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Dissertation

Molecular Epidemiology and Population Genetics of *Leishmania donovani* Strains Isolated from Different Ethiopian Visceral Leishmaniasis Endemic Areas

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Acronyms and abbreviations

AIDS	acquired immunodeficiency syndrome
ART	antiretroviral therapy
BAPS	Bayesian analysis of population structure
CL	cutaneous leishmaniasis
DNA	deoxyribonucleic acid
F_{IS}	inbreeding coefficient
F_{ST}	fixation index
GDA	genetic data analysis
*H*e	expected heterozygosity
HIV	human immunodeficiency virus
*H*o	observed heterozygosity
IRIS	immune reconstitution inflammatory syndrome
ITS	internal transcribed spacer
KE	Kenya
KO	Konso
MEGA	molecular evolutionary genetic analysis
MLMT	multilocus microsatellite typing
MSA	microsatellite analyzer
NB	Negele Borena
NE	North Ethiopia
NE/SD	*L. donovani* population consisting of strains from north Ethiopia and Sudan

NJ	neighbour joining
PCR	polymerase chain reaction
PKDL	post-kala-azar dermal leishmaniasis
PKDL/VL	para-kala-azar dermal leishmaniasis
RFLP	restriction fragment length polymorphism
SD	Sudan
SE	South Ethiopia
SE/KE	*L. donovani* population consisting of strains from south Ethiopia and Kenya
spp.	species

1. Abstract

Visceral leishmaniasis (VL) is a systemic disease caused by parasites of the *Leishmania donovani* complex and fatal if left untreated. In East Africa, Ethiopia is the second most affected country next to Sudan. Despite knowledge of the molecular epidemiology and population genetics of the etiologic agents of the VL is crucial for better understanding the dynamics of the disease as well as for implementing effective control measures little is known about the population structure of Ethiopian *L. donovani*. Whether the occurrence of VL epidemic outbreaks in highland areas, which were formerly considered non-endemic for VL, was related to introduction of parasites, epidemiological changes or a combination of both is also not yet clear.

Through a very thorough analysis of *L. donovani* strains from two main geographical areas in Ethiopia: (i) northwest foci (Humera and Metema) and (ii) south foci (Konso and Negele Borena) using 14 unlinked microsatellite markers, we found a hierarchical genetic population structuring with considerable genetic diversity. When compared with *L. donovani* strains from the neighbouring countries Sudan and Kenya, all strains from northwest Ethiopia grouped with strains from Sudan in one population named NE/SD whereas all strains from south Ethiopia and Kenya were assigned to another population named SE/KE. These two main East African *L. donovani* populations seem to correlate with the geographic distribution of the two principal sand fly vector species involved in the transmission of the parasite in East Africa. Both populations were further subdivided into two subpopulations which in turn consisted of 2 to 3 genetic clusters. Interestingly, one of the subpopulations in the NE/SD population contained all but three of the strains newly isolated from HIV-VL co-infected patients. In contrast to the NE/SD population, high levels of inbreeding were observed in the SE/KE population. Also, we detected putative hybrids resulting from natural hybridization/recombination events among the sympatric subpopulations in the NE/SD population. Most interestingly, almost all of these putative hybrid strains were isolated from HIV-VL co-infected patients. High inbreeding, implying recombination between similar and/or closely related strains, as well as the presence of natural hybrids at intra-species level represent strong arguments against the so far suggested strict clonality of *L. donovani* parasites.

In order to track the possible origins of the parasites responsible for an outbreak of VL in the highland of Ethiopia called Libo Kemkem, we analysed a panel of strains collected during and after the outbreak. Molecular characterization of strains from this newly emerging focus using

highly polymorphic microsatellite markers revealed a striking genetic heterogeneity among these strains which could be assigned to at least three genetically distinct clusters. All but three strains from this focus grouped together with strains from other foci of VL in northwest Ethiopia bordering Sudan. These findings support the hypothesis that the epidemic was related to multiple introductions of the parasite and not to the spread of a particular virulent parasite. An alternative explanation for the remarkable genetic variability could be that these parasites have been circulated locally for decades without being noticed. In this case it remains, however, an enigma why a sudden epidemic outbreak had occurred in 2004.

Disseminated cutaneous leishmaniasis (CL) caused by *L. donovani* in HIV/VL co-infected patients can be associated with immuno-suppression. Such cases can easily be misdiagnosed as diffuse CL, PKDL, PKDL/VL or lepromatous leprosy. Molecular characterization of strains isolated from both the viscera and skin lesions using ITS1-PCR-RFLP and MLMT, together with clinical, immunological and virological data, demonstrated that the skin lesions in these HIV-positive VL patients were due to dissemination of the viscerotropic *L. donovani* parasites as a consequence of the severe immuno-suppression. On the other hand, a case of PKDL/VL was diagnosed six months after ART commencement in a VL/HIV co-infected patient with stage IV disease as a consequence of immune restoration. Further studies using a combination of clinical, virological and molecular methods are required for a better management of HIV co-infected patients showing different clinical symptoms.

Zusammenfassung

Die viszerale Leishmaniose (VL) ist eine systemische Erkrankung, die durch Parasiten des *Leishmania donovani* Komplex hervorgerufen wird und unbehandelt tödlich verlaufen kann. Äthiopien ist in Ostafrika am zweithäufigsten, nach dem Sudan, betroffen. Obwohl Kenntnisse über die molekulare Epidemiologie und die Populationsgenetik des Erregers der VL essentiell für ein besseres Verständnis der Dynamik der Erkrankung und für die Implementierung effektiver Kontrollmaßnahmen sind, ist bisher wenig über die Populationsstrukturen von *L. donovani* in Äthiopien bekannt. Auch ob ein epidemischer Ausbruch in einem der Hochlandgebiete, die bisher als nicht endemisch für VL angesehen wurden, auf den Import von Parasiten, epidemiologische Veränderungen oder eine Kombination von beidem zurückzuführen ist, ist noch nicht klar.

Mit Hilfe einer detaillierten Analyse von 14 molekularen Markern bei *L. donovani*-Isolaten aus zwei geographischen Regionen in Äthiopien, i) aus den nordwestlichen Foci (Humera und Metema) und ii) den südlichen Foci (Konso und Negele Borena), haben wir hierarchische Populationsstrukturen und eine bemerkenswerte genetische Diversität von *L. donovani* in Äthiopien festgestellt. Bei einem Vergleich mit *L. donovani*-Stämmen aus den Nachbarländern Sudan und Kenia gruppierten alle Stämme aus Nordwest-Äthiopien mit den Stämmen aus dem Sudan in einer Population (NE/SD), während alle Stämme aus dem Süden Äthiopiens und aus Kenia einer anderen Population (SE/KE) zugeordnet wurden. Diese beiden hauptsächlichen *L. donovani*-Populationen in Ostafrika scheinen mit der Verbreitung der beiden prinzipiellen Vektorenarten (Sandmücken) zu korrelieren, die an der Übertragung der Parasiten in Ostafrika beteiligt sind. Beide Populationen lassen sich weiter in je zwei Subpopulationen aufteilen, die wiederum aus 2 bis 3 genetischen Gruppen bestehen. Es ist interessant, dass bis auf drei Stämme alle anderen Stämme, die von HIV-VL ko-infizierten Patienten isoliert wurden, zu der derselben Subpopulation in der NE/SD Population gehören. In der Population SE/KE wurde, im Gegensatz zu der Population NE/SD, eine hohe Inzuchtrate gefunden. Wir konnten auch die Existenz natürlicher Hybride nachweisen, die wahrscheinlich durch natürliche Hybridsierung/Rekombination zwischen den sympatrischen Subpopulationen in der NE/SD Population entstanden sind. Von großem Interesse ist, dass fast alle dieser Hybridstämme von HIV-VL ko-infizierten Patienten stammen. Eine hohe Inzuchtrate, die auf Rekombinationen zwischen ähnlichen und/oder eng verwandten Stämmen zurückzuführen ist, sowie die Existenz natürlicher intra-spezifischer Hybride sind deutliche Argumente gegen die bisherige Annahme strikter Klonalität bei *L. donovani*-Parasiten.

Um die mögliche Herkunft von Parasiten, die für einen VL Ausbruch im Hochland von Äthiopien verantwortlich waren, zu klären, wurde eine Reihe von Stämmen analysiert, die während oder nach dem Ausbruch isoliert worden waren. Die molekulare Charakterisierung der Stämme von diesem neuen Fokus mit Hilfe von hochpolymorphen Mikrosatellitenmarkern zeigte eine überraschende genetische Heterogenität dieser Stämme, die mindestens drei genetisch distinkten Gruppen zugeordnet werden konnten. Mit Ausnahme von drei Stämmen gruppierten alle Stämme von diesem Fokus mit Stämmen aus anderen Endemiegebieten für VL in Nordwest-Äthiopien an der Grenze zum Sudan. Dieses Ergebnis unterstützt die Hypothese, dass die Epidemie durch mehrfache Importe von Parasiten verursacht wurde und nicht durch die Ausbreitung eines spezifischen, besonders virulenten Stamms. Eine alternative Erklärung für die bemerkenswerte genetische Variabilität könnte jedoch auch sein, dass diese Stämme schon mehrere Dekaden unbemerkt in diesem Gebiet zirkulieren. In diesem Fall wäre es aber ein Rätsel, warum es im Jahr 2004 zu einem plötzlichen epidemischen Ausbruch kam.

Disseminierte kutane Leishmaniosen (CL) können im Zusammenhang mit Immunsuppression stehen, wie bei HIV/VL koinfizierten Patienten beobachtet wurde. Solche Fälle können leicht als diffuse CL, PKDL, PKDL/VL oder Lepra falsch diagnostiziert werden. Die molekulare Charakterisierung mit Hilfe der ITS1-PCR-RFLP und des MLMT von Stämmen, die aus viszeralen Organen und Hautläsionen des gleichen Patienten isoliert wurden, zusammen mit klinischen, immunologischen und virologischen Daten hat ergeben, dass die Hautläsionen bei den HIV-positiven VL-Patienten durch die Ausbreitung der viszerotropen *L. donovani*-Parasiten als Folge der schweren Immunsuppression bedingt waren. Andererseits wurde sechs Monate nach Beginn von ART bei einem VL/HIV-Patienten mit Phase IV AIDS eine PKDL/VL infolge der Immunrestoration diagnostiziert. Weitere kombinierte klinische, virologische und molekulare Untersuchungen sind für ein besseres Management von HIV-koinfizierten Patienten mit unterschiedlicher klinischer Symptomatik notwendig.

2. Introduction

Visceral leishmaniasis (VL) (also known as kala-azar) is a vector-borne disease which every year affects 500,000 people in Asia, east and northern Africa, southern America, and southern Europe. If left untreated, the disease is nearly always fatal. VL is one of the most neglected parasitic diseases and 90% of the global burden and the associated deaths occur in three of the poorest regions of the world: Indian subcontinent, East Africa and Brazil (http://www.who.int/topics/leishmaniasis). VL is caused by protozoan parasites of the *Leishmania donovani* complex, *L. donovani* and *L. infantum*. In east Africa and the Indian subcontinent, VL is caused by *L. donovani* and is thought to be anthroponotic; while in northern Africa, southern Europe and southern America it is a zoonotic disease caused by *L. infantum*.

In the horn of Africa, Ethiopia is, after Sudan, the second most affected country by the disease with an annual incidence of 4,000 cases [1]. Although the disease is thought to be distributed throughout the low lying regions of the country, the main and well-known classical foci endemic for VL are located in the northern (NE) and southern (SE) Ethiopian lowlands. While the NE foci are bordering Sudan, the SE foci are bordering Kenya [2, 3]. Sixty percent of the reported VL cases occur in the NE foci, where the VL cases are increasingly associated with HIV co-infection [4-6]. The foci in SE account for 20% of the cases and are rarely associated with HIV [6].

Despite the efforts to control VL in recent years, the disease is now expanding to highland areas formerly considered to be non-endemic. For instance, in Libo Kemkem, Amhara Region [7], and in Shiraro, Tigray Region (A. Hailu, pers. comm.) epidemic outbreaks occurred for the first time in 2004 and in 2010, respectively. However, it is not yet clear whether these areas represent old, previously unidentified foci or new foci emerging as a result of ecological changes and/or of movements of seasonal immigrant workers between these and the classical VL foci. This epidemiology is interesting and requires investigation.

The *L. donovani* parasites are thought to be transmitted by the bites of infected female sand flies of *Phlebotomus orientalis* in NE, as in Sudan [8, 9], and of *P. martini* and *P. celiae* in SE and Kenya [10]. A study in Brazil showed that differences in the biology and ecology of the insect vector species involved may influence the genetic make-up of the *L. braziliensis* populations they harbour and transmit [11]. Whether the ecological and biological differences of the principal phlebotomine sand fly vectors in East Africa may have influenced the genetic

variability of East African populations of *L. donovani* has not yet been investigated. Geographical variation was also reported for the efficacy of paromomycine treatment in East African countries, with the highest efficacies in southern Ethiopia followed by Kenya, and the lowest in northwest Ethiopia and Sudan [12]. The two main foci in Ethiopia are reported to differ in the prevalence of post-kala-azar dermal leishmaniasis (PKDL), which is higher in NE [13] compared to SE. PKDL is very high in Sudan bordering NE foci [14], whereas it occurs less frequently in Kenya bordering SE foci [15]. Such variations might either reflect differences in host immuno-genetic backgrounds, parasite genetics, or both.

In Ethiopia, PKDL is emerging as a major clinical complication of VL among patients with HIV/AIDS as a sequel of VL or concomitantly with VL [13]. The latter is recognised as a rare dermatosis called para-kala-azar dermal leishmaniasis (PKDL/VL). PKDL is thought to be a sign of immune recovery in endemic foci elsewhere and rarely seen in HIV-infected patients after successful antiretroviral therapy (ART). In VL-HIV co-infected patients on ART, PKDL may appear as a syndrome associated with immune reconstitution inflammatory syndrome (IRIS) [16]. Multiple skin lesions resembling PKDL as a consequence of the dissemination of *L. donovani* parasites from visceral organs have, however, been reported in VL/HIV co-infected patients [17-21] and confuse the clinical diagnosis of PKDL. Disseminated cutaneous leishmaniasis (disseminated CL) may also easily be misdiagnosed as diffuse cutaneous leishmaniasis (DCL) or lepromatous leprosy as both diseases are also endemic in Ethiopia. Thus, diagnosis of different cutaneous manifestations in HIV/AIDS patients with and without ART is challenging and warrants a proper identification of the causative agent together with a refinement of clinical, immunological and virological criteria to improve the care for HIV-VL co-infected patients.

Control of VL in Ethiopia is complicated by a surge of *Leishmania*-HIV co-infection, population migration, the shortcomings of the therapies and poor diagnostics [4, 5, 13, 22-24] together with a lack of proper epidemiological and population genetics studies. Intervention, as well as sustainable control strategies, should be designed based on epidemiological and population genetic studies of the causative parasites as well as the vectors. Characterization of the parasites using informative molecular tools will help to (i) monitor the emergence and spread of the disease, and (ii) unravel the presence or absence of associations between varying clinical manifestations, treatment outcomes and infecting *L. donovani* populations/subpopulations/genotypes. Additionally, population genetic studies of the parasites are useful to gain insights into their natural reproductive mechanism as well as their

transmission patterns [25], knowledge of which is crucial for the development of novel and effective control strategies.

Microsatellite markers containing simple repeat sequences are among the most variable types of DNA sequence in the genome [26]. Their polymorphism derives from variations in the number of repeats rather than in the primary sequence. Multilocus microsatellite markers combine many useful features, such as hyper-variability, ubiquitous occurrence, co-dominance, they are easy to assay, and the data can be stored and exchanged between different laboratories [27, 28]. Recent studies have demonstrated that they are the most powerful tool currently available for addressing key epidemiological and population genetics questions associated with leishmaniasis in different endemic regions of the world [25, 29-32]. Therefore, in the present studies we applied a panel of microsatellite markers to investigate the molecular epidemiology of VL and the population genetics of its causative agent, *L. donovani*, in Ethiopia.

3. Objectives

The aims of the present work were:

1. To define the population genetic structure of *L. donovani* strains from East Africa, in particular from Ethiopia, to investigate the genetic diversity, to resolve the mating pattern (clonality versus recombination), to search for natural hybrid genotypes, and to investigate whether particular populations/subpopulations/genotypes are predominant in HIV co-infected patients.
2. To assess the genetic diversity of *L. donovani* strains obtained from a new VL focus in Libo Kemkem in order to find out whether these parasites were introduced into the area by seasonal immigrant workers returning from classical VL foci in NE.
3. To perform molecular typing of paired *Leishmania* isolates obtained from cutaneous lesions and visceral organs of the same HIV/AIDS co-infected patients in order to identify the etiological agent and to clarify whether the skin lesions appear due to the dissemination of parasites from visceral organs to the skin, and to investigate whether parasites or host factors are important for development of skin lesions.

4. Materials and Methods

4.1 Strains studied

A total of 89 strains of *L. donovani* obtained from Ethiopian VL patients were used for the studies described herein. For the first and second objectives, 63 and 19 *L. donovani* strains isolated from VL patients with and without HIV/AIDS co-infection were used, respectively. For the third objective, seven strains (paired strains from viscera plus skin lesions of three HIV positive patients and one strain from skin lesions of a PKDL/VL patient) were used.

4.2 DNA extraction

Genomic DNA was extracted from cultured promastigotes using a standard phenol/chloroform method [33]. The DNA was precipitated by 96% ethanol, re-suspended in Tris-EDTA buffer and stored at 4^{o}C until use.

4.3 ITS1 PCR-RFLP, cloning and sequencing

Species identification was performed for all strains studied by using a PCR-RFLP approach targeting the ribosomal internal transcribed spacer 1 (ITS1) using the primers and reaction conditions previously described [34]. For selected strains, ITS1 PCR products were sequenced using the same primer pairs as those used for amplification. To check for inter-repeat variation in the ITS1 region the ITS1 PCR product was cloned using the pDrive Cloning kit and randomly selected clones bearing an ITS1 insert were sequenced using the M13rev standard primers. To further verify the existence of intra-genomic variants in the ITS1, the coding sequence of the heat shock protein 70 gene (*hsp70*) was analysed following the protocol described by Garcia et al. (2004) [35].

4.4 Multilocus microsatellite typing (MLMT)

DNA sequences of 14 microsatellite loci were amplified using primers, amplification reactions and cycling conditions as described previously [29, 36]. The forward primers were labelled with fluorescent dyes and allele length was sized using an automated ABI sequencer. Strain MHOM/IN/1980/DD8, for which the fragment size and repeat number were determined by direct sequencing of the 14 amplified microsatellite loci, was included as reference in each run of PCR and capillary sequencer. MLMT profiles for each strain were obtained by compiling all alleles at each locus.

4.5 Population genetic analysis

Bayesian statistics implemented in the STRUCTURE software version 2.1 [37] were used to disclose the major populations as well as the subpopulations within each population. The BAPS software [38] was then used to explore cryptic subdivisions which STRUCTURE was unable to detect in order to evaluate the degree of genetic differentiation resulting from the Wahlund effect.

Genetic distance representing the proportion of shared alleles (D_{AS}) among the strains studied was calculated for all three microsatellite datasets using the software programs MSA [39] and POPULATIONS 1.2.28 (http://www.legs.cnrsgif.fr/bioinfo/populations). From the resulting matrix, a neighbour-joining (NJ) tree was constructed using MEGA version 3 [40]. The bootstrap values for each tree were calculated by performing 1,000 re-samplings across the loci. Additionally, phylogenetic networks based on the neighbour-net distance were constructed using SplitsTree4 program [41]. Such networks can provide evidence for the presence of hybridization, horizontal gene transfer or recombination at inter- and intra-population and -subpopulation levels.

Population genetic structure was also investigated using Wright's F-statistics. The index of F_{ST} was computed with MSA version 3.0. F_{ST} measures the genetic differentiation and gene flow between the populations, subpopulations, and clusters inferred by STRUCTURE and BAPS as well as the subdivision of populations according to endemic focus or year of isolation. Its value ranges between 0 and 1. A value of greater than 0.25 for two paired genetic groups implies strong genetic differentiation. Inbreeding coefficient (F_{IS}) which measures the identity of alleles (homozygosity versus heterozygosity) within individuals belonging to subpopulations and clusters was calculated by GDA software (http://hydrodictyon.eeb.uconn.edu/people/plewis/software.php). F_{IS} ranges between −1 and 1. Negative values correspond to an excess of heterozygosity, e.g. found for clonal diploids, 0 is expected under panmixia and positive values are characteristic for heterozygote deficiency and inbreeding. In addition, GDA was used to compute allelic richness (A), mean number of alleles (MNA) and expected and observed heterozygosity (*He* and *Ho*, respectively).

5. Results

5.1 Population genetic structure of L. donovani strains isolated from the two classical VL foci in Ethiopia

For the population genetic study, a total of 63 strains of *L. donovani* (22 from SE and 41 from NE) newly isolated from human VL cases were investigated. The MLMT profiles obtained were compared to those of 60 previously characterized strains of *L. donovani* from Sudan, Kenya and India [29, 32]. A high genetic diversity with large numbers of unique MLMTypes (55/63) was demonstrated for both NE and SE strains. The two MLMTypes, which occurred more than once, were from the same patients at different episodes of the disease. By using different methods of analysis, based on Bayesian statistics and genetic distance measures, we identified two main populations for the East African strains of *L. donovani* designated as NE/SD and SE/KE. Both of them were genetically distinct from the population that comprised the majority of Indian *L. donovani* strains. The SE/KE population encompassed the 22 SE strains plus the 8 Kenyan strains included in the analysis. On the other hand, the NE/SD population comprised all strains from NE (41) and Sudan (21). Both East African populations were further divided into two sub-populations when re-analysed separately by STRUCTURE. The genetic differentiation between NE/SD and SE/KE populations was very strong (F_{ST} =0.494) with very limited gene flow between them. Relatively higher genetic diversity measured by *Ho* was found in NE (*H*o= 0.47) compared to SE (*H*o= 0.39). Clearly positive F_{IS} values were observed in SE (F_{IS} =0.34 to 0.86) and, less pronounced, in NE (F_{IS}=0.07 to 0.4), except in one cluster of NE/SD population which comprised putative hybrids. In the SE/KE population, the two subpopulations KO and NB/KE, and the clusters KO, NB, KE correlated with the geographical origin of the strains. This was, however, not the case for the two subpopulations of the NE/SD population, A and B, or for the clusters A1, A2, A3, B1, and B2. Instead, these genetic groups seem to be associated with host immune background and year of strain isolation. Strains isolated from HIV co-infected patients were predominantly found in the NE/SD subpopulation B. The high inbreeding coefficients observed in populations, subpopulations and clusters disclosed by STRUCTURE analysis, indicate that recombination is occurring between identical or closely related strains. This observation was further corroborated by the reticulated pattern seen in SplitsTree4 analysis. Interestingly, six putative natural hybrids with two corresponding hypothetical parents in the two NE/SD subpopulations A and B were identified in NE *L. donovani* strains.

These results have been published in the following paper:

Gelanew T, Kuhls K, Hurissa Z, Weldegebreal T, Hailu W, Kassahun A, Abebe T, Hailu A, Schönian G. 2010. Inference of population structure of *Leishmania donovani* strains isolated from different Ethiopian visceral leishmaniasis endemic areas. PLoS Negl Trop Dis. 4(11): e889.

5.2 Genetic diversity of L. donovani strains isolated during and after an epidemic outbreak in Libo Kemkem

Nineteen strains of *L. donovani* isolated during and after an epidemic outbreak in the Libo Kemkem area were characterized by MLMT and compared to those of previously characterized strains representing different genetic groups from different foci in NE (Humera, Metema and Belessa) as well as SE, Sudan and Kenya. Interestingly, no predominant but rather unique MLMTypes were found for all 19 strains of *L. donovani* from Libo Kemkem.

Visual inspection of NJ-tree, SplitsTree and STRUCTURE results revealed that none of these strains showed genetic relatedness to strains from SE and KE. At least three distinct genetic groups, A, B1 and B2, were identified by both genetic distance and Bayesian model analyses among all strains from foci in NE, including Libo Kemkem and Belessa highlands. Only three of the strains from Libo Kemkem formed an independent genetic group, B2, all other strains were scattered among the three different genetic clusters that encompassed previously characterized *L. donovani* strains from well-known foci in NE.

We have also analysed five strains isolated from VL patients in Libo Kemkem at the time of the first outbreak that had been previously reported to be *L. infantum* [7]. Neither MLMT nor ITS1 sequencing confirmed this result but showed that all strains, whether previously or newly isolated, belong to the species *L. donovani*. Like most other molecular studies, our investigation showed that *L. infantum* is not present in East African VL foci.

These results have been published in the following paper:

Gelanew T, Cruz I, Kuhls K, Alvar J, Cañavate C, Hailu A , Schönian G. 2011. Multilocus microsatellite typing revealed high genetic variability of *Leishmania donovani* strains isolated during and after a kala-azar epidemic in Libo Kemkem District, Northwest Ethiopia. Microbes Infect. 13:595-601.

5.3 Para kala-azar dermal leishmaniasis and disseminated CL due to L. donovani

MLMT profiles were obtained for paired *Leishmania* strains isolated from cutaneous lesions and visceral organs of the same patients with HIV/AIDS co-infection. First, these isolates were identified to the species level for two main reasons. It was necessary to rule out the possibility that the visceral disease was not a result of a dermatotropic *Leishmania* species, such as *L. aethiopica*, *L. tropica* and *L. major* which are co-endemic with *L. donovani* in Ethiopia [42-44]. In HIV-positive patients unusual clinical manifestations can occur, including VL caused by dermatotropic species [45, 46]. Furthermore, it had to be verified that the different cutaneous manifestations (PKDL, PKDL/VL and disseminated CL) did not result from a concomitant infection with dermatotropic *Leishmania* species. Both ITS1-PCR-RFLP and MLMT investigations demonstrated that the causative agent was *L. donovani* in all cases, irrespective of the disease phenotype. Besides, the three paired strains from viscera and skin lesions of the same patients were identical across the 14 microsatellite markers investigated. We found no association between the different phenotypes of the disease and parasite genotypes. By combining clinical, immunological, virological and molecular investigations, we described one case as PKDL/VL possibly associated with IRIS and three cases as disseminated CL which occurred concomitantly with VL in severely immuno-compromised HIV/AIDS patients. Interestingly, by using ITS1 cloning and sequencing, we found evidence for the presence of intra-genomic variants in the ITS1 region of the *L. donovani* strain isolated from the PKDL/VL case.

These results have been published in the following papers:

Gelanew T, Amogne W, Abebe T, Kuhls K, Hailu A, Schönian G. 2010. A clinical isolate of *Leishmania donovani* with ITS-1 sequence polymorphism as a cause of Para-kala-azar Dermal Leishmaniasis (PKDL/VL) in an Ethiopian HIV-positive patient on HAART. Br J Dermatol 163(4): 870-4

Gelanew T, Hurissa Z, Diro E, Kassahun A, Kuhls K, Schönian G, Hailu A. 2011. Disseminated Cutaneous Leishmaniasis Resembling Post-Kala-azar Dermal Leishmaniasis Caused by *Leishmania donovani* in Three Ethiopian Visceral Leishmaniasis and Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome Co-infected Patients. Am J Trop Med Hyg, 8(6):906-912.

6. Discussion

6.1 Population genetic structure of L. donovani strains isolated from two classical VL foci in Ethiopia

Unlike *L. donovani* strains from the Indian subcontinent which show a striking genetic homogeneity by MLMT [32], a remarkable genetic diversity was demonstrated for the Ethiopian strains of *L. donovani*. Our findings provide evidence for presence of hierarchically structured *L. donovani* populations in East Africa, and suggest that the two main populations identified correspond to the geographic distribution of the different sand fly species in two distinct epidemiological settings. This is further supported by identification of two genetically distinct populations in a single focus called south Omo where two sand fly species are sympatric (own unpublished data). The genetic differences found for the strains in NE and SE might explain the phenotypic differences that have been earlier observed between the two foci [6, 12, 13].

The East African *L. donovani* populations were found to be further sub-structured. The sub-populations identified in the SE/KE population corresponded to their geographic origin but with evidence of gene flow among them. This finding is consistent with low intertribal movement. By contrast, there was no association between the subpopulations in NE/SD populations and the geographic origin of the strains but rather with the immunological status of hosts and year of parasite isolation. The most interesting findings of this study were high inbreeding coefficients and identification of putative hybrids between genetic groups pointing to the possibility of genetic exchange among East African *L. donovani*. The high inbreeding found in the present and in other studies [25, 47] coupled with the growing evidence for the existence of natural hybrids at inter- and intra-species levels [48-53] challenge the assumption that the mode of reproduction of *Leishmania* parasites is overwhelmingly clonal. Our data suggest the co-existence of clonal and sexual reproduction in East African *L. donovani* strains. For parasites showing high inbreeding or undergoing recombination, an association between disease pathogenesis and parasite genotype would be unclear. This might explain the absence of clear correlations between phenotype of the disease and parasites genotype in the present and previous studies. However, strains from HIV-positive patients predominated in one of NE subpopulations. This might suggest that these strains are less virulent and cause only asymptomatic infections in immuno-competent individuals. The ongoing whole genome project may disclose candidate markers that could link the phenotype of the disease to the genotype of the parasite.

6.2 Genetic diversity of L. donovani strains isolated during and after the epidemic outbreak in Libo Kemkem

It was hypothesized that the epidemic outbreak of VL that had occurred 2004 in Libo Kemkem was related to multiple introductions of *L. donovani* parasites from different foci in NE. On the other hand, the epidemic could be associated with the emergence and the spread of a particular virulent strain. The latter has been shown for *L. tropica* strains isolated from CL patients during an epidemic outbreak in Turkey. *L. tropica* was shown to be a remarkably genetically heterogeneous species, but all outbreak strains presented a single MLMType, suggesting that the emergence of a new virulent strain was responsible for the outbreak [31]. By contrast, MLMT revealed a remarkably high genetic diversity for the Libo Kemkem strains, which is irreconcilable with the emergence of a particular strain related to the sudden epidemic outbreak.

With different microsatellite-based phylogenetic methods, the majority of the Libo Kemkem strains were found to be genetically related to parasites of the NE/SD population, in agreement with the hypothesis that these parasites could have been introduced by seasonal migrant workers. This is further corroborated by long travel history of Libo Kemkem residents to sesame growing lowland areas, Metema and Humera (classical foci for VL) [7]. An alternative hypothesis for such a remarkably high genetic diversity could be that the parasites have been circulating locally for several decades unidentified. In support of this hypothesis, few autochthonous VL cases were reported in a highland area called Belessa which is 130km away from the Libo Kemkem focus [42]. If this hypothesis is true, it remains unclear why a sudden epidemic had occurred in this area. Malnutrition due to famine, socio-epidemiological as well climatic changes might have contributed to epidemic outbreak [54] but this was not supported by the recorded data [7].

6.3 PKDL/VL and disseminated CL

By a combination of clinical, immunological, virological and molecular diagnostic methods we describe herein a few atypical leishmaniasis cases: one PKDL/VL and three disseminated CL cases due to *L. donovani* infections. The clinical distinction between PKDL, PKDL/VL and disseminated CL in HIV/AIDS patients in resource-poor endemic settings like in Ethiopia is challenging. This is, at least partly, related to the lack of widely accepted case definitions. Using ITS1 PCR-RFLP and MLMT we showed that the parasites isolated from the reticulo-endothelial system and the skin lesions of the same HIV-co-infected patients were always *L.*

donovani irrespective of the different forms of clinical dermatosis. The MLMT analysis revealed that the paired strains were genetically identical across the 14 loci.

Reports showed that PKDL is very uncommon in HIV-positive patients but, if it occurs, it seems to be related to the immune restoration as a consequence of ART-induced inflammation [16, 55], which is currently recognized as IRIS. The density of parasites in PKDL lesions is low. Only in few cases parasites could be demonstrated microscopically in smears from PKDL lesions [14, 56, 57]. In contrast, disseminated CL has been suggested to result from a lack of cell-mediated immunity to *Leishmania* antigens and/or parasites, leading to uncontrolled parasite growth and spread [58]. However, immune recovery was not observed in our HIV-VL co-infected patients before and during the onset of skin lesions even though two of them had been on ART. This is further corroborated by the heavy parasite loads in Giemsa-stained smears from both PKDL-like lesions and visceral organs. All virological, immunological, molecular and clinical data obtained from our patients support the classification of these cases as disseminated CL with concurrent VL instead of PKDL.

One of our patients, who had no past history of VL, developed a PKDL/VL 6 months after commencement of ART. Clinical and virological findings suggested that the dermatosis appeared as a consequence of ART-induced IRIS [16]. Although normally Ethiopian HIV infected individuals would benefit from an early commencement of ART, HIV patients living in VL endemic settings should be monitored carefully because they are at risk of developing VL or PKDL-associated with IRIS [16]. The single *L. donovani* isolate obtained from this patient presented intra-genomic variations in the ITS1 region. Schoenian et al (2001) [59] suggested the presence of such intra-genomic variations in *L. tropica* but this is the first report in *L. donovani.* The existence of intra-genomic variations could be explained by the fact that the different copies of ITS1 may accumulate mutations independently.

We did not find any evidence that parasites causing different forms of cutaneous diseases are genetically distinct from parasites that cause visceral disease only. This may indicate that interactions between host and parasite genotypes, rather than parasite genes on their own, play a critical role in determining the clinical symptoms. Our results may be underpowered by the small number of isolates from patients with dermal manifestations we investigated, but are in agreement with previous studies from India [32] and Sudan [60, 61].

Conclusions

In conclusion, the application of highly variable microsatellite markers for typing strains of *L. donovani* in different VL foci in Ethiopia has proven useful for addressing fundamental epidemiological questions associated with the disease in Ethiopia and for understanding the population genetics of East African *L. donovani* parasites. Two main populations of *L. donovani* parasites were found to circulate in East African foci, apparently corresponding to the geographic distribution of different sand flies vectors. This study provided also evidence for the existence of natural hybrids of the *L. donovani*, albeit their role in the epidemiology remains to be elucidated. It also revealed that *L. donovani* strains isolated during and after an epidemic outbreak in Libo Kemkem are genetically diverse and belong to different genetic groups, which supports the hypothesis that the epidemics might have resulted from multiple introductions of these strains into a previously non-endemic area. Furthermore, strains from viscera and skin lesions resembling PKDL in HIV/AIDS-VL co-infected patients analysis across the 14 microsatellite loci revealed the viscerotropic *L. donovani* parasite dissemination as consequence severe immuno-suppression. This finding and the diagnosis of PKDL/VL associated with IRIS will increase the physicians' awareness of atypical dermal manifestations as well as of future establishment of a clear-cut definition for different clinical dermal manifestations due to *L. donovani* infections in advanced HIV/AID patients.

The results of this study brought up some interesting research questions to be answered in the future. To mention the most important ones, knowledge on the epidemiological implication of the recombinant and/or hybrid strains (i.e., virulence or transmission potential) is crucial for control of the disease. Prior to this it is needed to verify whether the putative natural hybrids we identified are true hybrids or mixed genotypes. Currently, we are investigating these putative hybrids as well as representatives of their hypothetical parents using single cell cloning followed by multilocus sequencing typing and MLMT. It is of interest whether the two main populations of *L. donovani* in East Africa is truly a reflection of parasite-vector interaction. *L. donovani* parasites should be isolated from the two principal sand fly vectors *P. orientalis* and *P. martini* and characterized. The two populations of the *L. donovani* seem to correlate with the varying paromomycine treatment outcome observed in Ethiopia. Whether this truly is a reflection of variation in the parasites' genetics or host immuno-genetics (e.g., polymorphisms in genes encoding cytokines) deserves further investigation. .

The information obtained from this study together with that of future epidemiological and population genetic studies will be very useful for the design of effective parasite-targeted control strategies which aim to eradicate VL in East Africa.

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Agreement

This doctoral thesis (publication thesis) is based on the publications published in the journals listed below.

1 Gelanew T, Kuhls K, Hurissa Z, Weldegebreal T, Hailu W, Kassahun A, Abebe T, Hailu A, Schönian G. 2010. Inference of population structure of *Leishmania donovani* strains isolated from different Ethiopian visceral leishmaniasis endemic areas. PLoS Negl Trop Dis. 4(11): e889.

2 Gelanew T, Cruz I, Kuhls K, Alvar J, Cañavate C, Hailu A , Schönian G. 2011. Multilocus microsatellite typing revealed high genetic variability of *Leishmania donovani* strains isolated during and after a Kala Azar epidemic in Libo Kemkem District, Northwest Ethiopia. Microbes Infect. 13:595-601.

3 Gelanew T, Amogne W, Abebe T, Kuhls K, Hailu A, Schönian G. 2010. A clinical isolate of *Leishmania donovani* with its-1 sequence polymorphism as a cause of para-kala-azar dermal leishmaniasis (pkdl/vl) in an ethiopian hiv-positive patient on haart. Br J Dermatol 163(4): 870-4

4 Gelanew T, Hurissa Z, Diro E, Kassahun A, Kuhls K, Schönian G, Hailu A. 2011. Disseminated Cutaneous Leishmaniasis Resembling Post-Kala-azar Dermal Leishmaniasis Caused by *Leishmania donovani* in Three Ethiopian Visceral Leishmaniasis and Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome Co-infected Patients. Am J Trop Med Hyg, 8(6):906-912.

Erklärung über den Eigenanteil an den Publikationen

In %	Publikation/ Erläuterung des Anteils von Herrn Tesfaye Gelanew Taye	Nr.
80%	**Gelanew T**, Kuhls K, Hurissa Z, Weldegebreal T, Hailu W, Kassahun A, Abebe T, Hailu A, Schönian G. 2010. Inference of population structure of *Leishmania donovani* strains isolated from different Ethiopian visceral leishmaniasis endemic areas. PLoS Negl Trop Dis. 4(11): e889. TG conceived the idea, designed the study, took part in the field epidemiological data collection, did most of the strains isolation, performed the DNA extraction, ITS1 PCR-RFLP, MLMT, compiled the results, made the data analysis and interpretation, drafted the manuscript and revised subsequently and was corresponding author.	1.
70%	**Gelanew T**, Cruz I, Kuhls K, Alvar J, Cañavate C, Hailu A , Schönian G. 2011. Multilocus microsatellite typing revealed high genetic variability of *Leishmania donovani* strains isolated during and after a Kala Azar epidemic in Libo Kemkem District, Northwest Ethiopia. Microbes Infect. 13:595-601. TG conceived the idea, designed the study, did the DNA extraction, ITS1 PCR-RFLP, MLMT, compiled the results, made the data analysis, drafted the manuscript and revised subsequent of manuscript and was a corresponding author.	2.
60%	**Gelanew T**, Amogne W, Abebe T, Kuhls K, Hailu A, Schönian G. 2010. A clinical isolate of *Leishmania donovani* with its-1 sequence polymorphism as a cause of para-kala-azar dermal leishmaniasis (pkdl/vl) in an ethiopian hiv-positive patient on haart. Br J Dermatol 163(4): 870-4. TG conceived the idea, performed the DNA extraction, ITS, hsp70- PCR RFLP and PCR cloning sequencing, compiled the results, made the data analysis, writing the manuscript and its subsequent revisions and was a corresponding author.	3.
50%	**Gelanew T**, Hurissa Z, Diro E, Kassahun A, Kuhls K, Schönian G, Hailu A. 2011. Disseminated Cutaneous Leishmaniasis Resembling Post-Kala-azar Dermal Leishmaniasis Caused by *Leishmania donovani* in Three Ethiopian Visceral Leishmaniasis and Human	5.

	Immunodeficiency Virus/Acquired Immunodeficiency Syndrome Co-infected Patients. Am J Trop Med Hyg, 8(6):906-912. TG did the DNA extraction, ITS1-PCR RFLP, whole genome amplification and MLMT, compiled the results, made the data analysis, drafted and revised subsequently the manuscript and was corresponding author.	

Prof. Dr. Wolfgang Presber **Tesfaye Gelanew Taye**

Erläuterung des Impact Faktor in den publizierten Journalen

Impact factor	**Publikation des Anteils von Herrn T. Gelanew**	Nr.
4.69	**Gelanew T**, Kuhls K, Hurissa Z, Weldegebreal T, Hailu W, Kassahun A, Abebe T, Hailu A, Schönian G. 2010. Inference of population structure of *Leishmania donovani* strains isolated from different Ethiopian visceral leishmaniasis endemic areas. PLoS Negl Trop Dis. 4(11): e889.	1.
2.795	**Gelanew T**, Cruz I, Kuhls K, Alvar J, Cañavate C, Hailu A , Schönian G. 2011. Multilocus microsatellite typing revealed high genetic variability of *Leishmania donovani* strains isolated during and after a Kala Azar epidemic in Libo Kemkem District, Northwest Ethiopia. Microbes Infect. 13:595-601..	2.
4.2	**Gelanew T**, Amogne W, Abebe T, Kuhls K, Hailu A, Schönian G. 2010. A clinical isolate of *Leishmania donovani* with ITS-1 sequence polymorphism as a cause of Para-kala-azar dermal leishmaniasis (PKDL/VL) in an Ethiopian HIV-positive patient on HAART. Br J Dermatol 163(4): 870-4.	3.
2.795	**Gelanew T**, Hurissa Z, Diro E, Kassahun A, Kuhls K, Schönian G, Hailu A. 2011. Disseminated Cutaneous Leishmaniasis Resembling Post-Kala-azar Dermal Leishmaniasis Caused by *Leishmania donovani* in Three Ethiopian Visceral Leishmaniasis and Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome Co-infected Patients. Am J Trop Med Hyg, 8(6):906-912.	5.

Prof. Dr. Wolfgang Presber **Tesfaye Gelanew Taye**

Mein Lebenslauf wird aus daten schutzrechtlichen Gründen in der electornischen Version meiner Arbeit nich veröffentlicht.

Publications in peer-reviewed international journals

1 Zanger P, Kötter I, Raible A, **Gelanew T**, Schönian G, Kremsner PG. 2011. Successful treatment of cutaneous leishmaniasis due to *Leishmania aethiopica* with liposomal amphothericin B in an immunocompromised traveler returning from Eritrea. Am J Trop Med Hyg, 84(5): 692-4.

2 **Gelanew T**, Hurissa Z, Diro E, Kassahun A, Kuhls K, Schönian G, Hailu A. 2011. Disseminated Cutaneous Leishmaniasis Resembling Post-Kala-azar Dermal Leishmaniasis Caused by *Leishmania donovani* in Three Ethiopian Visceral Leishmaniasis and Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome Co-infected Patients. Am J Trop Med Hyg, 8(6):906-912.

3 **Gelanew** T, Cruz I, Kuhls K, Alvar J, Cañavate C, Hailu A , Schönian G. 2011. Multilocus microsatellite typing revealed high genetic variability of *Leishmania donovani* strains isolated during and after a Kala Azar epidemic in Libo Kemkem District, Northwest Ethiopia. Microbes Infect, 13(): 595-601.

4 Paniz Mondolfi AE, Stavropoulous C, **Gelanew T**, Loucas E, Perez Alvarez AM, Benaim G, Polsky B, Schoenian G, Sordillo EM. 2011. Successful treatment of Old World cutaneous leishmaniasis due to *L. infantum* with Posaconazole. Antimicrob Agents and Chemother, 55(4): 1774-6.

5 **Gelanew T**, Kuhls K, Hurissa Z, Weldegebreal T, Hailu W, Kassahun A, Abebe T, Hailu A, Schönian G. (2010). Inference of population structure of *Leishmania donovani* strains isolated from different Ethiopian visceral leishmaniasis endemic areas. PLoS Neglected Tropical Diseases. 4(11): e889.

6 **Gelanew T**, Amogne W, Abebe T, Kuhls K, Hailu A, Schönian G. (2010). A clinical isolate of *Leishmania donovani* with its-1 sequence polymorphism as a cause of para-kala-azar dermal leishmaniasis (PKDL/VL) in an Ethiopian HIV positive patient on HAART. British Journal of Dermatology, 163(4): 870-4.

7 Pozio E, Foggin CM, **Gelanew T**, Marucci G, Hailu A, Rossi P, Morales MA. (2007) *Trichinella zimbabwensis* in wild reptiles of Zimbabwe and Mozambique and farmed reptiles of Ethiopia. Vet Parasitol. 143:305-10.

8 **Gelanew T,** Lalle M, Hailu A, Pozio E, Cacciò SM (2007). Molecular characterization of human isolates of *Giardia duodenalis* from Ethiopia. Acta Trop.102(2): 90-99.

Oral presentations

1. **Tesfaye Gelanew** Katrin Kuhls, Asrat Hailu and Gabriele Schoenian. Microstaellite analysis of Ethiopian *Leishmania donovani* isolates. Presented at Kickoff Meeting for **Ecology and Transmission Dynamics of Visceral Leishmaniasis in Ethiopia** November 22-23, 2010, Mekele, Ethiopia**.**
2. **Tesfaye Gelanew**, Katrin Kuhls. Asrat Hailu and Gabriele Schoenian. Inference of Population Structure of *Leishmania donovani* Strains Isolated from Different Ethiopian Visceral Leishmaniasis Endemic Areas. Presented at **MEEGID X Conference .**3 – 5 November 2010, Amsterdam, The Netherlands**.**
3. **Tesfaye Gelanew**, Katrin Kuhls, Asrat Hailu, Gabriele Schoenian. Is vector-parasite interaction a determining factor for the Poulation sturucture of *L. donovani* in East Africa? Presented at **British Society for Parasitology**, March 29-April 1, 2010. Cardiff University, Wales, UK
4. Gabriele Schönian, Katrin Kuhls, Ahmad Amro, Mohammad Z. Alam, **Tesfaye Gelanew**. Epidemiologicaland population genetic studies in the *Leishmania. donovani* complex. Presented at **British Society for Parasitology**, March 29-April 1, 2010. Cardiff University, Wales, UK.

Poster presentations

1. Aysheshe Kassahun, Antony Moody, Asrta Hailu, **Tesfaye Gelanew**. Performance of four rapid diagnostic tests for the diagnosis of falciparum and non-falciparum malaria in endemic areas of Gondar region, Northern Ethiopia. Presented at ***MEEGID X Conference .****3 – 5 November 2010, Amsterdam, The Netherlands****.***
2. **Tesfaye Gelanew**, Israel Cruz, Katrin Kuhls, Jorge Alvar, Carmen Cañavate, Asrat Hailu and Gabriele Schönian. Multiple *Leishmania donovani* parasites introduction into Libo Kemkem, Addis Zemen Districts, Ethiopia. Presented at ***MEEGID X Conference .****3 – 5 November 2010, Amsterdam, The Netherlands. (***Best Poster Award**).
3. **Tesfaye Gelanew**, Katrin Kuhls, Asrat Hailu, Gabriele Schoenian. Is vector-parasite interaction a determining factor for the *Leishmania donovani* populations in East Africa? Presented at **Congress on Infectious Diseases and Tropical Medicine (KIT 2010)**, June 23-26, Cologne, Germany.
4. **Tesfaye Gelanew**, Katrin Kuhls, Asrat Hailu, Gabriele Schoenian. Is vector-parasite interaction a determining factor for the *Leishmania donovani* population in East Africa? Presented at **Conference on Arbeitsgemeinschaft für Gen-Diagnostik(AGD)**. October 8-9. Postdam, Germany. **Best Poster Award**
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Abstract

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Erklärung

„Ich, **Tesfaye Gelanew**, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema:

Molecular Epidemiology and Population Genetics of *Leishmania donovani* Strains Isolated from Different Ethiopian Visceral Leishmaniasis Endemic Areas

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 11.10.2011

Unterschrift

Tesfaye Gelanew Taye

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