Endemic burden of human leishmaniases in Europe

The leishmaniases are vector-borne diseases that have been endemic in southern Europe for centuries. They are transmitted by the bite of phlebotomine sandflies belonging to the genus *Phlebotomus* and most often exhibit one of two endemic clinical entities in humans, zoonotic visceral leishmaniasis (VL) and sporadic cutaneous leishmaniasis (CL). Four *Leishmania* species occur in the Mediterranean basin: *L. infantum*, the most frequent species, causing both VL and CL; *L. major*, occurring in North Africa and the Middle East, causing CL; *L. tropica*, found in Greece, Turkey, the Middle East and North Africa, causing CL; *L. donovani*, recently introduced in Cyprus, causing both VL and CL. These species are able to spread in new geographical areas where suitable sandfly vectors are present in sufficient numbers and under favourable ecological conditions. The risk is greater when the anthroponotic species, *L. tropica* and *L. donovani*, are involved, because reservoir hosts other than human are not required to complete the transmission cycle. It is widely accepted that the leishmaniases are dynamic diseases. As the conditions of transmission change (environmental, demographic, human behaviour and health), epidemiological studies and control measures to safeguard public health should be adapted for the application of successful monitoring measures.

The incidence of zoonotic VL caused by *L. infantum* in humans is relatively low (0.02–0.49/100,000 in the general population) with an average of about 700 clinical cases reported each year in southern Europe [1,2]. However, outbreaks or recrudescence may occur periodically in foci like the new focus in Spain where incidences increased up to 56 per 100,000 [3]. Incidence rates of sporadic CL, although generally accepted as high (in the range of one to a few 100), are not available because of poor notification.

Visceral leishmaniasis constitutes a problem in immunocompromised individuals. Starting from the early 1990s, the impact of co-infections with *Leishmania* and human immunodeficiency virus (HIV) was recognised as an alarming problem by international health authorities. Cases were reported from 35 countries worldwide, mostly in south-western Europe (France, Italy, Portugal and Spain), showing an association between the HIV pandemic and the zoonotic entity of VL caused by *L. infantum*. The cumulative number of co-infections recorded by a surveillance network from the World Health Organization (WHO) and UNAIDS was 692 by early 1995, 965 by 1998, and 1911 by early 2001. Spanish cases accounted for 57% of all co-infections worldwide, probably reflecting a relatively large area of *Leishmania*/HIV sympatry in Spain. The demonstration

of unusual modes of anthroponotic transmission (i.e. by syringe exchange) and the high rate of relapses following anti-leishmanial treatments, were alarming features indicating a trend toward an even higher incidence. By the end of the 1990s, however, several reports indicated that the *L. infantum*/HIV epidemic peak declined due to the introduction of the highly active antiretroviral therapy (HAART) which not only reduced the number of new cases of co-infection, but also the rate of VL relapses in individuals with restored immunological parameters (i.e. with a CD4+ count>200/µL). Currently, very few HIV-infected individuals with clinical VL are recorded annually in southern Europe, mainly in patients with acquired immunodeficiency syndrome (AIDS) who are unresponsive to HAART. Other countries, such as Germany, Greece, Switzerland and the United Kingdom currently report sporadic imported cases [4].

The spread of leishmaniases may be enhanced by globalisation, climatic change and other conditions which allow the parasite and its vectors to spread in space and time. Studies to foresee the effect of such changes have been undertaken by the EDENext EU FP7 (www.edenext.eu) project in order to safeguard unaffected areas by preventing the introduction, establishment and spread of the *Leishmania* pathogen and its vectors. Data on the disease and its spatial distribution in Europe and the Mediterranean basin were composed and made accessible online to researchers and public health officials (www.edenextdata.com) so that knowledge-based decisions could be made for monitoring the disease.

Increasing evidence suggests that elevated rates of asymptomatic *L. infantum* carriers are an indicator of the intense *Leishmania* circulation in southern Europe. Infection prevalences, as high as 10–47% in particular age groups, were recorded in healthy individuals from endemic foci of France, Greece, Italy and Spain by traditional and molecular methods [5]. On the other hand, CL cases, autochthonous or imported, may not seek treatment, especially in cases with mild clinical forms or older people and illegal immigrants. When the disease is introduced in new areas, physicians who are not familiar with the problem often do not consider CL in their differential diagnosis, and hence appropriate treatment is not given, allowing parasite circulation.

Literature search strategy

Scientific literature for the purposes of this review was searched in May 2012 by all participating co-authors, sourcing the PubMed and Scopus databases. An electronic search was conducted among articles from 1970 until recently, as well as a few relevant older references, cross-referencing the following combination of keywords: 'leishmaniasis' and '*Phlebotomus*' and 'emergence' and 'reservoirs'. Titles relevant to the scope of this review (an updated view of the establishment and spread of the leishmaniases in Europe with respect to newly emerging and re-emerging foci and the incrimination of neglected and new reservoir hosts) were obtained in full text and selected for inclusion. Unpublished data and titles from non-peer-reviewed literature were not considered.

Sandfly vectors

The vectorial status of phlebotomine sandfly vectors of *Leishmania* in Europe and the Mediterranean area was recently reviewed [6,7] and new species have recently been incriminated [8,9]. Nine proven, or potential, vector species (*Phlebotomus ariasi, P. perniciosus, P. perfiliewi, P. neglectus, P. tobbi, P. kandelaki, P. balcanicus, P. papatasi* and *P. sergenti*) are indigenous in Europe. In addition, species of questionable taxonomic status (*P. similis, P. syriacus*) or of possible but unproven vectorial capacity (*P. mascittii*) should be further studied. Traditionally, the limited number of known vectors was explained by the inability of some sandfly species to support the development of infective stages in their gut or because of unidentified ecological contact with reservoir hosts [6]. However, experimental infections, under laboratory conditions, revealed that only two tested sandfly species, *P. papatasi* and *P. sergenti,* are 'specific vectors'; they allow only the maturation of a single *Leishmania* species they transmit in nature (*L. major* and *L. tropica*, respectively) and do not support development of other *Leishmania* species [10-13]. Nonetheless, most sandfly species tested to date support development of multiple *Leishmania* spp. allowing them to mature in their midguts, thus falling into a category of the permissive vectors [9]. These species are members of the *Larroussius* and *Adlerius* subgenera, namely *P. perniciosus, P. arabicus* and *P. halepensis*. Although in nature, *P. perniciosus* is the proven vector of *L. infantum* in the western Mediterranean, P. arabicus the proven vector of *L. tropica* in Israel and *P. halepensis* the suspected vector of *L. infantum* in the Caucasus region, all three species supported full development of *L. major* and *L. tropica* under experimental conditions [14-16].

The broad vectorial competence of permissive sandfly species may have important epidemiological consequences and should be taken into account while estimating the risk of new leishmaniasis foci. The most important example is the introduction of *L. infantum* (syn. *L. chagasi*) from the Iberian Peninsula to Latin America, where it adapted to the local permissive sandfly *Lutzomyia longipalpis* [9]. Similarly, we can speculate that *L. tropica* could be transmitted by permissive vectors in the Mediterranean area, although *P. sergenti* was for a long time considered to be its sole vector. The vectorial capacity of *P. similis*, a sister species of *P. sergenti*, which is widely distributed in the north-eastern Mediterranean, is yet to be tested. *P. arabicus*, a proven vector in a CL focus in northern Israel [14], demonstrated a clear potential of permissivity to transmit the parasite.

While transcontinental import of new vectors to Europe by human activities appears improbable, due to the

fragile nature of sandflies in comparison to the rather robust invasive mosquito species, a shift of sandfly occurrence to northern areas of Europe, traditionally regarded as *Leishmania*-free, was recently well documented. In northern Italy, an increase in the density and geographical expansion of the *Leishmania* vectors *P. perniciosus* and *P. neglectus* was observed in 2003 and 2004 compared with the situation described in the 1960s and 1970s; this enabled the establishment and transmission of the parasite in the northern part of the country previously regarded as non-endemic [17]. In a similar manner, an increase in the incidence and distribution of canine leishmaniasis (CanL) was reported in 2007 from a new VL focus in southern France, a region outside the traditional endemic area of this disease. As no major changes in land use were observed, it was postulated that the increased CanL transmission could be attributed to vector dispersion (*P. perniciosus* and *P. ariasi*) due to an increase in the mean summer temperature during the two decades preceding the reported increase, a possible effect of global climate change [18]. A similar situation was described in Spain where the current distribution was compared to the predicted spreading of sandfly vectors based on expected climate changes [19]. In Germany, the detection of leishmaniasis cases in humans and animals (dogs, cats, horses) that had never travelled outside the country, has led to the hypothesis of a recent establishment of autochthonous transmission [20], suggesting a northward expansion of *L. infantum*, although entomological surveys have so far not provided solid evidence for the presence of competent vector species in Germany.

Reservoirs

Dogs, which may suffer from severe disease (CanL), are the primary domestic reservoir hosts of zoonotic VL caused by *L. infantum*. Canine infections are widespread in southern Europe, representing both a public health threat and a veterinary problem. Infections in cats and horses have also been reported in areas where CanL is present, and cats may suffer from feline leishmaniasis syndrome, which is less severe compared to CanL [21]. In Europe, a number of other indigenous mammal species have been found infected by *L. infantum*, including *Mus spretus* (Algerian mouse), *Apodemus sylvaticus* (European wood mouse), R*attus rattus* (black rat), *Rattus norvegicus* (brown rat), *Meles meles* (European badger), *Martes martes* (European pine marten), *Mustela nivalis* (weasel), *Geneta geneta* (common genet) and *Vulpes vulpes* (red fox) [6,21]. In addition to domestic dogs, the ability to transmit infection has been confirmed by xenodiagnosis in black rats and domestic cats [22], suggesting that they may represent a secondary reservoir host for *L. infantum*. The important question is: can any of these species serve as reservoir host, and can they participate in the establishment and spread of the parasite in new foci?

Zoonotic CL, caused by *L. major*, is not considered a threat for Europe, not even a very low risk, since its natural reservoir hosts, gerbils of the genera *Rhombomys, Psammomys* and *Meriones*, are not found in European countries [6]. However, the recent finding that voles of the species Microtus guentheri, a common rodent in Balkan countries, were infected by *L. major* in Israel [5], has challenged this assumption. In contrast, CL caused by *L. tropica* is universally believed to be anthroponotic because it is prevalent in urban settings. However, dogs have been reported as possible reservoirs or accidental hosts of *L. tropica* in some countries [21]. In a broader geographical context of the Mediterranean region, several zoonotic foci have been described, with rock hyraxes (*Procavia capensis*) as reservoirs in Israel [7,14] and *Ctenodactylus gundi* rodents found infected and probably serving as reservoirs in the area of Maghreb [23]. These two examples from neighbouring areas to Europe illustrate that the traditional terms used for the diseases caused by *L. tropica* and *L. major*, 'anthroponotic CL' and 'zoonotic CL', respectively, may not be fully appropriate.

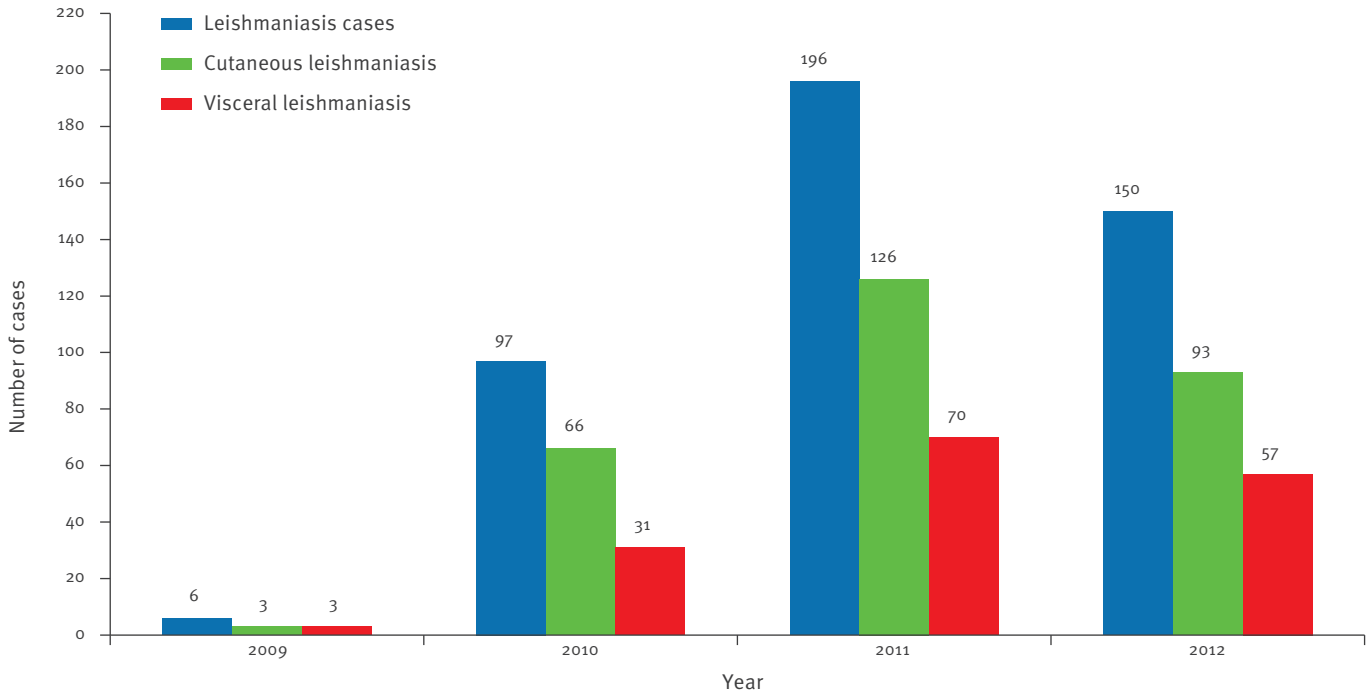
Spread of endemic *L. infantum* and the risk of introduction of non-endemic *Leishmania* species

Being previously confined to coastal Mediterranean biotopes, autochthonous leishmaniasis caused by *L. infantum* does not appear to be limited to these habitats anymore, suggesting an expansion towards new biotopes at northern latitudes and higher altitudes. During the period from 2002 to 2009, the northward spread of CanL was monitored in northern Italy, in newly endemic regions, with mean seroprevalences increasing from 1.8 to 4.7%, and reported human VL and CL cases [17]. In a region of the French Pyrenees, outside the traditional area endemic for leishmaniasis, CanL seroprevalence rates increased 10-fold over a period of 13 years between 1994 and 2007 [18]. In south-eastern Spain, a progressive increase in CanL seroprevalence rates was reported at elevated altitudes in the Alpujarras region, climbing from 9.2% in 1984 to 20.1% in 2006 [24]. Furthermore, a new CanL focus was recently detected in a Pyrenean area of north-western Catalonia [25].

It transpires, from both published and unpublished information that leishmaniasis cases due to *Leishmania* species that are not indigenous to Europe, are indeed frequent [6,21], occurring in migrants, visiting friends and relatives, or European citizens travelling to endemic countries outside Europe for tourism or work. Some 100 VL, 400 CL and 700 CanL imported cases have been diagnosed and published from traditionally non-endemic countries of Europe north of the regions with natural occurrence of leishmaniases (Germany, the Netherlands and the United Kingdom). Information about non-indigenous *Leishmania* importation in endemic areas of southern Europe is scarce because it is difficult to discriminate between autochthonous and non-autochthonous cases that share similar clinical aspects. For example in Italy, during 2011 and 2012, about 20 cases (16 of them immunocompetent CL patients) from four New World and 10 non-European

Figure 1

Human leishmaniasis outbreak of Fuenlabrada, Spain, 2009–2012 (n=449)



Old World countries were diagnosed, indicative of the intense circulation of parasites at global level. Most imported cases are CL forms caused by *L. tropica* or *L. major*, but also by neotropical parasites of the *L. braziliensis*, *L. amazonensis* or *L. guyanensis* complexes. Although adaptation of the latter parasites to Old World phlebotomine vectors and reservoir hosts does not seem probable, phlebotomine species susceptible to full development of *L. tropica* (*P. sergenti*) or *L. major* (*P. papatasi*) have been recorded in several southern European countries. Notably, the geographical range of P. sergenti extends to Spain, Portugal and Italy (Sicily), where genetic competence of local sandfly populations for anthroponotic *L. tropica* transmission has been suggested [26]. Because of the zoonotic nature of *L. major*, the probability of its introduction appears to be low. Nevertheless, hybrid *Leishmania* from Portugal have been reported, which share genetic traits from *L. infantum* and *L. major*, suggesting that adaptation of novel parasites to southern European vectors may take place in the future [27].

New foci in Spain, Crete and Cyprus

Spain

Recently, a new *L. infantum* focus has been described in Spain. Since late 2010, an unusual increase of human leishmaniasis cases (VL and CL) has been observed in the south-western Madrid region, mainly in Fuenlabrada (204,838 inhabitants) and was considered as an outbreak (Figure 1). The incidence rate in this municipality rose from 2.44/100,000 inhabitants in 2009 to 54.2/100,000 inhabitants in 2013. From July 2009 to December 2012, 449 leishmaniasis cases were diagnosed in Fuenlabrada and three affected neighbouring municipalities, Leganés, Getafe, and Humanes de Madrid, of which 158 (35.3%) were VL.This was the first reported outbreak of VL and CL of such magnitudein Spain.

From 2005 to 2011 a new periurban green park of around 450 hectares with an irrigation system was established very close to the residence of many of the VL and CL cases. This provided abundant food for hares in an area previously used for agriculture, now free of predators and hunters.

A survey revealed a large population of hares (*Lepus granatensis*) and a small population of rabbits living in this park, therefore the role of hares and rabbits as potential reservoirs of leishmaniasis in this focal area was studied during 2011 and 2012. Seroprevalence for *Leishmania*, studied during the same period in the same area in 2,070 dogs (by rK39 dipstick), was found to be 1.64% [28]. In addition, *Leishmania* was detected using a *Leishmania*-specific nested PCR (Ln-PCR) amplifying three targets (ITS1, ITS2, and *hsp70*) which proved to have 100% specificity for *Leishmania* [29]. Original R223 and R333 primers [30] used in this Ln-PCR assay detected *Leishmania* in four of 55 spleen samples from cats (7.3%) and in one of 66 spleen samples from rabbits (1.5%). However, the most interesting results were found in hares, as 43 of 148 animals studied (29%) were positive in Ln-PCR on spleen or

skin samples collected between December 2011 and July 2012. Xenodiagnosis assay, carried out on seven hares (using a *P. perniciosus* colony) revealed four positive animals [3], proving for the first time that sandfly vectors acquire *L. infantum* by feeding on apparently healthy hares. Direct sequencing of the positive ITS1, ITS2, and *hsp70* PCR products was performed [31]. Molecular characterisation, based on the ITS1 and ITS2 regions and the *hsp70* gene of 30 isolates, 24 from humans and six from hares (six positive sandflies after xenodiagnosis of three hares), were consistent with *L. infantum* and 100% identical to the sequence of the *L. infantum* strain isolated in Spain in 1987 from a patient with CL. Between December 2011 and February 2013, about 1,200 hares were captured in the park, representing a high population density of around 265 hares/km^2. A preliminary entomological study was conducted in September and October 2011, before starting the disease control measures, in order to analyse by PCR the blood feeding preferences of sandflies (based on the vertebrate *cytochrome b* gene), which showed a clear feeding preference for hares [32]. In the same study, the detection of *Leishmania* in the wild-caught *P. perniciosus* (studied by kDNA-PCR and cpb PCR) showed that 58.5% of flies were positive to *L. infantum*. This was the first evidence that hares can play a role as a reservoir of *L. infantum* in Europe, suggesting the existence of a sylvatic transmission cycle linked to the urban periphery. As noted above, the creation of the park resulted in an increase of hares as the reservoir host and sandfly populations, and thus led to the urbanisation of leishmaniasis. The new VL focus in Fuenlabrada is thus an example of leishmaniasis emergence due to environmental changes induced by man. The role of hares, and other possible sylvatic reservoirs, in the epidemiology of leishmaniasis deserves special attention in endemic sites.

Crete

In the island of Crete, Greece, CL was so common sixty years ago that it had a local name, 'Chaniotico spyri', meaning 'the skin lesion found in the area of Chania'. Yet, after DDT spraying against malaria vectors during World War II, sandfly populations were drastically reduced [33,34] and Crete remained a latent focus for CL for over 25 years [35]. Recently however, CL due to *L. tropica* has re-emerged and spread to all parts of Crete, with an average of five CL cases per year observed in the last three years. The parasite was isolated from relapsed patients, over 60 years-old, who reported that they had 'Chaniotico spyri' during childhood, as most people in their village at that time [35]. Of the 19 CL cases known in Crete during the last three years, 15 were over 60 years-old. Possibly age-related changes in the immune system of these patients allowed the parasite to become activated and cause new lesions. Such cases are expected to appear in larger numbers as people infected at childhood get older. Currently, *L. infantum* and *L. tropica* are found circulating in the island of Crete, a closed ecosystem of 8,336 km^2 and with a population of 601,131 (Greek statistics department 2001). They are involved in zoonotic and anthroponotic cycles, with an increasing number of human cases and a reported mixed infection in a dog. The two prevailing *Phlebotomus* species in Crete are *P. neglectus* and *P. similis*, the first a proven vector of *L. infantum* and the second a suspected vector of *L. tropica*. However, 10 *Phlebotomus* species are found in the island [35], and vectorial capacities of most of them have not yet been investigated. These species, like *P. mascittii*, may be able to transmit the local parasites but also other parasite species and/or strains that could be introduced to the island, to humans and other hosts, a situation that may complicate the epidemiology of the disease and its implications for public health in the future.

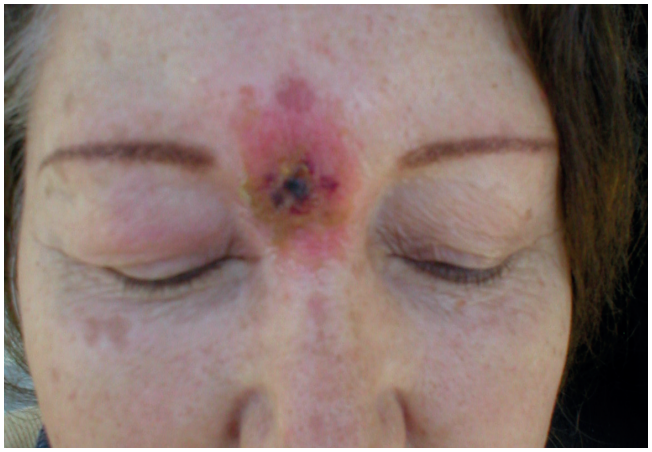
Cyprus

L. donovani is anthroponotic, causing VL, CL and post-kala-azar dermal leishmaniasis, depending on the geographical area. It is considered more aggressive than *L. infantum* and often does not respond to treatment with first-line drugs. For decades, *L. infantum* in Cyprus has been causing canine leishmaniasis without causing any human cases [36,37]. CanL was a serious veterinary problem until 1945 [38], but became latent after the mosquito eradication campaign [39] and the vast reduction in dog numbers (from 46,000 to 6,000) as a consequence of the successful anti-echinococcosis campaign between 1970 and 1975 [40,41]. Nevertheless, the reservoir host and vector populations for leishmaniasis gradually increased and CanL re-emerged on the island. In 1996, overall CanL seroprevalence was reported to be 1.7%; by 2006, it had increased six-fold, reaching 33.3% in some areas. Nevertheless, only one infantile VL case was reported during this period, in 1987 [42]. The situation is different in the northern part of the island not under effective control of the Government of the Republic of Cyprus, where an increasing number of human CL and VL, as well as CanL cases have been reported [43]. Although the population of the two parts of Cyprus has been free to cross the green line since 2003, leishmaniasis cases were not reported in the southern part until 2006, when three CL and two VL human cases were diagnosed.

For the typing of the isolates from Cyprus, a *K26*-PCR assay, which is specific for the *L. donovani* complex and discriminates between *L. donovani* and *L. infantum* [44], was used, together with multilocus enzyme electrophoresis (MLEE), the current reference method for characterising and classifying *Leishmania* strains [45], as well as microsatellite analysis [46]. All methods incriminated *L. donovani* MON-37 as the responsible strain. These isolates were found to be genetically very closely related to the Turkish *L. donovani* MON-37 and differed from the *L. donovani* MON-37 found in all other countries. This indicates that the parasite may have been introduced to Cyprus recently, probably from mainland Turkey, where human leishmaniasis is widespread, by Turkish immigrants and/or the army following the war in 1974. The fact that this strain was isolated from both human hosts and *P. tobbi* in Turkey

Figure 2

Cutaneous leishmaniasis due to *Leishmania donovani*, Cyprus, 2011



Photograph by Maria Antoniou. The patient agreed for the photograph to be published.

[46], strengthens the hypothesis that this vector may be responsible for the transmission of both *Leishmania* species in southern Cyprus, where no *P. neglectus* has been recorded so far [37]. However, further studies should investigate the capacity of other species to transmit *L. donovani* in Cyprus, such as P. galilaeus. Four new human CL cases (Figure 2) and one VL case, caused by *L. donovani*, have been diagnosed in Cyprus since. *L. donovani* was also found, as a mixed infection with *L. infantum*, in a dog (one of 20 dogs examined by *K26*-PCR) living in the same district as three CL patients [37].

All evidence indicates that two different transmission cycles are taking place on the island, one of *L. infantum* in dogs and one of *L. donovani* in humans. However, the mixed infection in the dog suggests that the cycles meet, demonstrating that some of the sandfly species found on the island bite both dogs and humans, contrary to what was believed [47]. The question remains: why do humans in the southern part of Cyprus not get infected by *L. infantum*? A seroepidemiolological study conducted in 600 people in two areas on Cyprus defined as high-risk in a seroepidemiological study conducted in dogs and one area defined as low-risk, did not reveal antibodies against the parasite [37]. However, a larger sample should be studied, to investigate the situation in depth before conclusions can be reached. It is also interesting to note that cases of VL have been reported in tourists visiting Cyprus [48,49] and that no *Leishmania* and HIV co-infections are known on the island. It is probable that genetic differentiation, in the parasite, the vector or the native population, has taken place, and these possibilities should be investigated to explain the Cyprus paradox [37].

Conclusion

It is apparent that the epidemiology of the leishmaniases in the Mediterranean basin is changing. Historical foci, silent for several decades, re-emerge and the threat of new strain/species introduction is evident. The new focus in Spain, with hares as reservoirs, clearly shows that hosts, neglected in previous epidemiological considerations, may play a major role in transmission cycles under changing conditions. At the same time, the concept of specific and permissive vectors draws attention to the possibility that a larger number of sandfly species could be incriminated in parasite transmission.Many reports indicate introduction and spread of exotic *Leishmania* species and zymodeme variants to areas of Europe that are already endemic. In areas where sandfly vectors are well established and circulating the local parasites, such introductions of for example a new *L. tropica* zymodeme in Crete and *L. donovani* in Cyprus [36], if able to support and transmit the new invaders, will enhance the possibility of genetic exchange between different species/strains of the parasite. As a result, new hybrids may be generated with different epidemiology, pathogenicity or drug resistance, a situation already shown in Portugal [27].

There is an urgent need to identify both *Leishmania* species and their vectors in detail. To safeguard public health, targeted control measures must be undertaken by local and European authorities. At the same time it is of vital importance for doctors in human and veterinary medicine to be well informed on the disease symptoms, therapy, and resistance of *Leishmania* to drugs. Scientific consortiums such as EDENext could prove appropriate platforms to accumulate, coordinate and integrate up-to-date knowledge and assist decision makers in assessing public health problems related to leishmaniases, appropriately.

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References

1. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS ONE. 2012;7(5):e35671. http://dx.doi.org/10.1371/journal.pone.0035671 PMid:22693548 PMCid:PMC3365071
2. Dujardin JC, Campino L, Ca-avate C, Dedet JP, Gradoni L, Soteriadou K, et al. Spread of vector-borne diseases and neglect of leishmaniasis, Europe. Emerg Infect Dis. 2008;14(7):1013-8. http://dx.doi.org/10.3201/eid1407.071589 PMid:18598618 PMCid:PMC2600355
3. Molina R, Jiménez M, Cruz I, Iriso A, Martín-Martín I, Sevillano O, et al. The hare (Lepusgranatensis) as potential sylvatic reservoir of Leishmania infantum in Spain. Vet Parasitol. 2012;190(1-2):268-71. http://dx.doi.org/10.1016/j.vetpar.2012.05.006 PMid:22677135
4. Alvar J, Aparicio P, Aseffa A, Den Boer M, Canāvate C, Dedet JP, et al. The relationship between leishmaniasis and AIDS: the second 10 years. ClinMicrobiol Rev. 2008;21(2):334-59. http://dx.doi.org/10.1128/CMR.00061-07 PMid:18400800 PMCid:PMC2292576
5. Michel G, Pomares C, Ferrua B, Marty P. Importance of worldwide asymptomatic carriers of Leishmania infantum (L. chagasi) in human. Acta Trop. 2011;119(2-3):69-75. http://dx.doi.org/10.1016/j.actatropica.2011.05.012 PMid:21679680
6. Ready P. Leishmaniasis emergence in Europe. Euro Surveill. 2010;15(10):pii=19505. Available from: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19505 PMid:20403308
7. Jacobson R. Leishmaniasis in an era of the conflict in Middle East. Vector Borne Zoonotic Dis. 2011;11(3):247-58. http://dx.doi.org/10.1089/vbz.2010.0068 PMid:20846030
8. Giorgobiani E, Lawyer PG, Babuadze G, Dolidze N, Jochim RC, Tskhvaradze L, et al. Incrimination of *Phlebotomus* kandelakii and *Phlebotomus* balcanicus as vectors of Leishmania infantum in Tbilisi, Georgia. PLoSNegl Trop Dis. 2012;6(4):e1609.
9. Volf P, Myskova J. Sand flies and Leishmania: specific versus permissive vectors. Trends Parasitol. 2007;23(3):91-2. http://dx.doi.org/10.1016/j.pt.2006.12.010 PMid:17207663 PMCid:PMC2839922
10. Killick-Kendrick R, Killick-Kendrick M, Tang Y. Anthroponotic cutaneous leishmaniasis in Kabul, Afghanistan: the low susceptibilty of *Phlebotomus* papatasi to Leishmania tropica. Trans. R. Soc. Trop. Med. Hyg. 1994;88(2):252-3. http://dx.doi.org/10.1016/0035-9203(94)90320-4
11. Pimenta PF, Saraiva EM, Rowton E, Modi GB, Garraway LA, Beverly SM, et al. Evidence that the vectorial competence of phlebotomine sand flies for different species of Leishmania is controlled by structural polymorphisms in the surface lipophosphoglycan. ProcNatlAcadSci U S A. 1994;91(19):9155-9. http://dx.doi.org/10.1073/pnas.91.19.9155 PMid:8090785 PMCid:PMC44766
12. Kamhawi S, Modi GB, Pimenta PF, Rowton E, Sacks DL. The vectorial competence of *Phlebotomus* sergenti is specific for Leishmania tropica and is controlled by species-specific, lipophosphoglycan-mediated midgut attachment. Parasitology. 2000;121(Pt 1):25-33. http://dx.doi.org/10.1017/S0031182099006125 PMid:11085222
13. Jecna L, Dostalova A, Wilson R, Seblova V, Chang KP, Bates PA, et al. The role of surface glycoconjugates in Leishmania midgut attachment examined by competitive binding assays and experimental development in sand flies.Parasitology. 2013;140(8):1026-3 http://dx.doi.org/10.1017/S0031182013000358 PMid:23611086
14. Svobodova M, Votypka J, Peckova J, Dvorak V, Nasseredin A, Baneth G, et al. Distinct transmission cycles of Leishmania tropica in 2 adjacent foci, Northern Israel. Emerg Infect Dis. 2006;12(12):1860-8. http://dx.doi.org/10.3201/eid1212.060497 PMid:17326936 PMCid:PMC3291354
15. Myskova J, Svobodova M, Beverly SM, Volf P. A lipophosphoglycan-independent development of Leishmania in permissive sand flies. Microbes Infect. 2007;9(3):317-24. http://dx.doi.org/10.1016/j.micinf.2006.12.010 PMid:17307009 PMCid:PMC2839925
16. Svarovska A, Ant T, Seblova V, Jecna L, Beverly S, Volf P. Leishmania major glycosylation mutants require phosphoglycans (lpg2-) but not lipophosphoglycan (lpg1-) for survival in permissive sand fly vectors. PLoSNegl Trop Dis. 2010;4(1):e580. http://dx.doi.org/10.1371/journal.pntd.0000580 PMid:20084096 PMCid:PMC2797086
17. Maroli M, Rossi L, Baldelli R, Capelli G, Ferroglio E, Genchi C, et al. The northward spread of leishmaniasis in Italy: evidence from retrospective and ongoing studies on the canine reservoir and phlebotomine vectors. Trop Med Int Health. 2008;13(2):256-64. http://dx.doi.org/10.1111/j.1365-3156.2007.01998.x PMid:18304273
18. Dereure J, Vanwambeke SO, Malé P, Martinez S, Pratlong F, Balard Y, et al. The potential effects of global warming on changes in canine leishmaniasis in a focus outside the classical area of the disease in Southern France. Vector Borne Zoonotic Dis. 2009;9(6):687-94. http://dx.doi.org/10.1089/vbz.2008.0126 PMid:19271999
19. Gálvez R, Descalzo MA, Guerrero I, Miró G, Molina R. Mapping the current distribution and predicted spread of the leishmaniosis sand fly vector in the Madrid region (Spain) based on environmental variables and expected climate change. Vector Borne Zoonotic Dis. 2011;11(7):799-806. http://dx.doi.org/10.1089/vbz.2010.0109 PMid:21417927
20. Naucke TJ, Menn B, Massberg D, Lorentz S. Sandflies and leishmaniasis in Germany. Parasitol Res. 2008;103(Suppl1):S65-8. http://dx.doi.org/10.1007/s00436-008-1052-y PMid:19030887
21. Gramiccia M, Gradoni L. The Leishmaniases of Southern Europe. In: Takken W, Knols B, editors. Emerging pests and vector-borne diseases in Europe, Ecology and control of vector-borne diseases, Wageningen: Academic Publishers; 2007. p.75-95.
22. Quinnell RJ, Courtenay O. Transmission, reservoir hosts and control of zoonotic visceral leishmaniasis. Parasitology. 2009;136(14):1915-34. http://dx.doi.org/10.1017/S0031182009991156 PMid:19835643
23. Jaouadi K, Haouas N, Chaara D, Gorcii M, Chargui N, Augot D, et al. First detection of Leishmania killicki (Kinetoplastida, Trypanosomatidae) in Ctenodactylusgundi (Rodentia, Ctenodactylidae), a possible reservoir of human cutaneous leishmaniasis in Tunisia.Parasit Vectors. 2011;4:159. http://dx.doi.org/10.1186/1756-3305-4-159 PMid:21834996 PMCid:PMC3162927
24. Martín-Sánchez J, Morales-Yuste M, Acedo-Sánchez C, Barón S, Díaz V, Morillas-Márquez F. Canine leishmaniasis in southeastern Spain. Emerg Infect Dis. 2009;15(5):795-8. http://dx.doi.org/10.3201/eid1505.080969 PMid:19402973 PMCid:PMC2687031
25. Ballart C, Alcover MM, Portús M, Gállego M. Is leishmaniasis widespread in Spain? First data on canine leishmaniasis in the province of Lleida, Catalonia, northeast Spain.Trans R Soc Trop Med Hyg. 2012;106(2):134-6. http://dx.doi.org/10.1016/j.trstmh.2011.11.001 PMid:22137192
26. Depaquit J, Ferté H, Léger N, Lefranc F, Alves-Pires C, Hanafi H, et al. ITS 2 sequences heterogeneity in *Phlebotomus* sergenti and *Phlebotomus* similis (Diptera, Psychodidae): possible consequences in their ability to transmit Leishmania tropica. Int J Parasitol. 2002;32(9):1123-31. http://dx.doi.org/10.1016/S0020-7519(02)00088-7
27. Volf P, Benkova I, Myskova J, Sadlova J, Campino L, Ravel C. Increased transmission potential of Leishmania major/Leishmania infantum hybrids. Int J Parasitol. 2007;37(6):589-93. http://dx.doi.org/10.1016/j.ijpara.2007.02.002 PMid:17376453 PMCid:PMC2839924
28. Vilas F, Carpintero J, Sevilla S, Martínez A, OrdobásGavín M, Bernal J, et al. Brote de leishmaniasis en la zonasuroeste de la Comunidad de Madrid. Medidas de investigación y control medioambiental.[Leishmaniasis outbreak in the south-western area of the Community of Madrid.Investigation measures and environmental control]. Prof Vet. 2012;17(79):6-15. Spanish.
29. Cruz I, Ca-avate C, Rubio JM, Morales MA, Chicharro C, Laguna F, et al. A nested polymerase chain reaction (Ln-PCR) for diagnosing and monitoring Leishmania infantum infection in co-infected patients with human immunodeficiency virus.Trans R Soc Trop Med Hyg. 2002;96(Suppl.1):S185-9. http://dx.doi.org/10.1016/S0035-9203(02)90074-X
30. van Eys GJ, Schoone GJ, Kroon NC, Ebeling SB. Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of Leishmania parasites. MolBiochemParasitol. 1992;51(1):133-42. http://dx.doi.org/10.1016/0166-6851(92)90208-2
31. Fraga J, Montalvo AM, De Doncker S, Dujardin JC, Van der Auwera G. Phylogeny of Leishmania species based on the

heat-shock protein 70 gene. Infec Genet Evol. 2010;10(2):238-45.
http://dx.doi.org/10.1016/j.meegid.2009.11.007
PMid:19913110

32. Jiménez M, González E, Iriso A, Marco E, Alegret A, Fúster F, et al. Detection of Leishmania infantum and identification of blood meals in *Phlebotomus* perniciosus from a focus of human leishmaniasis in Madrid, Spain. Parasitol Res. 2013;112(7):2453-9. http://dx.doi.org/10.1007/s00436-013-3406-3
PMid:23535889
33. Hertig, M. *Phlebotomus* and residual DDT in Greece and Italy. Am J Trop Med Hyg. 1949;29(5):773-809.
PMid:18141397
34. Hadjinicolaou J. Present status of *Phlebotomus* in certain areas of Greece. Bull World Health Organ. 1958;19(6):967-79.
PMid:20604045 PMCid:PMC2537816
35. Christodoulou V, Antoniou M, Ntais P, Messaritakis I, Ivović V, Dedet JP, et al. Re-emergence of visceral and cutaneous leishmaniasis in the Greek island of Crete. Vector Borne Zoonotic Dis 2012;12(3):214-22.
http://dx.doi.org/10.1089/vbz.2011.0004
PMid:22217163 PMCid:PMC3300062
36. Antoniou M, Haralambous C, Mazeris A, Pratlong F, Dedet JP, Soteriadou K. Leishmania donovani leishmaniasis in Cyprus. Lancet Infect Dis. 2008;8(1):6-7.
http://dx.doi.org/10.1016/S1473-3099(07)70297-9
37. Mazeris A, Soteriadou K, Dedet JP, Haralambous C, Tsatsaris A, Moschandreas J, et al. Leishmaniases and the Cyprus paradox. Am J Trop Med Hyg. 2010;82(3):441-8.
http://dx.doi.org/10.4269/ajtmh.2010.09-0282
PMid:20207870 PMCid:PMC2829906
38. Deplazes P, Grimm F, Papaprodromou M, Cavaliero T, Gramiccia M, et al. Canine leishmaniosis in Cyprus due to Leishmania infantum MON 1. Acta Trop. 1998;7(2):169-78.
http://dx.doi.org/10.1016/S0001-706X(98)00064-3
39. Tesh RB, Papaevangelou G. Effect of Insectiside spraying for Malaria Control on the incidence of sandfly fever in Athens, Greece. Am J Trop Med Hyg. 1977;26(1):163-6.
PMid:190909
40. Polydorou K. A short history of echinococcosis control in Cyprus.Hist Med Vet. 1984;9(3):61-4.
PMid:11621074
41. Economides P. Control of Zoonoses in Cyprus. Rev Sci Tech. 2000;19(3):725-34.
PMid:11107615
42. Minter DM, Eitrem UR. Sandflies and disease in Cyprus: 1944-1985. In Hart D.T. (Ed) Leishmaniasis: The current status and new strategies for control. Vol 3. NATO ASI series: Series A: Life sciences. New York: Plenum Press; 1989. P.207-16.
43. Howard MK, Ogunkolade W, Bryceson AD, Davidson RN, Moody AH, Miles MA. A DNA probe for human visceral leishmaniasis. Trans R Soc Trop Med Hyg. 1992;86(1):35-6.
http://dx.doi.org/10.1016/0035-9203(92)90430-K
44. Haralambous C, Antoniou M, Pratlong F, Dedet JP, Soteriadou K. Development of a molecular assay specific for the Leishmania donovani complex that discriminates *L. donovani*/Leishmania infantum zymodemes: a useful tool for typing MON-1. DiagnMicrobiol Infect Dis. 2008;60(1):33-42.
http://dx.doi.org/10.1016/j.diagmicrobio.2007.07.019
PMid:17889482
45. Rioux JA, Lanotte G, Serres E, Pratlong F, Bastien P, Perieres J. Taxonomy of Leishmania. Use of isoenzymes.Suggestions for a new classification. Ann Parasitol Hum Comp. 1990;65(3):111-25.
PMid:2080829
46. Gouzelou E, Haralambous C, Amro A, Mentis A, Pratlong F, Dedet JP, et al. Multilocus microsatellite typing (MLMT) of strains from Turkey and Cyprus reveals a novel monophyletic *L. donovani* sensulato group. PLoSNegl Trop Dis. 2012;6(2):e1507.
47. Leger N, Depaquit J, Ferte H, Rioux JA, Gantier JC, Gramiccia M, et al. [Phlebotominesandflies (Diptera-Psychodidae) of the isle of Cyprus. II--Isolation and typing of Leishmania (Leishmania infantum) Nicolle, 1908 (zymodeme MON-1) from *Phlebotomus* (Larroussius) tobbi Adler and Theodor, 1930].Parasite. 2000;7(2):143-6. French.
PMid:10887662
48. Valkoun A, Nádvorník V, Kostrhun L. [A case of visceral leishmaniasis imported from the Mediterranean]. CasLekCesk. 1985;124(51):1582-5. Czech.
PMid:4084964
49. Wheatley T, Sacks S, Flemans RJ, Rubenstein D. Visceral leishmaniasis: a rare imported disease.
J Infect. 1983;7(2):166-7.
http://dx.doi.org/10.1016/S0163-4453(83)90716-8

LeishMan: harmonising diagnostic and clinical management of leishmaniasis in Europe

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In 2012, an international group of experts from 12 institutions in seven European countries set up LeishMan (Leishmaniasis Management) [1], a project aiming to improve treatment of leishmaniasis on the basis of clinical presentation and molecular species differentiation.

The group of experts from currently 12 centres in Belgium, France, Germany, the Netherlands, Spain, Switzerland and the United Kingdom, aims to harmonise the diagnostic and clinical management of patients with cutaneous and mucosal leishmaniasis in Europe and has the following objectives:

- to conduct inter-laboratory comparisons and quality controls for diagnosis and parasite collection procedures;
- to establish and validate a consensus on molecular species typing;
- to address taxonomic problems in human-pathogenic species of the *Leishmania* genus;
- to implement permanent exchange between specialists and harmonise treatment recommendations in Europe;
- to collect accurate information on the treatment of cutaneous and mucosal leishmaniasis in Europe.

As different genotyping methods are in use in the various laboratories, a comparative analysis is required to assess whether they produce congruent results. To this end, a comparison of all currently applied species typing techniques is performed on the basis of a well-defined strain reference set. Development of standardised molecular tools is a further goal.

Sequence information from various parasite genome targets will be systematically collected from all clinical cases, and the outcome will be linked to the clinical parameters for final analysis of treatment success. Clusters of genotypes will be analysed with respect to clinical presentation and treatment outcome.

With the ongoing revision of the taxonomy of the genus *Leishmania* and after discussing difficulties in discriminating closely related species or species hybrids, the participants have agreed to form a working group with the aim to address these shortcomings.

A multicentre, multinational surveillance has started analysing leishmaniasis treatment protocols and treatment outcomes with respect to the infecting parasite genotype or species. All patients with parasitologically confirmed cutaneous or mucosal leishmaniasis are included in the participating centres. The clinical data (patient data, country where the lesion was acquired, localisation and description of the lesion, etc.) are assessed in a questionnaire and documented before and after treatment. Each physician applies their routine treatment schedules. However, suggestions for treatment guidance will be offered to all physicians. Patients will be followed at least until the lesion has healed. Follow-up examinations will be done according to the current guidelines.

References

1. European Network for Tropical Medicine and Travel Health (TropNet). LeishMan. Basel: TropNet. [Accessed 2 Jul 2013]. Available from: http://www.tropnet.net/index.php?id=103&fsize=%2Fetc%2Fpasswd%00