Variation of the parasite causing visceral leishmaniasis in East Africa

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Addis Ababa University, Ethiopia
Ecotypes of VL

1. *P. orientalis*, Acacia-Balanites woodland, black-cotton soil
   [Eastern Sudan, Northern, and North Eastern Ethiopia]

2. *P. martini/celiae*, eroded termite hills
   [South & SE Ethiopia, Kenya, Uganda, SE Sudan]

Map: Courtesy of HealthNet Intl, 2003
What we know, and don’t know

1. Disease phenotype
2. Response to PM treatment
3. Genetic diversity
4. *In vitro* drug susceptibility
5. Relationship between 3 and 4
# VL in East Africa:
Clinical presentation and diagnosis

<table>
<thead>
<tr>
<th></th>
<th>Sudan</th>
<th>N-Eth</th>
<th>S-Eth</th>
<th>India</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphadenopathy</td>
<td>72-86%</td>
<td>&lt;20%</td>
<td>&lt;15%</td>
<td>Low</td>
</tr>
<tr>
<td>PKDL</td>
<td>&gt; 50%</td>
<td>&lt;5%</td>
<td>&lt;5%</td>
<td>&lt;1%</td>
</tr>
</tbody>
</table>
Response to paromomycin treatment (East Africa & India)

Response to PM is variable between trial sites in Africa:
+ efficacy ranges of low – 96%

Efficacy is much lower in Sudan cf. India:
+ low cf. 93%
Hypothesis

East African visceral leishmaniasis is caused by geographically (and genetically) isolated populations of *Leishmania donovani*
Objectives

✓ To describe the genomic polymorphisms
✓ To determine if genotypic variations segregate by geographical location
✓ To describe if genotypic variation correlates with drug sensitivity / resistance (planned)
### Species and zymodemes within *Leishmania donovani* complex

<table>
<thead>
<tr>
<th>Species</th>
<th>Zymodemes</th>
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<tbody>
<tr>
<td><em>L. infantum</em></td>
<td>MON1, etc., MON-30, MON-81, MON-267, MON-278</td>
</tr>
<tr>
<td><em>L. archibaldi</em></td>
<td>MON-82, MON-257, MON-258</td>
</tr>
<tr>
<td><em>L. donovani</em></td>
<td>MON-18, MON-2, MON-32, MON-36, MON-37, MON-38, MON-274, MON-276, MON-277</td>
</tr>
</tbody>
</table>

*Both spp., not found in South Ethiopia and Kenya*
Phylogeny, parsimonious cladogram, based on MLEE

Pratlong et al., 2001

L. infantum complex

L. archibaldi complex

L. donovani complex

L. tropica complex

L. killicki complex

OTU from Sudan
Species of *L. donovani* complex

Recommendations, based on molecular tools:

- Only two species: "*L. archibaldi* is non-existent"
  - *L. infantum*
  - *L. donovani*

- *Leishmania donovani* is the only cause of VL in East Africa;

  "previous descriptions of *L. infantum* and *L. archibaldi* from this region are a consequence of convergent evolution in the isoenzyme data" [Jamjoom et al. *Parasitology*. 2004; 129, 399 – 409]
Targetted molecular markers

- **ITS-1** (Internal Transcribed Spacer) sequences
- **MER** (Mini Exon Repeats)
- **LEG** ($T_2B_4$)
- **CpbEF** (Cysteine proteinase b)
## Molecular markers analyzed

<table>
<thead>
<tr>
<th>Methods</th>
<th>S</th>
<th>Markers</th>
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<tr>
<td>PCR</td>
<td>1</td>
<td>CpbEF</td>
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<tr>
<td>PCR-RFLP</td>
<td>2</td>
<td>MER (Eae I)</td>
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<tr>
<td></td>
<td>3</td>
<td>MER (Nco I)</td>
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<td></td>
<td>4</td>
<td>MER (Hae III)</td>
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<tr>
<td></td>
<td>5</td>
<td>LEG (Hae III)</td>
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<td>6</td>
<td>ITS-1 (Hae III)</td>
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<tr>
<td>Analysis of sequences (PCR products)</td>
<td>7</td>
<td>MER</td>
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<tr>
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<td>ITS-1</td>
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<tr>
<td></td>
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<td>CpbEF</td>
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</table>
PCR amplifications (N=111)

- Majority of samples collected during LEAP0104 study
- n = 53 Sudan; n = 58 Ethiopia

<table>
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<tr>
<th>Markers</th>
<th>Sudan</th>
<th>N. Ethiopia</th>
<th>S. Ethiopia</th>
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<tr>
<td>MER</td>
<td>53/53</td>
<td>30/30</td>
<td>24/26</td>
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<td>CpbEF</td>
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Study areas
ITS-1

PCR, PCR-RFLP:

**PCR:** (320 bp); 1.0% agarose gel electrophoresis

**Primers:**
- L5.8S = 5’-TGATACCACTTATCGCACTT-3’
- LITSR = 5’-CTGGATCATTTTCCGATG-3’

**RFLP:** Hae III restriction; PAGE, 10% acrylamide
ITS RFLP

Marker  L. donovani  L. aethiopica  L. tropica  L. infantum  L. major

[Image of gel electrophoresis showing bands for each species]

[Map indicating regions such as Sudan and Ethiopia]
ITS sequence types

- 8x A
- 5x TA
- 7x A
- 6x TA
- 8x A
- 5x TA
- 7x A
- CC 6x A
- TC 6x TA
- 6x A

Map indicating countries:
- SUDAN
- ERITREA
- ETHIOPIA
PCR, PCR-RFLP:

PCR: (378-435 bp); 1.0% agarose gel electrophoresis

Primers: Fme2 = 5’-ACTTATTGGTATGCGAAACTTCCGG-3’
         Rme2 = 5’-ACAGAAACTGATACTTATATAGCGTTAG-3’

RFLP: Eae I, Nco I, Hae III (L. tropica, L. major)
       PAGE, 10% acrylamide
MER sequence types

- L. donovani
- L. donovani small gene variant
- L. major seq.
MER sequence types

L. donovani seq type A
- L. donovani seq type C
- L. donovani seq type B
- L. d Kenya small gene
- L. infantum France
- L. infantum Ibiza
- L. infantum Morocco
- L. infantum Malta
- L. infantum Italy
- L. major USSR 5ASKH

L. infantum small gene variant
- L. infantum large gene variant
LEG (T$_2$B$_4$)

PCR, PCR-RFLP:

**PCR:** (250 bp); 1.0% agarose gel electrophoresis

Primer T2: 5’-CGGCTTTCGCACCATGCGGTG-3’

Primer B4: 5’-ACATCCTGCCCACATACGC-3’

**RFLP:** Hae III restriction; PAGE, 10% acrylamide

L. dn = 2 bands: 180, 70 bp

L. in = 1 band: 250 bp

\(// = 3\) bands: 250, 180, 70 bp
LEG RFLP

L. donovani
L. aethiopica
L. tropica
L. infantum
L. major

Map showing prevalence in Sudan, Ethiopia, and Eritrea.
LEG sequence types
PCR:

CpbE (702); CpbF (741)
1.0 - 2.0% agarose gel electrophoresis

Primers:
Forward= 5’-CGTGACGCCGGTGGAAGAAT-3’
Reverse= 5’-CGTGCACTCGGCCGTCTT-3’

CpbE  = L. infantum

CpbF  = L. donovani/archibaldi
CpbEF sequence types

- L. donovani
- L. infantum

39 bp deletion
### Summary, multi-locus analysis

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**Summary, multi-locus analysis**

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Genotypes: Multi locus analysis

P. martini/celiae

P. orientalis

SUDAN

ERITREA

ETHIOPIA
**Summary:** main genotypes

<table>
<thead>
<tr>
<th></th>
<th>G-I</th>
<th>G-II</th>
<th>G-III</th>
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<td>South Ethiopia</td>
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</table>

- 2 major genotypes in E/Sudan
- 1 major genotype in N/Ethiopia
- 1 genotype in S/Ethiopia
Summary - Phase I study

**Multi-locus analysis**

- Genetic heterogeneity in Eastern Sudan & North Ethiopia
- Greater heterogeneity among isolates of Eastern Sudan
- *L. major* sequences within Sudanese *L. donovani* isolates
- Genetic homogeneity in southern Ethiopia
Evidences for distinct genotypes in Africa

Evidences from MLEE

African (Sub-saharan) *L. infantum* zymodemes
  = Not found in Mediterranean VL
  = Not found in S-Ethiopia, Kenya

Mediterranean *L. infantum* zymodemes
  = Not found in Sub-sahara Africa

African (Sub-saharan) *L. archibaldi* zymodemes
  = Not found in S-Ethiopia, Kenya
Evidences for distinct genotypes in Africa

**Evidences from molecular tools**

- **Oskam et al., 1998** - Restriction analysis and southern blotting
  - Microsatellites

- **Zemanova E. et al. 2004** - RAPD

- **Kuhls K. et al. 2007** - Microsatellite markers

- **Lukes J et al., 2007** - Multi-factorial (RAPD, RFLPs, Microsatellites, DNA Sequences)
Phylogenetic relationships, *L. donovani* complex

Sudan vs. Kenya vs. India

- gp63 intergenic region RFLP  
  Mauricio IL et al., 2001

- mitochondrial cytochrome oxidase II gene sequences  
  Ibrahim ME et al., 2001

- SCAR analysis  
  Lewin S et al., 2002
Ecotypes of VL

1. *P. orientalis*, Acacia-Balanites woodland, black-cotton soil
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2. *P. martini/celiae*, eroded termite hills
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Map: Courtesy of HealthNet Intl, 2003
Parasitology: next steps

Methods:  
- *In vitro* drug sensitivity testing (DST)
- Further genotypic characterization

- ✔  230 isolates of *Leishmania donovani complex* from VL patients
  
  230 isolates:  
  - E/Sudan (n= 75)
  - N/Ethiopia (n= 40)
  - S/Ethiopia (n= 55)
  - Kenya (n= 30)
  - Uganda (n= 30)

- ✔  Infection of peritoneal macrophages, CD1 mice
- ✔  IC$_{50}$ values: PM, AmBisome®, miltefosine, SSG
- ✔  Microscopy, Alamar blue (optional)
Acknowledgements

• Trial sites
  – Ethiopia: Arba Minch, Gondar
  – Sudan: Kassab

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  – Dr. Aldert Bart

• Cooperating institutions
  – IEND, AAU

• Funding
  – DNDi
Thank you