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## Special edition:

# Leishmaniasis in Europe

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- ***Eurosurveillance*** **special edition on leishmaniasis - painting a picture of the situation in Europe.**

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Illustration of sandfly, map of Europe

# Note from the editors: *Eurosurveillance* special issue on leishmaniasis painting a picture of the situation in Europe

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After the publication of a special issue on Chagas disease in September 2011, this is the second time we focus on diseases caused by pathogens belonging to the family Trypanosomatidae. Even though *Trypanosoma cruzi* and *Leishmania spp*. infections have different impact on health, they are (still) neglected and we would like to raise awareness for aspects related to public health.

At the end of March 2012, we invited contributions for a special issue with the main aim to contribute to the existing body of evidence and to make available data that can help paint a better picture of the epidemiological situation and burden of autochthonous leishmaniasis in Europe [1]. The initial response was limited and we prolonged the deadline for submissions until the end of August 2012. The prolongation coincided with the allocation the first impact factor for *Eurosurveillance* [2] and, in addition to this, a leading European expert kindly supported the call and spread the word among his peers. We are not able to judge which element had most influence, however, by the end of the August deadline we had received 35 contributions from 16 countries for the special issue.

The evaluation of these manuscripts was a challenge for the editorial team and the supporting experts. We needed to apply stricter criteria and select only those papers which we deemed of highest interest for the readers of Eurosurveillance. This led to favouring papers focusing on human disease and in particular surveillance.

In the selection process we were forced to reject also manuscripts of good quality, and after peer review we agreed to publish 12 articles from 10 countries in Europe to ensure a good geographical representation. The coordination of the special issue took some time and we thank all contributors, peer reviewers and supporters, in particular Luigi Gradoni from the Istituto Superiore di Sanità in Rome, Italy, for their engagement and patience.

In this first part of the special issue we present surveillance data from five endemic countries in southern Europe: Bulgaria, Greece, Croatia, Italy and France, together with a rapid communication on an increase in leishmaniasis cases in northern Italy in 2012-13. The second part of the special issue will be published on 25 July. It will feature an editorial by Luigi Gradoni and two papers from Spain on a recent outbreak in Madrid with some unusual findings. Data from the Netherlands, a non-endemic country where a series of cases imported from within Europe were detected, will also be presented. Moreover, it will cover various relevant topics such as the role of indigenous phlebotomine sandflies and mammals in spreading the pathogen as well as aspects related to treatment with tumour necrosis factor-alpha antagonists and new diagnostic methods.

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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.

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# Ongoing outbreak of visceral leishmaniasis in Bologna Province, Italy, November 2012 to May 2013

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**An increased number of autochthonous visceral leishmaniasis (VL) cases has recently been reported in Bologna Province in northern Italy. Over six months from November 2012 to May 2013, 14 cases occurred, whereas the average number of cases per year was 2.6 (range: 0–8) in 2008 to 2012. VL was diagnosed in a median of 40 days (range: 15–120) from disease onset. This delay in diagnosis shows the need for heightened awareness of clinicians for autochthonous VL in Europe.**

From November 2012 to May 2013, public health authorities, microbiologists and clinicians in Bologna Province, northern Italy, noted an upsurge in human cases of visceral leishmaniasis. During these six months, 14 cases were notified, an over five-fold increase compared with the annual average of 2.6 cases (range: 0–8) from 2008 to 2012. Here, we report preliminary epidemiological, microbiological and clinical findings.

## Background

Visceral leishmaniasis (VL) is a severe disease primarily affecting the host's reticuloendothelial system and is caused by parasitic protozoans belonging to the *Leishmania donovani* complex. VL is endemic in Mediterranean Europe, where the disease is caused by *L. infantum*. Transmission is mainly zoonotic and occurs via the bite of a phlebotomine sandfly species of the genus Phlebotomus [1,2]. In Italy, the Tyrrhenian littoral, the southern peninsular regions and the islands have been considered classical endemic zones for VL, whereas in continental northern regions, VL has mainly affected human immunodeficiency virus (HIV)-positive patients. Since the early 1990s, however, a northward spread of the disease to previously non-endemic Italian regions has been observed. These regions also exhibited a progressive decrease in HIV co-infection rates [3]. A recent survey conducted in a continental area of north-western Italy, which was previously considered to be non-endemic, detected anti-leishmanial antibodies in 7.4% of healthy adults; for half of the seropositives, ongoing infection was confirmed by PCR analysis [4]. Currently, the occurrence of both asymptomatic and symptomatic leishmaniasis seems to be underestimated in Italy.

## Outbreak description

In Italy, laboratory-confirmed cases of VL are reported by local public health departments to the regional authorities, which report cases to the Ministry of Health. The case definition for VL in Italy is based on the World Health Organization (WHO) case definition

**Figure 1**

Epidemic curve of human cases of visceral leishmaniasis, Bologna Province, northern Italy, November 2012–May 2013 (n=14)

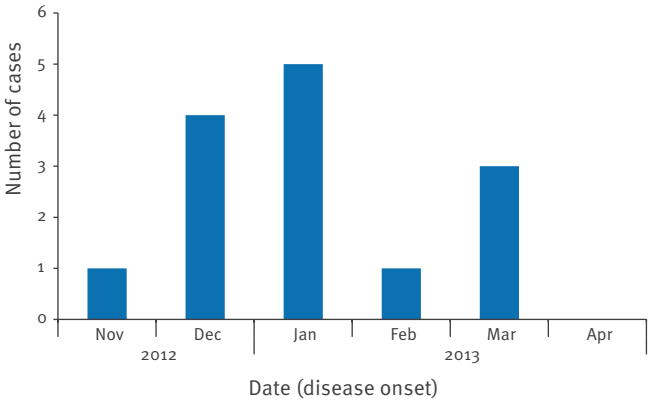
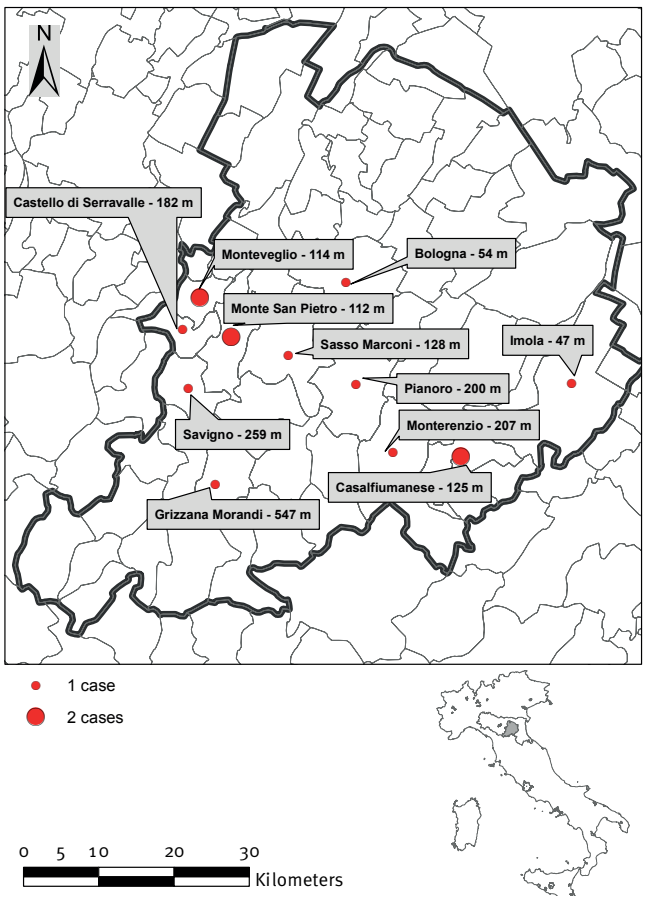


**Figure 2**

Geographical location of human cases of visceral leishmaniasis, Bologna Province, northern Italy, November 2012–May 2013 (n=14)




m: meters above sea level

and includes positive serology (indirect immunofluorescence antibody test (IFAT), ELISA, rK39-based immunochromatographic test (IC), direct agglutination test) and/or parasitology (microscopy, culture or PCR) for patients with suspected clinical symptoms [5].

From November 2012 to May 2013, 14 new cases of VL occurred in Bologna Province in northern Italy (Figure 1). Most patients resided in hilly, rural areas. Municipalities and their altitudes are reported in Figure 2.

The age range of patients was five months to 83 years, four cases were aged 18 months or younger, five were between 48 and 60 years-old, and five were between 62 and 83 years-old. The majority, 11 patients, were male. One patient was HIV-positive. Overall, five patients had known risk factors for VL, i.e. being under two years-old, (n=4) or being HIV-positive (n=1). All patients had symptoms compatible with VL, including a fever of unknown origin (n=13), mild to moderate anaemia (n=12), leukopenia (n=12), thrombocytopenia (n=10), hepatomegaly (n=7), splenomegaly (n=14) and weight loss (n=7). Interestingly, four of 14 VL cases presented with haemophagocytic syndrome (HS), a systemic hyperinflammatory disorder with severe dysfunction of immune homeostasis that may be secondary to VL [6,7].

VL was diagnosed in a median time of 40 days from disease onset (range: 15–120) and was supported by parasitological and serological means.

## Laboratory investigations

Bone marrow aspirate was performed in 12 cases, but smear was available for microscopic diagnosis only in 10 cases. The detection of amastigotes was positive in three of 10 cases investigated, and the culture was negative in all nine tested cases (Table), suggesting a low parasite load in the bone marrow [8]. As expected [9], molecular methods enhanced parasite detection in bone marrow samples. Leishmanial DNA was detected by nested PCR and/or real-time PCR in seven of 10 patients and in the peripheral blood of an additional patient who was HIV-positive; thus, molecular methods were positive in eight of 13 VL cases (not performed in one case).

Serology performed using an indirect haemagglutination assay (IHAT) was negative in all eight cases tested, whereas the rk39-based IC [10] and IFAT were positive in all tested patients (10 of 10 and 13 of 13, respectively, see Table). Thus, serodiagnosis by IFAT or IC in patients with suspected symptoms was fundamental.

Indeed, in four of five cases in whom a diagnosis of VL was posited based on only suggestive symptoms and serological tests, the response to anti-leishmanial treatment (liposomal amphotericin B, 10 mg/kg intravenous, single dose) was appropriate, suggesting that our diagnostic approach was correct. One case resolved symptoms without treatment and is currently under clinical and laboratory evaluation.

## Discussion

The risk of the emergence or resurgence of several exotic vector-borne pathogens in Europe, including chikungunya and dengue virus, has become a hot topic over the past decade, whereas other infections, such as leishmaniasis, have been neglected [11]. In fact, clinicians and microbiologists are often ill informed on the prevalence and symptoms of, and detection methods for VL, which may lead to initial misdiagnosis and a delay in diagnosis and treatment.

According to the epidemic curve (Figure 1), most cases of the ongoing outbreak in Bologna Province had disease onset in the winter months, indicating that patients were probably infected during summer and autumn 2012. Furthermore, half of the cases were diagnosed more than 40 days from disease onset and one of these cases was diagnosed 120 days after initial symptoms. This indicates a frequent delay in identifying the

**Table**

Parasitological and serological findings in visceral leishmaniasis cases, Bologna Province, Italy, November 2012–May 2013 (n=14)

| Case number | Time to diagnosis | Parasitological findings | Serological findings |
|---|---|---|---|
| 1 | 78 days | BM: microscopy - , PCR+ | IHAT-, IC+, IFAT+ (1:80) |
| 2 | 27 days | BM: microscopy- , culture-, PCR+ | IHAT-, IC+, IFAT+ (1:160) |
| 3 | 15 days | BM: microscopy -, culture-, PCR- | IHAT-, IC+, IFAT+ (1:160) |
| 4 | 42 days | BM: microscopy - , culture-,PCR- | IC+, IFAT+ (1:40) |
| 5 | 40 days | BM: microscopy+, culture-, PCR+ | IHAT-, IFAT+ (1:80) |
| 6 | 15 days | BM: microscopy -culture-, PCR- | IC+, IFAT+ (1:1,280) |
| 7 | 80 days | BM: microscopy -, culture-, PCR- | IHAT-, IC+, IFAT+ (1:5,120) |
| 8 | 40 days | PB: PCR+ | IHAT-, IC+, IFAT+ (1:10,240) |
| 9 | 120 days | PB: culture-, PCR- | IHAT-, IC+, IFAT+ (1:5,120) |
| 10 | 20 days | BM: PCR+ | IHAT- |
| 11 | 30 days | BM: microscopy + | IFAT+ (1:320) |
| 12 | 25 days | BM: microscopy - , PCR+ | IFAT+ (1:1,280) |
| 13 | 60 days | BM: microscopy+, culture-, PCR+ | IC+, IFAT+ (1:320) |
| 14 | 50 days | BM: culture- , PCR+ | IC+, IFAT+ (1:640) |

BM: bone marrow; PB: peripheral blood; IFAT: indirect immunofluorescence antibody test; IHAT: indirect haemagglutination test; IC: rk39-based immunochromatographic test; -: negative; +: positive.

parasitic infection. One third of the identified cases were revealed as having HS, and one child was erroneously diagnosed as having familial haemophagocytic lymphohistiocytosis (FHL). Symptoms for FHL and HS due to VL may overlap, and the differential diagnosis can be difficult [7, 12]. Thus, awareness of the increasing incidence of VL in areas previously considered to be at low risk is fundamental to avoid misdiagnosis, especially in infants in whom HS might be confused with FHL.

*L. infantum* is considered to be autochthonous in Bologna Province and *P. perfiliewi* is the predominant vector in this area [13]. In 1971–72, an outbreak of VL occurred in Bologna Province that affected 60 individuals, with 13 deaths [14]. Afterwards, the area remained endemic, with a low prevalence and an annual mean of 2.6 reported cases (range: 0–8 years) for the years 2008 to 2012 (R. Cagarelli, personal communication, May 2013). The reasons for the recent upsurge of VL cases in Bologna Province are unknown. Theoretically, current global warming in the Mediterranean area [15] may enhance leishmaniasis distribution due to the effect of temperature on parasite development and vector spread. However, according to WHO, there is no clear evidence indicating that sandfly and VL distribution in Europe have changed in response to climate change [2].

In conclusion, the increase in human VL cases in an area of northern Italy raises important public health concerns. Firstly, there is an urgent need to expand the existing control measures for canine leishmaniasis [13]. Secondly, healthcare professionals need to be informed of the upsurge in VL in the area to proceed with appropriate parasitological and serological tests in suspected cases, to promptly identify and treat cases of VL. Finally, the public needs to be aware of the potential exposure to sandfly bites in areas in which the parasite circulates, and to be educated in the use of appropriate preventive measures, such as mechanical and chemical repellents.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## Conflict of interest

None declared.

## Authors' contributions

Designed the study: SV and MPL. Wrote the first draft: SV. Collected, synthesised and analysed the data: RC, ACF, LA, CS, FM, MG, AS, TDM, RT, GAG, RR and RT. Interpreted the results critically and revised the article to ensure important intellectual content: PV, LG and MPL. All authors read and approved the final manuscript.

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# Geographical distribution and epidemiological characteristics of visceral leishmaniasis in Bulgaria, 1988 to 2012

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**Visceral leishmaniasis is a sporadic illness in Bulgaria. However, cases in humans are registered nearly every year. This study describes the geographical distribution of the disease in Bulgaria from 1988 to 2012, over a period of 25 years. Cases were analysed according to age, sex, and place of residence. A total of 122 cases were registered in 25 years, 118 of which were autochthonous and four of which were imported from endemic countries in southern Europe. The average annual incidence for the study period was 0.06 per 100,000 population, or an average of five cases per year (maximum 15 in 1989; no cases notified in 1991, 1995, 1996 and 2008). Cases of visceral leishmaniasis were recorded in 13 out of 28 regions in Bulgaria, mainly in the southern part of the country. The highest number of cases were registered in the regions of Blagoevgrad (n=36) and Stara Zagora (n=34). Data presented in this study show that there is ongoing transmission of visceral leishmaniasis in Bulgaria with a high mortality rate (1:7), affecting mostly children.**

## Introduction

Visceral leishmaniasis (VL) is a protozoan, vector-borne disease characterised by chronic course, remittent fever, hepatosplenomegaly, and anaemia to complete pancytopenia and secondary immunosuppression. *Leishmania infantum* is the causing agent of VL in the Mediterranean region. In areas endemic for VL, the disease tends to have a chronic course and children are especially affected [1]. The average incubation period of the disease varies from a few weeks to six months. Until recently, children aged between one and four years were the group most affected by endemic VL caused by *L. infantum* in southern Europe, North Africa, west and central Asia [2]. According to some authors, the ratio between children and adults with leishmaniasis in the Mediterranean region is 7:3 and the average age of the affected children is under four years [3]. However, in recent years, about a half of leishmaniasis cases in Europe have occurred in adults, following the appearance of the human immunodeficiency virus (HIV) infection and the increased number of patients receiving immunosuppressing treatments due to transplantation, malignancies or other underlying conditions [4].

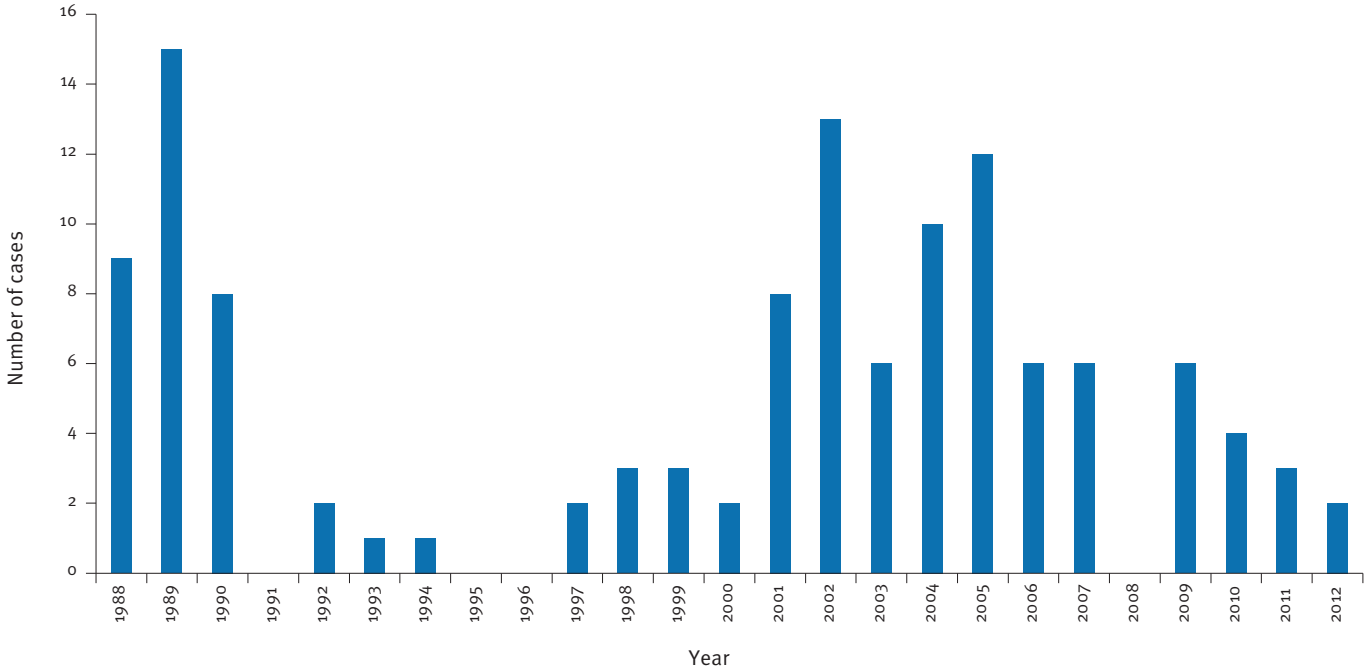
The first cases of VL in Bulgaria were reported by Mollov in 1921, who then described two clinical cases imported from Greece, and in 1937, the same author described the first autochthonous VL case in the country [5]. In the following 16 years, until 1953, a total of 57 autochthonous cases of VL were reported in Bulgaria, 50 of which were children. At that time, the disease occurred sporadically, mostly in the southern part of the country [6,7]. Between 1953 and 1988, only sporadic cases of VL were registered in the country. This was attributed to the mass use of dichlorodiphenyltrichloroethane, an organochlorine insecticide used during the eradication of malaria in Bulgaria, which led to the decrease in number and density of mosquitoes and phlebotomine sandflies that serve as vectors for leishmaniasis [8].

Studies on VL cases in Bulgaria were mainly focused on clinical aspects, diagnosis and treatment [9-13]. Over the last 15 to 20 years, the incidence of VL in Bulgaria increased significantly and the disease is now present in most parts of the country. Since 1988, autochthonous VL cases are registered almost annually.

Dogs are the principal reservoir hosts of *L. infantum* under domestic and peridomestic conditions [2]. In recent years, several studies on canine VL in Bulgaria were conducted. In 2004, a seroepidemiological screening for leishmaniasis among dogs was performed in 11 regions in Bulgaria: five regions in southern Bulgaria (Plovdiv, Stara Zagora, Yambol, Burgas, Blagoevgrad) and six regions in northern Bulgaria (Varna, Silistra, Ruse, Veliko Tarnovo, Pleven, Montana) (Figure 3) [14]. Sera from 220 dogs were tested by immunofluorescent assay, but none was seropositive. In 2006, clinical manifestations of leishmaniasis were observed and described in two domestic dogs in Petrich in southwestern Bulgaria [15]. In 2007, a seroepidemiological

**Figure 1**

Notified cases of visceral leishmaniasis, Bulgaria, 1988–2012 (n=122)




survey on the seroprevalence of *L. infantum* among dogs was carried out in two municipalities – Svilengrad and Petrich. Ten new cases of canine VL with clinical manifestations were diagnosed between November 2006 and November 2007 in these two municipalities [16].

There are no recent data on species composition of the phlebotomine sandfly fauna in Bulgaria. Few publications on the subject exist from the beginning of the 20th century and a single more recent study from 2011. Five species of the genus *Phlebotomus* were identified in the country so far: *Phlebotomus papatasi, Ph. sergenti, Ph. perniciosus* [17], *Ph. balcanicus* [18], *and Ph. tobbi* [19]. *Ph. perniciosus* is considered as a main vector, and *Ph. balcanicus* and *Ph. tobbi* are considered as potential vectors of VL [20].

The aim of the present study is to describe and summarise data from all registered cases of VL in Bulgaria from 1988 to 2012, by demographic information and geographic distribution and to compare the results with those published for other endemic European countries.

## Methods

According to the Bulgarian legislation, VL is a mandatorily notifiable disease since 1978 [21]. Notification and registration of all cases of VL are regulated in two ordinances [22, 23]. All cases of VL are reported to the regional health inspectorate (RHI) by the diagnosing physician. Each case must be confirmed by additional tests at the Department of Parasitology and Tropical Medicine (DPTM) at the National Centre of Infectious and Parasitic Diseases (NCIPD), Sofia. According to the legislation [23] cases of infectious diseases are classified into the following categories: possible, probable, and confirmed. Only confirmed cases of VL are subject to mandatory notification. RHI submits a summary of all recorded cases to the National Health Information Centre on a daily basis. The Centre processes the data collected from RHIs and sends a monthly summary of the situation to the Ministry of Health, the NCIPD, and the RHIs. Every year, the DPTM at NCIPD analyses the parasitic morbidity in the country. Based on this information, the Ministry of Health takes measures aimed at increasing the effectiveness of the surveillance system. Surveillance for VL comprises a set of activities including an epidemiological investigation of the cases on site and filling in a registration card. After treatment, the patients are subject to follow-up for one year.

For this study we used (i) data from registration cards of VL cases, (ii) data from the annual analyses of the parasitic morbidity in the country released by NCIPD each year and (iii) data from the clinical exams of patients checked at the DPTM at NCIPD.

The registration cards contain personal information, medical history, clinical and laboratory data, epidemiological and treatment information and data from parasitological laboratory tests.

We conducted a retrospective analysis of the medical records of the confirmed VL cases (both autochthonous and imported) in Bulgaria. The incidence per 100,000 population was calculated on the basis of

the information available from the National Statistical Institute about the number of the Bulgarian population per years [24, 25].

With the existing surveillance system in Bulgaria, underreporting of diagnosed VL cases is unlikely. Based on this, we consider the limitations or bias in this study as minimal. An approval from the ethical commission was not necessary for this study.

## Results

During the study period, 122 cases of VL were registered (Figure 1). Of these, 118 were autochthonous with patients from 51 settlements (urban and rural) in 13 of the 28 regions in Bulgaria. Four imported cases were recorded in Bulgarian citizens who had visited European countries in the Mediterranean region [9-11,26].

Cases of VL were registered in all years between 1988 and 2012, except for 1991, 1995, 1996 and 2008. The peaks of incidence were in 1989 (n=15 cases), 2002 (n=13), 2005 (n=12), 2001 (n=10) and 2004 (n=10).

Throughout the years we could observe that the incidence of VL was fluctuating. In different periods as in 1988–1990, 2001–2007 and 2009 the incidence was higher compared to the established average annual incidence of 0.06 per 100,000 population for the whole period (Figure 2).

### Age and sex distribution

Sixty-eight (56%) of the 122 patients were children and teenagers up to 18 years of age, and 54 (44%) adults. When analysing the age among children we found that the most affected group were children between one and two years of age (n=18), followed by children under one year (n=15). The group of children between zero and five years of age prevails among all persons with VL (n=48; 71% of cases among children and 39% of the total number of cases). The average age of the affected children in Bulgaria is 4.7 years. Ninety-one (75%) of the cases were male.

### HIV co-infection

Among the 54 adults diagnosed with VL, only one person was co-infected with human immunodeficiency virus (HIV).

### Mortality

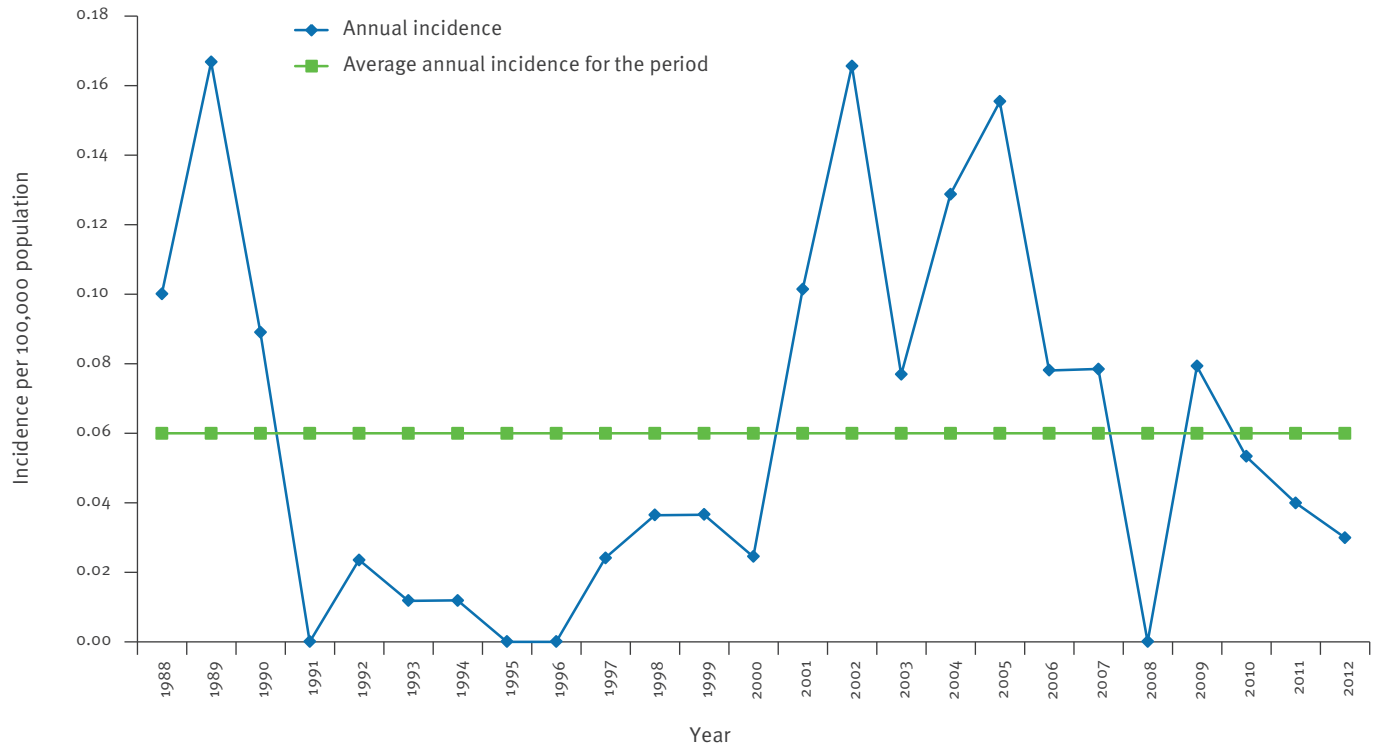
Seventeen people (13.9%) died from VL during the study period. The highest number of deaths (n=9) was registered in 1989. Average annual mortality for the whole period 1988 to 2012 was 0.01 per 100,000 population.

### Seasonality

Cases of VL were recorded in all months of the year. In more than a half of the cases (61 of the 118 autochthonous cases) the first clinical symptoms were noticed during the months from October to January.

**Figure 2**

Incidence of notified cases of visceral leishmaniasis per 100,000 population, Bulgaria, 1988–2012 (n=122)



## Table

Visceral leishmaniasis cases by age, sex, residence, and place of infection, Bulgaria, 1988–2012 (n=122)

| Characteristic | Age | | Sex | | Residence[a] | | Place of infection | |
|---|---|---|---|---|---|---|---|---|
| | Children | Adults | Men | Women | City | Village | Autochthonous | Imported |
| Number of cases | 68 | 54 | 91 | 31 | 82 | 36 | 118 | 4 |
| Percentage% | 56 | 44 | 75 | 25 | 69 | 31 | 97 | 3 |

[a] For autochthonous cases only (n=118).

## Figure 3

Autochthonous human cases of leishmaniasis (n=118) and results of seroepidemiological screening of dogs by region, Bulgaria, 1988–2012


FYROM: Former Yugoslav Republic of Macedonia.
Sources of data on dogs: [14,16].

## Autochthonous and imported cases

The majority of cases (n=118; 97%) were autochthonous. In 2006 and 2007 four (3%) imported VL cases were registered in Bulgarian citizens, two of whom had travelled to Italy and two to Spain.

## Residence and geographical distribution of cases

In terms of the type of residence in which the cases lived, 82 (69%) of them lived in urban and 36 (31%) in rural settlements (Table).

Cases of VL were recorded mainly in southern Bulgaria (Upper Thracian Plain and the Valley of Struma River), but cases of VL occurred also in northern Bulgaria indicating that the whole territory of the country is potentially endemic.

Figure 3 shows the notified autochthonous VL cases by region of residence. The highest number of autochthonous cases (n=96; 81%) was recorded in urban and rural settlements located at altitudes below 300 meters and typically with hilly landscape.

# Discussion

In this paper we studied the distribution of VL in Bulgaria over a 25-year period from 1988 until 2012. The vast majority of patients were autochthonous cases and this is convincing evidence for the presence of local transmission of VL in rural and urban areas. Although Bulgaria is not situated in the Mediterranean, there are favourable environmental conditions for local transmission of the disease. Our results showed that most cases were probably infected during the warmer months of the year (June-October) when phlebotomine sandflies are active.

The average age of the affected children in Bulgaria (4.7 years) is similar to the Mediterranean countries. Most of the patients lived in urban areas and a possible explanation for this finding could be that some of the sylvatic foci are located close to the cities with recorded cases. Most people living in cities in Bulgaria are closely linked to the rural settlements because they have relatives living there and visit them often (farming, hunting, recreational activities). The fact that they visit the villages often for various activities means that they are more frequently in contact with these foci.

Only one case of HIV/VL co-infection was recorded. Although HIV-testing was not performed in all patients, we consider this number correct because in the follow-up observation for at least one year, no cases of relapse have occurred and all adult patients were definitely cured. Furthermore, this assumption may be supported to some extent by the low number of HIV-positive people in Bulgaria. According to the National Program for Prevention and Control of HIV/acquired immunodeficiency syndrome (AIDS) at the Bulgarian Ministry of Health, by the end of 2012, the total number of officially registered persons living with HIV/AIDS in the country was 1,647 [27]. This is different from other countries in southern Europe where in the past up to 70% of the cases of VL in adults were associated with HIV infection, and HIV/VL co-infection was distributed mostly among adults where 77.3% of the recorded cases with co-infection affected the age group between 31-50 years old [4,28]. The incidence of new VL cases in HIV-positive patients dropped about 50 to 65% in the Mediterranean area after highly active antiretroviral therapy (HAART) was introduced [29].

Even though cases occurred in nearly all parts of the country, most VL cases during the study period (110 of the 118 autochthonous cases) occurred among residents of the southern part of the country and the highest number of cases was found in south-western Bulgaria (Blagoevgrad region) and central-southern Bulgaria (Stara Zagora). The presence of single cases of VL in the northern part of the country is probably due to the very similar climatic, geographic and faunistic features of the separate geographical zones in Bulgaria which define the epizootology and epidemiology of the disease. Another potential explanation could be the fact that people living in the northern part of the country have acquired the infection while travelling in the rural regions of the south.

Still considered by some as a tropical disease, zoonotic VL is endemic in a number of southern European countries. Cases of VL have been recorded in all countries neighbouring Bulgaria: Turkey, Greece, Romania, Serbia and the former Yugoslav Republic of Macedonia [30,31]. In southern Europe the incidence is relatively low: 0.02–0.49 per 100,000 population (estimated at around 700 new cases per year) [32]. Our data are in line with these estimates: the average incidence of VL from 1988 to 2012 is 0.06 per 100,000 population and new cases of the disease are recorded locally almost every year.

Although the incidence of VL in Bulgaria is relatively low, its severe course and the possibility of a lethal outcome is a reason to regard leishmaniasis as an illness with high impact on public health.


## Conflict of interest

None declared.

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# Epidemiology of human leishmaniasis in Greece, 1981-2011

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Leishmaniasis is endemic and mandatorily notifiable in Greece. Epidemiological surveillance data for leishmaniasis in Greece between 1981 and 2011 are presented. In 1998, the notification system began distinguishing between visceral and cutaneous leishmaniasis. The mean annual incidence of reported leishmaniasis cases between 1998 and 2011 was 0.36 per 100,000 population. Of a total 563 leishmaniasis cases reported after 1998, 523 (93%) were visceral leishmaniasis cases. Incidence of reported visceral leishmaniasis cases fluctuated during this period, generally decreasing after 2007, with a small re-increase in 2011. The mean annual incidence rate of reported visceral leishmaniasis cases was significantly higher in less than four year-olds (p <0.001). Leishmaniasis cases occurred both in the country mainland and islands. Between 1998 and 2011, Attica concentrated almost half of the reported visceral leishmaniasis cases, with incidence rates in western Attica and western Athens above 12.00 per 100,000 population. Compared to visceral leishmaniasis, cutaneous leishmaniasis had a rather sporadic distribution, with many prefectures appearing free of cases. From 2004, the notification also included risk factors and of 287 cases with known immune status, 44 (15%) were immunocompromised. Moreover having a dog at home was reported by 209 of 312 leishmaniasis cases (67%), whereas 229 of 307 cases (75%) reported the presence of stray dogs near their residence. Linking clinical surveillance data with laboratory data and improving collaboration with the veterinary public health sector are some of the future challenges for leishmaniasis surveillance in Greece.

## Introduction

Leishmaniasis is a vector-borne disease, caused by parasitic protozoans of the genus *Leishmania* and the disease is transmitted by phlebotomine sandflies [1]. Less common ways of infection include infected blood transfusion, congenital infection and parenteral transmission [2]. The most common forms of the disease in humans are the visceral and the cutaneous form. Visceral leishmaniasis causes a systemic disease characterised by fever, hepatosplenomegaly, anaemia and lymph node enlargement, and may be fatal without appropriate treatment, while cutaneous leishmaniasis mainly causes skin ulcers and is considered a less severe form of the disease [3].

Greece is considered to be an endemic country for both forms of the disease, with visceral leishmaniasis being the predominant form, endemic in nearly all geographical areas of the country and cutaneous leishmaniasis occurring sporadically [4,5]. *L. infantum* is the responsible species for the clinical manifestations of visceral leishmaniasis (and some cases of cutaneous leishmaniasis), while the vector species that transfer this type of parasite are *Phlebotomus neglectus, P. tobbi and P. perfiliewi* [6-9]. Anthroponotic cutaneous leishmaniasis is also present in Greece, caused by *L. tropica*, which is transmitted by P. sergenti [6]. Sporadic cases caused by *L. tropica* have been diagnosed both in the Greek mainland and in Greek islands [5,10,11].

The objective of this article is to present epidemiological surveillance data for human leishmaniasis in Greece, collected the last 30 years (1981–2011).

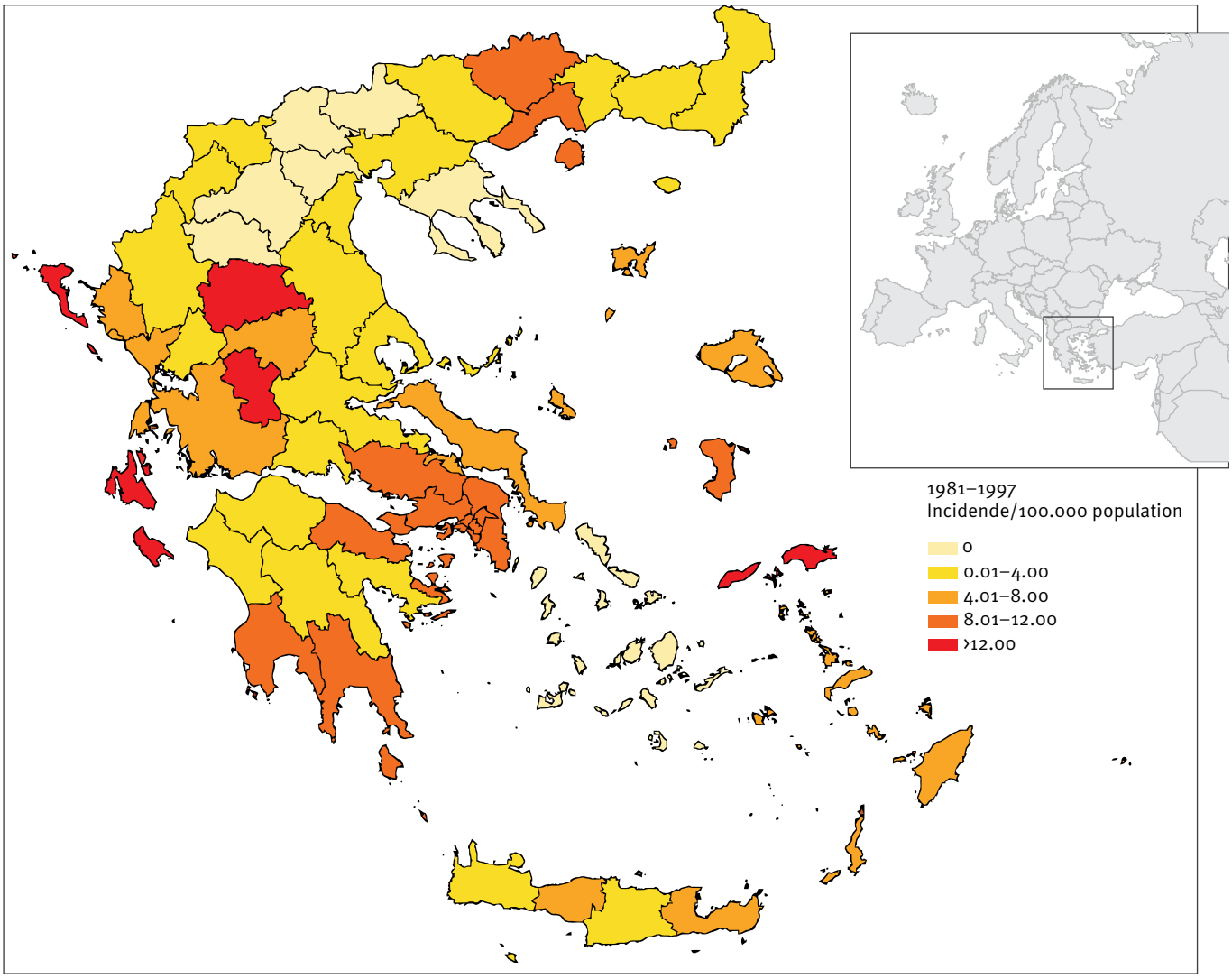
## Methods

Leishmaniasis is a mandatory notifiable disease in Greece. The national mandatory notification system is operating since 1998 under the auspices of the Hellenic Center for Disease Control and Prevention, which is responsible for the collection, processing and analysis of epidemiological data on communicable diseases in the country. Prior to 1998, aggregated leishmaniasis data were notified directly to the Hellenic Ministry of Health via the prefectures' public health directorates of the country.

In 2003, the mandatory notification system was redesigned both in the context of harmonising the national surveillance system with the European Union (EU)

**Figure 1**

Laboratory-confirmed leishmaniasis cumulative incidence rate per 100,000 population by prefecture of cases' residence, Greece, 1981–1997 (n=688)




surveillance framework and in the context of preparations for hosting the 2004 Olympic Games. The consequent changes regarding leishmaniasis surveillance were mainly the alteration of the notification time frame from a monthly to a weekly basis and the use of a redesigned notification form that included risk factors for infection, as well as clinical manifestations and laboratory findings.

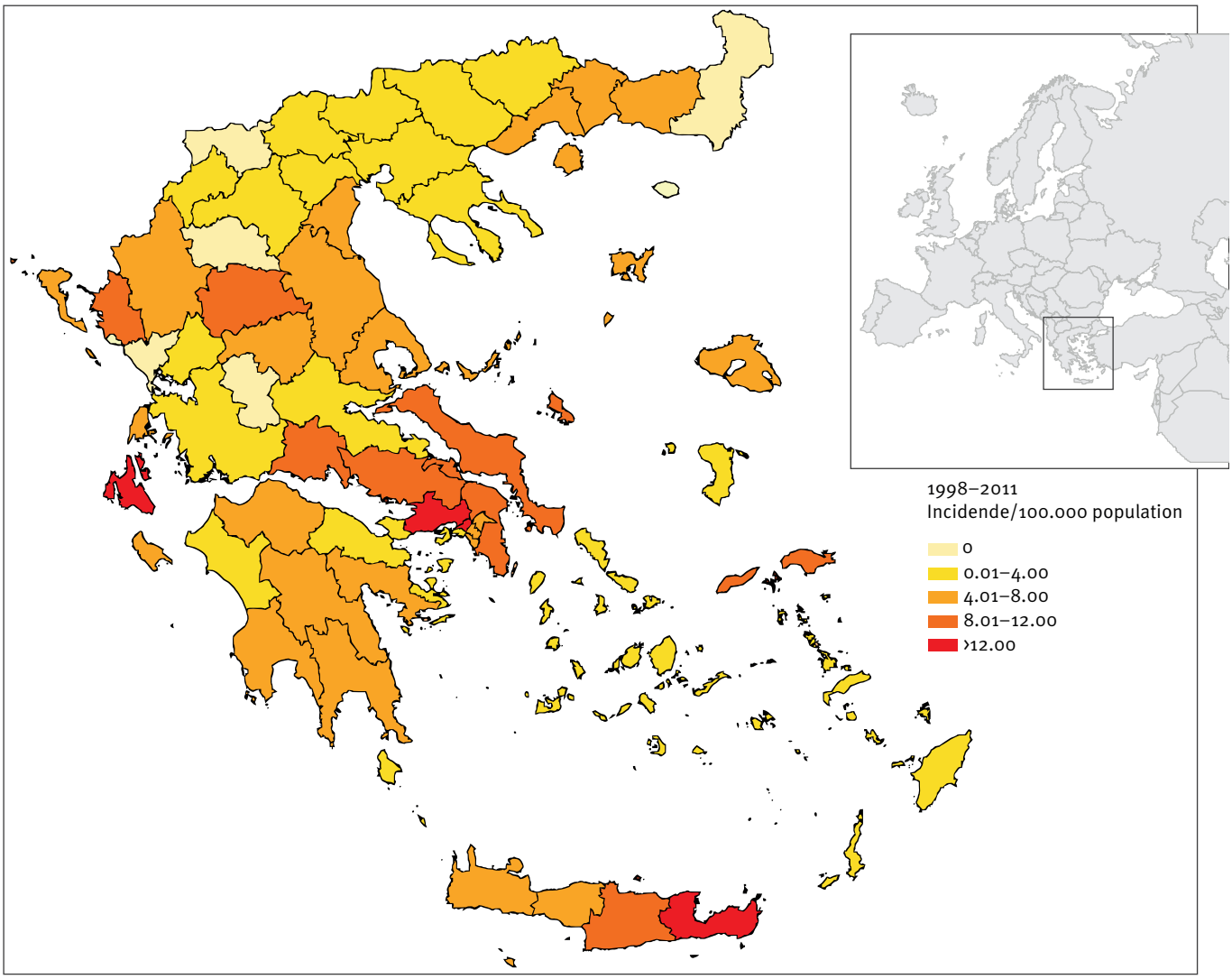
According to the Hellenic mandatory notification system, a confirmed visceral leishmaniasis case is an individual with clinical manifestations compatible with visceral leishmaniasis (persistent fever, splenomegaly, substantial weight loss, anaemia, lymph node enlargement) and laboratory confirmation via serology and/or detection of the pathogen on clinical samples. A confirmed cutaneous leishmaniasis case is an individual with clinical manifestations compatible with cutaneous leishmaniasis (appearance of skin lesions – nodular or ulcerative – usually on exposed areas of the body, which can be followed in some cases by the appearance of mucosal lesions) and laboratory confirmation via detection of the pathogen on clinical samples (in case of presence of mucosal lesions only, laboratory confirmation is performed via serology).

From 1981 to 1997, aggregated data on laboratory-confirmed leishmaniasis cases were derived from the Hellenic Ministry of Health records, while data on laboratory-confirmed leishmaniasis cases from 1998 to 2011 were derived from the national mandatory notification system. The distinction between visceral and cutaneous leishmaniasis cases was introduced in the notification process in 1998.

For a period limited to between 2004 and 2009, leishmaniasis cases were also reported from the Laboratory of Clinical Bacteriology, Parasitology, Zoonoses and

**Figure 2**

Laboratory-confirmed visceral leishmaniasis cumulative incidence rate per 100,000 population by prefecture of cases' residence, Greece, 1998–2011 (n=558)




Of a total 614 visceral leishmaniasis cases reported to the surveillance system in Greece between 1998 and 2011, the figure shows the 558 with available information on prefecture of residence.

Geographical Medicine of the Medical School of the University of Crete and from the Reference Laboratory for Opportunistic Infections of the Department of Parasitology, Entomology and Tropical Diseases of the National School of Public Health. Data on age, sex and risk factors were not available for these cases.

Data about leishmaniasis cases were compiled from 1998 through 2011 with respect to age, sex, Greek citizenship and hospitalisation. In addition, for the period from 2004 to 2011, during which the reformed mandatory notification system was operational, data were compiled regarding risk factors for the disease (owning a dog, presence of sandflies in the area of residence, presence of stray dogs in the proximity of the patients' residence, being immunocompromised), clinical manifestations and laboratory findings.

To assess temporal variation, annual incidences per 100,000 population were calculated for the period from 1998 to 2011, using data from the mandatory notification system and population data from the 2001 census population. Cumulative incidence per 100,000 population was calculated by prefecture of cases' residence, for the period from 1981 to 1997 (1991 census population), based on aggregated data from the records of the Hellenic Ministry of Health. On the other hand, cumulative incidence per 100,000 population was calculated by prefecture of cases' residence for the period between 1998 and 2011, based on data derived from the national mandatory notification system, and also including the cases reported from the Laboratory of Clinical Bacteriology, Parasitology, Zoonoses and Geographical Medicine of the Medical School of the University of Crete and the Reference Laboratory for Opportunistic Infections of the Department of

Parasitology, Entomology and Tropical Diseases of the National School of Public Health.

Incidence rate ratios were tested for significance using the chi-squared test. A p value ‹0.05 was considered significant. Data were analysed with Stata v 12.1., and area maps were created using Epi Map (EpiInfo v 3.4.3).

## Results

### Cumulative incidence rates of leishmaniasis per prefecture from 1981 to 1997

From 1981 through 1997, a total of 688 aggregated laboratory-confirmed cases of leishmaniasis were reported. The respective period's cumulative leishmaniasis incidence rate by prefecture of cases' residence is depicted in Figure 1.

From 1981 to 1997, in the mainland, the prefectures with cumulative leishmaniasis incidence rate of reported cases above eight per 100,000 population are located mainly in central Greece, Thessaly, southern Peloponnese, and eastern Macedonia. In the islands, cumulative incidence rates of reported cases above 12.00 per 100,000 population are observed for the islands of Corfu, Kefallonia and Zakynthos in the Ionian Sea, and in Chios and the island complex of Samos in the Aegean Sea.

### Cumulative incidence rates of leishmaniasis per prefecture from 1998 to 2011

From 1998 to 2011, 563 laboratory-confirmed leishmaniasis cases were reported through the national mandatory notification system. An additional 101 cases were reported from the Laboratory of Clinical Bacteriology, Parasitology, Zoonoses and Geographical Medicine of the Medical School of the University of Crete and the Reference Laboratory for Opportunistic Infections of the Department of Parasitology, Entomology and Tropical Diseases of the National School of Public Health, for the years 2004 to 2009 (an additional 39% to the 260 cases reported via the mandatory notification system in the same period). From the total of 664 cases, 614 were visceral leishmaniasis cases and 50 were cutaneous leishmaniasis cases.

Figure 2 presents the 1998 to 2011 cumulative visceral leishmaniasis incidence rate by cases' prefecture of residence for a total of 558 cases for which residence was known.

During the years between 1998 and 2011, in the mainland, the prefectures with cumulative incidence rate of visceral leishmaniasis reported cases above eight per 100,000 population are located mainly in central Greece, with the Attica region, concentrating almost half of the reported visceral leishmaniasis cases (253 cases). In the islands, cumulative incidence rates of reported cases above eight per 100,000 population are observed mainly for the island of Kefallonia in the Ionian Sea (7 cases), for the Samos island complex of the Aegean Sea (5 cases), and for the island of Crete (Heraklion prefecture: 24 cases, Lasithi prefecture: 12 cases).

Of a total 50 cutaneous leishmaniasis cases reported to the surveillance system in Greece between 1998 and 2011, information on prefecture of residence was available for 47. In the mainland, the prefectures reporting cutaneous leishmaniasis cases were located in Peloponnese (Achaia, Arkadia, Ilia, Argolis, Lakonia), in central Greece (Aitoloakarnania, Phthiotis, Attiki, Evia), Thessaly (Trikala) and Macedonia (Thessaloniki and Serres). In the islands, cutaneous leishmaniasis cases were reported in Crete (Heraklion, Lasithi) and in Chios and Samos in the Aegean Sea. All of these prefectures had a cumulative incidence rate of cutaneous leishmaniasis reported cases below four per 100,000 population, with the exception of the Lakonia prefecture, which exceeded this value (6 reported cases in total).

### Annual incidence rates of leishmaniasis

For the period from 1998 to 2011, the mean annual incidence rate of laboratory-confirmed leishmaniasis reported cases, based on data from the national mandatory notification system, was 0.36 per 100,000 population. Of the 563 laboratory-confirmed cases reported through the mandatory notification system, 523 cases (93%) were visceral leishmaniasis cases. The annual incidence rates of reported laboratory-confirmed cases of visceral leishmaniasis for the years between 1998 and 2011 (n=523) is depicted in Figure 3.

Visceral leishmaniasis annual incidence rate of reported cases presents fluctuations (mean annual incidence rate: 0.34, range: 0.17–0.46) and the lowest values are recorded in 1998 and 2003 (0.21 and 0.17 per 100,000 population, respectively). The low incidence in 1998 coincides with the beginning of reporting of leishmaniasis via the national mandatory notification system, while in 2003, the low incidence coincides with a reform of this system, whereby notification forms requiring information on risk factors for the disease, clinical manifestations and laboratory findings were introduced. From 2007, a decrease in the incidence rate below 0.36 per 100,000 population was observed, with a small re-increase in 2011.

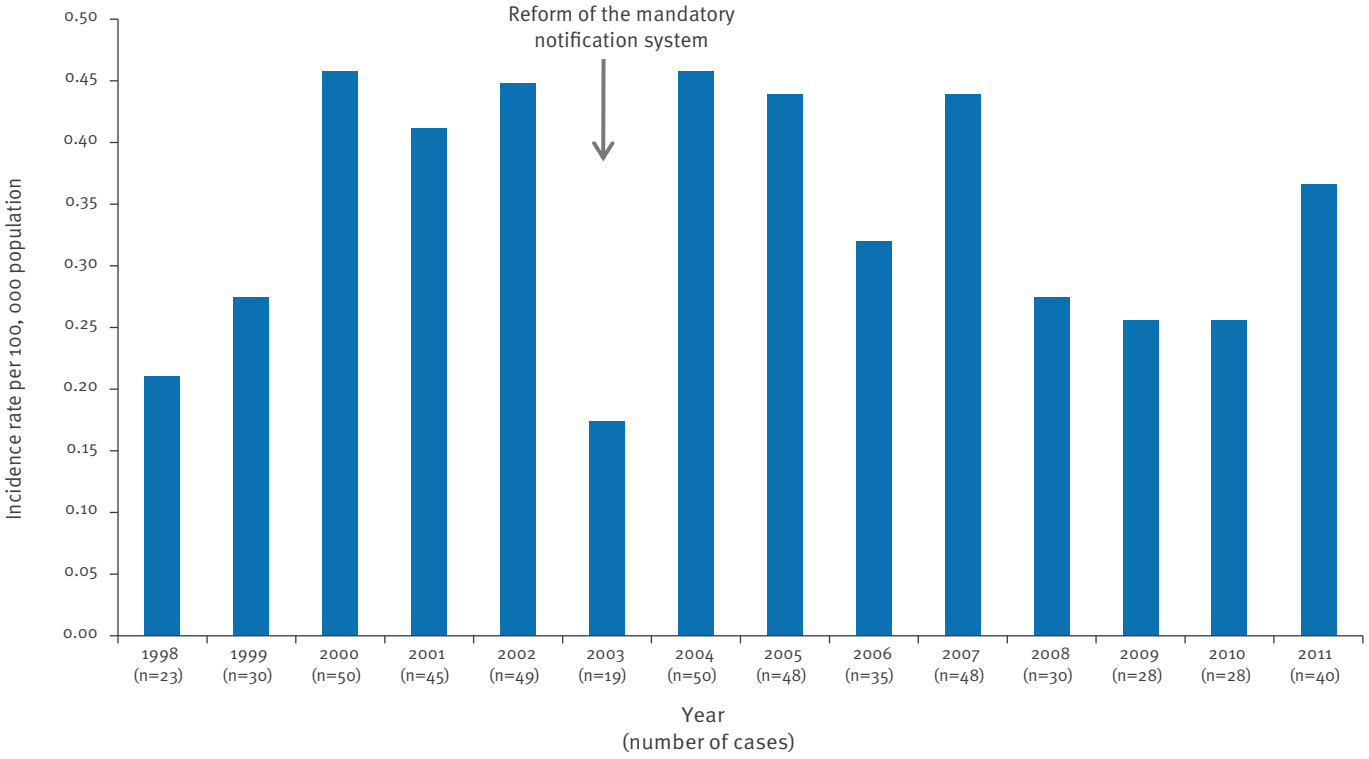
### Age and sex distribution and origin of leishmaniasis cases

Information on sex and age was available for 500 (96%) of the visceral leishmaniasis cases, among which 330 (66%) were male. Distribution by sex and age is presented in Figure 4.

The disease was present in all age groups. The age group comprising individuals below four years-old had a statistically significantly higher mean annual incidence rate compared to every other age group (p value‹0.001 in all comparisons). The majority of cases in all age groups were of male sex.

**Figure 3**

Annual incidence rate of laboratory-confirmed reported cases of visceral leishmaniasis per 100,000 population, Greece, 1998–2011 (n=523)




The total 523 visceral leishmaniasis cases represented in the Figure are those reported by the mandatory notification system in Greece from 1998 to 2011.

Four hundred and forty seven cases of the 523 reported visceral leishmaniasis cases had Greek citizenship and 70 were of foreign origin (for 6 cases the relevant information was unknown). Among the 482 visceral leishmaniasis cases (92%) for which information about hospitalisation was known, 461 were hospitalised (96%). The number of reported visceral leishmaniasis cases showed no apparent seasonal trend. Cases were almost equally distributed by month of reporting (median number of reported cases by month: 43.6, range: 33–54).

A total of 40 of 563 cases reported by the mandatory notification system were cutaneous leishmaniasis cases. Information on sex and age was available for 38 cases, among which 22 were male. The age group comprising five to 14 year-olds had the highest mean annual incidence rate (0.044 per 100,000 population), followed by the age group with 15 to 24 year-olds, with a mean annual incidence rate of 0.032 per 100,000 population. Twenty four cases were Greek citizens and 16 were of foreign origin. Twenty one cases were hospitalised.

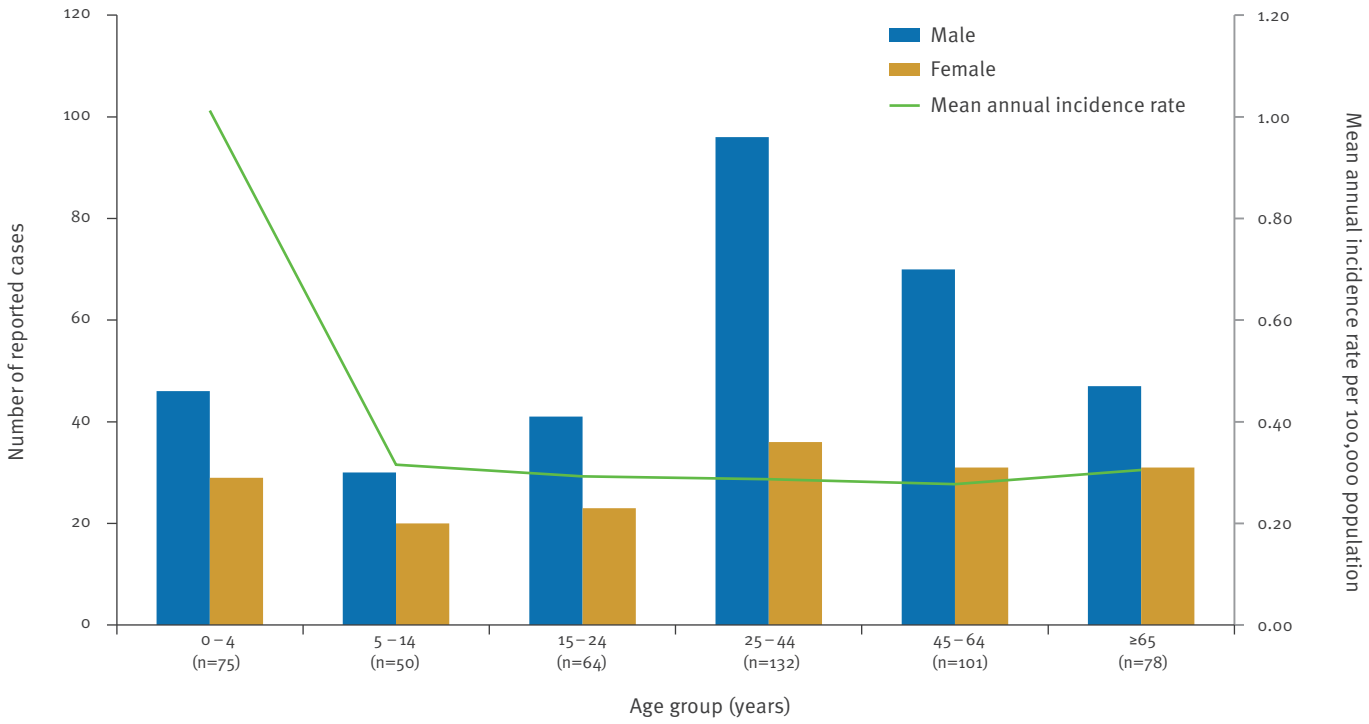
## Clinical manifestations, laboratory findings and risk factors for leishmaniasis

From 2004 to 2011, a total of 330 leishmaniasis cases were reported via the reformed mandatory notification system. Information on immune status was available for 287 cases (87%), of which 44 (15%) were reported as immunocompromised. Information on the risk factor 'owning a dog' was available for 312 cases (94%), with a total of 209 (67%) cases reporting having a dog at home. Presence of sandflies in the area of residence was reported for 216 of the 298 cases for which information was available (72%). The respective percentage for the presence of stray dogs in the proximity of the patients' residence was 75% (229 of 307 cases, for which information was available).

Among the 330 cases reported from 2004 through 2011, a total of 307 (93%) were cases of visceral leishmaniasis. Two hundred and twelve (69%) of the latter were confirmed via serological testing and 121 (39%) via detection of the pathogen on clinical samples. Regarding clinical manifestations, 253 (82%) cases were reported with persistent fever, 260 (85%) with hepatomegaly or splenomegaly, 53 (17%) with

**Figure 4**

Age and sex distribution of reported visceral leishmaniasis cases, Greece, 1998–2011 (n=500)




The mean annual incidence is the mean annual number of leishmaniasis cases per 100,000 of the age group under consideration.
The 500 leishmaniasis cases represented in the Figure are those from a total of 523 reported by the mandatory notification system in Greece for which information on sex was available.

lymphadenopathy, six (2%) with cutaneous nodular lesions, two (1%) with cutaneous ulcerative lesions and five (2%) with mucosal lesions.

Among the 23 cases of cutaneous leishmaniasis reported from 2004 through 2011, five (22%) were confirmed via serological testing and 21 (91%) via detection of the pathogen on clinical samples. Information on clinical manifestations was available for all 23 cases. Of these 11 (48%) were reported with cutaneous nodular lesions, 14 (61%) with cutaneous ulcerative lesions and three (13%) with mucosal lesions.

## Discussion

This report aims to provide epidemiological information for leishmaniasis in Greece during the last 30 years (1981–2011), by analysing national epidemiological surveillance data.

During this period, there were two important alterations in the way the disease is reported in the country; one in 1998 (which involved change from aggregated data collection to case by case data collection) and one in 2003 (which consisted in a reform of the national mandatory notification system, whereby disease specific notification forms were introduced, that included information on risk factors, as well as clinical manifestations of the disease and laboratory findings). Both alterations aimed to improve the disease's surveillance via a more thorough collection of information. We believe that once the introduced changes were incorporated in the system and assimilated by the reporting physicians, they contributed to a better description of the disease's epidemiological features.

The mean annual incidence of reported leishmaniasis cases per 100,000 population for the years 1998 to 2011 in Greece was 0.36. According to data from the Centralized Information System for Infectious Diseases/World Health Organization (CISID/WHO), the 1998 to 2010 mean annual incidence of reported leishmaniasis cases per 100,000 population for Italy and Spain was 0.23, whereas the respective number for France, for the years 2003 to 2010 was 0.24 [12]. The comparatively higher incidence in Greece may be the result of a number of factors, including for example warm climate, a high background of canine leishmaniasis and changes in agricultural pesticide practices that in the past contributed to sandfly population suppression [13]. In particular, high prevalence of canine

leishmaniasis, is becoming a crucial risk factor for leishmaniasis in humans [14], while serological screening in canine populations is thought to generally underestimate the existing prevalence of the infection [15].

Comparisons between countries are of limited value if not accompanied by an estimation of the magnitude of underreporting. In Greece, the magnitude of underreporting remains unknown, being crudely estimated by WHO as mild (1.2–1.8 fold) [16]. In this study, between 2004 and 2009, the Laboratory of Clinical Bacteriology, Parasitology, Zoonoses and Geographical Medicine of the Medical School of the University of Crete and from the Reference Laboratory for Opportunistic Infections of the Department of Parasitology, Entomology and Tropical Diseases of the National School of Public Health reported 101 leishmaniasis cases in addition to the 260 reported by the mandatory notification system. The total number of 361 cases during the 2004 to 2009 period corresponds to approximately 1,4 fold the number of cases reported to the mandatory system. Considering that the laboratories reporting these extra cases are two of the biggest performing *Leishmania* identification in the country, the value of 1.4 fold, as an estimation of the magnitude of underreporting seems to be consistent with the WHO estimation.

The annual incidence rates of the reported visceral leishmaniasis cases are presented starting from 1998, the year of the first reform of the Hellenic surveillance system. With the exception of the year 2003, where a low in the incidence rate (0.17 per 100,000 population) could be attributed to the reporting healthcare workers adapting to the newly introduced, redesigned notification forms, the annual incidence rate of the reported cases remained in general stable, with a decrease occurring after 2007 followed by a slight re-increase in 2011.

Only 40 (7%) of the 563 cases reported from 1998 to 2011 were cutaneous leishmaniasis cases. It is notable that a considerable number of cutaneous leishmaniasis reported cases (16 of 40) were of foreign origin, with a possibility of being imported.

Data from the mandatory notification system cannot be considered a reliable source of information regarding the responsible pathogens, as the relevant field on the mandatory notification form is rarely completed by the reporting physicians. In a survey conducted in the island of Crete, covering the period from 1986 to 2010, all isolated strains (n=16) from visceral leishmaniasis patients were of *L. infantum* type, while isolated strains from cutaneous leishmaniasis patients (n=5) were of *L. infantum* (n=3) and *L. tropica* type (n=2) [5].

Regarding visceral leishmaniasis, all age groups were affected, with 375 of 500 (75%) of the cases being older than 14 years-old. This is a finding that does not seem to conform to the findings of studies in other Mediterranean countries, such as Turkey and Malta, where the majority of visceral leishmaniasis cases is below this age [17,18]. Compared to every other age group, the age group comprising less than four year-olds in Greece had a statistically significantly higher mean annual incidence rate. Cutaneous leishmaniasis infection is reported to be more frequent in the age groups of five to14 and 15 to 24 year-olds, a finding that seems to be in line with data from Turkey, where the infection is reported to be more frequent in the age group of 10 to 19 year-olds [18].

During the period from 1998 to 2011, Attica concentrates almost half of the reported visceral leishmaniasis cases, with western Attica and western Athens presenting incidence rates above 12.00 per 100,000 population, whereas their incidence rate was lower in the first study period from 1981 to 1997. This observed cumulative incidence rate increase between the two periods could be probably explained by an increase in seroprevalence in dogs in Attica [19]. In the island of Kefallonia in the Ionian Sea and in the island complex of Samos in the Aegean Sea, stable cumulative incidence rates of the disease's reported cases of above 8.00 per 100,000 population are observed across the two study periods, a finding that should be interpreted with caution, as in the case of rare diseases, areas with small populations appear to have high incidence rates even when a small number of cases occurs. Another similar example is that of the Evritania prefecture, with a population of approximately 20,000 people. Although Evritania has a zero cumulative incidence rate after 1998, it appears as a high cumulative incidence rate prefecture before 1998, although only three cases of the disease were reported since 1981.

A cumulative incidence rate increase after 1998 is observed in the prefectures of the island of Crete, which could be explained by an increasing tendency in seroprevalence and incidence in dogs in Crete [20]. Another possible explanation could be a reporting and diagnostic bias, due to the location in the island of the Laboratory of Clinical Bacteriology, Parasitology, Zoonoses and Geographical Medicine, which was established there in the early 1990s.

Finally, comparing visceral and cutaneous leishmaniasis geographical distribution during the period from 1998 to 2011, it is notable that cutaneous leishmaniasis has a rather sporadic geographical distribution, with a large number of prefectures appearing free of cases.

In 2003, notification forms were redesigned to include laboratory data and risk factors related to leishmaniasis infection. Being immunocompromised was reported by 15% of the cases for which immune status was known, although no data is collected through the mandatory notification system regarding co-infection with human immunodeficiency virus. 75% of cases for which relevant information was available, reported presence of stray dogs in the proximity of their residence, whereas the percentage of cases owning a dog was lower (67%).

As stray dogs live outdoors, the possibility of exposure and infection is expected to be much higher than in pets that are used to stay indoors [17]. According to data from the Hellenic Veterinary Association, the total number of owned dogs in Greece is estimated to be around 500,000, leading to a crude estimation of approximately 15% of the general population having a dog at home. Analytical studies could shed more light regarding interdependence between presence of dogs and acquisition of human infection.

The emergence of leishmaniasis in non endemic European countries as well as the re-emergence of the disease in the Mediterranean region of Europe have recently been identified as possible scenarios, whereas there are indications that the disease has been more or less neglected at the public health policy level [6,18]. In order to be able to perform an effective risk assessment at the European level, the availability of data about leishmaniasis and its spatial distribution in Europe and the Mediterranean region is crucial. Having robust and effective national surveillance systems is an important step in this direction and efforts to improve surveillance should be systematic and continuous. Linking laboratory data with clinical surveillance, as well as coordinating the exchange of information between the human public health and the veterinary public health sector are some of the challenges that the Greek surveillance system has to meet in the future.

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# Asymptomatic *Leishmania infantum* infections in humans living in endemic and non-endemic areas of Croatia, 2007 to 2009

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**The prevalence of asymptomatic leishmaniasis in the general population of Croatia has not been studied to date. To assess the prevalence of *Leishmania infantum* specific IgG antibodies among immunocompetent residents of Croatia, sera from 2,035 persons (eastern coast of Adriatic Sea, n=1,186; Adriatic islands, n=653; mainland, n=196), were tested by an enzyme immunoassay. A total of 231 (11.4%) persons had anti-*Leishmania* antibodies. Multivariate analysis revealed that seropositivity was associated with geographic location and age. Residents of coastal areas and islands were significantly more seropositive than mainland residents (odds ratios (OR) 20.37 to 28.51). Moderate to high anti-*Leishmania* seroprevalence was found throughout the eastern Adriatic coast and islands (4.0% to 22.2%) including the sites previously considered non-endemic. A highly endemic focus was identified in central coastal Dalmatia (seroprevalence 22.2%; OR: 1.72; 95% confidence interval (CI): 1.33-2.22). Regarding age, children aged 0-9 years were the most vulnerable group for asymptomatic *Leishmania* infection (OR: 2.19; 95% CI: 1.16-4.14).**

## Introduction

Leishmaniasis is caused by *Leishmania* spp., hemoflagellate protozoa belonging to the family Trypanosomatidae. Infected phlebotomine sandflies serve as vectors for the transmission of all *Leishmania* species. Human leishmaniasis can be divided into three main disease manifestations: (i) visceral leishmaniasis (VL), (ii) cutaneous leishmaniasis (CL) and (iii) mucocutaneous leishmaniasis (ML). The strain of the infecting organism and the host's immunologic status greatly influence clinical manifestations.

Zoonotic VL caused by *Leishmania infantum* is endemic in Mediterranean countries of Europe and domestic dog is the main reservoir [1-3]. During the last decade, there have been reports of spreading leishmaniasis northward into previously non-endemic areas of central and northern Europe [1-3]. It is well known that, besides clinical cases of VL, asymptomatic infections are common in endemic areas [2,3].

Croatia encompasses 56,538 km2. According to the 2011 census, it had a population of 4,284,889 [4]. Geographically, Croatia is composed of three areas: the Adriatic coastal zone with islands in the south, the Pannonian Plain in the north, and the mountainous region in-between. The Croatian littoral comprises a relatively narrow land belt with islands along the eastern coast of the Adriatic Sea (Figure). The littoral is traditionally divided into two large areas based on geography, ecology and cultural heritage: the northern (Istria and Primorje counties) and the southern part (Dalmatia) which is further subdivided into northern, central and southern Dalmatia. The Adriatic islands and the coastal zone are characterised by Mediterranean climate which provides good living conditions for sandfly vectors of *Leishmania* [5,6]. Continental Croatia has temperate continental or continental climate and is usually considered free of phlebotomine vectors of *Leishmania*.

Human VL and canine VL has been reported in central and southern coastal and insular Dalmatia (from Split to Dubrovnik) since 1930 [7]. From 1931 to 1957, 398 human VL cases were diagnosed in this region [8]. A case of CL was firstly recorded in 1945 and 201 CL cases were recorded by 1957. After the late 1950s, the number of VL cases declined, probably because of mass spraying with antimalarial insecticides [8]. Since 1990, studies have identified re-emerging foci of both human and canine VL in central and southern Dalmatia [9-11].

In Croatia notification is compulsory for both VL and CL, although these diseases are not included in the decisions of the European Parliament for reporting communicable diseases [12, 13]. Medical practitioners are notifying human leishmaniasis cases through

Croatia's health information system or directly to the epidemiologist in the regional public health institutes. Cases are defined as *probable* which is a case clinically compatible in endemic region or with epidemiological connection to a *confirmed* case, and confirmed that is a case laboratory-confirmed by positive parasitological (microscopy or cultivation) and serological (IFA, ELISA) tests. Case definitions for CL are similar, excluding the serological tests [13]. According to data periodically published by the Croatian National Institute for Public Health there were between one and four new cases of VL reported each year in Croatia in the last decade [14]. The estimated mean annual incidence of human leishmaniasis is 0.4 per 100,000 population [5]. Leishmaniasis in Croatia is described as predominately paediatric disease: almost half of VL patients are children up to the age of 10 years and the disease is more often found in men [9,10]. Most of the reported cases occurred among inhabitants of the Croatian coast and islands [9,10]. Besides this, few VL cases were diagnosed in Austrian [15] and Hungarian [16] tourists after returning from the Dalmatian littoral.

Recently, three *Phlebotomus* species, known to serve as vectors for *L. infantum* were found in central and southern coastal and insular Dalmatia [5]. A veterinary seroepidemiologic survey conducted in central Dalmatia among apparently healthy dogs using dot-ELISA, found canine seroprevalence ranging from zero to 42.85%, which was in accordance with previous recognition of central and southern coastal and insular Dalmatia as a high endemic foci of *L. infantum* for dogs [11]. In contrast to high seroprevalence ratios in dogs, no information about the prevalence of the infection in otherwise healthy human inhabitants of different Croatian regions is available.

The aim of this study was to assess the prevalence of IgG antibodies to *L. infantum* among healthy people living in different regions of Croatia, and to compare the seroprevalence in endemic regions with that in non-endemic regions. This is the first investigation on asymptomatic leishmaniasis in residents of Croatia.

## Methods

The target population for our study was the apparently healthy general population in previously recognised endemic and non-endemic areas for leishmaniasis in Croatia. The studied areas known as endemic areas: insular Croatia, and central and southern Dalmatia, as well as areas previously considered nonendemic but with favourable Medieranean climate: northern Dalmatia, Istria and Primorje. Two counties in northern Croatia with continental climate where sandflies were considered rare or absent: Brod-Posavina County (centre Slavonski Brod) and the most north-western Međimurje County (centre Čakovec), were also included.

### Serological survey

Serum samples were collected from 2007 to 2009 to examine the prevalence of IgG antibodies to *L. infantum* in Croatia. We used 'residual' sera of apparently healthy, immunocompetent individuals who did not show any symptoms of leishmaniasis and came to hospitals or clinics for routine laboratory check-ups or for blood donation. Participating hospitals' or clinics' laboratories were selected according to their geographic location: 14 served population along the Adriatic coast and two were from non-endemic parts of Croatia. Laboratories provided data on age, sex and site of residence for the study participants.

**Table 1**

Sex distribution in the study population (n=2,035) compared with that of the total Croatian population

| | Individuals tested | Male | Percentage (%) | (95% CI) | | Census population | Male | Percentage (%) |
|---|---|---|---|---|---|---|---|---|
| **All** | | | | | | | | |
| Adriatic coast | 1,186 | 571 | 48.15 | 45.30% | 50.99% | 1,247,133 | 605,605 | 48.56 |
| Adriatic islands | 653 | 268 | 41.04 | 37.27% | 44.81% | 113,875 | 56,658 | 49.75 |
| Croatian mainland[a] | 196 | 137 | 69.90 | 63.48% | 76.32% | 2,923,881 | 1,404,072 | 48.02 |
| **Region/Site of residence** | | | | | | | | |
| Adriatic coast | | | | | | | | |
| Istria and Primorje | 117 | 47 | 40.20 | (31.32% | 49.08%) | 467,678 | 226,115 | 48.35 |
| Nothern Dalmatia | 159 | 84 | 52.80 | (45.04% | 60.56%) | 255,158 | 124,849 | 48.93 |
| Central Dalmatia | 571 | 293 | 51.30 | (47.20% | 55.40%) | 419,131 | 203,513 | 48.56 |
| Southern Dalmatia | 339 | 147 | 43.40 | (38.12% | 48.68%) | 105,166 | 51,128 | 48.62 |
| Croatian mainland | | | | | | | | |
| Brod-Posavina county[a] | 107 | 96 | 89.70 | (84.0% | 95.5%) | 158,575 | 77,115 | 48.60 |
| Međimurje county | 89 | 41 | 46.10 | (35.7% | 56.4%) | 113,804 | 55,601 | 48.90 |

CI: confidence interval.

[a] Healthy donor effect bias present in Brod-Posavina county as blood donors constituted the majority of the sample.

To determine the target sample size for each region we used published statistical tables, which provide minimal sample sizes that are necessary for given combinations of precision, confidence levels, and variability.

A minimum of 1,100 individuals were selected as the target sample size for the coastal region with previously recognised endemic foci in order to reach the precision level of ±3% under assumption of the confidence level of 95%, p=0.5 and the size of population greater than 100,000 inhabitants. For islands, the targeted precision on population with more than 100,000 inhabitants was ±5% resulting in a minimum sample size of 400, whereas for the reference non-endemic continental region, under the assumption of maximum seroprevalence of 2%, the targeted sample size was 84 to reach the ±3% precision level.

Convenience sampling was used to select respondents in the most economically, technically, and operationally feasible method [17].

To estimate whether the study sample represents the research setting population, we compared the percentage of men estimated from the study sample with the 2011 census data (Table 1).

We classified sites based on their seroprevalence as 'high endemic focus' and 'moderate seroprevalence'. We derived these definitions combining the data from the literature with the results of this study. In particular, in a study by Federico et al. a seroprevalence of *L. infantum* infection of 4% in the Rome and Caltanissetta area was marked as 'moderate' [18] whereas Marty et al. detected a seroprevalence of 38% in the high endemic focus of Alpes-Maritimes, using Western blot technique, which is more sensitive than the technique used in this study [19]. The seroprevalence rate in the majority of our coastal areas was around 7% and defined as 'moderate seroprevalence'. The one area with a seroprevalence of 22% had a significantly higher seroprevalence in comparison with surrounding areas, all of which were classified as 'moderate seroprevalence'. Therefore, that site was defined as a 'high endemic focus' and the definition was further supported by epidemiological data since the majority of cases of VL in Croatia are reported from 'high endemic foci' [5,9-11].

Sera were tested for the presence of IgG antibodies to *L. infantum* by commercial enzyme-linked immunosorbent assay (NovaLisa *Leishmania infantum* IgG, NovaTec Immunodiagnostica GmbH, Dietzenbach, Germany), according the manufacturer's instructions. Declared specificity and sensitivity of the test were 85% and 91% respectively. A serum was considered positive when the ratio between the optical density (OD450) value of the serum and the cut-off was >1.1.

The study was approved by the ethics committees of the Split University School of Medicine and of the Split University Hospital. Individual informed consent was not required according to the ethical committee.

## Statistical analyses

Data were analysed with statistical package SPSS 19.0 (SPSS; Chicago, Illinois, US). Associations of seropositivity with sex or geographic region were tested by Pearson's chi-square test whereas the significance of difference in median age of participants by seropositivity was tested by Mann-Whitney U test. The strength of association between seropositivity and the site of residence was estimated by odds ratio (OR), 95% CI and p-values. For each site observed seroprevalence was compared to unweighted average rate in the accompanying region (i.e. coastal or continental) and ORs were calculated from the standard 2x2 table.

A multiple logistic regression analysis was used to evaluate the potential risk factors associated with *Leishmania* infection, including age as categorical variable, sex, and region of residence. Significance level was set at 0.05. In case of multiple testing, we adjusted the p-values with the Bonferroni correction.

## Results

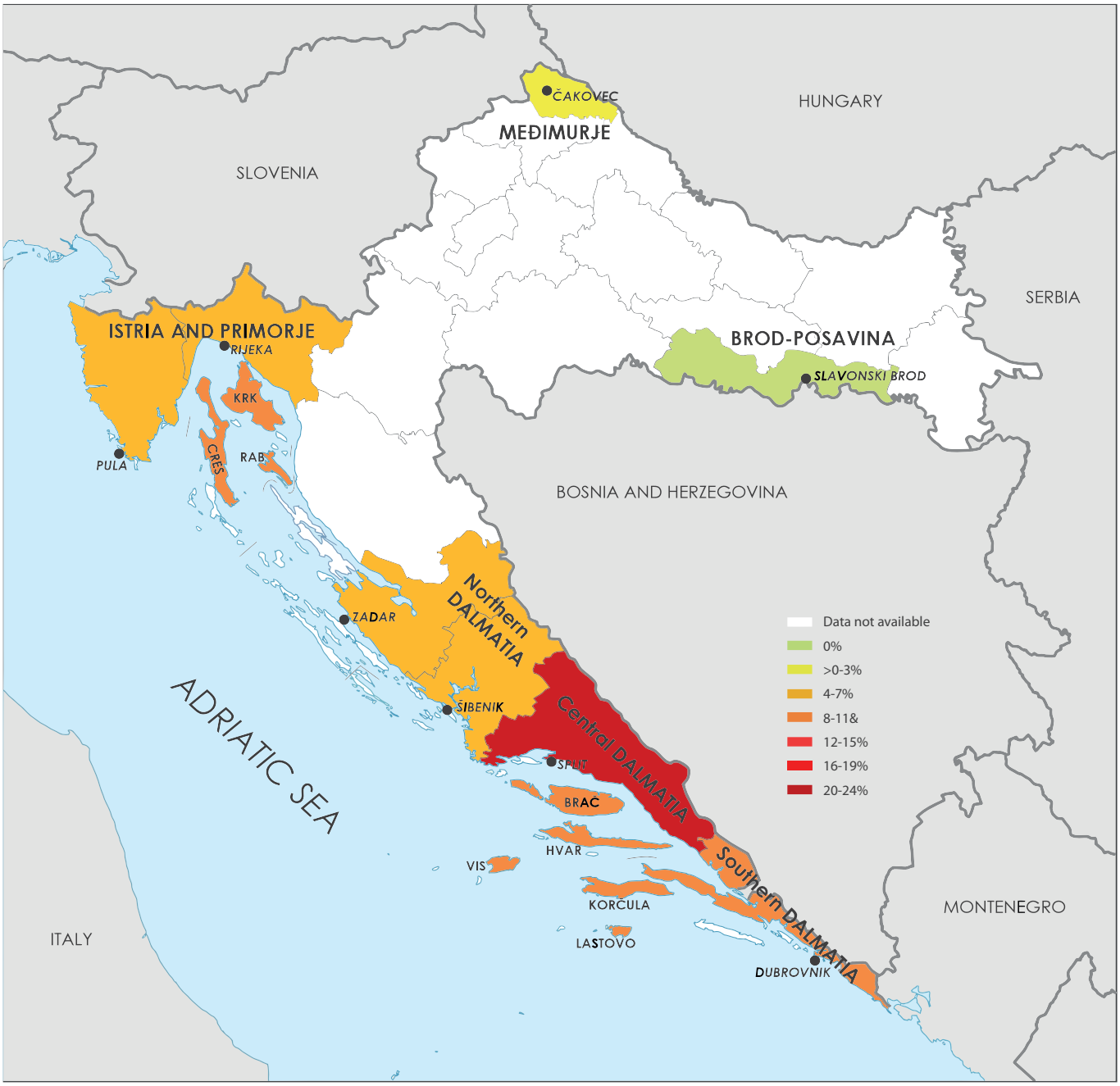
Blood specimens from 2,035 persons (975 men and 1,060 women) were collected. These included 1,186 persons in the coastal area from Istria to the Montenegro border, 653 on Adriatic islands and, for comparison, 196 residents of two northern continental Croatian counties (Table 2). The survey enrolled persons from all age groups, ranging from eight months to 88 years, with the median of 42 years (interquartile range (IQR): 21-59). The large deviation from the census data was observed only in the reference site in Brod-Posavina County, in which blood donors were overrepresented.

Of the total of 2,035 sera of healthy residents, anti-*L. infantum* IgG antibodies were found in 231 (11.4%). Seroprevalence differed significantly among Croatian sites ranging from 0.0% in Brod-Posavina County to 22.2% in central Dalmatia ($x2=112.24$; $df=13$; $P<0.001$) (Figure, Table 1). Overall, according to the place of residence, we found a strong association between seropositivity and residence in island, coast, or continental areas ($x2=35.41$; $df=2$; $P<0.001$). Post hoc chi-square analysis revealed that inhabitants of coastal areas had significantly higher seroprevalence than islanders ($x2=9.27$; $df=1$; $Pcorr=0.007$) or those from the two continental regions ($x2=29.43$; $df=1$; $Pcorr<0.001$). Furthermore, islanders had significantly higher seroprevalence than continental inhabitants ($x2=17.37$; $df=1$; $Pcorr<0.001$).

To analyse the spatial distribution pattern of *Leishmania* infection in more detail, we estimated strength of associations of seropositivity with the site of residence in particular regions: coastal, continental, or islands (Table 3). Only in Adriatic coastal counties seroprevalence of sites differed significantly from the average regional rate. While central Dalmatia inhabitants had

## Figure

Geographic distribution of seroprevalence for anti-*Leishmania* IgG in asymptomatic healthy individuals, Croatia, 2007–2009 (n=2,035)



the highest prevalence of antibodies and the highest risk for *Leishmania* infection (OR: 1.72; 95% CI: 1.33 to 2.22; p<0.001), residents of the rest of coastal areas had lower risk (OR: 0.27 to 0.52; p-value: 0.002 to 0.006) as compared to the average rate in the coastal region. Although these results clearly indicated central Dalmatia as a high endemic focus for *Leishmania* infection, the observed seroprevalence in other coastal areas (ranging from 4.3% to 8.0%) also indicated these areas as endemic sites where the seroprevalence was higher than in continental Croatia.

Regarding sex and age as potential risk factors, we found no association of seroprevalence with sex (x2=0.11; df=1; p=0.739). Among 975 men, antibodies were found in 113 (11.6%) sera, while among 1,060 women, antibodies were found in 118 (11.1%) sera. This finding was further confirmed by the multivariate model with seropositivity as dependent, and age, sex and geographic location as independent variables. Multivariate logistic regression analysis of *Leishmania* seropositivity in association with age, sex and geographic location is shown in Table 4.

In contrast to sex, we found significant differences in the median age of seropositive and seronegative persons: 40 years (IQR: 16-58), and 42 years (IQR: 22-60), respectively (Mann-Whitney U test, p=0.039). This indicated age as a risk factor for *Leishmania* infection. Additionally, in the multivariate model with age, sex and geographical region as covariates, age was a significant predictor of seropositivity (overall significance p=0.022). The rates of seroprevalence for each age group of participants as well as the associated OR with 95% CI adjusted for covariates are shown in Table 4. Results show that *Leishmania* seropositives are most likely in 0 to 9 year-olds (17.5%; OR: 2.19; 95% CI: 1.16–4.14). It is noted that anti-*Leishmania* antibodies were found in nine of a subgroup of 71 children under the age of four, including one girl aged one year old. The data also show that *Leishmania* seropositivity does not continuously change with age. Instead, a bimodal distribution is indicated with comparable high risk of asymptomatic infection in those aged 0 to 9, 10 to 19 and 40 to 49 years (OR: 1.84 to 2.19; all p-values <0.05). In contrast, people of all other age groups had similar risk for *Leishmania* infection to those in the 30 to 39-year-old reference age group (OR: 1.00 to 1.33; p-values: 0.374 to 0.992).

## Discussion

To our knowledge, this is the first study of the prevalence of asymptomatic *Leishmania* infection in the general population of Croatia. In order to determine the *Leishmania* infection distribution, residents of various geographical and ecological areas were included in a serological survey. The findings of this study reveal a strong association of seropositivity with geographic region (east Adriatic coastal and islands areas) and age group. The presence of seropositive people in northwestern coastal and island regions (Istria and

**Table 2**

Age and sex and seropositivity for anti-*Leishmania* IgG of study population by area of residence, Croatia, 2007–2009 (n=2,035)

| | Number of sera | Participant age groups (years) | | | | | | | Information on age missing | Age range | Male (%) | Seropositive (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0-9 | 10-19 | 20-29 | 30-39 | 40-49 | 50-59 | 60-69 | >70 | | | | |
| **Total** | **2,035** | **177** | **272** | **250** | **246** | **242** | **315** | **205** | **290** | **38** | **0 - 88** | **975 (47.9)** | **231 (11.4)** |
| Region/Site of residence | | | | | | | | | | | | | |
| Adriatic coast | 1,186 | 149 | 206 | 133 | 131 | 146 | 189 | 97 | 107 | 28 | 0 - 86 | 569 (48.0) | 169 (14.2) |
| Istria and Primorje | 117 | 12 | 13 | 11 | 27 | 17 | 17 | 10 | 10 | 0 | 0-83 | 47 (40.2) | 5 (4.3) |
| Northern Dalmatia | 159 | 10 | 29 | 19 | 15 | 21 | 36 | 15 | 14 | 0 | 3-84 | 84 (52.8) | 10 (6.3) |
| Central Dalmatia | 571 | 112 | 117 | 74 | 59 | 63 | 67 | 31 | 33 | 15 | 0-82 | 293 (51.3) | 127 (22.2) |
| Southern Dalmatia | 339 | 15 | 47 | 29 | 30 | 45 | 68 | 42 | 50 | 13 | 0-86 | 145 (42.8) | 27 (8.0) |
| Adriatic islands | 653 | 17 | 41 | 70 | 61 | 69 | 110 | 97 | 181 | 7 | 1-88 | 268 (41.0) | 61 (9.3) |
| Croatian mainland | 196 | 11 | 25 | 47 | 54 | 27 | 17 | 10 | 2 | 3 | 0 -72 | 137 (69.9) | 1 (0.5) |
| Brod-Posavina county | 107 | 0 | 12 | 25 | 34 | 19 | 10 | 4 | 0 | 3 | 18-62 | 96 (89.7) | 0 (0.0) |
| Međimurje county | 89 | 11 | 13 | 22 | 20 | 8 | 7 | 6 | 2 | 0 | 0 -72 | 41 (46.1) | 1 (1.1) |

**Table 3**

Seroprevalence of anti-*Leishmania* IgG by residence compared with unweighted average in the associated region, Croatia, 2007–2009, (n=2,035)

| | p-value | OR | 95% CI |
|---|---|---|---|
| Region/Site of residence | | | |
| Adriatic coast | Reference: seropositivity 14% (95% CI, 12-16%) | | |
| Istria and Primorje | 0.002[a] | 0.27 | 0.11-0.67 |
| Northern Dalmatia | 0.006[a] | 0.4 | 0.21-0.78 |
| Central Dalmatia | <0.001[a] | 1.72 | 1.33-2.22 |
| Southern Dalmatia | 0.002[a] | 0.52 | 0.34-0.80 |
| Adriatic islands | [b]seropositivity 9% (8-11%) | | |
| Croatian mainland | Reference: seropositivity 0.2% (0.1-0.9%) | | |
| Brod-Posavina county | 0.758 | 0 | NA |
| Međimurje county | 0.849 | 2.22 | 0.14-35.83 |

CI: confidence interval; NA: not applicable; OR: odds ratio.

[a] Significant at level 0.01.

[b] Site samples were too small for reliable comparison.

Primorje) where cases of VL have not been reported yet, suggests that *Leishmania* transmission toward north may have occurred.

A highly endemic focus in central Dalmatia was confirmed in accordance with previous reports of both canine and human leishmaniasis in central and southern Dalmatia's coast and islands [5, 7-11]. However, the northwestern part of the Adriatic littoral, including Istria, Primorje, and northern Dalmatia, was considered a non-endemic region [5, 7-11]. In 2002, a case of VL was diagnosed in a patient who had no history of travel to known endemic regions and apparently had contracted the infection during his stay on the Velebit Mountain in northern Dalmatia [20]. Our results confirm this observation as they indicate that asymptomatic *Leishmania* infection is found throughout the eastern Adriatic coast and islands. Depending on the geographical location, a moderate to high (4%-22%) prevalence of asymptomatic infection has been observed.

Data from seroepidemiological studies conducted in other Mediterranean countries have also shown a variable prevalence (from 0.5 to 56%) of *Leishmania* infections depending on the geographic regions studied and on the test used for detection [3,21]. In our study we used a commercial ELISA as a relatively simple method for testing a large number of sera. Convenience sampling was used for selecting respondents. Despite some of its limitations, in a study by Kelly et al. it was shown that a convenience sample of sera from diagnostic laboratories was an appropriate sampling strategy to provide population immunity data to inform country's health policies [17]. Although the healthy donor effect bias was present in Brod-Posavina County, blood donors represented only a minority in other sites and are not expected to have influenced results.

The highest prevalence of 22.2%, significantly higher than in other coastal zones, was found in central Dalmatia. In comparison with other coastal areas, central Dalmatia has a high background of canine leishmaniasis and sandflies [5,6,11] which are likely to be associated with high prevalence of asymptomatic *Leishmania* infection among residents in this area. Central Dalmatia is also an active focus with the highest number of human VL cases in Croatia [9,10,14]. Most of VL cases diagnosed in Croatia during the study period from 2007 to 2009 occurred in central Dalmatia: seven cases were diagnosed with a mean annual incidence of 0.5 per 100,000 population (unpublished data). In southern Dalmatia, *Leishmania* exposure was higher than expected (8% seroprevalence), despite the lack or small number of clinical cases and apparently lower risk of infection than observed for central Dalmatia.

As expected, the seroprevalence was significantly lower in residents of continental areas of Croatia; in fact all but one person (a 40 year-old woman) were seronegative. As these regions are not considered to be endemic for sandflies, we cannot exclude the possibility that the one seropositive person could have been infected while traveling to the Croatian littoral where people from continental areas often spend their summer vacations.

The same possibility of travel-acquired infection with consecutive seroconversion has to be taken in account when the seemingly northward spread of asymptomatic leishmaniasis is interpreted. One could also speculate that autochthonous *Leishmania* infection may occur due to the observed spread of sandflies to

**Table 4**

Seroprevalence of anti-*Leishmania* IgG by age, sex and region of residence, Croatia, 2007–2009 (n=2,035)

| Age group (years) | Sera tested | IgG positive (%) | p value | OR | 95% CI |
|---|---|---|---|---|---|
| 0-9 | 177 | 31 (17.5) | 0.016[a] | 2.19 | (1.16-4.14) |
| 10-19 | 272 | 39 (14.3) | 0.048[a] | 1.84 | (1.01-3.38) |
| 20-29 | 250 | 18 (7.2) | 0.992 | 1.00 | (0.50-2.01) |
| 30-39 | 246 | 17 (6.9) | | Reference | |
| 40-49 | 242 | 37 (15.3) | 0.015[a] | 2.13 | (1.16-3.93) |
| 50-59 | 315 | 30 (9.5) | 0.621 | 1.17 | (0.63-2.19) |
| 60-69 | 205 | 19 (9.3) | 0.631 | 1.18 | (0.60-2.36) |
| >70 | 290 | 30 (10.3) | 0.374 | 1.33 | (0.71-2.51) |
| Sex | | | | | |
| Men | 975 | 113 (11.6) | 0.61 | 1.08 | (0.81-1.44) |
| Women | 1,060 | 118 (11.1) | | Reference | |
| Region | | | | | |
| Adriatic islands | 653 | 61 (9.3) | 0.003[a] | 20.37 | (2.78-149.2) |
| Adriatic coast | 1,186 | 169 (14.2) | 0.001[a] | 28.51 | (3.95-205.79) |
| Continental sites | 196 | 1 (0.5) | | Reference | |

CI: confidence interval; OR: odds ratio.

[a] Significant at level p<0.05.

some regions at the North of Croatia [1,2], especially in light of the recent finding of *Phlebotomus* spp. in southern Hungary near the Croatian border [16].

In agreement with other reports [21-24] our study shows that there is no difference in the prevalence of asymptomatic *Leishmania* infection between men and women.

In respect to age, our prevalence results differ from findings in other studies. In the present study a bimodal distribution of *Leishmania* seropositivity by age, with peaks in young (0-19 years) and middle-aged (40-49 years) population, was suggested for asymptomatic population. Several studies reported the age distribution of seroprevalence to *Leishmania* in a healthy human population so far. The authors mainly noted a higher prevalence in older people suggesting that susceptibility to *L. infantum* infection increases with age [21-23] or they claimed no association with age [24,25]. Furthermore, Davies et al. suggested seroprevalence drops rapidly with age [26]. In some of these studies [21,23] age groups under 18 years were not included and/or samples were not equally distributed by age, resulting in different precision of seroprevalence estimate between different age groups. A study in Brazil, using a non-commercial ELISA found that 28.5% sera of 638 tested children aged between 0 and 5 years were positive, and concluded that infection is associated with the age of ≥2 years [27]. In our study, seropositives were most likely to be aged 0 to 9 years. Since in Croatia VL is still predominantly paediatric disease [6,7] it cannot be excluded that some of the seropositives in this age group might become symptomatic, therefore further differently designed studies with follow up of such participants are needed.

It can be concluded that compared to other parts of Croatia, seroprevalence is significantly higher in central and southern coastal as well as insular areas. This indicates the presence of asymptomatic *L. infantum* infection in humans and confirms the endemicity of these areas. This finding may be of particular importance in light of the increasing popularity of the Croatian coast and islands, from Istria to Dubrovnik, as a holiday destination for travellers from *Leishmania*-free areas or countries. Our data should alert physicians to consider leishmaniasis in the differential diagnosis of conditions such as unexplained febrile illness especially in immunocompromised subjects returning from these endemic areas. In addition, seropositivity observed in non-endemic areas and the higher seroprevalence in children should be investigated in the future. Therefore, further studies including clinical, parasitological, epidemiological and entomological investigation are required for elucidating the cycle of transmission, the maintenance and the role of *Leishmania* in human and animal health in different Croatian regions.

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# The burden of visceral leishmaniasis in Italy from 1982 to 2012: a retrospective analysis of the multi-annual epidemic that occurred from 1989 to 2009

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**Starting from 1989 Italy experienced an increase of visceral leishmaniasis (VL) cases over a baseline of 10 to 30 cases reported annually. The number of cases peaked in 2000 and 2004 with more than 200 cases/year, and subsequently declined to reach on average one third of the 2000 peak value in the period after 2010. A retrospective analysis from 1982 to 2012 showed that the multi-annual epidemic consisted of major components including (i) an outbreak involving infants and immunocompetent adults in parts of the Campania region (southern peninsular Italy) and that appears to have declined naturally, (ii) a second outbreak affecting human immunodeficiency virus (HIV)-infected individuals throughout the country, that declined owing to the use of highly active antiretroviral therapies (HAART), (iii) a generalised increase of VL cases in immunocompetent individuals and patients affected by associated conditions other than HIV from endemic regions of peninsular and insular Italy (other than Campania), which was due to a geographical spreading of VL foci, with no major case-clusters or outbreak features. A minor component consisted in the appearance of a few autochthonous cases in formerly non-endemic areas, starting from the early 1990s. Epidemic determinants and reasons for VL decline in the Campania region remain largely unexplained, despite the information available on canine reservoir and phlebotomine vectors in Italy.**

## Introduction

Visceral leishmaniasis (VL) is a protozoan disease transmitted by phlebotomine sandflies and caused by members of the *Leishmania donovani* complex. The disease results from the systemic intracellular infection of the macrophage-rich organs. The incubation period is long (an average of 3–8 months, reaching up to >10 years in case of reactivation from latent infections) and the chronic appearance of signs and symptoms makes a clinical suspicion difficult in low or non-endemic areas. Epidemiological features and determinants of VL outbreaks are largely diverse. Epidemics of anthroponotic VL caused by *L. donovani sensu stricto* (a species recently introduced in the Mediterranean [1]) have long been known to occur as multi-annual waves with, as seen in outbreaks in India and East Africa, thousands of cases in wide areas, followed by inter-epidemic periods of five to 20 years [2-5]. On the other hand, zoonotic VL, a disease caused by *L. infantum* for which domestic dogs act as the main reservoir hosts, exhibits a typical pattern characterised by isolated or small localised clusters of cases (usually less than 10), with large epidemics uncommon in the Mediterranean area [6]. The first documented outbreak of zoonotic VL occurred within the 1971 to 1972 period, near Bologna, Italy, with 60 clinical cases (13 deaths) diagnosed from villages where only a total of four cases had been documented in the previous 50 years [7]. Determinants of this outbreak have remained unexplained. Investigations led to the discovery and first worldwide description of asymptomatic VL cases [8]; indeed, chronic *L. infantum* asymptomatic infections have been shown thereafter to be widespread in southern European countries [9]. Very recently (2010–2012) another localised VL outbreak with more than 100 cases has occurred near Madrid, Spain, where major determinants have been identified in environmental changes and the unusual role of hares as reservoir hosts [10].

Between 1989 and 2009, Italy experienced an increase in the number of VL cases over a baseline of about 10 to 30 cases reported annually since the 1950s. The number of cases peaked in 2000 and 2004, when more than 200 cases/year were observed and thereafter began to decline. The bell shape of the epidemic wave, of 20-years in width, and the substantial lack of aggressive control programmes that could justify the general decline of VL incidence, strongly resemble in trend the historical *L. donovani* multi-annual epidemics, although the number of cases involved is dramatically lower. Indeed, there is still not enough knowledge on the epidemiology of *L. infantum*, and our surveillance data show that epidemics can present in a different way than expected. In this paper, we have retrospectively analysed both published and unpublished

information collected by our unit within the frame of VL epidemiological research and surveillance in Italy, in order to identify possible determinants explaining the recent trend of the disease in the country.

## Methods

### Study design

Human VL data collection was based on available notifications, review of published literature and unpublished information. Basic data for all patients included at least the year of diagnosis and the patient's residence at the level of the first administrative unit (region). More detailed information regarding selected patient groups were analysed from both published and unpublished data collected by our unit since 1982. Canine reservoir data collection was based on the review of published literature and unpublished information available at our unit or obtained from public veterinary reference centres in Italy.

### Human data

VL is a compulsory notifiable disease in Italy since 1956. Diagnosed cases are recorded at the provincial local health units to which hospitals belong; notifications are gathered at regional level and subsequently centralised at the Ministry of Health. However under/late reporting may occur from some provinces or when VL is diagnosed in patients suffering from associated conditions such as human immunodeficiency virus (HIV) co-infection, or organ transplant.

Online notifications centralised at the Italian Ministry of Health (last update: 2009 [11]) and notifications available upon request at the regional or provincial local health units registries until 2012, were integrated with information stored in the database of our unit at Istituto Superiore di Sanità, which holds detailed patient's information from an average of 50% (annual range: 43–69%) of cases annually notified in Italy, thanks to centralised VL diagnosis and medical surveillance activities performed by our laboratories [12]. Diagnosis in clinically-suspected patients was routinely performed on clinical samples sent by hospitals (paediatrics, internal medicine, and infectious diseases wards) from throughout the country. Serology (immunofluorescent antibody test, IFAT) and microscopy, culture and polymerase chain reaction (PCR) (the last technique being employed routinely since 1997) on bone-marrow and/or peripheral blood (buffy coat) material were used in combination as recommended by World Health Organization guidelines [13,14]. If VL was confirmed, relevant information on patients was obtained, which included demographic data, place of hospitalisation, putative place of infection, concomitant/underlying conditions, drug regimens used and post-therapy results. Isolation of parasites from VL clinical samples was also routinely performed, resulting in a large collection of 871 *Leishmania* strains from different Italian areas and patient categories. Parasites were identified by multi-locus enzyme electrophoresis (MLEE) following the standard Montpellier (MON) nomenclature [15], and/or by molecular techniques suitable for *L. infantum* identification including PCR-restriction fragment length polymorphism (RFLP) of a repetitive DNA sequence [13] or by internal transcribed spacer 1 (ITS-1) nested-PCR-RFLP [16].

### Data on canine leishmaniasis

Besides the analysis of published literature, that was employed to map historical canine leishmaniasis (CanL) seroprevalence rates, recent information on diagnosis in dogs was obtained for the period from 2005 to 2011 (and updated in 2012) from the network of 10 institutes for zooprophylaxis as well as from six collaborating faculties of veterinary sciences. The main objective, to be accomplished in the frame of the European seventh framework programme (FP7) project EDENext, consisted in the country-wide mapping of communes (lowest administrative level) with undisputable presence of autochthonous CanL as an indicator of endemic transmission.

## Results

### General trend of visceral leishmaniasis in Italy

Laboratory-confirmed recorded cases of VL in Italy from 1982 to 2012 are shown in Figure 1. Between 1950 and 1981, the number of annual cases ranged between 10 and 30 cases/year [17]. Starting from 1989 (44 cases), a steady increase was observed with a peak in 2000 and again in 2004 involving 215 and 204 cases, respectively. A progressive decline in the annual number of cases occurred during the subsequent eight years, reaching on average one third of peak cases in the period between 2010 and 2012.

### Major components of the epidemic trend

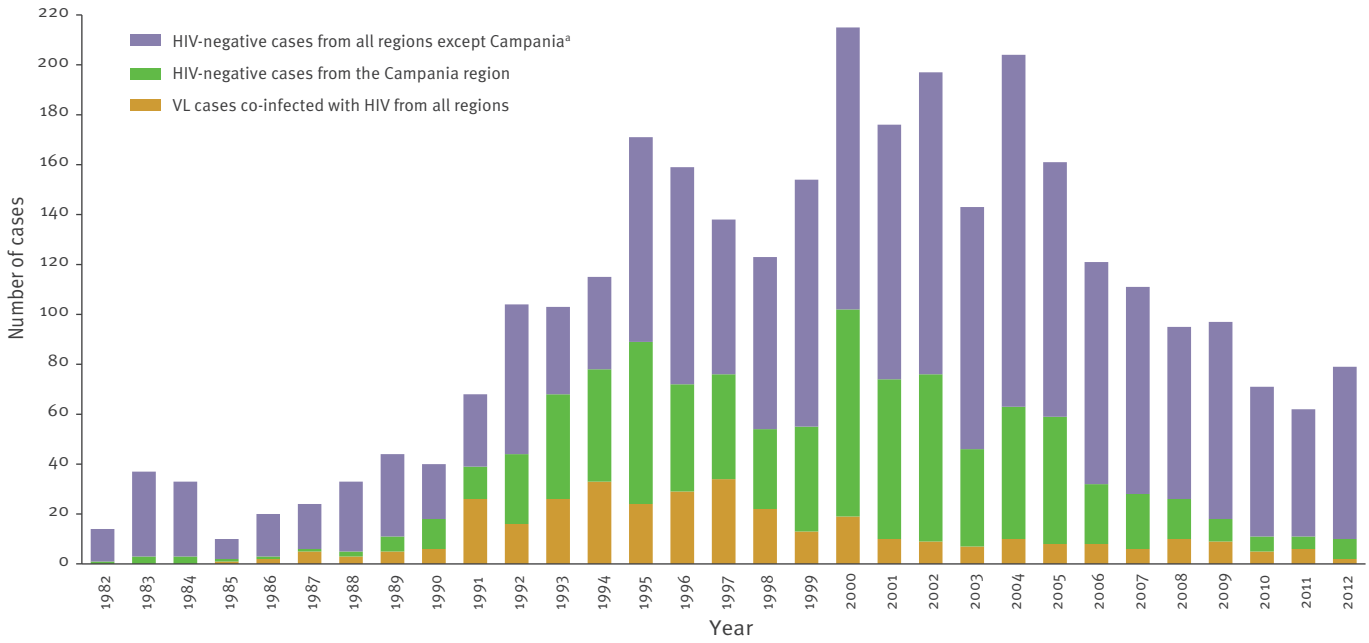
Three major components of the epidemic curve were identified including (i) an outbreak involving parts of three provinces of the Campania region, (ii) an HIV–VL co-infection epidemic and (iii) a generalised increase of VL in regions of peninsular and insular Italy.

### Epidemic involving parts of three provinces of the Campania region

Starting from 1989, an increase of VL cases occurred, resulting in not only major hospitals with experience in VL management, receiving cases. As some cases were misdiagnosed and/or received inappropriate treatment [18], a dedicated surveillance was implemented in the Campania region consisting in VL testing centralised at Istituto Superiore di Sanità in Rome. The surveillance disclosed the beginning of an epidemic trend that, from 1989 to 1990, involved areas of three provinces, namely Naples (Vesuvius area), Caserta (inland area) and Salerno (coastal area). Case clusters were identified in several villages or peri-urban districts reaching an incidence of two VL cases/1,000 population in some years. A striking feature was that this epidemic was unrelated to HIV co-infections [19], which were occurring also in

**Figure 1**

Distribution of annual number of laboratory-confirmed visceral leishmaniasis cases in Italy, 1982–2012 (n=3,122)




HIV: human immunodeficiency virus; VL: visceral leishmaniasis.

[a] Each year the largest part (>90%) of the HIV-negative cases from all regions other than Campania, included patients from regions of peninsular and insular Italy, the remaining cases being from regions of northern continental Italy.

the form of an epidemic in other Italian regions in the same period. Parasite identification carried out on 225 strains up to the year 2003, revealed that about half of the cases (110 strains) were due to a novel zymodeme of *L. infantum* (MON-72) found also in dogs and in phlebotomine vectors from Campania foci [20,21]. The analysis of clinical records did not suggest any particular virulence of this zymodeme as compared to the commonest agent of Mediterranean VL, zymodeme MON-1.

Altogether 789 cumulative cases were diagnosed during the long outbreak period from 1989 to 2008 (Figure 1). After a peak of 83 cases in 2000, the annual number of cases dropped slowly to less than 10 cases/year after 2009. Since, the disease has become sporadic in all age groups, including children, and disappeared from most of the former 'outbreak villages and districts' although it emerged with a few cases in the remaining two provinces of Campania region (Avellino and Benevento).

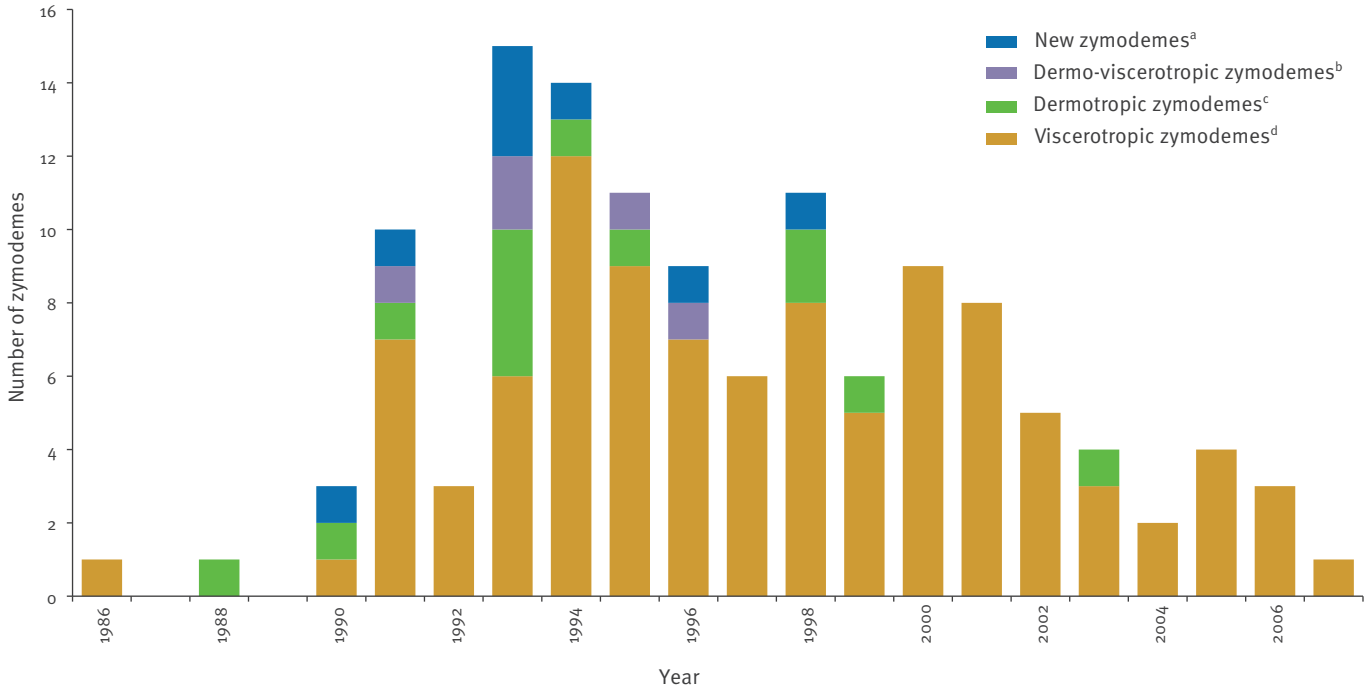
## Human immunodeficiency virus–visceral leihmaniasis co-infection epidemic

A dedicated surveillance network centralised at World Health Organization headquarters, Geneva, was established after a meeting held at Istituto Superiore di Sanità, Rome, in 1994. From the beginning, our unit participated in the network by reporting information collected through a national monitoring system [22]. First HIV–VL cases were recorded in 1985. A sharp increase in the annual number of cases was observed in 1991 followed by two peaks in 1994 (33 cases) and 1997 (34 cases). The introduction and generalised use of highly active antiretroviral therapy (HAART) for HIV infection at a time between 1997 and 1998 resulted in a clear decrease in the incidence of HIV–VL co-infections. Since 2001, a plateau of about 10 cases/year or less has been observed until present (Figure 1). Cases were recorded from several regions of Italy, with higher incidences in Sicily, Lombardy and Latium. In all the epidemic phases, clinical and epidemiological features of HIV–VL were very similar to those of other Mediterranean affected countries, such as Spain and France, and have been reviewed for all cases recorded up to 2006 [23]. VL relapses, which occurred invariably in acquired immunodeficiency syndrome (AIDS) individuals before the HAART era, became less frequent in these patients following the epidemic decline. Of note, these relapses still characterise a number of co-infected patients, resulting in a significant impact on the public healthcare system.

The characterisation of *L. infantum* strains isolated from HIV-patients in Italy until 1998 has been extensively reviewed [15], and shows the high level of genetic polymorphism of these strains. In subsequent years, 51 new strains were isolated and identified by MLEE and/or molecular methods. Altogether, 206 strains (163

**Figure 2**

Yearly composition of zymodemes of *Leishmania infantum* isolated from human immunodeficiency virus-infected patients with visceral leishmaniasis (primary infection), Italy, 1986–2007 (n=126)




MON: Montpellier nomenclature.

[a] New zymodemes are represented by MON-136, MON-185, MON-188, MON-190, MON-201, MON-228, MON-183 var. MDH100 and MON-189 var. NH140 (1 strain each).

[b] Dermo-viscerotropic zymodemes are represented by MON-34 (4 strains) and MON-80 (1 strain).

[c] Dermotropic zymodemes are represented by MON-11 (1 strain), MON-24 (6 strains), MON-29 (2 strains), MON-78 (2 strains) and MON-189 (1 strain).

[d] Viscerotropic zymodemes are represented by MON-1 (102 strains) and MON-72 (1 strain).

from primary infections, the remaining from disease relapses) were typed from cases which occurred during the 1986 to 2007 period. *L. infantum* was confirmed as the main agent responsible of the co-infections, whereby only one case of co-infection with *L. donovani* was recorded and this case was imported. The annual composition of zymodemes from primary infections was more diversified in the group of strains obtained before HAART therapy introduction than in the group obtained after HAART. The pre-HAART group was composed of 84 strains collected up to 1998, and the post-HAART group included 42 strains typed between 1999 and 2007 (MLEE analysis data are not available after this year). As shown in Figure 2, the former group consisted of 16 zymodemes, dominated by the viscerotropic MON-1 (60/84, 71%). The remaining zymodemes, represented by one to six strains each, showed variations in one to four enzyme patterns and were identified as the already known dermotropic (n=4), viscerotropic (n=1) and dermo-viscerotropic (n=2) zymodemes, and eight new zymodemes never isolated from HIV-negative leishmaniasis patients. Conversely, the post-HAART group consisted only in three zymodemes, mainly composed by MON-1 (40/42, 95%) and by two known dermotropic zymodemes (1 strain each). The difference in zymodeme composition between the two strain groups scored as difference in the proportion of strains with the MON-1 zymodeme 'MON-1' versus those with zymodemes other than MON-1 'non-MON-1' in each group (60 vs 24 strains, and 40 vs 2 strains in pre-HAART and post-HAART period, respectively) was highly significant (Fisher exact test, P<0.001). Of note, HIV–VL cases were referred to our unit from the same hospitals distributed throughout the country during the whole period from 1986 to 2007. These findings suggest that HAART therapy, while conferring an immune restoration, may also operate a negative selection towards less virulent agents of VL in treated immunocompromised patients.

## Generalised increase of visceral leishmaniasis in regions of peninsular and insular Italy

Taking the Campania region aside, the peninsular regions of the Tyrrhenian, Ionian and Adriatic coast, as well as Sicily and Sardinia islands (all historically endemic for VL) showed a generalised increase of VL in HIV-negative individuals, which was remarkable from 1995 (79 cases) to 2007 (68 cases) with a peak of 130

**Table**

Visceral leishmaniasis cases recorded among human immunodeficiency virus-negative individuals in regions other than the Campania region, Italy, 1995–2007 (n=1,032)

| Region | 1995 | 1996 | 1997 | 1998 | 1999 | 2000 | 2001 | 2002 | 2003 | 2004 | 2005 | 2006 | 2007 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Liguria | 8 | 6 | 1 | 1 | 6 | 5 | 6 | 3 | 5 | 4 | 6 | 3 | 9 |
| Tuscany | 1 | 3 | 3 | 2 | 2 | 5 | 4 | 9 | 8 | 10 | 6 | 4 | 10 |
| Latium | 13 | 18 | 15 | 17 | 16 | 27 | 23 | 21 | 23 | 23 | 29 | 22 | 23 |
| Abruzzo | 0 | 1 | 2 | 2 | 2 | 5 | 0 | 1 | 2 | 5 | 6 | 3 | 0 |
| Apulia | 5 | 11 | 10 | 2 | 12 | 11 | 17 | 6 | 4 | 7 | 1 | 6 | 2 |
| Calabria | 10 | 4 | 6 | 4 | 6 | 1 | 7 | 7 | 2 | 6 | 5 | 9 | 6 |
| Sardinia | 7 | 13 | 2 | 5 | 4 | 8 | 5 | 9 | 5 | 5 | 2 | 4 | 3 |
| Sicily | 33 | 27 | 17 | 24 | 26 | 36 | 27 | 52 | 29 | 43 | 25 | 18 | 7 |

Visceral leishmaniasis cases recorded in human immunodeficiency virus-negative individuals in the most endemic regions of peninsular and insular Italy – other than the Campania region – in 1995–2007, corresponding to the period of highest incidence among this population. Cells reporting the two highest values of numbers of cases recorded by region are shaded in grey, showing the uneven distribution of the hot spots.

cases in 2004 (Figure 1). Case analysis showed that such increase was not associated to large case clusters in particular geographical areas, but rather to a generalised spreading of transmission foci involving one or more endemic regions depending on the years (Table) and affecting different provinces.

## Minor component of the trend: visceral leishmaniasis in northern continental Italy

Starting from 1990, regions from northern continental Italy traditionally considered free from *Leishmania* transmission had become focally endemic for *L. infantum*. This was definitely shown through active investigations involving canine serosurveys and phlebotomine sandfly monitoring during the 2003 to 2006 period in six regions (Piedmont, Valle d'Aosta, Lombardy, Veneto, Trentino Alto-Adige and Friuli-Venezia Giulia) [24]. With regard to human VL, cases had been regularly diagnosed between 1982 and 2012 in residents from all the above regions (a range of 5–15 cases/year, including HIV-co-infected individuals) mainly from cities of Lombardy and Piedmont. Travel histories were not available for cases notified to the Ministry of Health and most of them should be considered as imported from highly endemic southern regions of Italy or other Mediterranean counties as the consequence of travels during summer holidays. On the other hand, a few indisputable cases classified as autochthonous have been identified during the above active investigations [24] and also diagnosed thereafter by our laboratories, consisting of HIV-negative individuals from newly endemic areas of Piedmont, Lombardy and Veneto.

## Individual factors: age and associated conditions

To investigate further on determinants of the Italian epidemic trend, we analysed the age distribution and underlying/concomitant conditions associated to VL. Full data were available for patients diagnosed from 1987 to 2005, thus representing both pre- and late epidemic phases. Records from 1,296 patients were considered. The resulting population is shown in Figure 3, distributed by arbitrarily 'VL-driven' age groups. 755 cases (about 60%) were adults (›17 years of age), however infants (‹ 2 years-old) were the most numerous homogeneous group (329 cases; 25% of all cases, 61% of paediatric ones, i.e. 0–16 years-old). Cases in preschool and young school children (3–6 years-old) and in children grouped with young adolescents (7–16 years-old) were considered separately to show the relatively decreasing risk for VL associated to age increase in childhood (indeed the two age-groups have 100 cases each, yet the 3–6 years-old group spans only a total of 4 years of age, compared to 10 years spanned by the 7–16 years-old group). Adults and middle aged individuals (17–50 years-old) were considered as a single group because these formed the age group at higher risk for HIV infection, and accounted for 575 patients (44% of all cases, 76% of adults›17 years-old). The age group between 51 and 70 years accounted for 140 cases, and the elderly group (›70 years-old, including several individuals over 80 years of age) for 40 cases.

Patients without any recorded underlying/concomitant conditions represented the greatest majority of the population, including 1,049 cases (81%). 179 HIV co-infected patients were recorded, representing 14% of total cases (n=1,296) and 31% of the 17–50 age-group patients (n=575). One hundred and ninety-seven (99%) of these HIV patients were adults. Finally, associated conditions other than HIV infection were recorded in 68 patients representing 5% of total cases. Fifty-seven of the 68 patients were adults, representing 8% (57/755)

of any adults. Twenty-three underlying/concomitant conditions other than HIV infection were recorded. They were single in 54, or associated in 14 patients. The most frequent conditions were organ transplantation (14 cases), hepatitis C and liver cirrhosis (12 cases each), pregnancy (9 cases), leukaemia (7 cases), lymphoma (5 cases), hepatitis B and systemic lupus erythematosus (4 cases each), thymoma and diabetes (3 cases each), and haemophagocytosis (2 cases). Conditions found in one case each were chronic glomerulonephritis, multiple sclerosis, pneumonia, primitive CD4 deficit, infection with cytomegalovirus, Wegener's granulomatosis, rheumatoid arthritis, thalassaemia, splenectomy, pericarditis and chronic renal failure.

Our findings point out that even if 'physiologically less immunocompetent' groups like infants and the elderly were excluded (for a total of 369 patients), the large majority of remaining cases (680/927, 73%) still consisted of immunocompetent individuals for whom susceptibility to VL disease remains unexplained.

## Canine leishmaniasis data

### General distribution

CanL is widespread in southern Europe, but the highest values of predicted seroprevalence are found in the Italian peninsula. The median prevalence calculated from 377 canine serosurveys performed in Italy from 1971 through 2006, involving about 424,000 dogs, was 18% with a range of 11–21% in different decennia when the surveys were performed [25]. Of 494 *L. infantum* canine strains characterised by MLEE, the greatest part belong to zymodeme MON-1 (457 strains), found distributed throughout the country; a large group of strains (n=36) from the Campania region was found to belong to the variant zymodeme MON-72. These homogeneous groups of canine zymodemes did not reflect the elevated zymodeme polymorphism detected among the agents of human disease (both VL and cutaneous leishmaniasis), leaving the role of dogs as reservoir for all *L. infantum* zymodemes endemic in Italy unexplained.

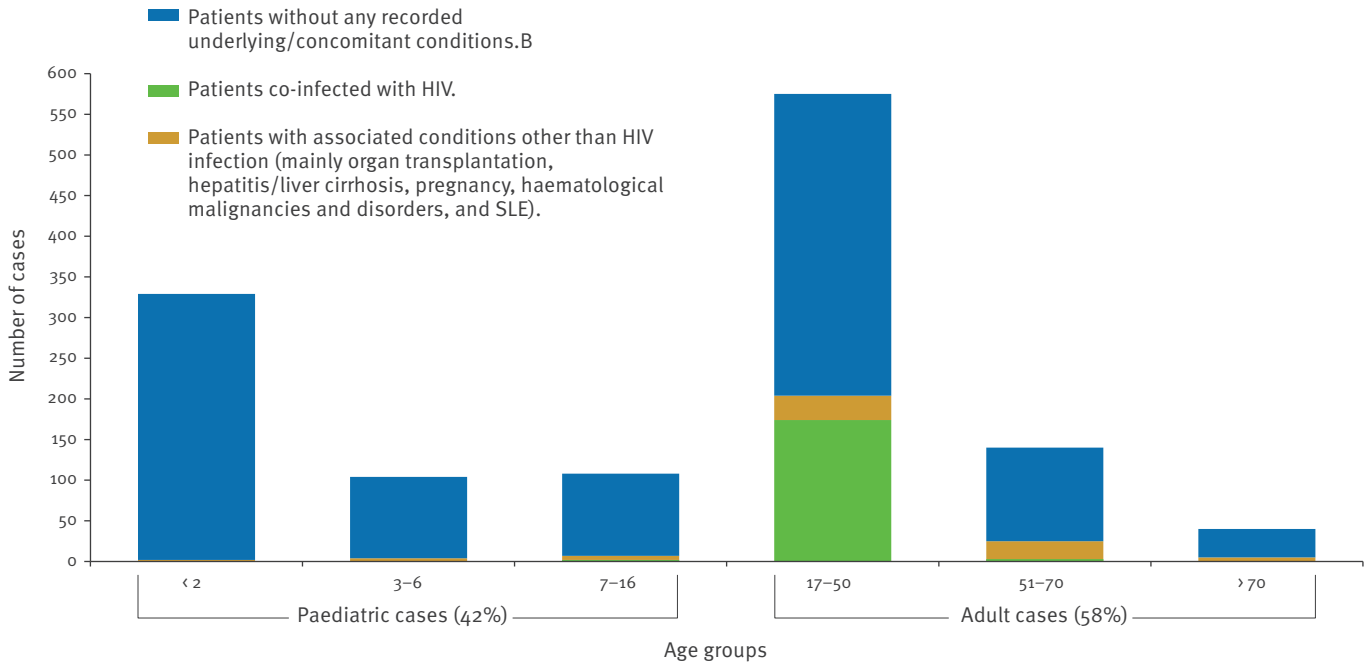
A 2005 to 2012 data mapping on the presence of autochthonous CanL among approximately 8,100 Italian communes is shown in Figure 4. Although still incomplete because of the lack of investigations in several areas, about 2,700 communes (33%) have been found endemic for CanL so far.

### Canine reservoir in the Campania region

CanL has long been known to be endemic at high prevalences in the Campania region. In 1999, one year before the peak of 83 VL cases recorded in the region, the local institute for zooprophylaxis had examined 1,675 dogs and found 251 seropositives at IFAT titres ›1/80, corresponding to a seroprevalence of 15% [26].

**Figure 3**

Age distribution and underlying/concomitant conditions recorded in 1,296 patients affected by visceral leishmaniasis in Italy, 1987–2005




HIV: human immunodeficiency virus; SLE: systemic lupus erythematosus.

In the 2005 to 2009 period, corresponding to the drop of VL incidence in that region, the same Institute reported 9,723/70,557 seropositive dogs at the same IFAT titre range, corresponding to a seroprevalence of 14% i.e. very similar to the value recorded in 1999 (2010, data from Istituto Zooprofilattico Sperimentale del Mezzogiorno, Portici, Naples). These findings suggest that canine seroprevalence rates cannot per se explain the epidemic trends of human VL.

## Discussion

At country-wide level, leishmaniasis epidemics may consist of an increase of disease cases over the expected baseline in the general population throughout the territory, or represent the additive outcome of different outbreaks occurring in particular areas and/or affecting specific groups of individuals. Epidemic trends have a typical bell shape where the increasing slope represents the natural epidemic onset, whereas the decreasing slope may either be natural or due to aggressive human interventions to control the disease. Our analysis indicates that the VL epidemic in Italy was a complex phenomenon, in which more than one component was represented: (i) an outbreak involving infants and immunocompetent individuals in parts of the Campania region, that appears to have declined naturally; (ii) a second outbreak affecting HIV-infected individuals throughout the country, that declined via the use of HAART therapies, which also probably applied a negative selection pressure on less virulent *Leishmania* genotypes originally involved in the epidemic. Hence, the contribution of the HIV–VL epidemic to the general VL trend in Italy has been limited in time, so that the two trends peaked and started to decline in different years. As mentioned above, the HIV–VL trend did not affect greatly the Campania VL trend, since the occurrence of co-infected cases in this region was negligible.; (iii) a generalised increase of cases due to disease spreading within traditionally endemic areas (a major contribution in cases) as well as the appearance of cases in previously non-endemic areas (a minor contribution in cases).

While the appearance of autochthonous VL in northern continental Italy could reasonably be explained by the de novo colonisation of these areas by phlebotomine vectors along with the frequent importation of Leishmania-infected dogs from the endemic south [24], and the generalised increase of cases in endemic areas by an increase in transmission potential due to changes in vector density, both the onset and the natural decline of the outbreak in the Campania region, traditionally endemic for VL, will probably remain unexplained. One reason for the decline could be searched in the immunity levels against *Leishmania* acquired by the population during the epidemic peaks, however this hypothesis is difficult to verify because of the sporadic nature of the disease that would require large population cohorts to be examined prospectively.

**Figure 4**

Map (ArcView GIS 10) showing the distribution of communes (in blue) where autochthonous cases of canine leishmaniasis have been recorded, Italy, 2005–2012

GIS: geographic information system.

Other investigated epidemiological compartments of the zoonotic VL cycle, the canine reservoir and phlebotomine vectors, did not help in elucidating the causes of the Italian epidemic. First of all, no aggressive programmes to control the increase of VL, that may justify the general drop in incidence, were ever put in place at country level. Indeed, only some regions have implemented guidelines and rules to monitor infections in pets and/or kennelled stray dogs, recommending drug treatment of infected animals and the use of topical insecticides against sandflies. However, as far as pet dogs are concerned, both measures were left at the owners' expenses resulting in the limited coverage of the dogs to control. Because canine topical insecticides have an impact on CanL incidence only after repeated mass use [27] and drug treatments may have some efficacy in decreasing transmission only when administered during the sandfly season, the measures actually undertaken were most probably not very effective in reducing the *Leishmania* transmission potential.

Incidence of CanL may vary considerably within endemic areas, with focal distribution. To date there is no clear evidence for a direct association of CanL prevalence values and incidence of human VL disease in a given territory. Indeed, several examples are available in literature where very elevated prevalences recorded in dogs did not result in human VL cases at all [28]. It implies that although dogs are efficient sentinel hosts for the *Leishmania* transmission in a given territory, and hence the finding of autochthonous CanL cases in previously non-endemic areas can be of value to predict the occurrence of human cases, the prevalence rate of canine infections does not appear an useful parameter to explain determinants of human VL trends in endemic areas, like we observed in Campania region.

With regards to the phlebotomine vectors, Italy is endemic for four Phlebotomus species proven to transmit *L. infantum*, *P. perniciosus* being the most widespread and efficient vector. A recent atlas based on validated bibliographical records on phlebotomine sandflies from 1985 to 2009 reported the presence of this species in 134 of approximately 8,100 Italian communes throughout the country. Among the other vector species, *P. perfiliewi* was recorded in 50 communes of peninsular Italy, *P. neglectus* in 41 communes from southern and northern, but not from central Italy, and P. ariasi in four communes at the French border [29]. Hence, considering the huge disproportion with available CanL data, information on phlebotomine vector distribution in Italy is still largely incomplete. On the other hand, like for CanL, to date there is no clear evidence for a direct association of phlebotomine vector presence and the incidence of human VL in a given territory. Indeed, many parameters necessary to a robust definition of vectorial capacity in phlebotomine species, such as standardised density measurements, dog- versus man-biting rates, number and frequency of blood meals/ovodepositions, infected vs infectious sandfly ratio, are still lacking from the scientific literature worldwide. Hence, while the presence of competent vectors in a given territory can be predictive for the occurrence of human VL when CanL cases are found too, current entomological data are still of low informative value for the analysis of epidemic VL trends.

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# Surveillance of leishmaniases in France, 1999 to 2012

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**Leishmaniasis is endemic in the south of France, where autochthonous disease is caused by *Leishmania infantum*, and affects both humans and dogs. The prevalence of canine leishmaniasis is between 3 and 66% depending on the region and the methods used. Human leishmaniases are also imported into France, mainly from French Guiana and North Africa. The surveillance of autochthonous and imported human leishmaniases is based on passive notification to the National Reference Centre for Leishmaniases (NRCL) created in 1998. Between 1999 and 2012, 317 autochthonous and 1,154 imported cases were notified to the NRCL. The average number of autochthonous cases notified per year was 22.6, mainly cases of visceral leishmaniasis (84.5%). All cases were infected in the south of France. Leishmaniasis incidence is 0.22 per 100,000 inhabitants in the endemic area. Imported cases were more frequent (annual mean of 82.4 cases) and consisted predominantly in cutaneous leishmaniasis (CL) cases (91%), essentially *L. major* CL imported from Maghreb and Sub-Saharan Africa, and *L. guyanensis* CL from French Guiana. This national notification system allowed a better understanding of the incidence and distribution of the disease; it is also useful to assess the temporal-spatial evolution of the disease in France, which appears relatively stable.**

## Introduction

In Europe, leishmaniasis is a zoonosis endemic in countries surrounding the Mediterranean Basin. In France, the French Ministry of Health supported the creation of the National Reference Centre for Leishmaniases (NRCL) in 1998 in Montpellier (http://www.parasitologie.univ-montp1.fr/english_vers/en_index.htm), with the aim of better understanding the epidemiological situation of the human disease at the national level. Its true incidence was unknown and the suspected increase of imported cases and of cases in immunocompromised patients needed to be confirmed. In this context, one of the first activities of the NRCL was to set up a system for notifying autochthonous and imported human leishmaniasis cases in France. This retrospective study reports the results of fourteen years of this surveillance.

## Epidemiological situation of leishmaniases in France

In France, the endemic area of leishmaniases is restricted to the south of the country. Several foci are clearly identified along the Mediterranean coast from the Spanish to the Italian border: the eastern Pyrénées, the Cévennes, the Provence, the Alpes-Maritimes and Corsica. The transmission is generally rural but two large cities, Nice and Marseille, are known to comprise endemic foci within their boundaries [1,2]. Dogs constitute the main reservoir of the pathogen, and *Leishmania infantum* is the species responsible for all autochthonous cases. Human cases due to this species are reported every year. However, symptomatic visceral leishmaniasis (VL) human cases represent only 'the tip of the iceberg' [3]. Indeed, individuals living in endemic areas of *L. infantum* are frequently exposed to biting by the sandfly vector. Epidemiological studies conducted worldwide in endemic areas of *L. infantum* and using leishmanin skin test, serology, blood cultivation or polymerase chain reaction (PCR), strongly suggest that the frequency of asymptomatic carriers is high [reviewed in 3].

Before 1999, there was no established notification of human leishmaniasis cases to the French health system. It was therefore difficult to have a precise picture of the incidence and prevalence of leishmaniases in France. Yet, the mean annual incidence of autochthonous VL in France was estimated for the years from 1989 to 1995 at around 1.3, 0.66 and 0.22 cases per

100,000 inhabitants for the foci in the Pyrénées-Orientales, the Alpes-Maritimes and the Cévennes, respectively [1,4]; however, these values do not reflect the strong variations between micro-foci, which can be evidenced using classical [5] or modern [2, 6, unpublished data] epidemiological tools.

## The phlebotomine sandfly vector

In France, the disease is spread by sandflies of the genus *Phlebotomus*, specifically *P. perniciosus* and *P. ariasi*, which have a seasonal activity, generally from May/June to September/October [7,8]. In southern France, *P. perniciosus* represents the most common vector species: it is mainly present in rural and in peri-urban areas and preferentially at altitudes less than 600 m above sea level. *P. ariasi*, in contrast, is found preferentially in rural areas at altitudes between 200 and 1,400 m above sea level; it represents the main vector in the Cévennes and Pyrénées-Orientales foci [9, reviewed in 10].

## The canine reservoir

Canine leishmaniasis, affecting essentially the domestic dog (Canis familiaris), is endemic in the regions confined by a triangle of which the apex corresponds to the departments (French administrative territorial divisions) of Ardèche and Drôme and the base to the Mediterranean coast. Two national surveys, performed in 1987 and 2004 and exclusively based on reports from veterinary clinics [11,12], led to the creation of a map displaying the endemic geographical areas and the changing profile of the disease, by comparing maps over an interval of almost 20 years. The information was completed by a retrospective database search and mapping about canine leishmaniasis covering the period 1965 to 2007 [13]. The results show that the disease is still prevalent in southern France, including Ardèche, and that new endemic areas emerge, contiguous to pre-existing endemic foci. Overall, 25 of 95 departments are affected; but for several of them, very low numbers of cases were reported [13,14], questioning the endemic nature of the disease in these areas and reducing the main endemic region to the 12 departments closest to the Mediterranean Sea. The seroprevalence in dogs in the latter ranges from 8.1 to 28% [11, reviewed in 1].

In the Cévennes focus, a study conducted in 1997, showed that among 253 domestic dogs tested serologically, 29.6% were positive and 70% of them presented clinical signs of leishmaniasis [6]. However, in the same survey, using an ultrasensitive PCR assay, the overall prevalence of parasite carriage was found at 80%; and at least 65% of asymptomatic dogs were found harbouring circulating parasites in their blood [6]. Thus, asymptomatic dogs can act as a reservoir of the parasite and seem to allow the transmission and spread of leishmaniasis [reviewed in 15-17]. Another study modelling previous surveys of canine leishmaniasis estimated the prevalence between 5.4 and 20.3% [18].

## Human cases

It is not easy to get an accurate picture of the disease progression in humans in southern France during the twentieth century [1]. According to literature data, at least 200 cases of leishmaniasis were recorded between 1918 and 1975, and a further 22 cases from 1975 to 1984 and 65 cases from 1985 to 1992 [unpublished data, 1, 4, 19]. To our knowledge, a single attempt was made in the past to prospectively record all national cases of VL during two years (1986-87) [20]: a total of 89 patients were recorded, of which 70 acquired the disease in France.

The objectives of the present study are to update these data and to report the results obtained by the NRCL in the surveillance of the human disease in France between 1999 and 2012.

# Methods

All leishmaniasis cases notified in France between 1999 and 2012 were analysed according to the place of infection, clinical presentation of the disease, age and risk factors for immunosuppression. For reasons of data completeness, only the period from 2007 to 2012 was analysed for risk factors.

French Guiana (a French overseas territory located in South America) is an endemic area for leishmaniasis [21-23]. Although the Parasitology-Mycology Department of the University Hospital of Cayenne is associated with the NRCL, the focus of the analyses presented here is Europe, and thus cases diagnosed in French Guiana are not included in this study. However, cases imported from French Guiana and diagnosed in France are included.

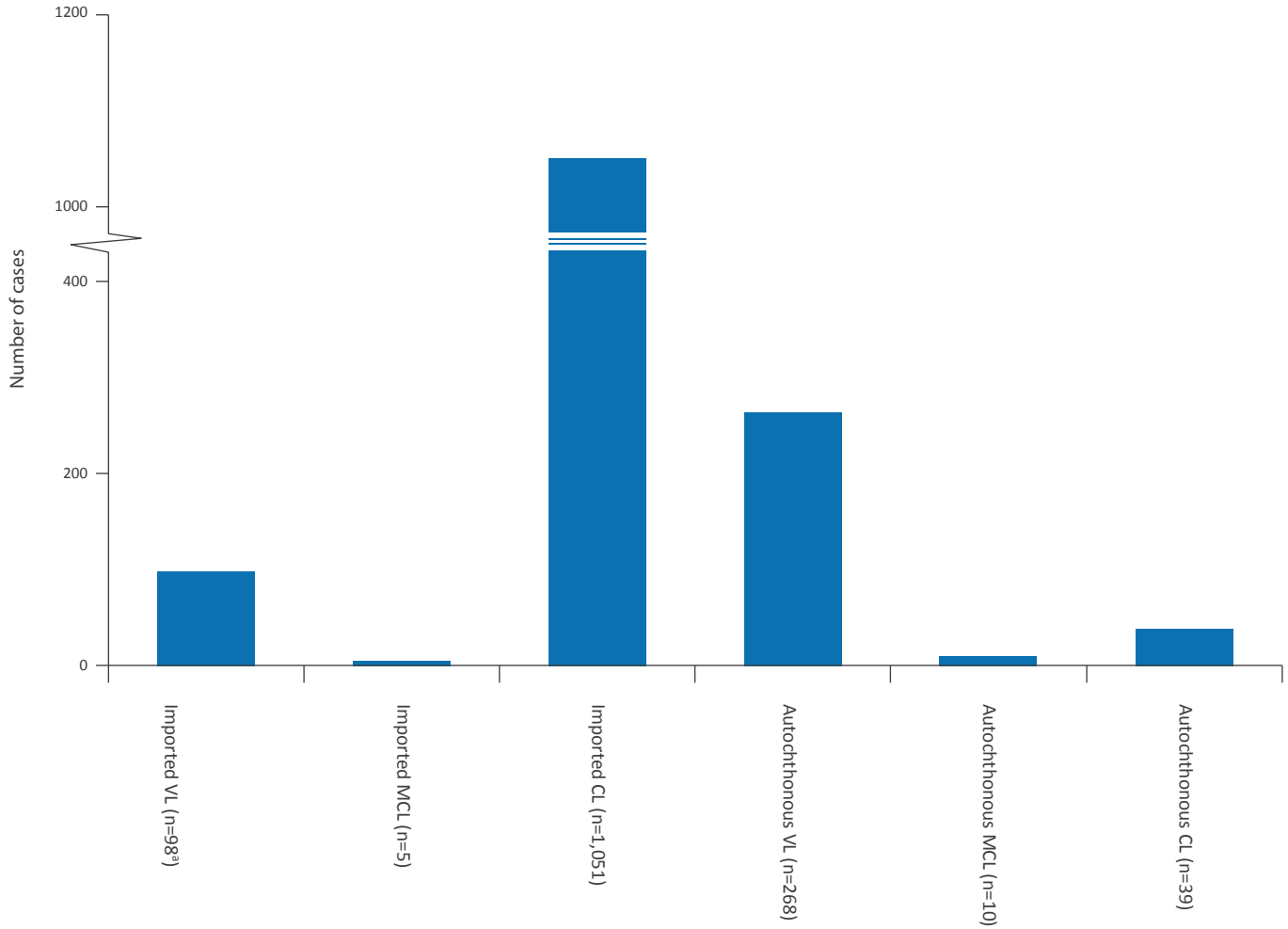
Cases confirmed by at least one specific biological test could be notified to the CNRL. Data were obtained via the standard reporting form created in 1998 at the NRCL. This form is available online (http://www.parasitologie.univ-montp1.fr/doc/Declaration_pub_2011.pdf) and can be sent back after anonymisation of the data, by mail or email but it cannot be filled online. The notification is not compulsory. It is made on a voluntary basis and relies mainly on care facilities supporting patients. The following characteristics are specified on the form: age (children defined as <16 years old), sex, risk factors with particular reference to immunosuppressive conditions such as organ or bone marrow transplantation, human immunodeficiency virus (HIV) infection, immunosuppressive therapy, leukaemia, solid organ cancer, clinical features (for VL: pancytopenia, splenomegaly, hepatomegaly, fever, weight loss; for CL or MCL: number of lesions, localisation, type of lesions such as ulceration, nodule) duration of symptoms, the presumed place of infection and laboratory tests performed for diagnosis.

# Results

During a period of 14 years between 1999 and 2012, the NRCL received notifications of 317 and 1,142

**Figure 1**

Notified autochthonous and imported leishmaniasis cases, France, 1999–2012 (n=1,471)




CL: cutaneous leishmaniasis; MCL: mucocutaneous leishmaniasis; VL: visceral leishmaniasis.

[a] Including 12 VL cases of undetermined origin.

autochthonous and imported cases of leishmaniasis, respectively, as well as 12 (visceral) cases of undetermined origin (Figure 1). More than 70 health centres notified cases: they were mostly university hospital centres but also general hospital centres, the health services of the French army, and occasionally private medical clinics or even a few practitioners.

## Autochthonous human leishmaniasis cases

Of the 317 cases of autochthonous leishmaniasis 268 (84.5%) were VL cases, 39 (12.3%) cutaneous leishmaniasis (CL) and 10 (3.1%) mucocutaneous leishmaniasis (MCL) cases.

The ratio of men to women was 1.8 and the disease affected mostly adults (222 cases; 70%); among those, 50 were over 60 years old; 73 patients (23%) were less than five years old; the mean age of the patients was 35.5 years and the median 39 years (range1 to 89 years).

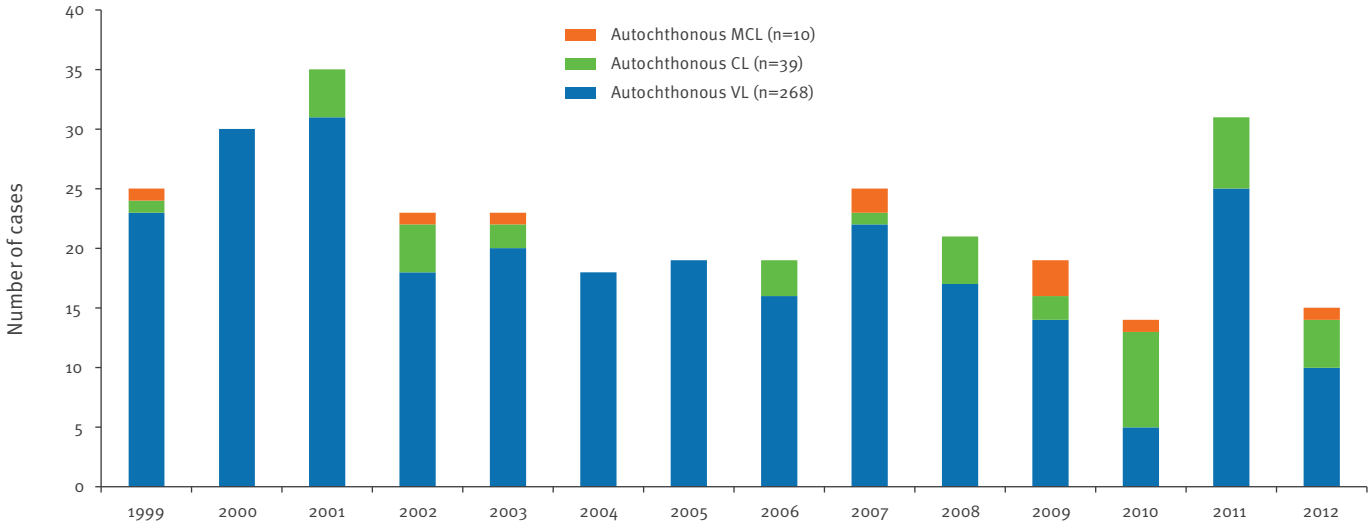
Figure 2 shows the number of autochthonous annual cases from 1999 to 2012 according to the clinical presentation. For the whole metropolitan France, the average number of notified cases per year was 22.6 (ranging from 14 in 2010 to 35 in 2001). Infection occurred in the south of France in all cases. The mean annual incidence of leishmaniasis that may be inferred from these data is 0.21 per 100,000 inhabitants for the endemic area and 0.26 for the eight most affected departments (Figure 3); it varies from 0.64 (Alpes Maritimes) to 0.01 (Aude) per 100,000 inhabitants.

## Visceral leishmaniasis cases

VL was the predominant clinical presentation (268/317; 84.5%), with a mean of 19.1 (ranging from 5 to 31) cases per year, and a peak at 32 VL cases in 2001. The two

**Figure 2**

Annual number of notified autochthonous cases of leishmaniasis by clinical picture, France, 1999–2012 (n=317)




CL: cutaneous leishmaniasis; MCL: mucocutaneous leishmaniasis; VL: visceral leishmaniasis.

departments with the highest number of notified VL cases are the Alpes-Maritimes (97 cases) and Bouches-du-Rhône (46 cases) (Figure 3).

An analysis of our data over the period 2007 to 2012 shows that VL cases occurred in 62% (90/145) in men and more frequently (46.9%; 68/145) in the age group 20 to 60 years; 19.3% (28/145) of the cases were observed in people over 60 years (Figure 4).

## Cutaneous leishmaniasis and mucocutaneous leishmaniasis cases

Since 1999, only 39 cases of CL and 10 of MCL have been reported. Autochthonous CL, with a mean of 2.8 cases (ranging from 0 to 8) per year. With respect to MCL, only 10 cases have been reported, including three from Eastern Pyrénées. MCL was essentially a primary condition of mucosae, with neither visceral, nor purely cutaneous involvement.

## Imported leishmaniasis cases

Between 1999 and 2012, 1,154 imported cases were reported to the NRCL, a mean of 82.4 cases (range 37 to 148) annually. Only 98 of these were VL cases, resulting in an annual mean of seven cases. CL cases represented 91% (1,051/1,154) of the total: 41.9% (440/1,051) of these were from Africa, originating from North Africa in 30.9% (325/1,051) of cases and in sub-Saharan Africa in 11% (115/1,051). Imported CL cases acquired in French Guiana represented 41.7% (438/1,051) of the notified cases (only 44 cases, 4.2% of them having been infected in the rest of Latin America). These three geographical areas correspond to the main migratory movements to metropolitan France.

Molecular strain typing from the Old World cases for the years 2009 to 2011, identified a large majority (141 cases; 88%) of *L. major*, followed by *L. tropica* (18 cases; 11%); for the New World cases, *L. guyanensis* was largely predominant (170 cases; 83%), followed by *L. braziliensis* (27 cases; 13%). It is of note that imported cases from French Guiana reported to the NRCL are not necessarily representative of the epidemiological situation of this region.

## Autochthonous and imported visceral leishmaniasis cases and immunocompromising conditions

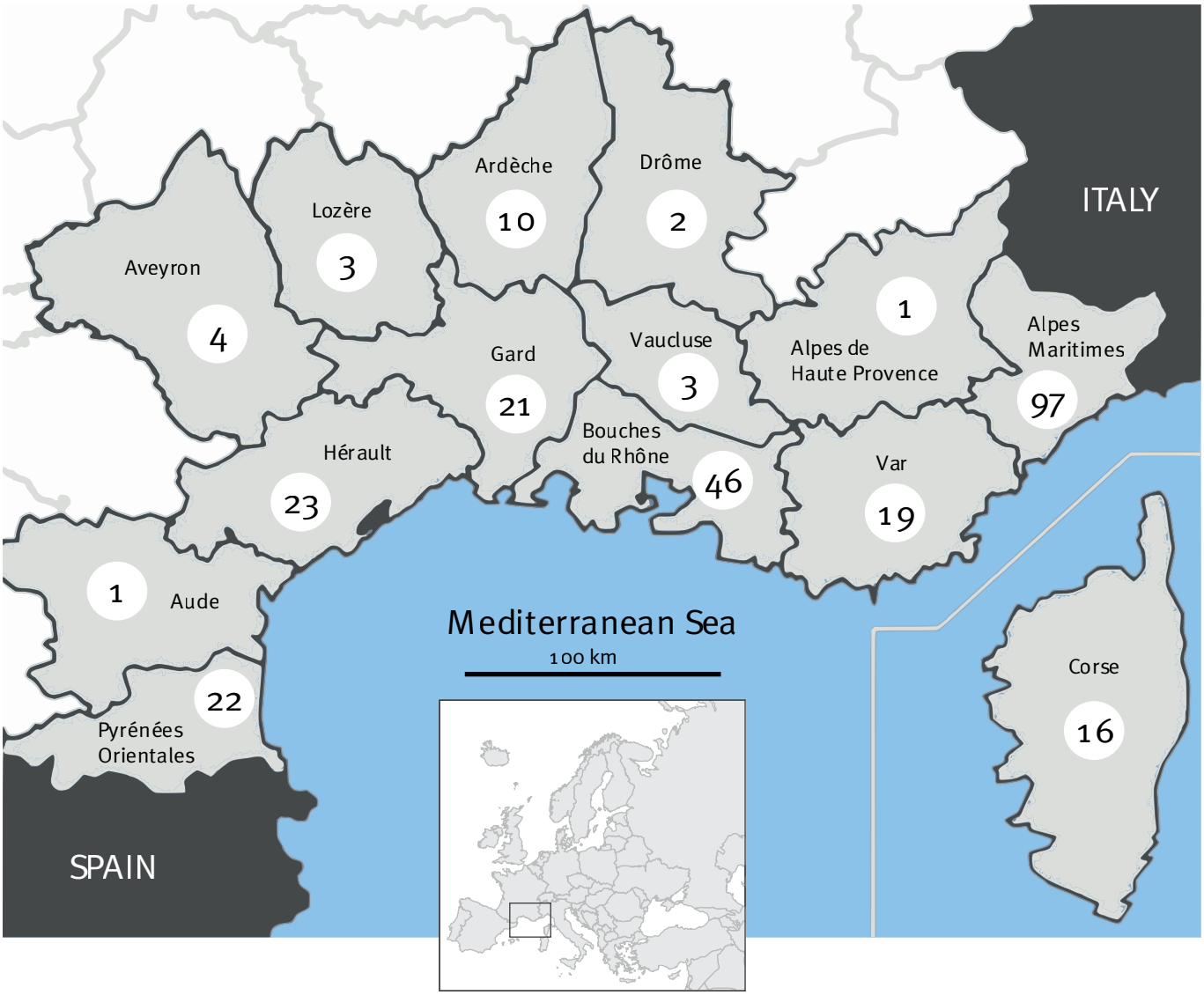
Taking into account all VL cases (n=366) reported to the NRCL from 1999 to 2012, 268 autochthonous cases and 98 imported cases, data analysis showed the association of an immunocompromising condition in 44.3% of cases (162/366): 31.4% (115/366) were HIV infected, 9.6% (35/366) had an immunosuppressive treatment and 3.3% (12/366) had received an organ or bone marrow transplant.

## Discussion and conclusion

In France, autochthonous leishmaniasis is due to *L. infantum* and is endemic mainly in the Mediterranean region. Canine leishmaniasis remains widespread in these foci and is the subject of studies by the ANSES (National Agency for Health Security of Food and Environment) as well as several national veterinary (Lyon, Maisons-Alfort, Nantes) or medical (Nice, Marseille) faculties, often in collaboration with the NRCL. With respect to human cases, the creation of a notification system at the NRCL in 1999 has allowed a better understanding of the incidence and distribution

**Figure 3**

Cumulative number of notified autochthonous visceral leishmaniasis cases in the most affected departments in France, 1999–2012 (n=268)

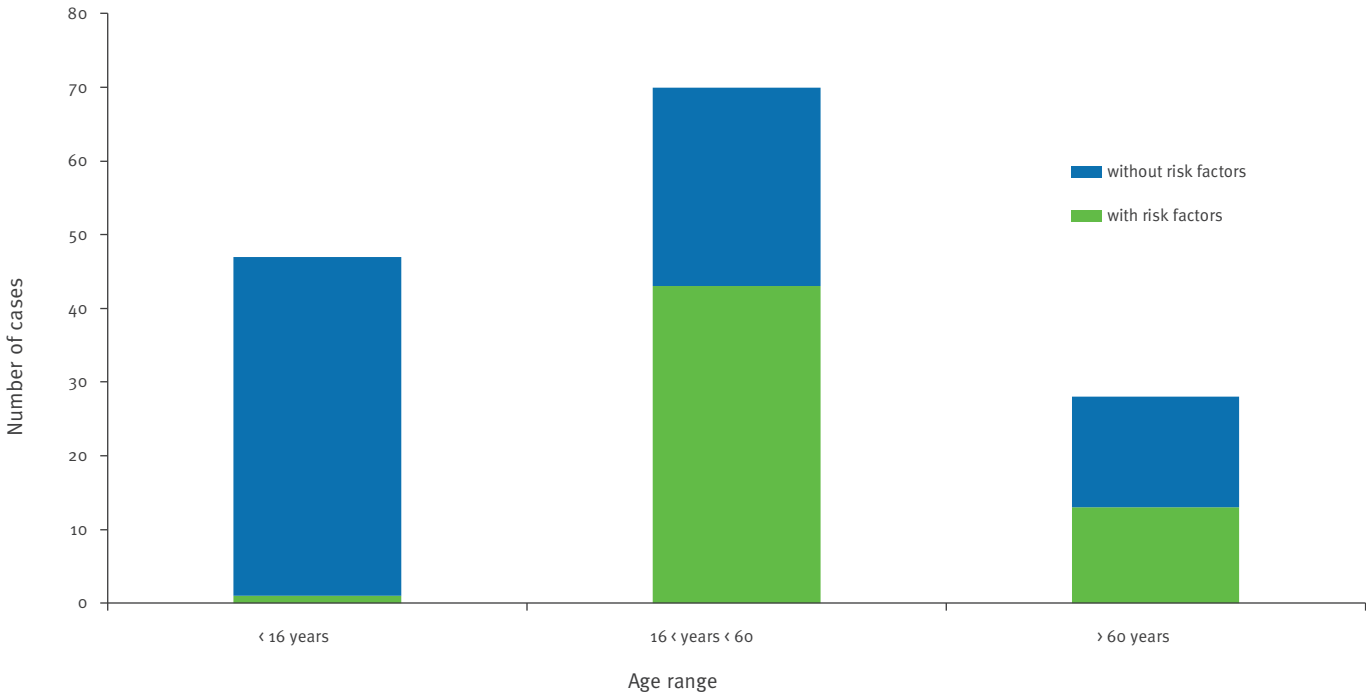



of the disease. Although an increase in incidence or an extension of the endemic areas can be anticipated in view of global warming [24,25], it appears that autochthonous cases remain relatively rare (annual mean 22.6) in France with a clear predominance of VL (84.5%).

VL has long been considered a disease of young children. The first case of Mediterranean kala-azar in a child in France was reported in 1918 [26]. A national survey conducted in 1986-87 showed a predominance of the disease in children, which constituted 51% of cases, with equal distribution between both sexes [20]. However, over the last decades, with the emergence of immunosuppression and primarily of HIV / acquired immunodeficiency syndrome (AIDS), the epidemiology of the disease has changed: in particular, the incidence in adults has increased significantly. Indeed, between 1975 and 2004, children accounted for only 30% of the cases [1]. This can be partly correlated with the increasing number of cases of leishmaniasis/HIV co-infection: HIV-positive patients are at high risk, and leishmaniasis is considered an opportunistic disease [27,28]. Between 1996 and 1998, 50% of new VL cases in southwestern Europe (including France) involved HIV-positive patients [27]. The mean age in our adult patients with VL was 36 years [28]. Nevertheless, the occurrence of cases among the elderly was not exceptional, which may be explained by ageing, declining of immune system, association with other pathologies such as tumours, inflammatory diseases, VL can also affect patients with no apparent immunosuppression nor risk factor, irrespective of their age. Overall, the incidence of VL during the study period was relatively stable, but a decrease of the incidence can be noted if compared to the period from 1993 to 1997 [1, 2], this

**Figure 4**

Notified autochthonous leishmaniasis cases by age group and risk factors, France, 2007–2012 (n=145)




For reasons of completeness of the data, only 2007–2012 was analysed for risk factors.

being most likely linked with the introduction of highly active antiretroviral therapy in AIDS patients.

In contrast to VL, autochthonous cases of CL and MCL are rare in France [29] and appear sporadic. CL is probably underdiagnosed and certainly undernotified, to a great extend because cutaneous lesions due to *L. infantum* are often small, painless and spontaneously self-curing within a few months; hence these benign lesions are essentially seen by general practitioners or dermatologists, which generally do not notify cases. The number of MCL cases notified to the NRCL, however, reflects almost the whole of the total seen in France, as this atypical clinical presentation necessitates the implementation of a specific laboratory diagnosis and initiation of anti-*Leishmania* drug treatment.

Compared to autochthonous cases, the number of imported cases is relatively high with an annual mean of 82.4 cases, mainly consisting in CL cases due to *L. major* and *L. guyanensis*, while for VL cases the annual mean of seven is not far from the nine cases per year reported in 1986-87 [20].

The monitoring by the NRCL is also useful to assess the temporal-spatial evolution of the disease. It is difficult to infer from these data whether the incidence of autochthonous leishmaniasis is declining in France or not: certain data sets suggest a medium-term (over decades) tendency to decline [1,2,20], but our data show that it is currently relatively stable. As to the risk of seeing the emergence of 'exotic' or hybrid parasites [30] which would be transmitted locally by permissive phlebotomine vectors, we consider that it is almost null. Indeed, (i) there is no evidence for any permissivity of *P. perniciosus* and *P. ariasi*; (ii) the probability of this occurring in nature appears extremely low, as, on top of permissivity, it requires gathering a number of factors allowing transmission (southern France, summer season, absence of treatment, etc.).

## The Working Group for the Notification of Human Leishmanioses in France

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## Conflict of interest

None declared.

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# 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.

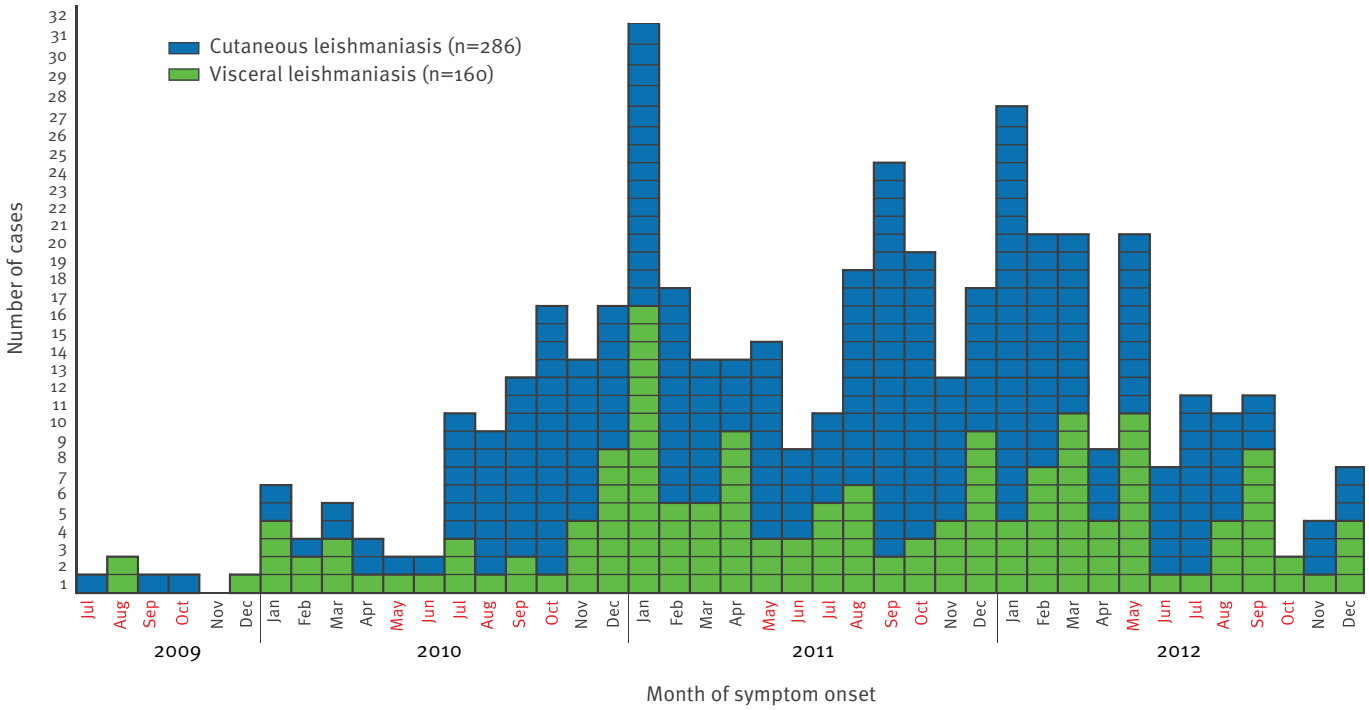
A specific case definition was established for the outbreak: a case was a person who met the clinical and laboratory criteria for leishmaniasis defined by the Epidemiological Surveillance Network, with residence in the towns located on the south-west area of the region of Madrid and with onset date of 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**

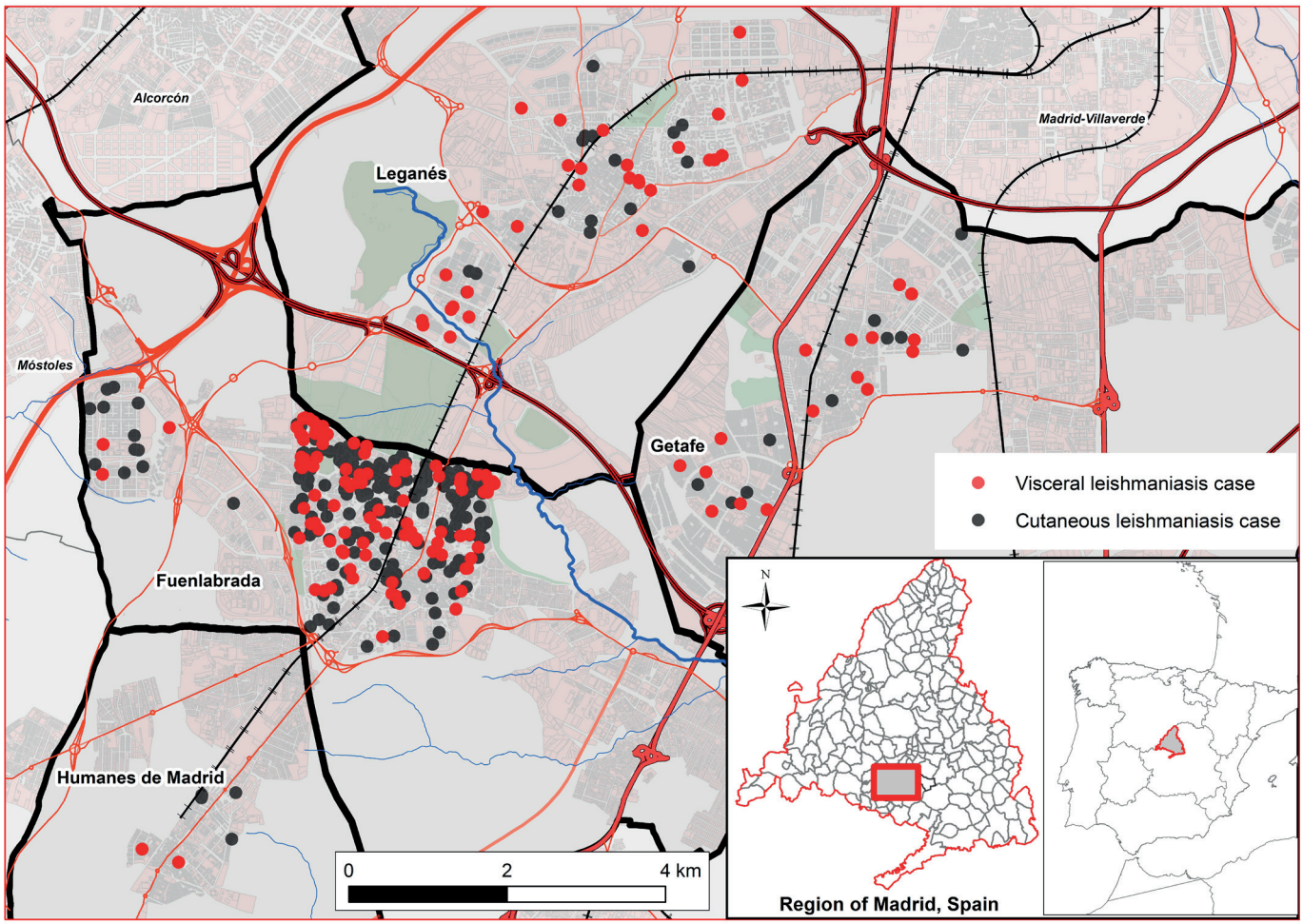
Outbreak cases of leishmaniasis by month of symptom onset and clinical presentation, region of Madrid, Spain, July 2009–December 2012 (n=446)




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

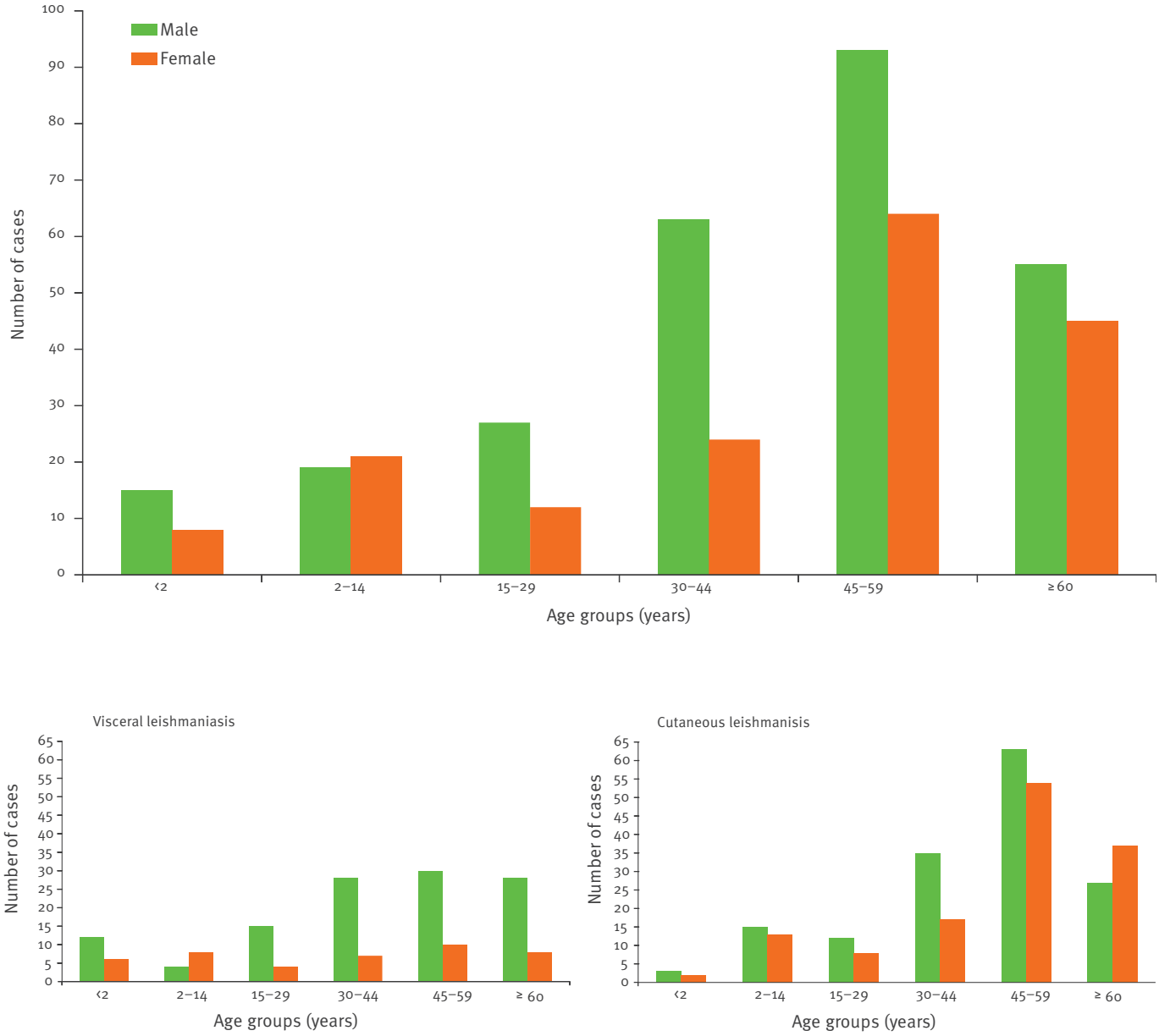
From 1 July 2009 to 31 December 2012, 542 cases of leishmaniasis were reported in the region of Madrid to the Epidemiological Surveillance Network, of which 446 (82.3%) met the outbreak case definition: 6 were identified in 2009, 97 in 2010, 196 cases in 2011 and 147 cases in 2012. The mean incidence 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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# 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.

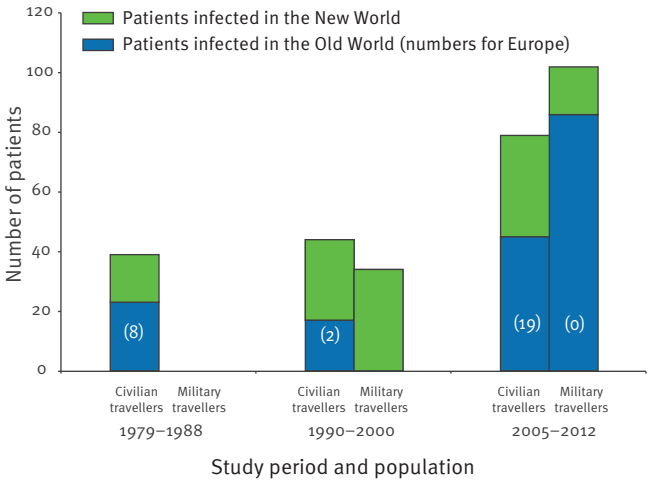
# Results

## 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)



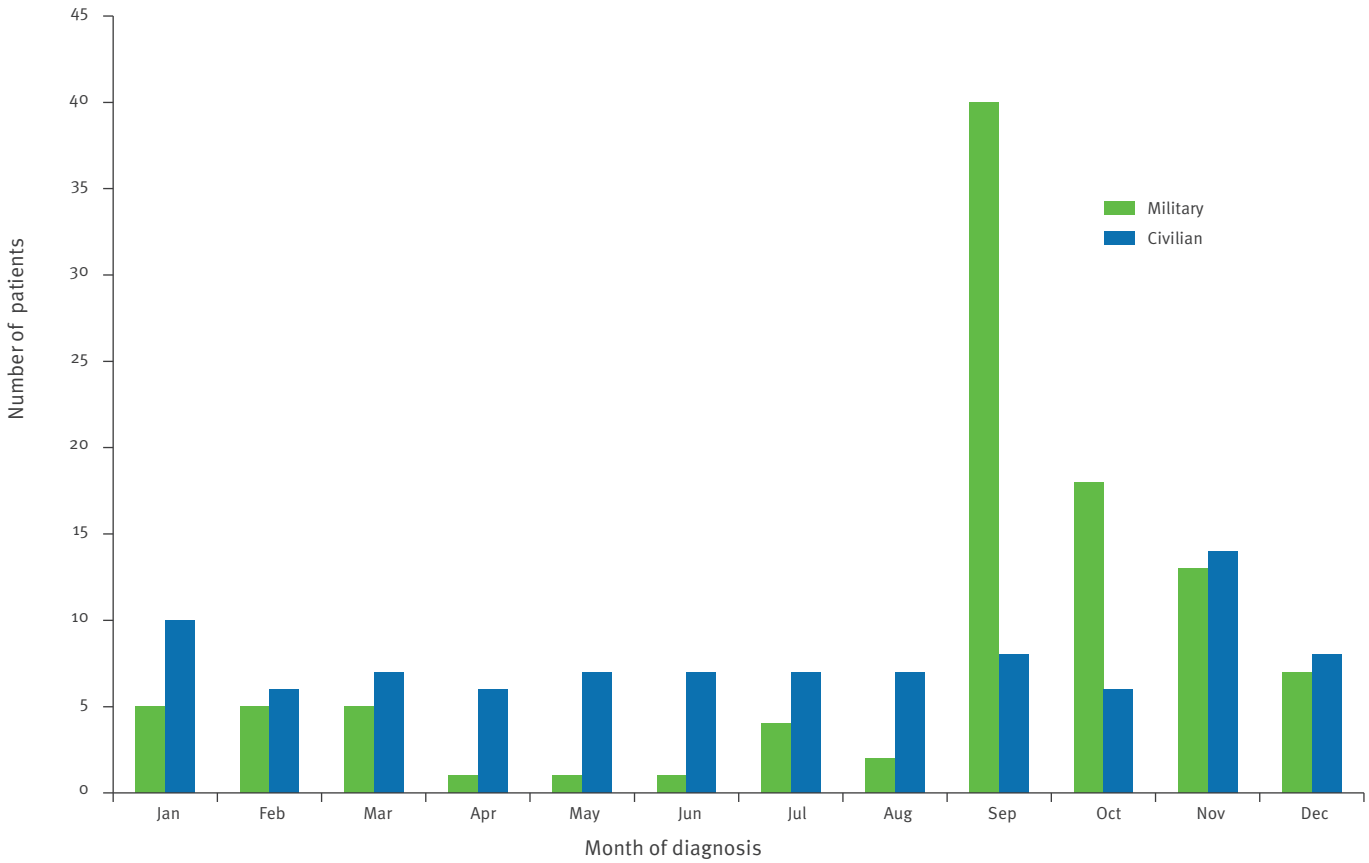

New World refers to the Americas. Old World comprises Africa, Asia and Europe.

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)

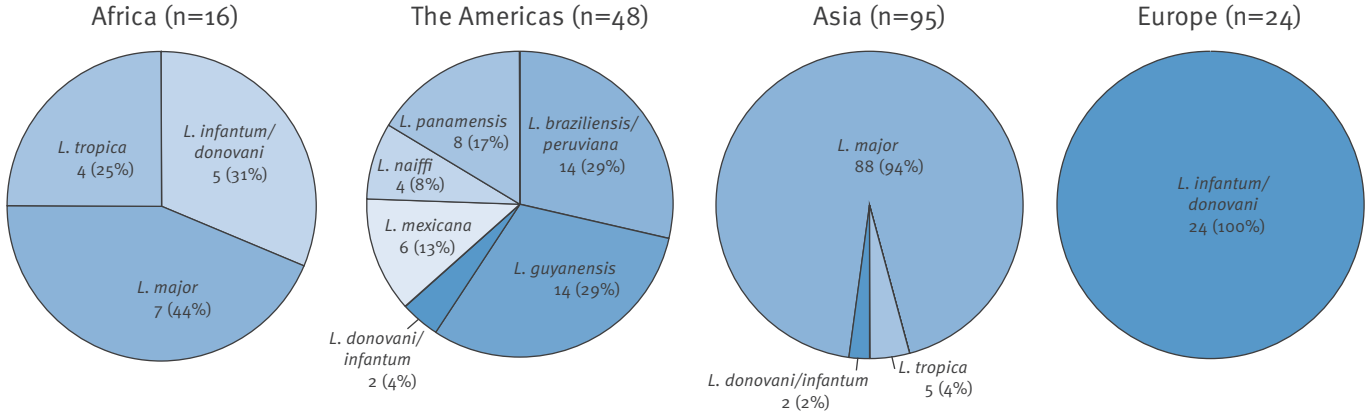


**Table 3**

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.

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# 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.

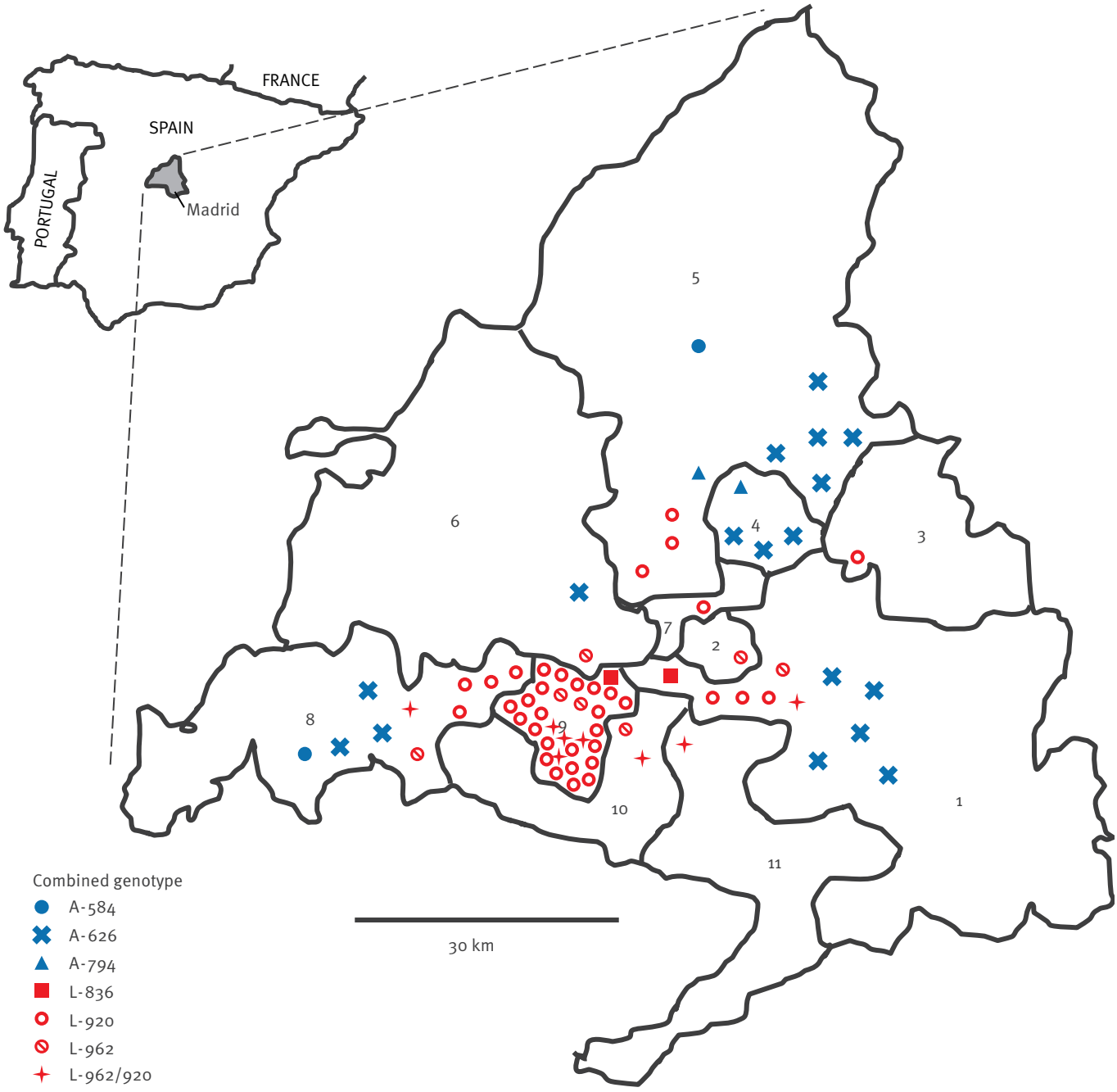
[a] Isolates ordered by year of isolation and Health Area.

[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)

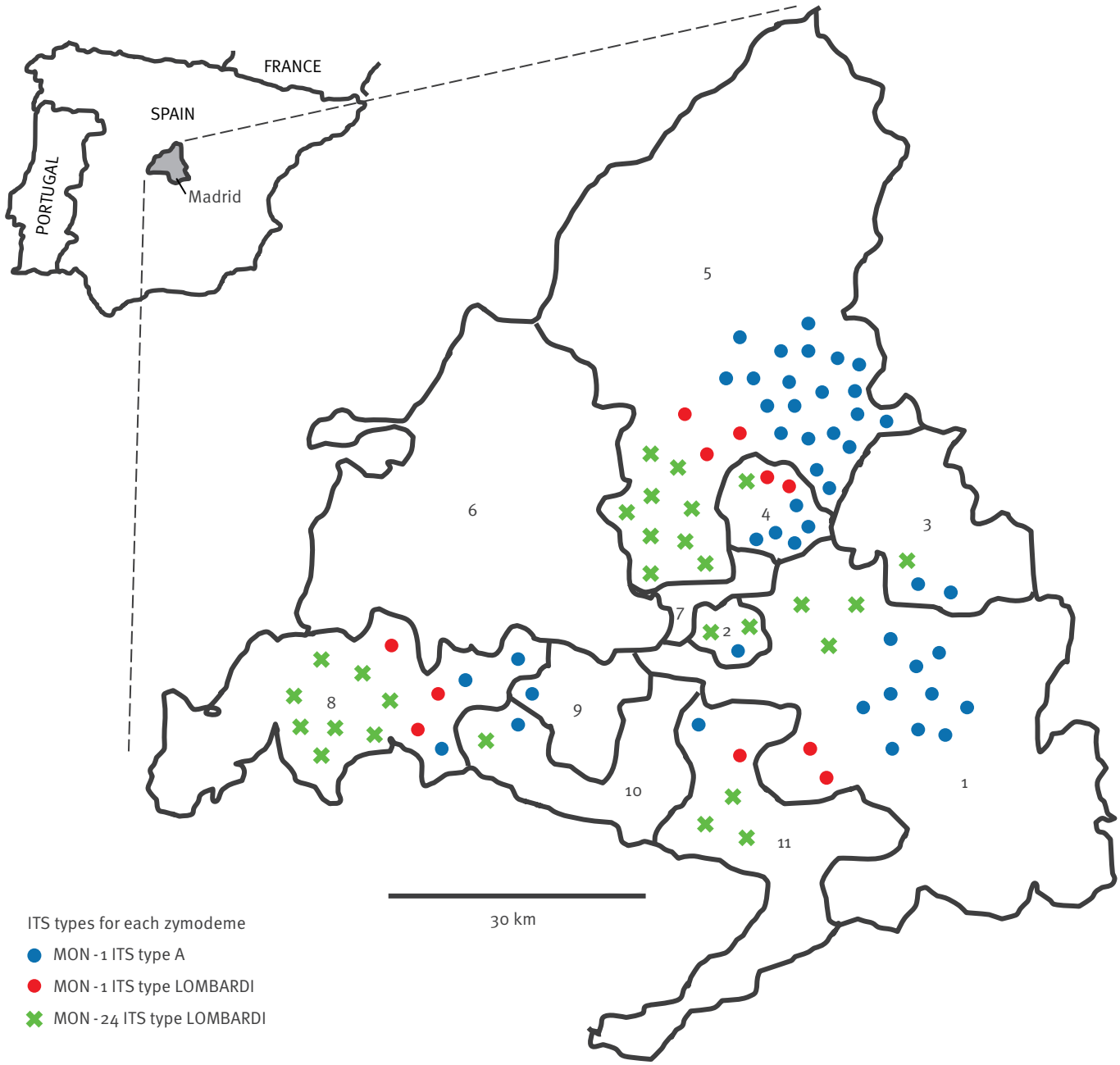
| Isolate WHO code[a] | Year of isolation | Health Area of isolate origin | Clinical form of leishmaniasis | HIV | Age group in years | Zymodeme | ITS type |
|---|---|---|---|---|---|---|---|
| MHOM/ES/2003/LLM-1327 | 2003 | 5 | VL | NEG | ‹5 | MON-1 | A |
| MHOM/ES/2003/LLM-1258 | 2003 | 8 | VL | NEG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

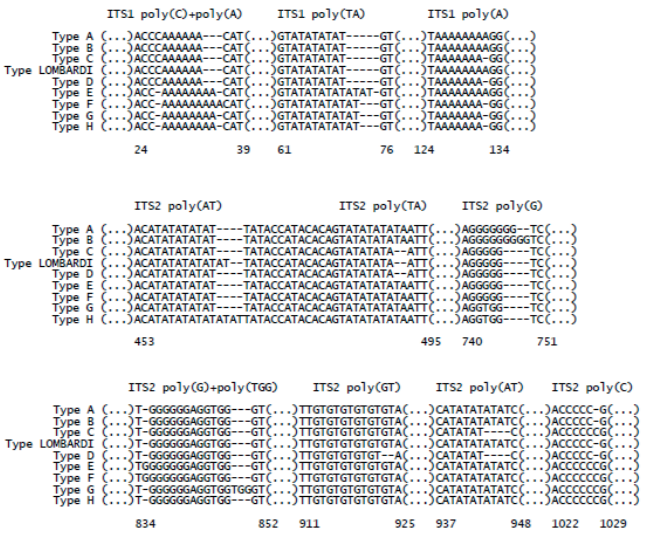
Distribution and ITS type of analysed *Leishmania infantum* isolates from Madrid, Spain, 1988–2005 (n=83)




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.

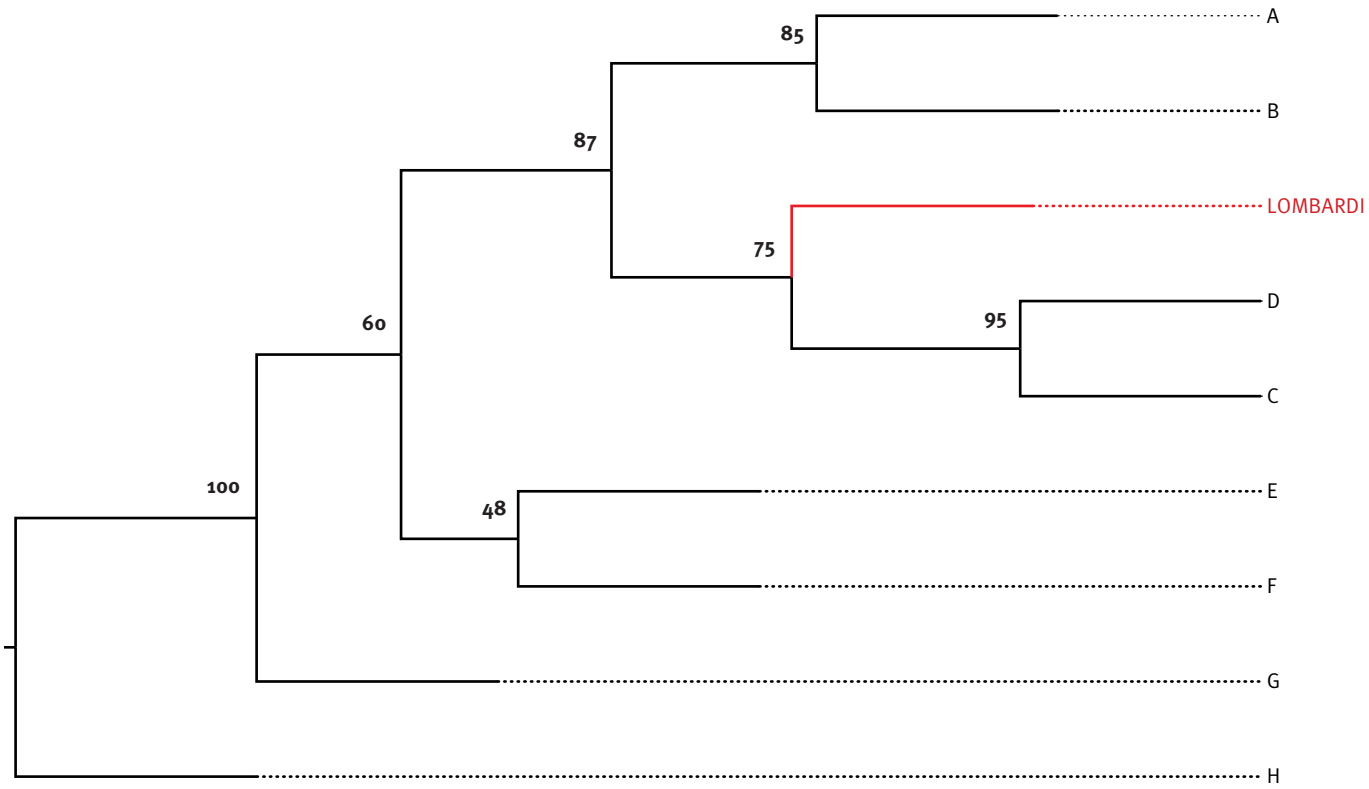
Our phylogenetic analysis included ITS-LOMBARDI in a cluster – supported by a bootstrap value of 87% – with ITS types A, B, C and D. However, it is further separated from types A and B in a subsequent subcluster (Figure 4).

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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# *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].

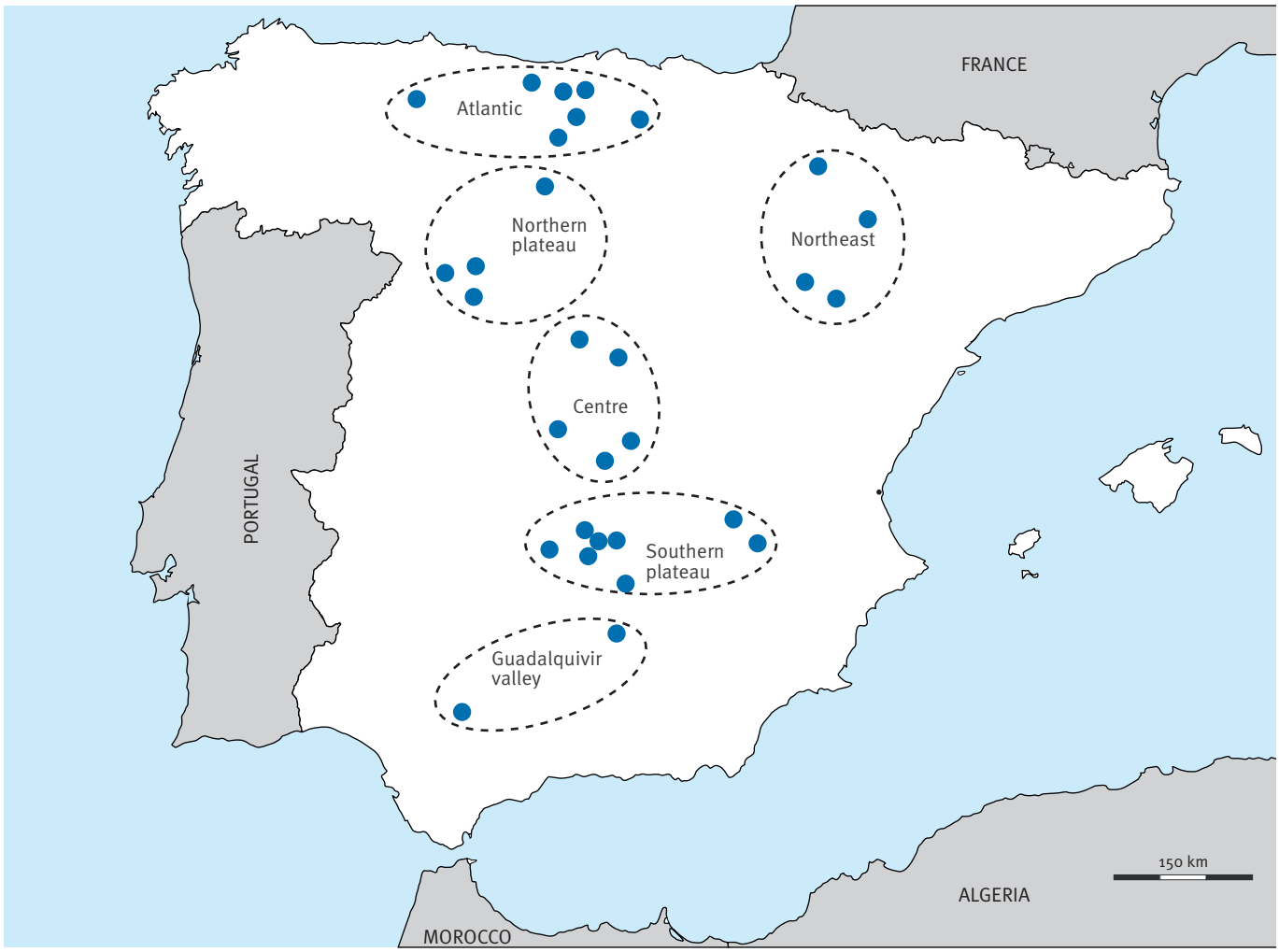
Three different hare species inhabit the Iberian Peninsula: (i) the Iberian hare that is present in vast areas of the Iberian Peninsula, (ii) the European brown hare (*Le. europaeus*) that lives in northern and northeastern Spain, and (iii) the Broom hare (*Le. castroviejoi*) that can be found in the 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.

## Acknowledgements

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# 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

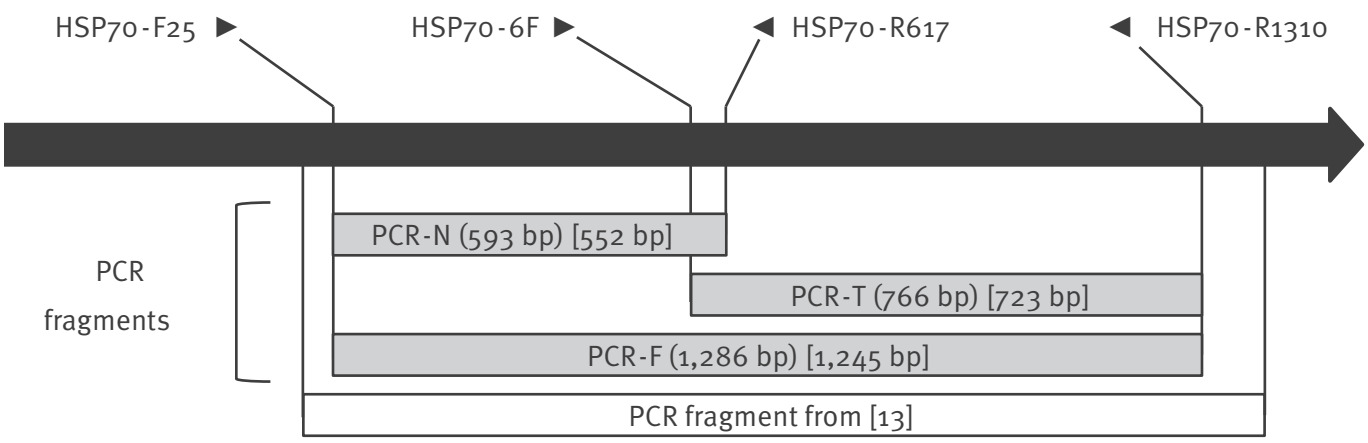
As a result of current human mobility, European infectious disease clinics are occasionally confronted with leishmaniasis patients who got infected in an area endemic for *Leishmania* outside their own country. Typically it concerns tourists, expatriates, military staff, migrants, and relatives visiting 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.

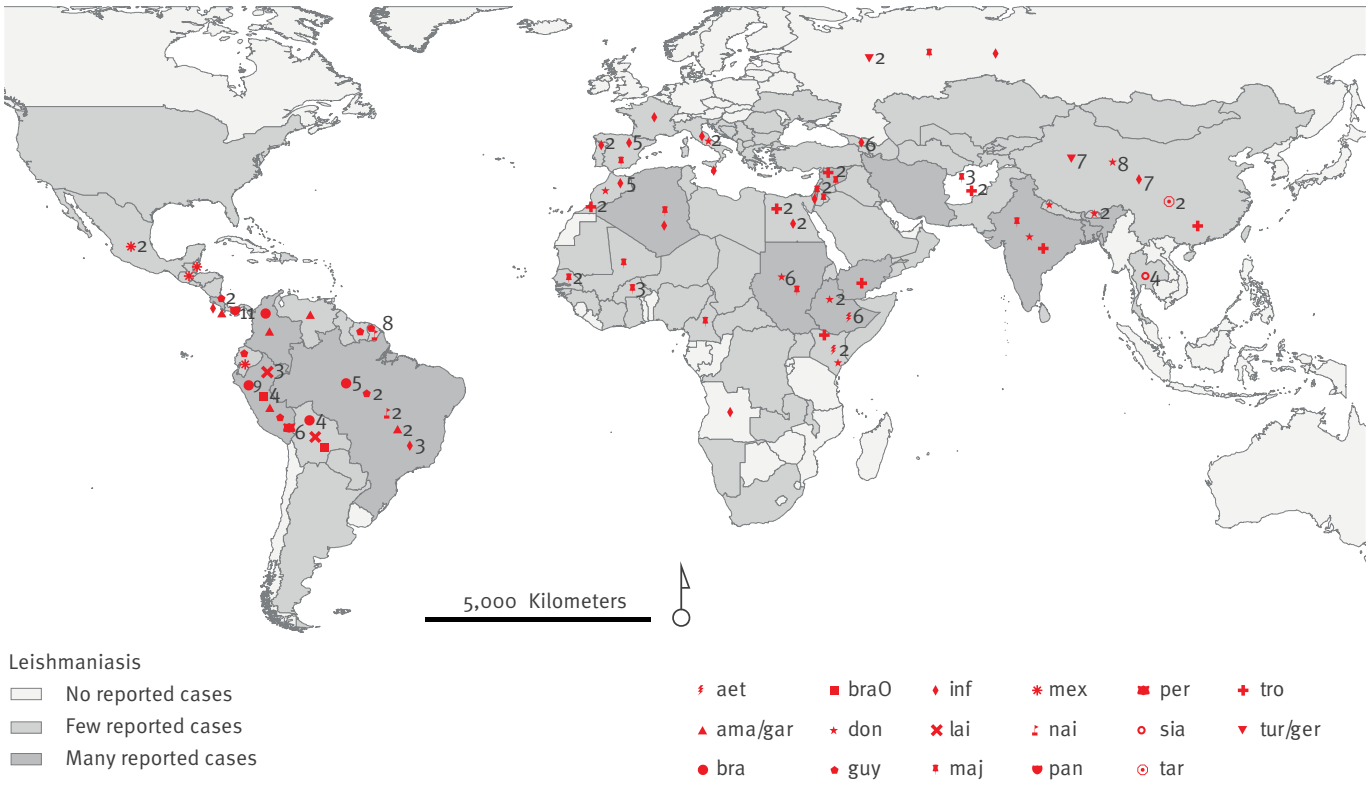
The clustering of a representative selection of the *hsp70* sequences is depicted in Figure 3. A dendrogram of the complete set of available *hsp70* sequences can be found at www.itg.be/LeishmaniaHSP70. The medically relevant clusters indicated in bold in these figures could be easily discriminated, 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)

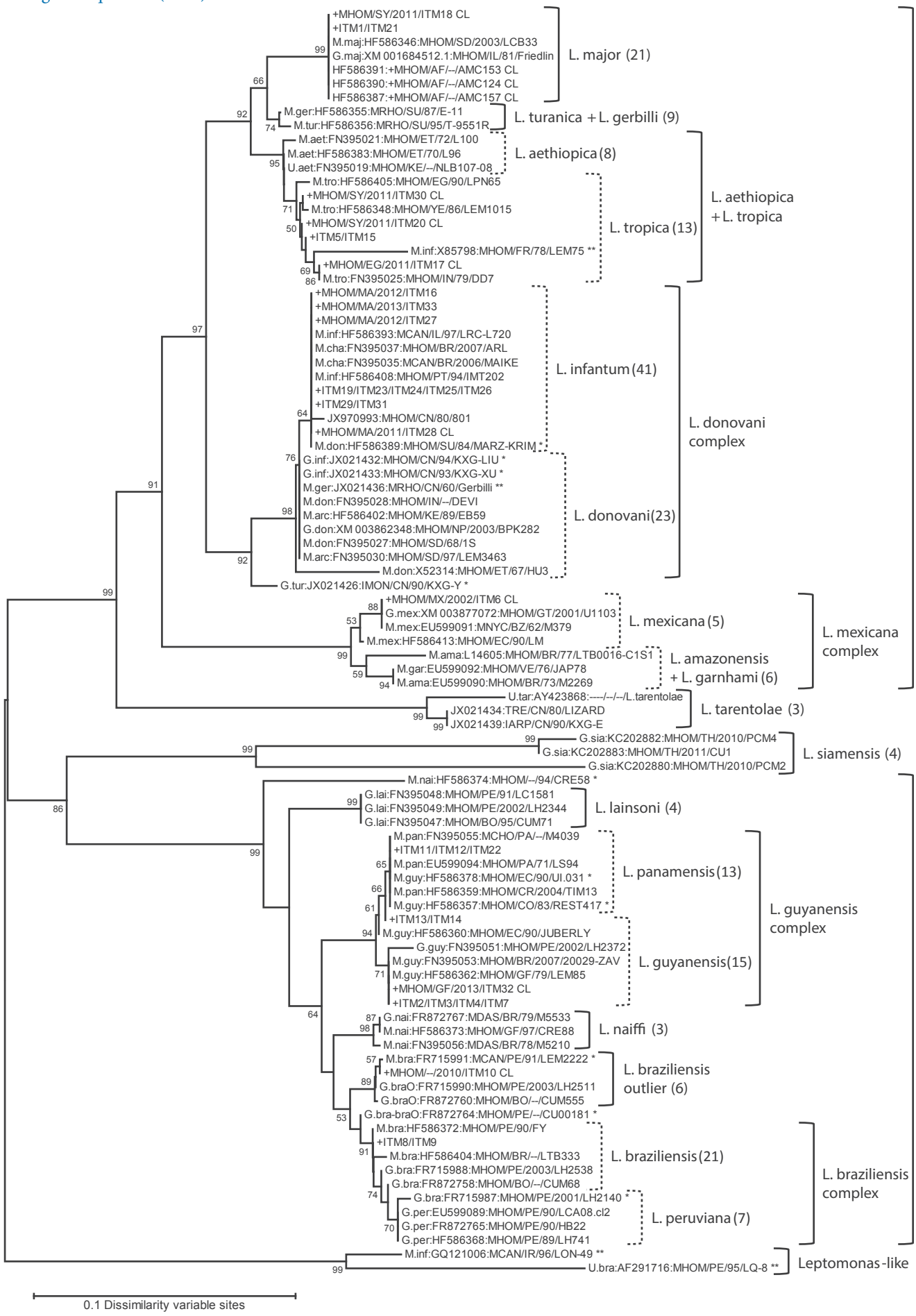


**Figure 3 notes**

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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# 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<br>• Ensure uniform methodology and data collection<br>• Enhance national drug registers in countries endemic for leishmaniasis (where necessary)<br>• Host online platform for case registration by non-endemic countries (i.e. travellers, migrants)<br>• Increase awareness among policy-makers and professional societies |
| National drug registers hosted by professional societies in rheumatology | • Implementation of cohort studies<br>• 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<br>• Detection of cases outside endemic countries<br>• Increase awareness / case reporting among clinicians |
| Pharmaceutical industry | • Data on drug sales with sufficient detail allowing geographically high resolution in leishmaniasis endemic areas<br>• 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.

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# 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.

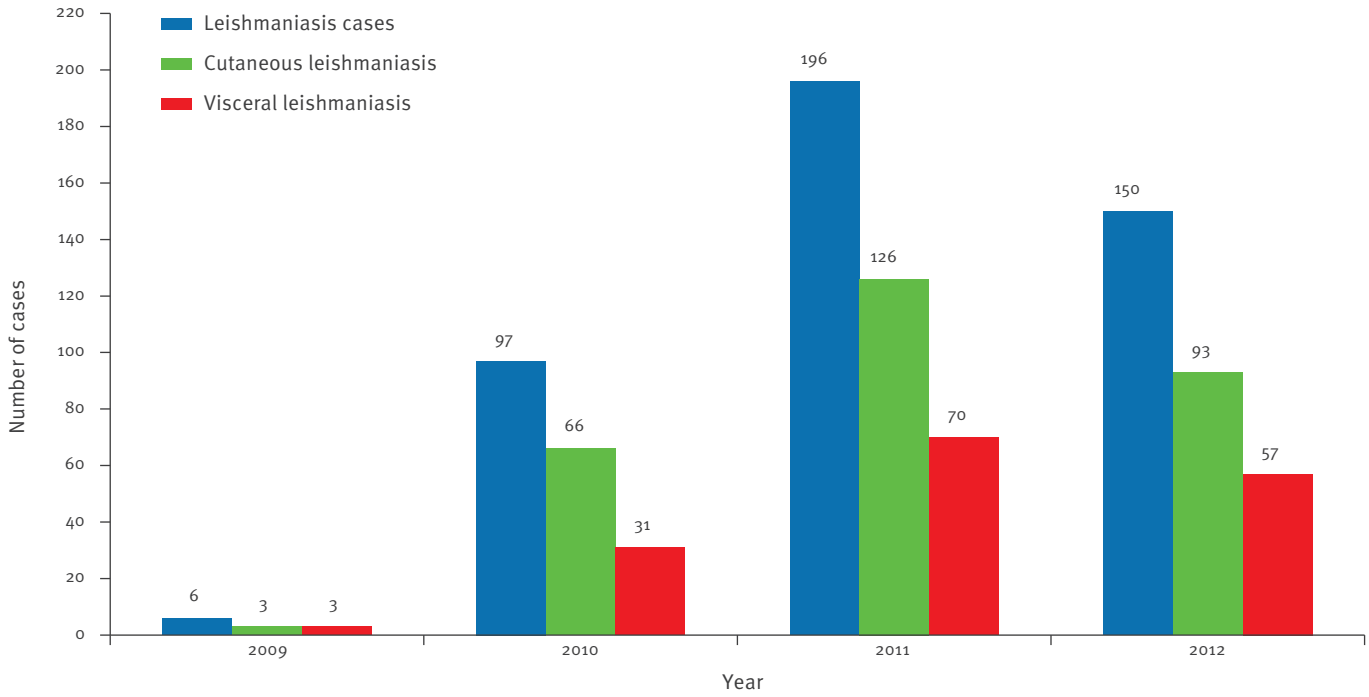
## Spread of endemic *L. infantum* and the risk of introduction of non-endemic *Leishmania* species

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.

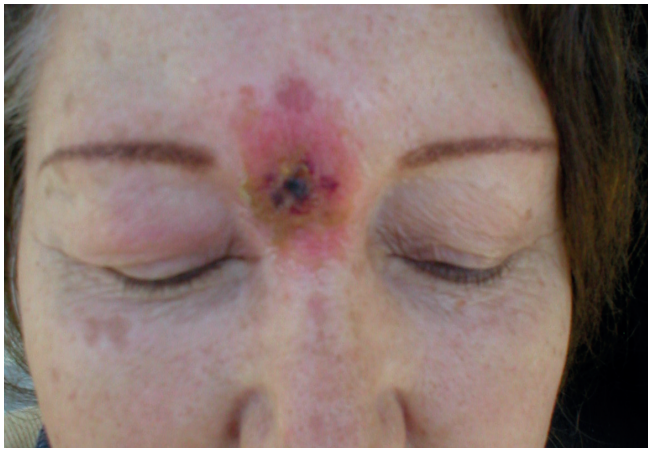
## Cyprus

*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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# 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.

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http://www.ncipd.org/

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http://www.iss.it/publ/noti/index.php?lang=1&tipo=4

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