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Studies on epidemiology and genetic diversity of *Leishmania infantum* endemic in the Southern Mediterranean

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List of abbreviations

CL	cutaneous leishmaniasis
CVL	canine visceral leishmaniasis
ELISA	enzyme-linked immunosorbent assay
*F*is	inbreeding coefficient
F_{ST}	F-statistics
*H*e	expected heterozygosity
*H*o	observed heterozygosity
ITS	internal transcribed spacer
MLEE	multilocus enzyme electrophoresis
MLMT	multilocus microsatellite typing
MNA	mean number of alleles per locus
MON	Montpellier
NJ	neighbour-joining tree
OR	Odds ratio
P	proportion of polymorphic loci
PCR	polymerase chain reaction
PMOH	the Palestinian Ministry of Health
RFLP	restriction fragment length polymorphism
SM	Southern Mediterranean
VL	visceral leishmaniasis
WB	the West Bank of Jordan
WHO	World health organisation
ΔK	ad hoc quantity

1. Abstract

Leishmania infantum is known to cause visceral and cutaneous leishmaniasis around the Mediterranean. The parasite is transmitted to humans and animals by the bite of phlebotomine sand fly. The clinical manifestations are highly diverse, humans and dogs are infected, and the diseases are associated with several epidemiological conditions and risk factors that are not yet fully understood. This study was aimed to investigate the molecular characteristics and genetic heterogeneity of strains of *L. infantum* in the Southern Mediterranean (SM) in order to understand their role in disease diversity and epidemiology.

The molecular epidemiological parameters of VL in Hebron district, Palestine were studied. Seventy-six cases were reported between 1993 and 2007. All cases were in children less than 9 years old. The ITS1-RFLP analysis revealed *L. infantum* as the causative agent and isoenzyme analysis identified two investigated isolates as zymodeme MON-1. A serological survey of 455 children revealed 8.4% seropositivity for *L. infantum.* The study identified two groups at high risk for attracting VL: child household members of VL patients and families having dogs and/or other domestic animals. Out of seven *Phlebotomus* species present, two putative vector species were identified: *Phlebotomus syriacus* (45%) and *Ph. tobbi* (10%).

A multilocus microsatellite typing (MLMT) approach, based on 14 hypervariable markers, was applied to investigate the population structure and genetic variability of *L. infantum* strains from the SM. They were isolated from human VL and CL, and from canine VL cases. The strains belonged to both MON-1 and non MON-1 isoenzyme types according to their MLEE profile. Bayesian model-based approach and phylogenetic analysis based on genetic distances were used to analyse the MLMT data. Two main populations were inferred from 44 Israeli and Palestinian strains. These results demonstrate similar disease dynamics in these areas. The re-emergence of VL in central Israel and Palestine is more likely due to increased dog and human contact with sylvatic cycles of parasitic infection rather than to recent introduction from the older foci.

Three populations were identified for 55 Algerian and 4 for 27 Tunisian strains, respectively. These populations were consistent with the classification based on isoenzymes, dividing the strains into MON-1 and MON-24/MON-80 groups. Further sub-division into several populations was found for MON-1 strains, largely correlating, albeit not completely, with their geographical distribution. MLMT results did not correlate to host origin as strains from humans and dogs are grouped together. A clear relationship could be demonstrated between the clinical presentation of leishmaniasis and parasite genotype. Moreover, the existence of hybrid strains between the MON-1 and MON-24/MON-80 groups has been shown and verified by analysis of clones of one of these strains. To our knowledge, this is the first report describing relationships between clinical picture and parasite genotype, as well as the existence of hybrids between zymodemes MON-1 and MON-24/MON-80.

Zusammenfassung

Leishmania infantum ist der Erreger viszeraler und kutaner Leishmaniosen im gesamten Mittelmeerraum Der Parasit wird durch den Stich infizierter Sandmücken der Gattung *Phlebotomus* auf Mensch und Tier übertragen. Die Krankheitsbilder sind sehr verschiedenartig, Menschen und Hunde sind infiziert, und die Erkrankungen werden mit verschiedenen epidemiologischen Bedingungen und Risikofaktoren assoziiert, die noch nicht vollständig geklärt sind. Das Ziel der vorliegenden Arbeit war es, molekulare Eigenschaften und die genetische Heterogenität von *L. infantum*-Stämmen aus dem südlichen Mittelmeerraum (SM) zu untersuchen und zu einem besseren Verständnis ihrer Rolle für die Diversität der Krankheitsbilder und der Epidemiologie der Erkrankung beizutragen..

Im Gebiet Hebron, Palästina, in dem 76 VL-Fälle zwischen 1993 and 2007 dokumentiert wurden, wurden epidemiologische Parameter der VL untersucht. Alle Patienten waren Kinder jünger als 9 Jahre alt. Mit Hilfe der ITS1-RFLP-Methode wurde *L. infantum* als der Erreger bestätigt und mittels Isoenzymanalyse wurden 2 der Isolate als Zymodem MON-1 identifiziert. Eine serologische Studie von 455 Kindern ergab eine Seropositivität von 8.4% für *L. infantum.* Zwei Gruppen hatten ein hohes Risiko an VL zu erkranken: Kinder, die entweder im gleichen Haushalt wie VL-Patienten oder in Familien mit Hunden und/oder anderen Haustieren leben. Zwei der im Gebiet Hebron identifizierten *Phlebotomus*-Arten sind potenzielle Vektoren von *L. infantum*: *Phlebotomus syriacus* (45%) und *Ph. tobbi* (10%).

Die Multilocus-Mikrosatelliten-Typisierung (MLMT), basierend auf 14 hypervariablen Markern, wurde für die Untersuchung der Populationsstrukturen und der genetischen Variabilität von *L. infantum*-Stämmen des Mittelmeerraums angewandt. Die Stämme stammten von humanen VL- und CL-Fällen sowie von erkrankten Hunden. Sie gehörten sowohl zu dem Zymodem MON-1 als auch zu verschiedenen nicht-MON-1 Zymodemen, entsprechend ihrer MLEE-Profile. Eine "Bayesian model-based"-Methode und phylogenetische Analysen der genetischen Distanz wurden für die Auswertung der MLMT-Daten benutzt. Die 44 israelischen und palästinensischen Stämme konnten dabei zwei Hauptpopulationen zugeordnet werden. Die Ergebnisse zeigten eine ähnliche Dynamik der Erkrankung in diesen Gebieten. Die Rückkehr der VL in Zentralisrael und Palästina ist höchstwahrscheinlich eher auf einen intensiveren Kontakt von Hunden und Menschen mit sylvatischen Zyklen der parasitären Infektion als auf einen kürzlichen Import aus älteren Foci zurückzuführen.

Drei Populationen wurden für 55 algerische und 4 für 27 tunesische Stämme identifiziert. Die MON-1- und MON-24/MON-80-Stämme wurden, in Übereinstimmung mit der auf Isoenzymmustern basierten Klassifikation, verschiedenen Populationen zugeordnet. Die MON-1-Gruppe war in mehrere Populationen unterteilt, weitestgehend, aber nicht total, in Korrelation mit der geographischen Verbreitung der Stämme. Eine Korrelation zwischen MLMT-Profilen und Wirtshintergrund der Stämme wurde nicht beobachtet, da die Stämme von Menschen und Hunden immer zusammen gruppierten. Die Beziehung zwischen dem klinischen Bild der Leishmaniose und dem Genotyp des Parasiten war aber deutlich. Außerdem wurde die Existenz von Hybrid-Stämmen, zwischen den MON-1- und MON-24/MON-80-Gruppem nachgewiesen

und durch die Klonierung eines dieser Stämme verifiziert. Soweit uns bekannt, ist dies der erste Bericht über eine Korrelation zwischen dem klinischen Bild der Leishmaniose und dem parasitären Genotyp sowie über die Existenz von Hybriden zwischen den Zymodemen MON-1 und MON-24/MON-80.

2. Introduction

Leishmania infantum, belonging to the *L. donovani* complex, is the causative agent of human visceral (VL), cutaneous (CL) and canine visceral (CVL) leishmaniasis in all Mediterranean countries. The disease is zoonotic and transmitted to humans from its reservoir hosts, mainly dogs [1], by the bite of sand flies of genus *Phlebotomus* [2].

In Palestine and Israel, *L. infantum* causes exclusively VL. Preliminary reports from the PMOH indicated that VL is highly prevalent in Hebron district, which is located in southern West Bank (WB). Between 1990 and 1999 the VL cases occurring in Hebron accounted for 25.2% of the total VL cases reported in the WB [3]. In Israel the disease is endemic to central and northern areas [4]. However, human VL and CVL, essentially, disappeared from central Israel between the years 1950 and 1994 and re-emerged thereafter [5], whilst sporadic human, canine, and asymptomatic VL cases have been reported in north Israel (the old focus) over the last few decades [6-8]. The PMOH records indicated that the disease emerged in the WB in the early 1990s; the situation is similar in the neighboring Israeli foci. The vectors of *L. infantum* have not been identified in these areas, but several putative vectors, *Phlebotomus perfiliewi*, *Ph. neglectus*, *Ph. syriacus* and *Ph. tobbi*, were abundant in the endemic foci in the northern WB [9]. Based on the MLEE, the current standard typing method of *L. infantum*, 28 zymodemes were described around the Mediterranean with zymodeme MON-1 being the predominating type [10], also in Israel and Palestine [11].

In Algeria and Tunisia, three main zymodemes of *L. infantum* have been identified: MON-1 and MON-24, which were more frequent, and MON 80 [12, 13]. The correlation between the clinical presentation of disease and the zymodemes remains controversial, as the predominating MON-1 zymodeme, mostly associated with VL cases, was also found to cause CL [13, 14]. Moreover, the dermotropic MON-24 zymodeme [14] was occasionally isolated from VL cases [13], whereas zymodeme MON-80 was linked with both VL and CL [13, 15].

L. infantum strains isolated from dogs in both countries were predominantly of MON-1 type [16], however, other enzymatic variants (MON-34, MON-24, MON-77) were isolated occasionally from Algerian CVL cases [17]. The reservoir host of MON-24 and MON-80 has not yet been elucidated. Two sand fly species were found to be infected with *L. infantum*. *P. perniciosus*, is the principal vector of the viscerotropic *L. infantum* zymodeme MON-1 in

Algeria and Tunisia. *P. perfiliewi* was found to be infected by *L. infantum* MON-24 in northern Algeria and was abundant in northern part of Tunisia [18].

L. infantum parasites are associated with diverse clinical and eco-epidemiological situations in SM. The association with different clinical forms of the disease, VL and CL, with different hosts and the wide geographical distribution in each country raised the question about the role of parasite diversity in the clinical manifestation and epidemiology of the disease.

MLEE typing is considered as gold standard for typing *L. infantum* strains. However it has some disadvantages, as it needs bulk cultivation of parasites, post-translational modifications may influence the mobility of the protein, synonymous nucleotide substitutions may not be observed and different allozymes can have coincident mobilities. Other typing methods, for review see Schönian *et al* [19], are limited in the intrinsic level of polymorphism they can detect and are, only in exceptional cases, able to differentiate strains in the zymodeme MON-1. For that, we applied a multilocus microsatellite typing approach (MLMT) which is considered most discriminating and adequate for typing strains of the *L. donovani* complex, even within single zymodemes like MON-1. It has the advantage of generating reproducible results that can be stored in databases and exchanged among laboratories, and has recently proved useful in evolutionary investigations and a taxonomic revision of the *L. donovani* complex [20, 21].

Main Objectives of the work described here are:

1. To study epidemiological parameters and parasitological features of paediatric VL in Hebron District, Palestine between 1993 and 2007. This includes detection and identification of parasites from retrospective and new VL cases in that focus, determination of disease prevalence in the human population, assessment of risk factors for VL in this area, and investigation of the putative transmitting vector species.
2. To investigate and compare the genetic structure and gene diversity of *L. infantum* strains isolated from north Israel (the old focus), central Israel (the re-emerging focus), and from Palestinian foci. To correlate the diversity/ homogeneity of these strains with their geographical distribution and the corresponding environmental and ecological conditions existing in the various locations to clarify the disease transmission and re-emergence in these foci.
3. To apply a multilocus microsatellite typing (MLMT) approach for population studies of *L. infantum* isolated from different endemic foci in Tunisia and Algeria, representing different

clinical forms (VL and CL), and different zymodeme types (MON-1,24 and 80). The focus of this work was to investigate the degree of polymorphism within *L. infantum* and its zymodemes, the existence and geographical distribution of particular *L. infantum* populations, the existence of gene flow between those, and correlations to the clinical pictures.

3. Materials and Methods

3.1 Leishmania samples, sero-survey and epidemiological data collected from Hebron District

Two cultures and 36 archived Giemsa-stained bone-marrow aspirates from VL patients were used for molecular characterization of VL in Hebron District. To identify the parasites at species level the ribosomal internal transcribed spacer 1 (ITS1) was amplified using the primer pair L5.8S and LITSR, and the PCR product (~300 bp) was digested with the restriction endonuclease *Hae*III [22]. The two cultures were typed with MLEE by determining the mobility of 15 enzymes in starch gel electrophoresis [23].

For studying sero-prevalence in human population, blood samples were collected from 455 children between 6 months and 12 years old and screened for serum anti-*Leishmania* antibodies. The sero-survey was done using an in-house enzyme-linked immunosorbent assay (ELISA) based on crude leishmanial antigen [5, 6]. For all study participants, demographic data including gender, age, area, being a household member of previous VL cases, and the presence of domestic dogs and/or other animals were collated for risk assessment.

Sand flies were collected and examined for *Leishmania* infection. For taxonomic identification, heads and genitalia were removed and mounted on microscope slides in Berlese's fluid and identified using several keys [9]. Females belonging to *Phlebotomus* spp. were identified by the structures at the base of the spermathecal ducts. For parasite isolation, females that were blood-fed or had swollen abdomens were used to obtain parasite cultures in semi-solid agar supplemented with 10% rabbit blood.

The PMOH records were screened for VL cases reported in the district between 1991 and 2007. The collected data included age, gender, date of admission, method of diagnosis, treatment regime and response, and the exact geographical origin of each case. Statistical analysis was carried out by SPSS 16.0 software. Fisher's exact test was used to examine the significance of the association between the variables and sero-positivity for VL. A p-value of less than 0.05 was

considered to be significant. Calculation of odds ratios (ORs) with confidence intervals (CIs) were used to quantify the risk.

3.2 Multilocus microsatellite typing (MLMT)

For population structure analysis, a total of 126 samples, either cultures or archived Giemsa-stained slides isolated from human VL and CL and CVL cases, were investigated. This included 17 Palestinian and 27 Israeli samples, 55 strains from Algeria and 27 from Tunisia. The population structure of *L. infantum* was investigated in each country individually. In all cases, reference strains from different Mediterranean countries representing different zymodemes were included for comparison.

Genetic polymorphism and population structure of *L. infantum* were investigated using 14 hyper-variable unlinked microsatellite markers that were shown to be highly discriminatory for strains of the *L. donovani* complex [21, 24]. Fragment size variation of the amplified products has been screened by either 4% MetaPhor agarose gel electrophoresis [24] or automated fragment analysis on a capillary sequencer [25]. This technique permits the exact determination of the number of repeats for the amplified microsatellite markers.

Population structure was investigated using a Bayesian model-based clustering method (program STRUCTURE 2.2) [26]. The admixture model was used, which correlated allele frequencies among populations. Microsatellite profiles were also used to define genetic distances. Phylogenetic trees based on the proportion of shared alleles distances (D_{AS}) were constructed using the neighbour-joining (NJ) method with the help of the MSA 3.0 [27], POPULATIONS 1.2.28 and MEGA version 3.1 software [28]. The software GDA [29] was applied to analyse the microsatellite data with respect to allelic diversity which is the number of allelic variants per marker (A), mean number of alleles (MNA) per population which is considered an indicator of genetic variation within a population, proportion of polymorphic loci (P), expected (H_e) and observed (H_o) heterozygosity and inbreeding coefficient (F_{IS}). Genetic differentiation and gene flow were assessed by calculating F_{ST} values with corresponding *p*-values (confidence test), using the MSA software. F_{ST} values higher than 0.25 indicate strong genetic differentiation [30]. Recombination has been evaluated through Neighbor Joining networks (NeighborNet) obtained by SplitsTree [31] and by the population membership coefficients obtained using STRUCTURE.

4. Results

4.1 Epidemiology of paediatric visceral leishmaniasis in Hebron District, Palestine

Seventy-six VL cases in children, aged between 6 months and 9 years, were recorded in 13 different villages located west of Hebron city between 1993 and 2007. Primary diagnosis was based on clinical symptoms and direct microscopy of bone marrow aspirates. ITS1-RFLP and sequencing, performed for two cultured strains and 36 archived Giemsa slides of bone marrow aspirates all from Hebron District, confirmed identification as *L. infantum.* Isoenzyme typing using the two cultured isolates revealed a MON-1 isoenzyme profile.

Thirty-eight (8.3%) of the 455 individuals screened for serum antileishmanial antibodies were sero-positive. No difference was seen between males and females. Sero-positivity was highest for child household members of previous VL cases and among people who had domestic dogs and/or other animals.

A total of 120 Phlebotomine sand flies were investigated. The following species were identified: *Ph. major syriacus* (45%), *Sergentomyia spp.* (19%), *Ph. sergenti* (17%), *Ph. tobbi* (10%), *Ph. papatasi* (5%), *Ph. halepensis* (2%), and *Ph. mascitti canaaniticus* (2%). The blood feed female sand flies were dissected and microscopically examined. No parasites were seen and cultures remained negative up to 1 month.

These results have been published in the following paper.

***A. Amro**, K. Azmi, G. Schönian, A. Nasereddin, M.B. Alsharabati, S. Sawalha, O. Hamarsheh, S. Ereqat, Z. Abdeen, Epidemiology of paediatric visceral leishmaniasis in Hebron district, Palestine, Transactions of the Royal Society of Tropical Medicine and Hygiene2009. 103(7): p. 731-6.*

4.2 Population structure of *L. infantum* in Israel and Palestine

For MLMT, 44 strains of *L. infantum* isolated from human and canine VL cases were analysed together with the WHO reference strain MHOM/TN/1980/IPT1 and 22 strains from Portugal, Spain, France, Italy, Greece and Turkey belonging to zymodeme MON-1 and to different non-MON-1 zymodemes. Most of the Israeli and Palestinian strains had their individual MLMT profiles.

Both Bayesian model-based clustering method and distance-based analysis detected 4 main populations: Population A, containing all but one Palestinian strains, and strains from central

Israel and two Israeli settlements in the West Bank; population B, containing all strains from northern Israel and some strains from central Israel; population C, containing the European and Turkish MON-1 strains; and population D containing all European strains of non MON-1 type.

Genotype data of strains representing populations A and B were re-analysed separately. No further population sub-structure was found for population A, but three sub-populations were exposed in population B. Sub-populations B1 and B2 consisted of strains from central Israel and one Palestinian strain from Jenin District, and B3 of strains from old foci in northern Israel.

F-statistics revealed great genetic isolation between the 4 main populations. Sub-populations B1, B2, and B3 were also genetically differentiated according to their F_{ST} values, however, not significantly (*p*-values > 0.05), probably due to the small sample size of the sub-populations.

The genetic diversity was lowest for population B and higher for population A. Expected heterozygosity was much higher than observed and the inbreeding coefficients suggested a high degree of 'inbreeding' within each population.

These results have been published in the following paper.

***A. Amro**, G. Schönian, M.B. Alsharabati, K. Azmi, A. Nasereddin, Z. Abdeen, L.F. Schnur, G. Baneth, C.L. Jaffe, K. Kuhls, Population genetics of Leishmania infantum in Israel and the Palestinian Authority through microsatellite analysis, Microbes and Infection 2009. 11(4): p. 484-92.*

4.3 Population structure of *L .infantum* in Algeria

Fifty-five Algerian strains of *L. infantum* isolated from human VL and CL and from canine VL cases and belonging to three zymodemes, MON-1, MON-24, and MON-80, were subjected to MLMT.

STRUCTURE and distance analyses identified the same 3 populations: Population A, comprising mostly Algerian MON-1 strains; population B, consisting of MON1 strains from Algeria and different European foci; and population C, comprising strains of MON-24/MON-80 which are mainly from CL cases. No correlation was found between a particular MLMT profile and the host background of strains (canine vs. human). Three strains had mixed A/B genotypes; and 8 strains mixed A/C genotypes.

F_{ST} and corresponding p-values indicated significant genetic differentiation between the three populations. Gene flow was evident between populations A and C, and A and B, respectively.

When used to characterize strains isolated from three patients during different episodes of the disease, MLMT made it possible to differentiate between relapses and/or re-infections.

These results have been published in the following paper.

*N. Seridi, **A. Amro**, K. Kuhls, M. Belkaid, C. Zidane, A. Al-Jawabreh, G. Schönian, Genetic polymorphism of Algerian Leishmania infantum strains revealed by multilocus microsatellite analysis, Microbes and Infection . 2008. 10(12-13): p. 1309-15.*

4.4 Population structure of *L. infantum* in Tunisia

Twenty-seven Tunisian *L. infantum* strains isolated from human VL and CL and from CVL cases and belonging to three zymodemes, MON-1, MON-24 and MON-80 were investigated with MLMT.

Four populations have been identified by STRUCTURE and genetic distance analyses. F_{ST} values varied between 0.335 and 0.627, confirming that these four populations are genetically differentiated. Population 1 consists of MON-1 strains from Tunisia; population 2 of MON-1 strains from different European foci and two Tunisian MON-1 strains; population 3 of all MON-24/MON-80 strains isolated from VL cases; and population 4 of all MON-24 isolated from CL cases. Gene flow was detected between the European and the Tunisian MON-1 populations, as well as between the Tunisian MON-1 and the combined MON-24 populations.

Four strains, three of MON-24 and one MON-80, had mixed 1/3 genotypes and were heterozygous in 10-14 markers. In order to test whether they might represent real hybrids, one strain was cloned and six of the clones obtained were re-analysed by MLMT. All clones showed MLMT profiles identical to that of the original strain. Alleles specific for both populations 1 and 3 were present in hybrid strains and the clones, indicating that at least the cloned strain is a real 1/3 hybrid.

The degree of recombination was assessed from distance data by inferred a NJ network of the SplitsTree software. A reticulate pattern was seen mainly between populations 1 and 3, with the hybrids showing intermediate positions, and within population 4.

Population 4 was found to be most diverse, whereas population 1 was least diverse although it included the highest number of strains. Inbreeding coefficients were highest in the two MON-1 populations, indicating clonal propagation.

This work has been published in the following paper.

*N. Chargui, **A. Amro**, N. Haouas, G. Schönian, H. Babba, S. Schmidt, C. Ravel, M. Lefebvre, P. Bastien, E. Chaker, K. Aoun, M. Zribi, K. Kuhls, Population structure of Tunisian Leishmania infantum and evidence for the existence of hybrids and gene flow between genetically different populations, International Journal for Parasitology. 2009. 39(7): p. 801-11.*

5. Discussion

5.1 Leishmaniasis in Hebron District, Palestine

So far, VL diagnosis in Hebron district was based on clinical symptoms and direct microscopy of bone marrow aspirates. Serological and molecular diagnostics were first introduced in 2005. The application of these methods has improved the accuracy of case detection.

Species identification by ITS1 PCR-RFLP showed that *L. infantum* is the causative agent of VL in Hebron District, which is identical to the species causing VL around the Mediterranean [4]. The two isolates were typed as MON-1 zymodeme. These results are not surprising since MON-1 is the predominating zymodeme around the Mediterranean, though zymodeme MON-281 has been described in North Palestine [32].

The serological survey indicates asymptomatic circulation of the parasite among the population. The associations between sero-positivity and being household member of a previous VL case, as well as between sero-positivity and dog and/or other domestic animal ownership were significant and identified these two groups as being at high risk for VL. Their monitoring would help in disease prevention by early diagnosis and subsequent treatment. While previous VL cases and dogs may serve as source of infection, the role of other animals has to be established. They might attract sand flies by their presence and thus increase the probability of being bitten and infected.

A preliminary investigation of sand fly fauna revealed the abundance of two putative vector species, *Ph. syriacus*, the proven vector of Mediterranean VL (WHO 1984), and *Ph. tobbi*, which was found to transmit *L. infantum* (zymodeme MON 1) in Cyprus [33]. The distribution of sand flies in Hebron District differs from that in the VL focus in the northern WB, Jenin District. There, *Ph. perfiliewi* was most abundant followed by *Ph. tobbi* and *Ph. syriacus* [9]. Interestingly, *Ph. perfiliewi* was not abundant in the southern WB which might be due to differences in altitude, as vector survival might differ at high altitude as in Hebron sites (600-1011 m) from that at lower altitude in Jenin District (altitude 300-500 m). The results of the

study in Hebron District are, however, limited because only a small sample of sand flies was investigated.

In conclusion, the focus of VL in Hebron District was shown to follow the epidemiological patterns of paediatric disease characteristic for the Mediterranean region. Zoonotic transmission is most likely, although reservoir studies have not yet been performed. Stray and domestic dogs, which were found to be infected with *L. infantum* in Jenin District [3] and in central Israel [5], are abundant. Also the possible role of anthroponotic transmission has to be elucidated. New sensitive molecular techniques allow for testing whether and how many asymptomatically infected individuals are present in an endemic region that could serve as source of infection. A survey of blood donors in southern France showed that the number of asymptomatic carriers was underestimated in the past [34]. It is necessary to collect and analyse more epidemiological and demographic data to identify risk factors associated with the incidence of VL, and to investigate the parasite host and vector in Hebron District in order to build effective health policies for a better control of the disease.

5.2 Genetic diversity of Leishmania infantum endemic in the Southern Mediterranean

MLMT was applied to investigate genetic polymorphism and population structure of SM *L. infantum* strains isolated from VL, CL and CVL, representing Mon-1 and non MON-1 zymodemes types. European strains were added in each study for comparison. It was possible to detect significantly different populations with considerable polymorphisms by using the Bayesian statistics-based clustering method and construction of NJ trees.

As general trait in SM, MON-1 strains grouped differently from the non MON-1 ones, indicating that MLMT results were in agreement with the MLEE. However, MLMT detected further division of the MON-1 group into several populations. The MON-1 populations are characterized by a lesser degree of genetic diversity, presence of gene flow, and a higher degree of inbreeding compared to non MON-1, indicating clonal propagation [35].

The MON-1 populations correlated largely, however not completely, with geographical distribution. For instance, SM strains were different from the European ones with the exception of some Algerian and Tunisian MON-1 strains that grouped with European strains. This might be a result of the close relationship between people from south Europe and SM and frequent migration between these places.

So far, only few Palestinian and Israeli strains were typed by MLEE and were mostly identified as zymodeme MON-1 profile. By MLMT all strains from Palestinian and Israeli foci grouped together in the MON-1 group and none with the non MON-1 group. Thus all strains from this area not identified by MLEE seem to belong to MON-1, or to be closely related isoenzyme-patterns. When the MON-1group was analysed separately, further substructures have been seen with the Palestinian and Israeli strains clearly secluded from the European MON-1 strains. The geographical distribution of the two populations of which one was further subdivided into 3 sub-populations enabled us to understand VL dynamics in central Israel and Palestine. The re-emergence in central Israel and Palestine is more likely due to increasing parasite transmission and infectivity, and dog and human contact with the sylvatic cycle rather than to recent introduction from the old foci in north Israel. The latter scenario could be, however, true for the B-sub-populations found in few foci in central Israel. However, potential changes in animal reservoir by creating new habitats for them and sand fly vectors, as described previously for *L. tropica* in north Israel [4, 36], and in immune status of the exposed population [8] should not be excluded. MLMT results did not correlate to host origin as strains from humans and dogs are grouped together. This finding emphasises the key role of dogs as reservoirs.

In Tunisia, and partially in Algeria, strains isolated from VL and CL cases were found to belong to different populations. Thus, for the first time a relationship between clinical presentation of leishmaniasis and parasite genotype could be demonstrated. The role of host susceptibility towards infections by *L. infantum* and of sand fly factors needs to be elucidated in order to better understand the importance of genetic differences between parasites causing VL and CL in Tunisia and Algeria.

The most striking finding of this study was evidence for the existence of MON-1/MON-24-80 hybrids. Hybrids seem to occur quite frequently, as in total 12 MLMT profiles (14.6% of the 82 Algerian and Tunisian strains) were suggestive of hybrid genotypes. To exclude that heterozygous alleles are due to mixed infections or eventual bias occurring during cultivation of parasites as shown previously [37, 38], one Tunisian strain was cloned and proven as hybrid.

Since multiple heterozygous loci were identified among hybrids and gene flow was detected between different populations, recombination between strains with different alleles seems to be the most parsimonious explanation [39]. The occurrence of gene flow and genetic recombination is increasingly reported in *Leishmania* spp [40-43] although the mechanism of genetic exchange

remains to be established. These findings raise new questions that have to be addressed, such as (i) what is the mechanism of recombination in *Leishmania* spp., (ii) does recombination occur in the mammalian host or in the vector, and (iii) do hybrids have a selective advantage over parent strains? A recently described *L. infantum*/*L. major* hybrid was found to have a higher transmission potential in relation to the respective vectors [44].

Conclusion

Our results demonstrate the usefulness of MLMT for strain typing and population genetic analysis of *L. infantum*. Moreover, it was possible to address significant epidemiological questions such as detecting hybrid strains and investigating gene flow between different populations, differentiating between relapses and re-infections for patients suffering from multiple episodes of the disease, and correlating genotypes with geographical origins, clinical presentation and age of the patients. MLMT was useful in understanding the disease transmission and emergence in closely related endemic foci. In order to improve our knowledge about the population structure of *L. infantum* in the whole Mediterranean area, the role and relatedness of different populations, and the impact of genetic recombination and gene flow in creating genetic diversity, we are currently investigating strains from Morocco, Libya, Egypt, Lebanon, and Turkey. An overall analysis will be done in the near future for the whole region to understand the disease epidemiology at regional and global levels.

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Agreement

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1) ***A. Amro**, K. Azmi, G. Schönian, A. Nasereddin, M.B. Alsharabati, S. Sawalha, O. Hamarsheh, S. Ereqat, Z. Abdeen, Epidemiology of paediatric visceral leishmaniasis in Hebron district, Palestine, Transactions of the Royal Society of Tropical Medicine and Hygiene (2009) (in press).*

2) ***A. Amro**, G. Schönian, M.B. Alsharabati, K. Azmi, A. Nasereddin, Z. Abdeen, L.F. Schnur, G. Baneth, C.L. Jaffe, K. Kuhls, Population genetics of Leishmania infantum in Israel and the Palestinian Authority through microsatellite analysis, Microbes and Infection. 2009. 11(4): p. 484-92.*

3) *N. Seridi, **A. Amro**, K. Kuhls, M. Belkaid, C. Zidane, A. Al-Jawabreh, G. Schönian, Genetic polymorphism of Algerian Leishmania infantum strains revealed by multilocus microsatellite analysis, Microbes and Infection. 2008. 10(12-13): p. 1309-15.*

4) *N. Chargui, **A. Amro**, N. Haouas, G. Schönian, H. Babba, S. Schmidt, C. Ravel, M. Lefebvre, P. Bastien, E. Chaker, K. Aoun, M. Zribi, K. Kuhls, Population structure of Tunisian Leishmania infantum and evidence for the existence of hybrids and gene flow between genetically different populations, International Journal for Parasitology. 2009. 39(7): p. 801-11.*

Erklärung über den Eigenanteil an den Publikationen

Nr.	**Publikation/ Erläuterung des Anteils von Herrn A. Amro**	**In %**
1.	***A. Amro**, K. Azmi, G. Schönian, A. Nasereddin, M.B. Alsharabati, S. Sawalha, O. Hamarsheh, S. Ereqat, Z. Abdeen, Epidemiology of paediatric visceral leishmaniasis in Hebron district, Palestine, Transactions of the Royal Society of Tropical Medicine and Hygiene 2009. 103(7): p. 731-6.* A. Amro is the ***corresponding author*** of this paper. He designed the study, collected blood samples from the field, performed the serological tests, extracted DNA from cultures and slides, typed the strains by ITS1-RFLP, collected and dissected the sand fly vectors, collected and analyzed the demographic data and drafted the manuscript.	**80%**
2.	***A. Amro**, G. Schönian, M.B. Alsharabati, K. Azmi, A. Nasereddin, Z. Abdeen, L.F. Schnur, G. Baneth, C.L. Jaffe, K. Kuhls, Population genetics of Leishmania infantum in Israel and the Palestinian Authority through microsatellite analysis, Microbes and Infection. 2009. 11(4): p. 484-92.* A. Amro is the ***corresponding author*** of this paper. He extracted DNA from the Palestinian strains, amplified all Palestinian, Israeli and European strains with 14 microsatellite markers, did the fragment length analysis, performed population structure and NJ analysis, compiled and analyzed the results, and drafted the manuscript.	**70%**
3.	*N. Seridi, **A. Amro**, K. Kuhls, M. Belkaid, C. Zidane, A. Al-Jawabreh, G. Schönian, Genetic polymorphism of Algerian Leishmania infantum strains revealed by multilocus microsatellite analysis, Microbes and Infection. 2008.10(12-13): p. 1309-15.* A. Amro is the ***corresponding author*** of the paper and ***contributed equally to the first author***, amplified all strains with 12 out of 14 markers, did the fragment length analysis, performed population structure and NJ analysis, compiled the results and made the analysis, drafted the materials- methods and the results chapters in the manuscript.	**45%**
4.	*N. Chargui, **A. Amro**, N. Haouas, G. Schönian, H. Babba, S. Schmidt, C. Ravel, M. Lefebvre, P. Bastien, E. Chaker, K. Aoun, M. Zribi, K. Kuhls, Population structure of Tunisian Leishmania infantum and evidence for the existence of hybrids and gene flow between genetically different populations, International Journal for Parasitology. 2009. 39(7): p. 801-11.* A. Amro amplified the strains with 7 out of 14 markers, amplified the cloned strains with 14 markers, did the fragment analysis, population structure and NJ analysis, compiled the results and took part in revising the manuscript.	**40%**

Prof. Dr. Wolfgang Presber **Ahmad Amro**

Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht

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This work is dedicated to my parents, my wife, Suha, and my kids, Mahmoud, Amir and Salma

Erklärung

„Ich, **Ahmad Y Amro**, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema:

Studies on epidemiology and genetic diversity of *Leishmania infantum* endemic in the Southern Mediterranian

selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die unzulässige Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Berlin, den 17.07.2007 Unterschrift

Ahmad Amro