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DISSERTATION

Molecular characterization of parasites of the *Leishmania donovani* complex causing visceral leishmaniasis in South and Central Asia

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1 List of Abbreviations

CE	Capillary electrophoresis
CL	Cutaneous leishmaniasis
FCA	Factorial correspondence analysis
GDA	Genetic data analysis
He	Expected heterozygosity
Ho	Observed heterozygosity
ITS1	Internal transcribed spacer 1
kDNA	Kinetoplastid deoxyribonucleic acids
MLEE	Multilocus enzyme electrophoresis
MLMT	Multilocus microsatellite typing
MNA	Mean number of allele per locus
MON	Montpellier system, France
NJ	Neighbour-joining tree
P	Proportion of polymorphic loci
PCR	Polymerase chain reaction
PKDL	Post kala-azar dermal leishmaniasis
RFLP	Restriction fragment length polymorphism
VL	Visceral leishmaniasis
Δ K	Adhoc quantity

2 Abstract

Visceral leishmaniasis (VL) in Asia is caused by parasites of the *Leishmania donovani* complex- *L. donovani* sensu stricto in the Indian subcontinent, and *Leishmania infantum* in China and Central Asia. Transmission of VL due to *L. donovani* is thought to be anthroponotic whereas VL by *L. infantum* is clearly zoonotic. But many other aspects of epidemiology of VL in Asian foci, such as genetic diversity of parasites, distribution of the two species, and presence of genetically different population are not fully understood. The aim of this study was to characterize parasites from different Asian regions at species and strain levels by using molecular approaches.

Species identification has been performed by amplification of the ribosomal internal transcribed spacer 1-PCR (ITS1-PCR) and subsequent RFLP or sequence analyses using DNA extracted from bone marrow aspirates and skin exudates spotted on filter paper or glass slide. Out of 39 specimens collected from VL and PKDL suspects in Bangladesh, 38 specimens were positive by ITS1-PCR whereas only 26 were positive by microscopic examination. The causative agent in all Bangladeshi VL cases was *L. donovani*. With the same approach *L. infantum* was confirmed as causative agent of VL in Uzbekistan and Tajikistan. This had been suspected earlier but could never been proven so far because of the failure to cultivate these parasites *in vitro*. ITS1-PCR was thus shown to be superior to the diagnosis of leishmaniasis compared to parasitological confirmation by microscopy due to its higher sensitivity and capacity for direct species identification.

A multilocus microsatellite typing (MLMT) approach has been used to characterize strains of *L. donovani* and *L. infantum* from different Asian VL foci. This method has been shown to be highly discriminatory and reproducible, and the results can be stored in data bases and exchanged between different laboratories. Recently, MLMT proved to be useful for molecular epidemiological and population genetic studies in the *L. donovani* complex.

In this study, MLMT was applied to investigate the genetic diversity of strains of *L. donovani* from the Indian subcontinent, and of strains belonging to the most widespread isoenzyme type of *L. donovani*, MON-37, as well as to define the relatedness of the *L. infantum* parasites isolated from Uzbeki and Tajiki VL cases to other members of the species complex. The majority of isolates from the Indian subcontinent were assigned to a very homogeneous population regardless of their geographical origin, clinical manifestation, and their *in vitro* or *in vivo* susceptibility to antimonial drugs. The circulation of a single homogeneous population of *L. donovani* is, most probably, related to the epidemic spread of VL in this area. When strains of zymodeme MON-37 from India, Sri Lanka, Kenya, Israel and Cyprus were compared by MLMT they were assigned to different distantly related genetically defined groups, according to their geographical origin. The zymodeme MON-37 was found to be paraphyletic and not pertaining to a single genetic entity. Finally, MLMT showed that *L. infantum* from Uzbekistan and Tajikistan belong to the predominating zymodeme MON-1 but form a separate genetic group distinct from MON-1 populations from Europe, the Middle East and North Africa. Parasites circulating in the Uzbeki and Tajiki foci most probably have been restricted there for a long time rather than having been recently introduced from elsewhere by human or animal reservoir migration.

The present study demonstrates the usefulness of molecular methods that allow for differentiation of *Leishmania* parasites at species and strain levels for addressing key epidemiological questions that are of importance for improving VL control.

2 Abstrakt

Die viszerale Leishmaniose (VL) in Asien wird durch Parasiten des *Leishmania donovani*-Komplex hervorgerufen, *L. donovani* sensu stricto auf dem indischen Subkontinent und *Leishmania infantum* in China und Zentralasien. Die Übertragung der VL verursacht durch *L. donovani* ist vermutlich anthroponotisch, während die VL verursacht durch *L. infantum* klar zoonotisch ist. Viele andere epidemiologischen Aspekte der VL in asiatischen Foci, so z.B. die genetische Diversität der Parasiten, die Verbreitung der beiden Spezies und das Vorkommen von genetisch unterschiedlichen Populationen sind noch nicht vollständig geklärt. Das Ziel dieser Arbeit war es, die Parasiten aus verschiedenen asiatischen Regionen auf Spezies- und Stamm-Ebene mit Hilfe molekularer Methoden zu charakterisieren.

Die Spezies-Identifizierung wurde durch die Amplifizierung des ribosomalen "internal transcribed spacer" 1 (ITS1-PCR) mit nachfolgender RFLP- oder Sequenzanalyse durchgeführt. Dazu wurde DNA verwendet, die von Knochenmarksaspiraten und Hautexudaten auf Filterpapier oder Glasobjektträgern extrahiert wurde. Von den 39 Proben, die von VL- und PKDL-Patienten in Bangladesh erhalten wurden, waren 38 Proben positiv in der ITS1-PCR während sich nur 26 als positiv in der mikroskopischen Untersuchung erwiesen. Das infektiöse Agens in allen VL-Fällen aus Bangladesh war *L. donovani*. Mit der gleichen Methode wurde *L. infantum* als der Verursacher der VL in Usbekistan und Tadshikistan bestätigt. Das war früher bereits vermutet worden, konnte jedoch noch nicht überprüft werden, weil bisher alle Versuche, den Erreger zu kultivieren, fehlschlugen. Die ITS1-PCR hat sich auf Grund der höheren Sensitivität sowie ihrer Kapazität zum direkten Nachweis der *Leishmania*-Spezies als überlegen gegenüber dem mikroskopischen Nachweis der Parasiten erwiesen.

Die Methode der Multilocus-Mikrosatelliten-Typisierung (MLMT) wurde benutzt, um *L. donovani*- and *L. infantum*-Stämme aus verschiedenen asiatischen VL-Foci zu charakterisieren. Diese Methode ist hoch diskriminierend und reproduzierbar, und ihre Resultate können in Datenbanken niedergelegt und zwischen Laboratorien ausgetauscht werden. Vor kurzem haben mehrere Studien die Nützlichkeit der MLMT für molekular epidemiologische und populationsgenetische Untersuchengen des *L. donovani*-Komplex bewiesen.

In dieser Arbeit wurde die MLMT angewandt, um die genetische Diversität von *L. donovani*-Stämmen vom indischen Subkontinent und von Stämmen, die zu dem am weitesten verbreiteten Zymodem von *L. donovani*, MON-37, gehören, zu untersuchen, sowie um das Verhältnis der *L. infantum*-Parasiten von usbekischen und tadshikischen VL-Fällen zu anderen Vertretern des Spezies-Komplex zu definieren. Die meisten der Isolate vom indischen Subkontinent wurden der gleichen sehr homogenen Population zugeordnet unabhängig von ihrer geographischen Herkunft, der klinischen Manifestation der Erkrankung und der in vitro oder in vivo Suszeptibilität gegenüber Antimonium-Präparaten. Die Zirkulation einer einzigen homogenen Population von *L. donovani* ist wahrscheinlich für die epidemische Ausbreitung der VL in diesem Gebiet verantwortlich. Der Vergleich von Stämmen des Zymodems MON-37 aus Indien, Sri Lanka, Kenia, Israel und Zypern mit Hilfe der ergab, dass sie zu verschiedenen nur entfernt verwandten genetischen Gruppen gehören, die mit ihrer geographischen Herkunft korrelieren. Das Zymodem MON-37 war paraphyletisch und stellte keine genetisch einheitliche Gruppe dar. Schließlich zeigte die MLMT, dass *L. infantum* aus Usbekistan und Tadshikistan zu dem dominierenden Zymodem MON-1 gehören, aber eine separate genetische Gruppe bilden, die sich von den MON-1-Populationen in Europa, dem Mittleren Osten und Nordafrika unterscheiden. Die Parasiten, die in den usbekischen und tadshikischen Foci zirkulieren, waren wahrscheinlich schon längere Zeit beschränkt auf diese Region und wurden nicht erst kürzlich durch die Migration von Menschen oder Tieren importiert.

Diese Arbeit demonstriert den Nutzern molekularer Methoden, die die Differenzierung der *Leishmania*-Parasiten auf Spezies-und Stammebene gestatten, für die Klärung wichtiger epidemiologischer Fragen, die für die Verbesserung der VL-Kontrolle von Bedeutung sind.

3 Summary of publication thesis

3.1 Introduction

Visceral leishmaniasis (VL) is a vector borne (re)-emerging protozoan disease. It has been reported from 51 countries around the world with an annual incidence of 500,000 cases. It is one of the most-neglected poverty-related diseases and fatal if left untreated. It is caused by *Leishmania donovani* complex- *L. donovani* sensu stricto in the Indian subcontinent and East Africa and *Leishmania infantum* in Europe, Asia, North Africa and South America.

The clinical manifestation of disease, mode of transmission and lack of animal reservoir are similar in eastern India, Bangladesh and Nepal due to the common aetiological agent, *L. donovani*. Detection of *Leishmania* parasites in clinical materials is necessary to confirm a suspected case of leishmaniasis. Most commonly used methods for direct detection of parasite are microscopic examination, which has only low and highly variable sensitivity (Herwaldt, 1999), and different serological tests for antileishmanial antibodies. Molecular diagnostic approaches are rarely used in India, Bangladesh and Nepal.

In Bihar, India, almost 70% of VL cases do not respond to pentavalent antimonials, the first-line treatment (Croft et al., 2006). From Nepal and Bangladesh only 24% (Rijal et al., 2003) and 12% (Shamsuzzaman, pers. comm.) of treatment failure, respectively, has been reported. Antimony- resistant parasites have been identified (Lira et al., 1999; Rijal et al., 2007), but the reasons for the emergence of antimonial resistance and its geographic patterns are not all understood. Post kala-azar dermal leishmaniasis (PKDL), a sequel of VL, occurs in the Indian subcontinent and East Africa (Thakur and Kumar, 1992; Zijlstra et al., 2003). However, it is not known whether PKDL is caused by parasites persisting after apparent cure from VL infection or due to re-infection. Foci of cutaneous leishmaniasis (CL) due to *L. donovani* have recently emerged in Sri Lanka (Karunaweera et al., 2003) and in the western Himalayas in northern India (Sharma et al., 2005). It is unknown whether specific genetic traits of *L. donovani* strains are related to drug resistance or to different clinical manifestation of the disease, VL, PKDL or CL.

Strains of *L. donovani* belonging to the zymodeme MON-37 have been isolated from cases of VL and CL from widely separated geographical locations in the world, including India, Sri Lanka, Kenya, Israel and Cyprus. In the latter, it was the first report of VL and CL caused by *L. donovani* sensu stricto anywhere in Europe (Antoniou et al., 2008). It was assumed that the

Mon-37 strains have been introduced through infected immigrants from the Indian subcontinent and got established because of the presence of indigenous competent vectors in Cyprus.

Human VL was recorded as occurring widely in the towns and rural areas of the Central Asian Republics of former Soviet Union, and the Caucasus. Its epidemiology suggested that it was a zoonosis possibly caused by *Leishmania infantum* (Ponirovskii et al., 2006*)*. So far, identification of the causative agent of VL has been impossible owing to the failure to culture strains. For Uzbekistan, the continuing existence of stable foci of VL in the Namangan and Fergana regions has been documented in epidemiological studies but nothing is known from other Central Asian countries.

The gold standard method for typing *Leishmania* is still Multilocus Enzyme Electrophoresis (MLEE), with the Montpellier system (MON) most widely used (Rioux et al., 1990). Because this method requires cultured parasites, and is very laborious and time-consuming, only very few strains from the study area have been typed so far. Of the genotyping methods that have been developed to overcome the disadvantages of MLEE, kDNA PCR-RFLP and multilocus microsatellite typing (MLMT) were most powerful for discriminating closely related strains of the *L. donovani* complex (Botilde et al., 2006). Microsatellites are tandemly repeated stretches of short nucleotide motives of 1-6 bp ubiquitously distributed in eukaryotic genomes and mutate much faster than the bulk of DNA. Recently developed panels of highly polymorphic and co-dominant microsatellite markers which have proven to be useful for strain typing and population genetics studies in the *L. donovani* complex (Kuhls et al., 2007; Ochsenreither et al., 2006), were applied in the present study.

3.2 Objectives

The aim of the present work was:

i) to compare the sensitivity of internal transcribed spacer 1- PCR (ITS1-PCR) assay for parasite detection with that of microscopic detection in clinical materials from VL and PKDL suspects in Bangladesh.

ii) to type strains of *L. donovani* from different parts of the Indian subcontinent using a multi-locus microsatellite typing (MLMT) approach and to test whether specific genetic traits of *L. donovani* can be related to drug resistance or to different clinical manifestation of the disease.

iii) to test whether MLMT can be applied directly to clinical materials without prior cultivation of parasites.
iv) to identify the origin of the *L. donovani* MON-37 strains from Cyprus by comparing their MLMT profiles with MON-37 strains from the Indian subcontinent and from Africa.
v) to identify the causative agent of VL in Uzbekistan and Tajikistan by amplifying and sequencing the ITS1 using DNA extracted from archived Giemsa-stained smears of human bone marrow aspirates
vi) to perform epidemiological strain typing of *Leishmania* isolates from Uzbekistan and Tajikistan using MLMT.

3.3 Materials and methods

Samples from VL and PKDL suspects in Bangladesh used for direct microscopy and ITS1-PCR diagnosis: Thirty-nine patients suspected on clinical grounds for suffering from VL or PKDL were referred to the Department of Microbiology, Mymensingh Medical College from various regional hospitals and health centers. Bone marrow aspirates from the 35 KA suspects and skin exudates from 4 PKDL cases were taken by physicians. Giemsa-stained smears were prepared and examined microscopically for the presence of amastigotes. Bone marrow aspirate and skin exudates were also spotted on filter paper and subjected to ITS1-PCR.

Strains from Indian subcontinent subjected to MLMT: One hundred thirty-two of *L. donovani* strains originated from Bangladesh (n=21), India (n=89), Nepal (n=20), and Sri Lanka (n=2) were typed by using a panel of 15 microsatellite markers. Previously obtained MLMT profiles of 15 East African (Sudan, Ethiopia, Kenya) *L. donovani* strains (Kuhls et al., 2007) were included for comparison. Most of the strains used for this study were obtained as promastigote cultures. We also used nine bone marrow samples from Bangladesh spotted on filter paper or glass slides.

MON-37 *L. donovani* strains characterized by MLMT: Fifteen strains belonging to zymodeme MON-37 originated from 5 different countries, namely India, Sri Lanka, Kenya, Israel and Cyprus. The multilocus microsatellite profiles of these strains were compared with each other and with those of 32 strains of *L. donovani* of different origin and zymodemes.

***Leishmania* isolates from Central Asia**: In total, 13 Giemsa-stained bone marrow smears were analysed that were taken for diagnosis from patients, 11 from different foci in Uzbekistan and 2 from Tajikistan, and brought to the Isaev Institute in Samarkand during the period from 2006 to 2008. Identification of the causative species was performed by ITS1-PCR and typing strain level by MLMT.

ITS1-PCR assay: ITS1 was amplified from the DNA extracted from filter papers and Giemsa-stained slides using the primers LITSR (5`-CTG GAT CAT TTT CCG ATG-3`) and L5.8S (5`-TGA TAC CAC TTA TCG CAC TT-3`) and PCR conditions described elsewhere (Schonian et al., 2003). PCR-RFLP was performed with *Hae* III restriction endonuclease to identify the *Leishmania* species. Alternatively, the ITS1-PCR products (~320 bp) were commercially sequenced employing the same primers as for the PCR. The sequences obtained were processed and aligned using the multiple alignment programme BioEdit (Hall, 1999) and edited manually.

PCR amplification of microsatellite markers: Fifteen microsatellite markers (Li22-35, Li23-41, Li41-56, Li45-24, Li46-67, Li71-5, Li71-7, Li71-33, Lm2TG, Lm4TA, TubCA, CS19, CS20, LIST7031, and LIST7039) were amplified applying the conditions previously described (Kuhls et al., 2007; Ochsenreither et al., 2006). Microsatellite-containing fragments were analyzed by either using MetaPhor agarose gels or capillary electrophoresis (CE) by the CEQ 8000 automated genetic analysis system of Beckman Coulter, USA or commercially available capillary electrophoresis (SMB Services in Molecular Biology Berlin) on an automated sequencer ABI PRISM GeneMapper (Applied Biosystems).

MLMT data analysis: Population structure was investigated by the STRUCTURE software (Pritchard et al., 2000), which applies a Bayesian model-based clustering approach. This algorithm identifies genetically distinct populations on the basis of allele frequencies. Microsatellite-based genetic distances were calculated using either the software MICROSAT (Minch et al., 1995) or MSA (Dieringer, 2002) and POPULATIONS (http://www.legs.cnrs-gif.fr/bioinfo/populations). Neighbour-joining (NJ) trees, including the test for confidence intervals by bootstrapping (100 replicates) based on the resulting distance matrix, were constructed with the programme PAUP version 4.0b8 (Swofford, 2000) or POPULATIONS and MEGA (Kumar et al., 2004). For visualising the genetic substructure at population and individual level we applied a factorial correspondence analysis (FCA) implemented in the

GENETIX software (Dawson and Belkhir, 2001). Microsatellite data were analyzed with respect to diversity of alleles (A), mean number of alleles (MNA), expected heterozygosity (H_e), and observed heterozygosity (H_o) by using the GDA software (http://hydrodictyon.eeb.uconn.edu/people/plewis/software.php).

3.4 Results

Microscopy and ITS1-PCR with clinical materials from VL and PKDL suspects in Bangladesh: Out of 39 specimens collected from 35 VL and 4 PKDL suspects, 26 were positive by microscopic examination of smears from bone marrow and skin exudates. The ITS1-PCR was positive in 34 of the 35 bone marrow aspirates and in all 4 skin exudates spotted on filter paper. ITS1-PCR products were obtained for 12 of the 13 patients with negative microscopy. All *Leishmania* isolates were identified as belonging to the *L. donovani* complex by ITS1-PCR-RFLP.

These results have been published in the following paper:

Alam, M.Z., Shamsuzzaman, A.K.M., Kuhls, K. and Schönian, G. (2009). PCR diagnosis of visceral leishmaniasis in an endemic region, Mymensingh district, Bangladesh. *Tropical Medicine and International Health*, 14(5): 499-503.

MLMT of *L. donovani* strains in the Indian subcontinent: Sixty-nine of the strains from India (mainly from Bihar), Bangladesh and Nepal presented an identical microsatellite profile. All but one of 40 additional strains from Indian VL and PKDL patients had the same fragment sizes for the 11 markers tested, identical to that found for the predominating microsatellite profile. Three strains from India, MHOM/IN/71/LRC-L51a, MHOM/IN/54/LRC-L51p and MHOM/IN/54/SC23, shared an identical genotype which was, however, clearly different from those described before. The two strains from Sri Lanka showed identical MLMT profiles, significantly different from all the previous ones. Two Indian strains (MHOM/IN/83/CHANDIGARH and MHOM/IN/61/L13) had unique microsatellite profiles. A Bayesian model-based clustering algorithm implemented in the STRUCTURE was used to infer the population structure of *L. donovani* strains in the Indian subcontinent based on these microsatellite data. In total, 85 strains from Bangladesh, Bihar (India) and Nepal formed a very homogeneous population regardless of geographical origin, clinical manifestation, and whether or not they presented *in vitro* or *in vivo* susceptibility to antimonial drugs. Interestingly, three old strains from India were grouping with East African strains. A NJ

phylogram displayed exactly the same population pattern as STRUCTURE. Minor differences concerned the single Indian strain (MHOM/IN/83/CHANDIGARH) from the Himachal Pradesh focus and the two strains (MHOM/LK/2002/L60b and MHOM/LK/2002/L60c) from Sri Lanka, which were assigned to the same population by STRUCTURE but did not form a cluster in the tree. According to their position in the NJ tree these strains are most closely related to the Bangladesh/India-1/Nepal group. One single strain from India, MHOM/IN/61/L13, grouped in the Sudan/Ethiopia 2 cluster, as also found by STRUCTURE. The cluster including 85 *L. donovani* strains from Bangladesh, India and Nepal, was statistically highly supported. Homozygous allele combinations were found to predominate in this population. These findings confirmed that strains predominating on the Indian subcontinent were least diverse when compared to other populations of *L. donovani*, especially to those from East Africa.

These findings have been published in the following paper:

Alam, M.Z., Kuhls, K., Schweynoch, C., Sundar, S., Rijal, S., Shamsuzzaman, A.K.M., Raju, B.V.S., Salotra, P., Dujardin, J.C., Schönian, G. (2009). Multilocus microsatellite typing (MLMT) reveals genetic homogeneity of *Leishmania donovani* strains in the Indian subcontinent. *Infection, Genetics and Evolution,* 9(1): 24–31.

MLMT of *L. donovani* MON-37 strains: Nine of the 15 MON-37 strains had their own specific microsatellite profiles. The strains MHOM/CY/2006/CH33 and MHOM/CY/2006/CH35 from Cyprus shared the same profile whereas the other three Cypriot strains (MHOM/CY/2006/CH32, MHOM/CY/2006/CH34 and MHOM/CY/2006/CH36) had unique profiles. The strains MHOM/LK/2002/L60b and MHOM/LK/2002/L60c from Sri Lanka had identical profiles and so did the Indian strains MHOM/IN/2003/LEM4537 and MHOM/IN/2003/LEM4527 that were isolated from the same patient. The NJ phylogram constructed using the Chord-distance matrix displayed the same three main clusters revealed by (Alam et al., 2009; Kuhls et al., 2007): (1) India-1/Bangladesh/Nepal, (2) Sudan/Ethiopia with two sub-clusters, and (3) Kenya/India-2, as well as a fourth cluster containing the two strains from Iraq and a single strain from China. None of the MON-37 strains grouped with the strains of *L. donovani* in the Sudan/Ethiopia cluster, nor were they assigned to a common genetic group within the tree. The Cypriot strains formed a clearly distinct cluster in the tree. The Bayesian model-based clustering approach implemented in STRUCTURE identified different distantly related genetically defined subgroups of MON-37 strains according to their geographical origin. MON-37 strains from Cyprus and Israel clearly differed not only from

each other but also from all the other MON-37 strains studied here. The graphical representation of the factorial correspondence analysis (FCA) of the MLMT data confirmed the assignment of the MON-37 strains to different, clearly separate genetic groups, except for those from Sri Lanka and India. FCA analysis underlines that strains from Cyprus belong to an unique genetic group and are clearly differentiated from the other MON-37 strains.
These observations have been published in the following paper:
Alam, M.Z., Haralambous, C., Kuhls, K., Gouzelou, E., Sgouras, D., Soteriadou, K., Schnur, F., Pratlong, F. and Schönian, G. (2009). The paraphyletic composition of *Leishmania donovani* zymodeme MON-37 revealed by multilocus microsatellite typing. *Microbes and Infection*, 11(6-7): 707-715

ITS1 sequencing and MLMT of *Leishmania* isolates from Central Asia: Amplification and sequencing of ITS1 region using DNA extracted from Giemsa-stained smears of human bone marrow aspirates identified *L. infantum* as the causative agent of VL in Central Asia. In STRUCTURE analysis, the 13 Uzbek and Tajik DNA samples were found to be most closely related to strains of zymodeme MON-1 from other endemic areas. Within the MON-1 group, Uzbek and Tajik parasites were, however, assigned to a distinct cluster genetically clearly separated from populations of MON-1 from Europe, the Middle East and North Africa.
These results have been published in the following papers:
Alam, M.Z., Kovalenko, D.A., Kuhls, K., Nasyrova, R.M., Ponomareva, V.I., Fatullaeva, A.A., Razakov, S.A., Schnur, L.F. and Schönian, G. (2009). Identification of the agent causing visceral leishmaniasis in the Namangan Region of Uzbekistan by analysing parasite DNA extracted from patients' Giemsa-stained tissue preparations. *Parasitology*, in press, doi: 10.1017/S0031182009006465.

3.5 Discussion

Microscopy and ITS1-PCR with clinical materials from VL and PKDL suspects in Bangladesh: At present, definitive diagnosis of VL relies mainly on demonstration of parasites in bone marrow or splenic aspirates, or serological tests. However, the scarcity of parasites in bone marrow aspirates from many patients complicates parasitological confirmation and the sensitivity with microscopy is very low (Al-Jawabreh et al., 2006). Serology is not always available in diagnostic centres even in hospitals of Bangladesh. Therefore, the minimum basis for starting treatment of KA is a positive rK39

immunochromatographic test which is often used as field test in endemic areas. However, the sero-diagnosis might be negative in early acute stage of the disease and does not differentiate between active, past or subclinical infection. Moreover, serological tests remain positive well beyond the time of cure, which limits their usefulness for the diagnosis of relapses or re-infection (Chappuis et al., 2007). PCR might be an alternative when the clinical signs and symptoms suggest VL but microscopic scanning and serological tests give negative results. Furthermore, PCR allows a highly sensitive and specific (up to 100%) detection of the *Leishmania* parasite irrespective of species or genus (Cruz et al., 2006; Reithinger and Dujardin, 2007). In our study, ITS1-PCR was superior to confirmation of leishmaniasis by visualization of amastigotes using direct microscopy. Therefore, inclusion of PCR as diagnostic tool in Bangladesh is suitable for achieving accurate diagnosis of VL suspected cases.

MLMT of *L. donovani* strains in the Indian subcontinent: Most strains from the Indian subcontinent presented a very homogeneous population that included all strains previously typed as MON-2. The identification of a single cluster of genetically almost identical strains of *L. donovani* in the regions of Bangladesh, Bihar and Nepal highly endemic for VL suggests that this population emerged only recently and underwent a very short evolutionary process since then. The most plausible explanation for the great genetic homogeneity of strains of *L. donovani* from Bangladesh, Bihar and Nepal is a bottleneck event that exterminated the original *L. donovani* population(s) leaving only a small pocket of survivors. In the 1960s, kala-azar had virtually disappeared from the Indian subcontinent as a collateral effect of insecticide spraying under the Malaria Control Program. However, the completion of this campaign resulted in a dramatic resurgence of the disease in Bihar in the late 1970s (Sen Gupta, 1975) which then spread downstream to Bangladesh and to bordering regions in Nepal. However, it can not be ruled out that Indian *L. donovani*, being parasites of humans only, may have undergone a long process of adaptation to human physiology during which they have lost their intraspecies diversity as previously suggested (Pandey et al., 2007). The great homogeneity of MLMT profiles of the strains isolated from patients in these areas is nevertheless surprising especially bearing in mind the different clinical manifestations that were caused by the strains investigated and the varying refractoriness to standard antimonial treatment encountered in different countries of the Indian subcontinent (Laurent et al., 2007; Sundar et al., 2000). Thus, strains from KA and PKDL patients on one hand, as well as antimony-resistant and antimony-sensitive strains from Nepal (Laurent et al., 2007) and India

on the other showed the same MLMT profiles. The mechanisms underlying treatment failure, so far only explored for antimonial drugs (SSG), are far from being fully understood. In Bihar, parasite resistance *in-vitro* was consistently found to be correlated with SSG treatment failure (Sundar, 2001) but this was not the case in Nepal (Rijal et al., 2007). The first studies on naturally resistant strains of *L. donovani* point to two possible mechanisms leading to SSG unresponsiveness: up-regulated expression of genes encoding oxidative stress protective proteins (Decuypere et al., 2005) and specific alteration of macrophage transport functions (Mookerjee Basu et al., 2008). An important outcome of this study is that bone marrow aspirates spotted on filter papers or glass slides are a suitable material for microsatellite typing. Parasite culture is not easy to perform, especially under field conditions, and often not successful. Therefore, assays that can be carried out directly on clinical materials are of great advantage for surveys involving high numbers of isolates. All three strains that were isolated in the study area before the start of insecticide spraying differed significantly from the strains in the main cluster that were isolated between 1977 and 2007. This would support the bottleneck-hypothesis mentioned above. Interestingly, the three old strains were grouping with East African strains. We can only speculate whether this might be due to human migration or other reasons because there is no epidemiological information available about these strains. However, the number of strains collected before insecticide spraying is very small and we cannot exclude cross-contamination as the reason for their clustering with East African strains. In conclusion, our results demonstrate a remarkably homogeneous single clone of *L. donovani* related to the epidemic spread of VL in the Indian subcontinent.

MLMT of *L. donovani* MON-37 strains: MON-37 is perhaps the most widespread zymodeme of the species *L. donovani.* Typing of strains by scoring variation in 14 polymorphic microsatellite markers revealed that strains are genetically diverse and do not belong to one genetic entity. They appeared as paraphyletic groups in a phylogenetic tree based on their genetic distances and were assigned to five different genetic groups by using a Bayesian model-based clustering algorithm. The different genetic groups seem to correlate to the geographical origin of the strains. Of note is that three MON-37 strains from Cyprus although they possess an almost identical MLMT genotype caused VL (two of the strains) and CL (one strain).

In Cyprus, isoenzyme analysis showed that human cases of VL and CL that occurred recently were not caused by strains of *L. infantum*, as one might have suspected or assumed, but by

MON-37 strains of *L. donovani*. Importation from countries outside Europe through migrants, perhaps from South Asia, was suggested as one possible scenario for their emergence (Antoniou et al., 2008). However, MLMT analysis assigned the Cyprian strains to a separate genetic group which was only distantly related to MON-37 strains from other endemic areas. The differences between these strains and strains from the Indian subcontinent and East Africa were too substantial for assuming a very recent introduction by immigrants or even by infected vectors. However, since Cyprus is the stepping stone from Africa and Asia to Europe, the possibility of a recent introduction of these strains from another endemic area - not examined in the present study - cannot be excluded. On the other hand, if these strains have been circulating for a long time in Cyprus, why was the southern part of the island free of human leishmaniasis with only two infantile VL cases reported since 1935 (Minter and Eitrem, 1989)? Are the lack of clinicians' awareness for the disease and the non-availability of good diagnostic/typing molecular tools that have been only recently developed sufficient to explain this? The above suggests that the epidemiology of leishmaniases caused by *L. infantum* and *L. donovani* in the Eastern Mediterranean Region needs to be studied more deeply by applying the discriminating molecular biological approaches.

Microsatellite analysis revealed that the zymodeme MON-37 is paraphyletic and does not reflect the genetic relationship between strains of different geographical origin, which do not pertain to a single genetic entity. It was recently shown that strains with the same phenotype for a given enzyme had different protein sequences, and that changes in charge or molecular mass could in some cases compensate each other (Mauricio et al., 2006). As a result, indistinguishable phenotypes can be produced by distinct genotypes. Whether this is true in the case of MON-37, should be clarified for strains of this zymodeme originating from different locations.

ITS1 sequencing and MLMT of *Leishmania* isolates from Central Asia: This molecular biological study showed that *L. infantum* is the causative agent of VL currently occurring in Uzbek and Tajik foci, confirming past suspicions owing to the zoonotic nature of the disease. This was done without the need to isolate and culture the leishmanial parasites, a process that has always failed regarding the parasites causing VL in Uzbekistan. By comparison, the MLMT profiles of the DNA samples obtained from thirteen Uzbek and Tajik cases of VL showed that the parasites grouped with strains belonging to the most predominating zymodeme MON-1. Within this group *L. infantum* from Uzbekistan and Tajikistan were,

however, assigned to a distinct cluster genetically clearly separate from strains of zymodeme MON-1 from Europe, the Middle East and North Africa. The existence of a genetically homogeneous but distinct group of *L. infantum* MON-1 indicates that the parasites circulating in the Uzbeki and Tajiki foci studied have been restricted there for a long time rather than having been recently introduced from elsewhere by human or animal reservoir migration.

In conclusion, this study demonstrated the usefulness of ITS1-PCR to elucidate the cases of VL in the current diagnosis of the disease in endemic areas and proved applicable for differentiation of *Leishmania* parasites at species level. Furthermore, our present study addressed several key epidemiological questions for the first time for *L. donovani* and *L. infantum* in the South and Central Asia because of the high discriminatory power of microsatellite markers, thus creating a basis for further epidemiological investigations. Altogether these data show that MLMT is a sensitive and specific method for *Leishmania* strain typing. It is easier to perform than MLEE and may even discriminate within groups, which appear homogeneous in MLEE the today's gold standard of *Leishmania* typing. This supports the discussion on whether MLMT should substitute MLEE in the future for discrimination of *Leishmania* strains in epidemiological and population genetic studies.

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4 Declaration of own contributions

The contributions to the publications by the doctoral candidate are as follows:

Publication 1

Alam, M.Z., Shamsuzzaman, A.K.M., Kuhls, K. and Schönian, G. (2009). PCR diagnosis of visceral leishmaniasis in an endemic region, Mymensingh district, Bangladesh. *Tropical Medicine and International Health*, 14(5): 499-503.

IF: 2.312 (2008)

70 Percent contribution

Contribution in details:

The doctoral student was responsible for designing of the study, extraction of DNA from clinical materials, performing ITS1-PCR and restriction digestion. Manuscript preparation and review procedures were performed by the doctoral student with the help of G. Schönian.

Publication 2

Alam, M.Z., Kuhls, K., Schweynoch, C., Sundar, S., Rijal, S., Shamsuzzaman, A.K.M., Raju, B.V.S., Salotra, P., Dujardin, J.C., Schönian, G. (2009). Multilocus microsatellite typing (MLMT) reveals genetic homogeneity of *Leishmania donovani* strains in the Indian subcontinent. *Infection, Genetics and Evolution,* 9(1): 24–31.

IF: 2.792 (2008)

60 Percent contribution

Contribution in details: The doctoral candidate performed mass cultivation of promastigotes and extraction of DNA from promastigotes and clinical materials. He carried out the MLMT approach for the strains of *L. donovani* from the Indian subcontinent; this included amplification of microsatellite markers, preparation of PCR products for fragment analyses, determination of repeat numbers and analysis of microsatellite data. The doctoral candidate was responsible for manuscript writing and review process.

Publication 3

Alam, M.Z., Haralambous, C., Kuhls, K., Gouzelou, E., Sgouras, D., Soteriadou, K., Schnur, F., Pratlong, F. and Schönian, G. (2009). The paraphyletic composition of *Leishmania*

donovani zymodeme MON-37 revealed by multilocus microsatellite typing. *Microbes and Infection*, 11(6-7): 707-715.
IF: 2.801 (2008)
60 Percent contribution

Contribution in details: The doctoral student was responsible for MLMT of Cypriot *L. donovani* strains, including PCR, preparation of samples for fragment analyses and estimation of repeat numbers. He also performed part of the microsatellite data analysis and manuscript writing.

Publication 4

Alam, M.Z., Kovalenko, D.A., Kuhls, K., Nasyrova, R.M., Ponomareva, V.I., Fatullaeva, A.A., Razakov, S.A., Schnur, L.F. and Schönian, G. (2009). Identification of the agent causing visceral leishmaniasis in the Namangan Region of Uzbekistan by analysing parasite DNA extracted from patients' Giemsa-stained tissue preparations. *Parasitology*, in press, doi: 10.1017/S0031182009006465.
IF: 2.071 (2008)
50 Percent contribution

Contribution in details: The doctoral candidate performed DNA extraction from clinical materials. He amplified ITS region of the DNA, prepared the PCR product for sequencig and performed alignment of ITS-PCR products in order to identify the causative *Leishmania* species. He amplified the DNA obtained from Uzbeki and Tajiki samples with 14 microsatellite markers, prepared the PCR products for fragmant analyses and estimated the repeat numbers. The doctoral candidate was responsible for analysis of microsatellite data and manuscript writing.

Mohammad Zahangir Alam — Prof. Dr. Wolfgang Presber
Doktorand — Betreuender Hochschullehrer

5 List of selected publications

This doctoral thesis (publication thesis) is based on the publications, which are published in the following journals:

1) **Alam, M.Z**., Shamsuzzaman, A.K.M., Kuhls, K. and Schönian, G. (2009). PCR diagnosis of visceral leishmaniasis in an endemic region, Mymensingh district, Bangladesh. *Tropical Medicine and International Health*, 14(5): 499-503. **IF**: 2.312 (2008)

2) **Alam, M.Z**., Kuhls, K., Schweynoch, C., Sundar, S., Rijal, S., Shamsuzzaman, A.K.M., Raju, B.V.S., Salotra, P., Dujardin, J.C., Schönian, G. (2009). Multilocus microsatellite typing (MLMT) reveals genetic homogeneity of *Leishmania donovani* strains in the Indian subcontinent. *Infection, Genetics and Evolution,* 9(1): 24–31. **IF**: 2.792 (2008)

3) **Alam, M.Z.,** Haralambous, C., Kuhls, K., Gouzelou, E., Sgouras, D., Soteriadou, K., Schnur, F., Pratlong, F. and Schönian, G. (2009). The paraphyletic composition of *Leishmania donovani* zymodeme MON-37 revealed by multilocus microsatellite typing. *Microbes and Infection*, 11(6-7): 707-715. **IF**: 2.801 (2008)

4) **Alam, M.Z**., Kovalenko, D.A., Kuhls, K., Nasyrova, R.M., Ponomareva, V.I., Fatullaeva, A.A., Razakov, S.A., Schnur, L.F. and Schönian, G. (2009). Identification of the agent causing visceral leishmaniasis in the Namangan Region of Uzbekistan by analysing parasite DNA extracted from patients' Giemsa-stained tissue preparations. *Parasitology*, in press, doi: 10.1017/S0031182009006465. **IF:** 2.071(2008)

6 Curriculum Vitae

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

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I would like to express my deepest sense of gratitude, sincere appreciation and indebtedness to Frau Dr. Gabriele Schönian, for her scholastic guidance, tremendous support, valuable suggestions, constant encouragement and overall supervisions throughout the study that making this dissertation real. I am also grateful to Professor Dr. Wolfgang Presber for his administrative support, valuable advices and recommendation. I would like to express my cordial thanks to Dr. Katrin Kuhls for her constant cooperation, suggestion and inspiration. Sincere thanks to Frau Carola Schweynoch and all other members of Dr. Schönian's group for their assistance, time and cooperation during the research period.

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Many thanks go as well to Bangladesh Agricultural University for providing study leave to pursue my PhD degree in Berlin, Germany.

I also would like to expresses my heartfelt gratitude to my beloved parents, my wife, Reema and my son, Rafi for their blessings and sacrifices throughout the period of my study.

8 Declaration

„Ich, Mohammad Zahangir Alam, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: **Molecular characterization of parasites of the *Leishmania donovani* complex causing visceral leishmaniasis in South and Central Asia** 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 30 June 2009 Mohammad Zahangir Alam