Identification of *Leishmania* spp. and a report of novel species in Thailand

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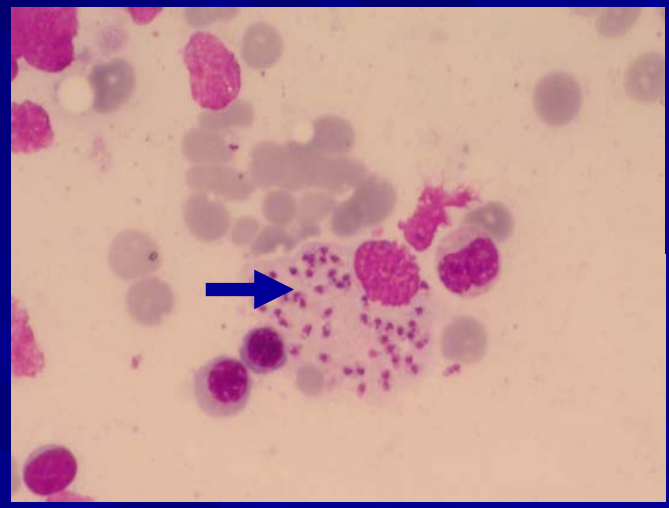
Diagnosis of Leishmaniasis

- Traditional diagnostic methods
- Serological diagnosis
- Molecular diagnosis
- Species identification

Traditional diagnostic methods

Microscopic examination

- Examination of intracellular amastigotes from Giemsa-stained lesion biopsy smears (CL), or lymph node, bone marrow aspiration (VL)
- remains the gold standard in leishmania diagnosis
- High specificity



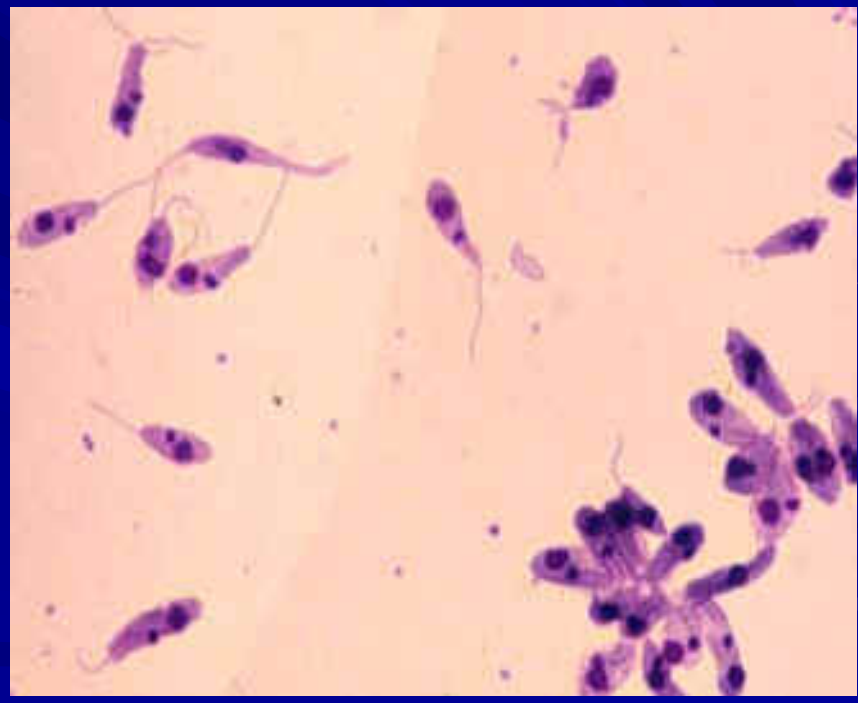
Intracellular amastigotes

Disadvantages

- Operator dependent
- low sensitivity
- procedures for demonstration of the parasite are invasive

Traditional diagnostic methods

- **Culture**
  - Growth of promastigotes in days to weeks
- **Sensitivity of 50-70%***



* Weigle KA et al, Am J Trop Med Hyg 1987; 36: 489-496

Cultivation of Promastigotes (1)

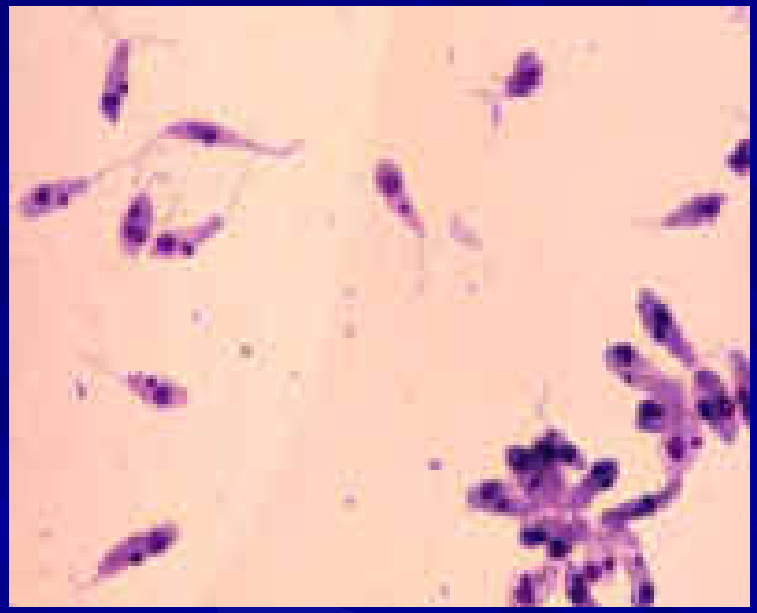
- **Undefined medium**
  - NNN medium with Locke's solution overlays
  - NNN medium with medium-199 as a liquid phase
- **Partially or Semi-defined liquid media**
  - Grace's medium
  - Schneider's Drosophila medium,
  - M199

All require a supplement of 10-20% heat inactivated bovine serum or calf serum

Cultivation of Promastigotes (2)

- **Define liquid media (serum-free media)**
  - Enriched Synthethic Medium (ESM)
  - Medium 199+
  - RPMI+

Culture conditions : 26° C or room temperature, adequate aeration



Promastigote, flagellate form

Serological Diagnosis

- Indirect fluorescence antibody (IFA)
- ELISA

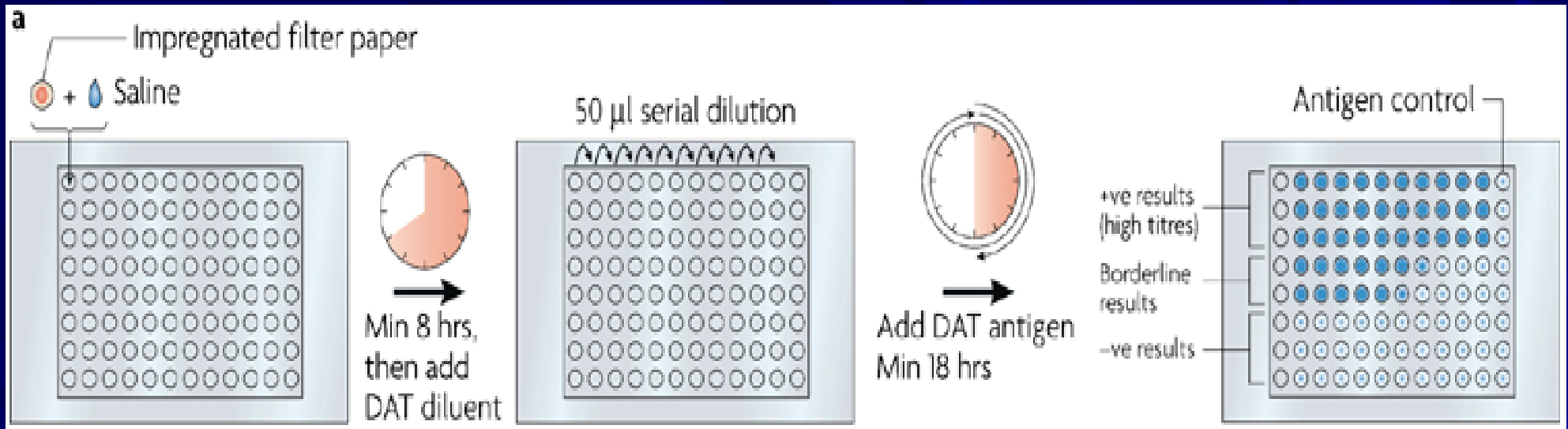
Not suitable for field conditions

Field use

- Direct Agglutination Test (DAT)
- rK39
- *Fast Agglutination Screening Test (FAST)

DAT and rK39 dipstick

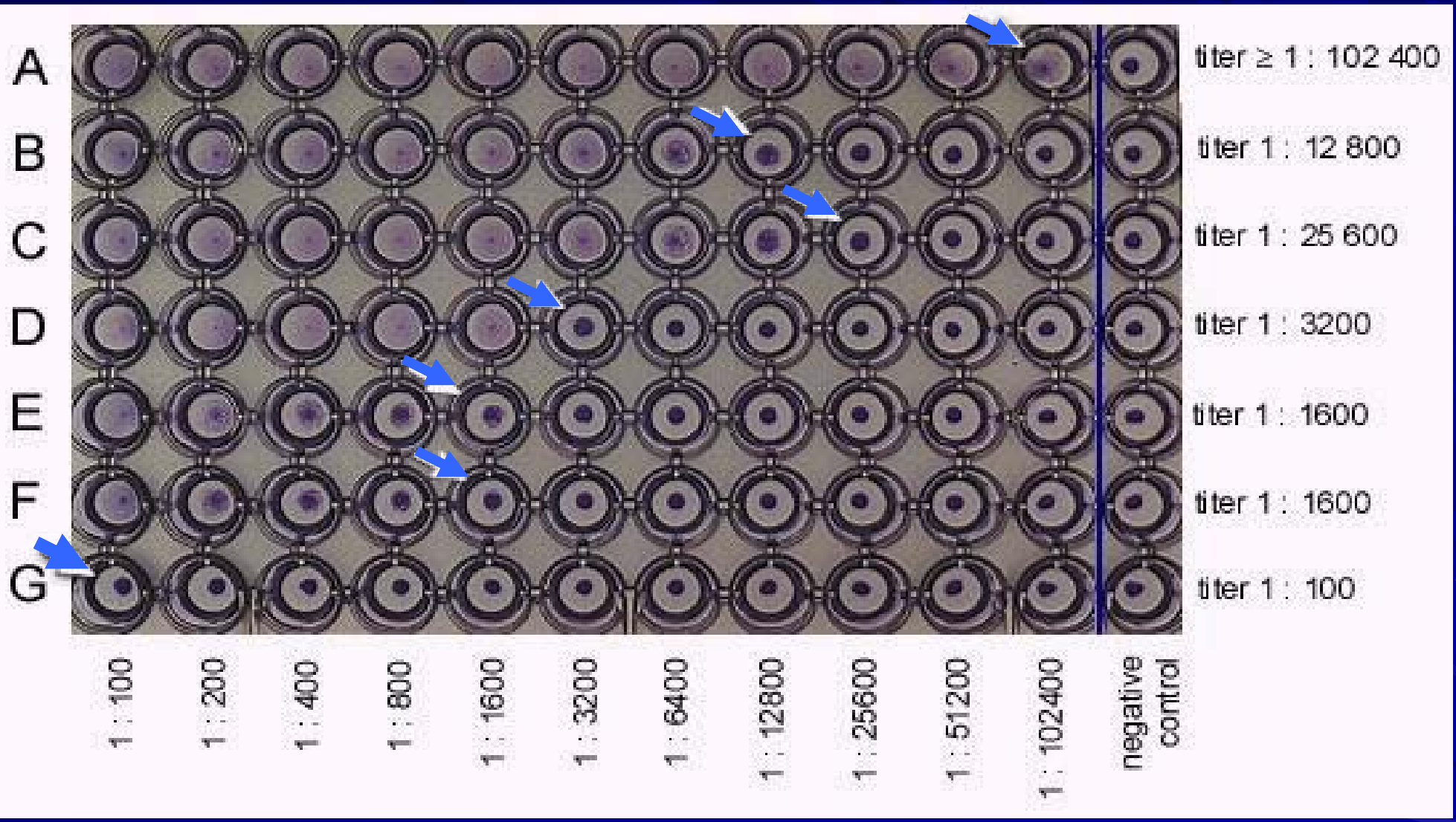
- Easy to use
- Require minimal technological expertise or laboratory set up
- Ideal for both field and laboratory use



Direct Agglutination Test (DAT)

- A semi-quantitative test
- using dilutions of patient's serum and stained killed *L. donovani* promastigotes
- Agglutination is visible after 18 hours with the naked eyes
- Develop for field use

DAT



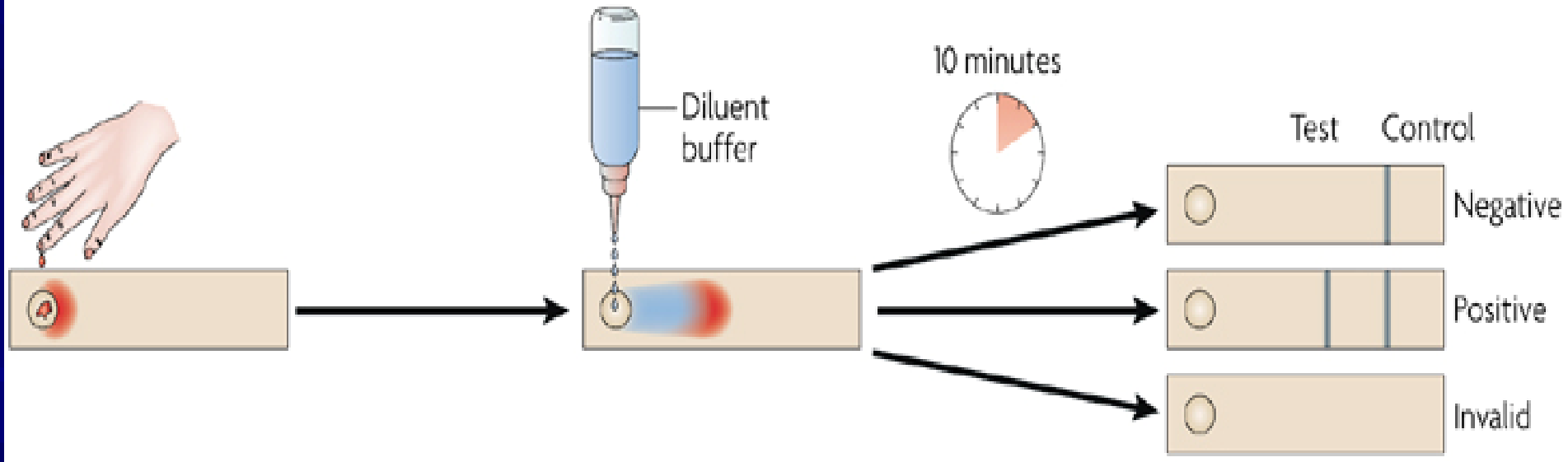
Limitation of use: Agglutinating antibodies may persist for a long time after treatment. The DAT does not discriminate between current and past infection.

rK39

- Recombinant kinesin-related protein of 39 kDa called rK39
- a part of a kinesin-related protein in *L. chagasi* which is conserved within *L. donovani complex*
- antibody in cutaneous and mucocutaneous leishmaniasis can not be detected

rK39 : immunochromatographic test strip

- developed to dipstick



Evaluation of DAT and rK39 dipstick for visceral leishmaniasis

- The Meta analysis of 30 studies (1986-2004)*
- Similar diagnostic performance
  good → excellent
- Seem comparable
- Sensitivities
  - DAT 94.8% (95%CI, 92.7-96.4%)
  - rK39 dipstick 93.9% (95%CI, 87.7-97.1%)
- Specificities
  - DAT 85.9% (95%CI, 93.9-98.7%)
  - rK39 dipstick 90.6% (95%CI, 66.8-97.9%)

**Chappuis F et.al., BMJ 2006;333: 723*

rKRP42

- a promising new antigen
- part of an *L. donovani* kinesin-related protein
- rKRP42 having 39 more amino acids than rK39.

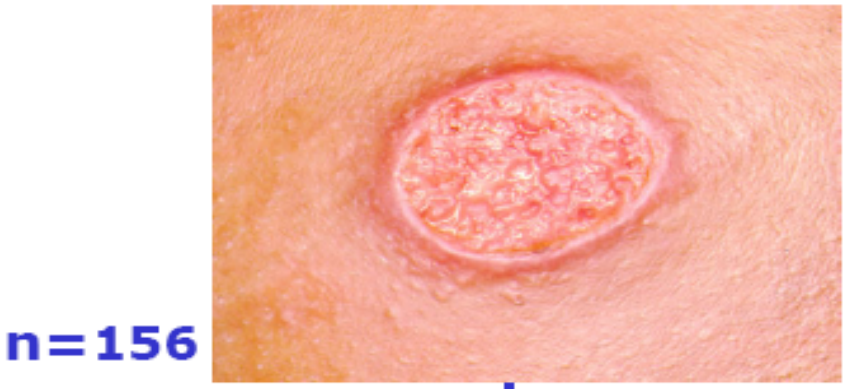
Test for the sensitivity and specificity

sensitivity	94.6% (70 /74 VL samples)
specificity	99.3% (148/149 negative samples)

- sensitivity of rK39 dipstick test was 93.2% (69 /74 VL patients).

Takagi H. et al., Am J Trop Med Hyg. 2007 May;76(5):902-5.

Leishmaniasis Diagnosis: Comparison of different conventional methods and PCR/Hybridization



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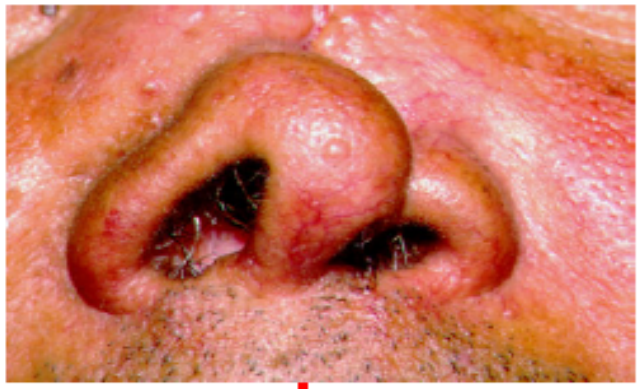

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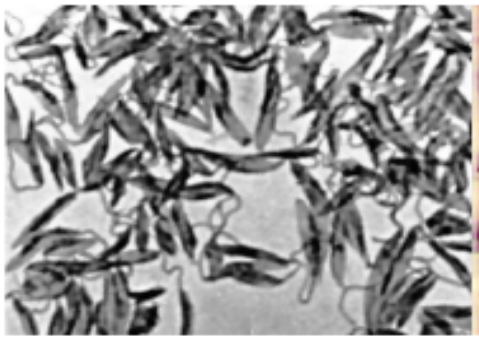

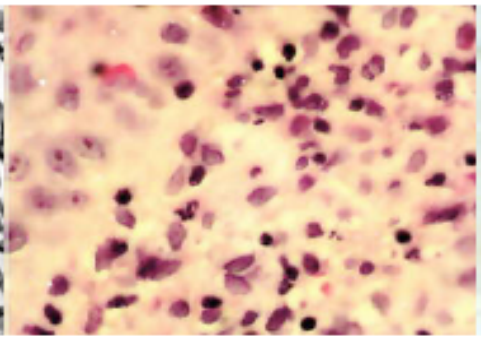

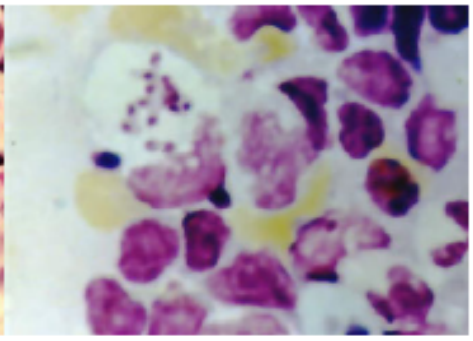

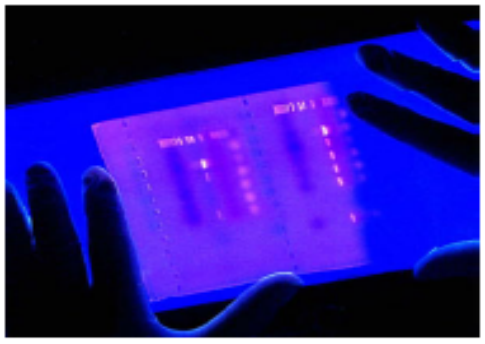

	Culture	HE	TP	PCR/Hyb
LCL	44%	50%	35%	94%
ML	10%	26%	0%	70%

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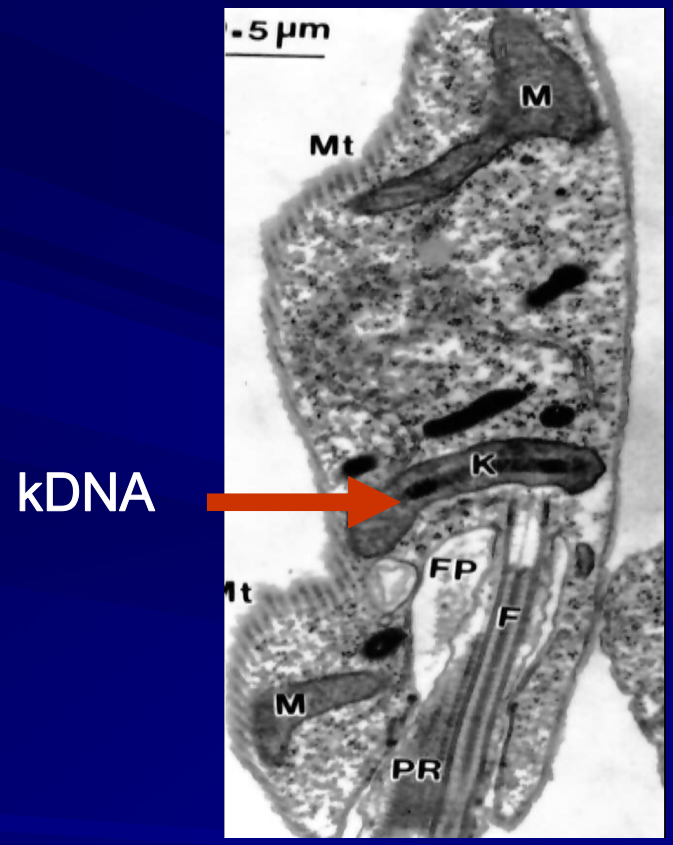
Ministry of Health, Rio de Janeiro, Brasil

PCR and Leishmaniasis

- Over 400 articles published on PCR and leishmaniasis since 1989
- High-copy-number target genes are chosen
  - rRNA genes
  - ITS1 region of rRNA genes
  - Kinetoplast DNA minicircles
  - Mini-exon genes
- Other genes
  - Gp63 genes
  - hsp70 genes
  - Cysteine proteinase genes

kDNA minicircle gene



- An excellent target for a sensitive and rapid detection method
- present at thousands of copies per cell
- show the highest sensitivity by PCR

PCR (kDNA gene) & Hybridization

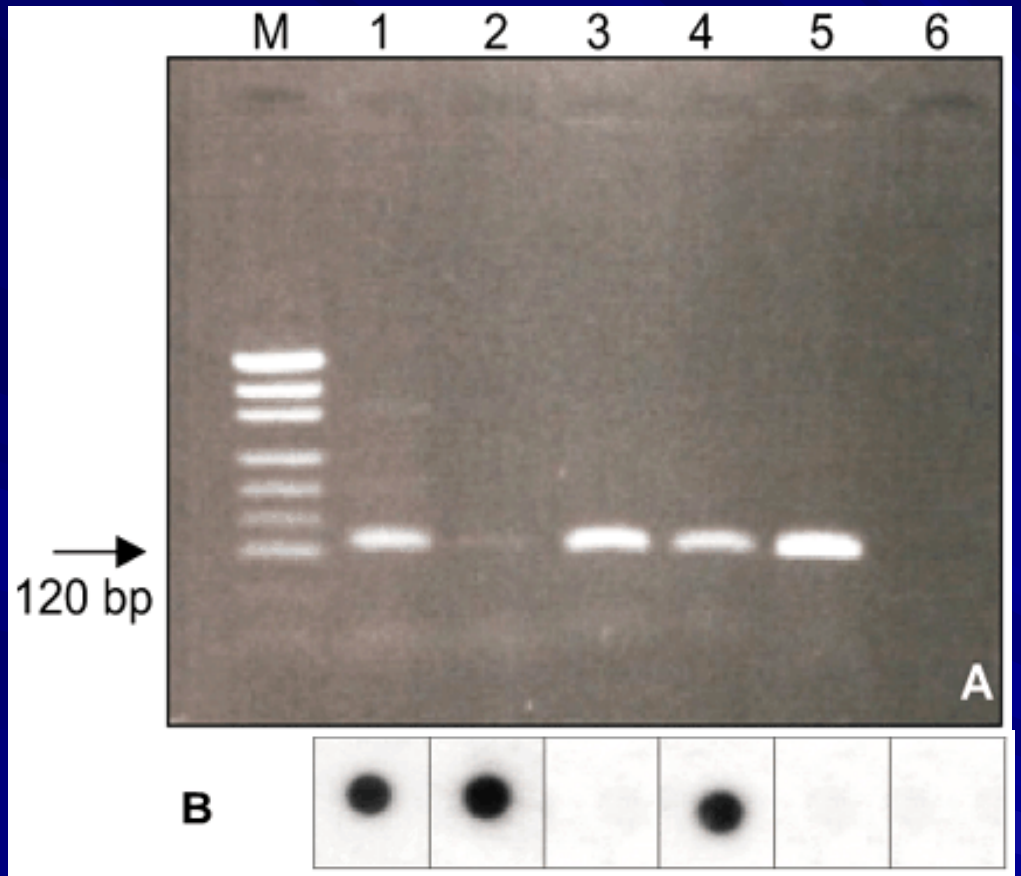
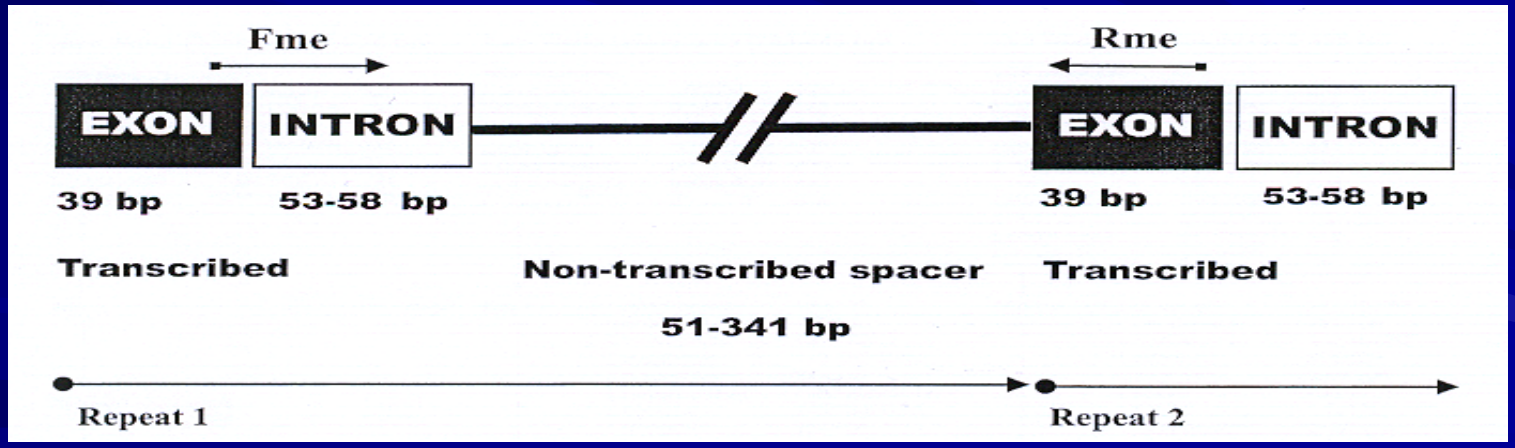


Fig. 2 - A. PCR products obtained with primers of the genus *Leishmania* visualized *after* 2% agarose gel electrophoresis stained with ethidium bromide. MW 50base-pair DNA ladder; lanes 1 and 2: bone marrow and blood from patient, lane 03: DNA of *L. chagasi*, lane 4: DNA of *L. braziliensis*, lane 5: DNA of *L. amazonensis* and lane 6: negative control. B. Hybridization with a cloned minicircle from *L. braziliensis*.

Mini-exon (spliced leader) gene repeat

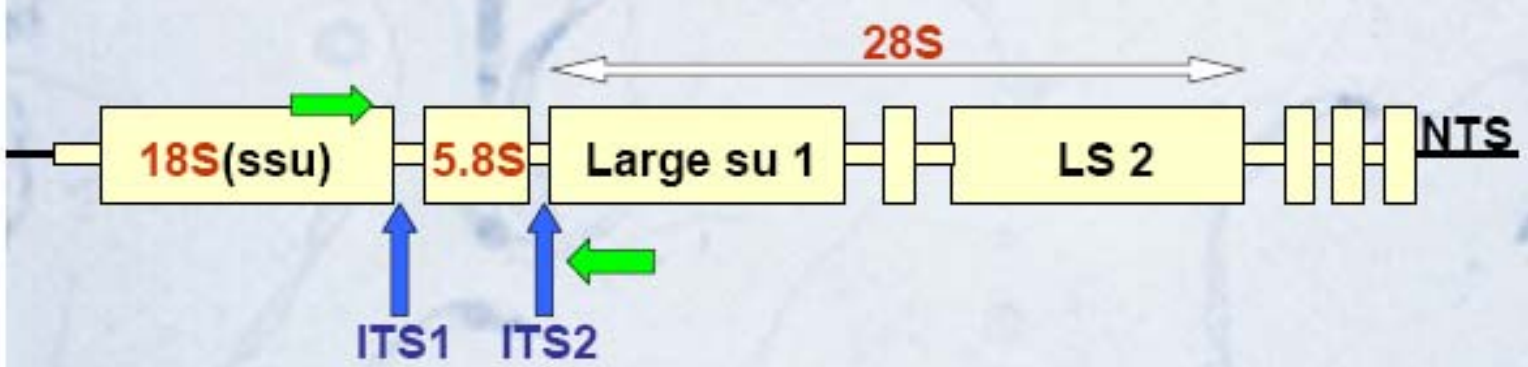
- Involve in the transsplicing process of nuclear mRNA
- Present 100-200 tandemly repeated copies per nuclear genome
- Sequence variation of non-transcribed spacer was observed between individual species



Marfurt J et. al., J Clin microbiol 2003; 41: 3147-3153.

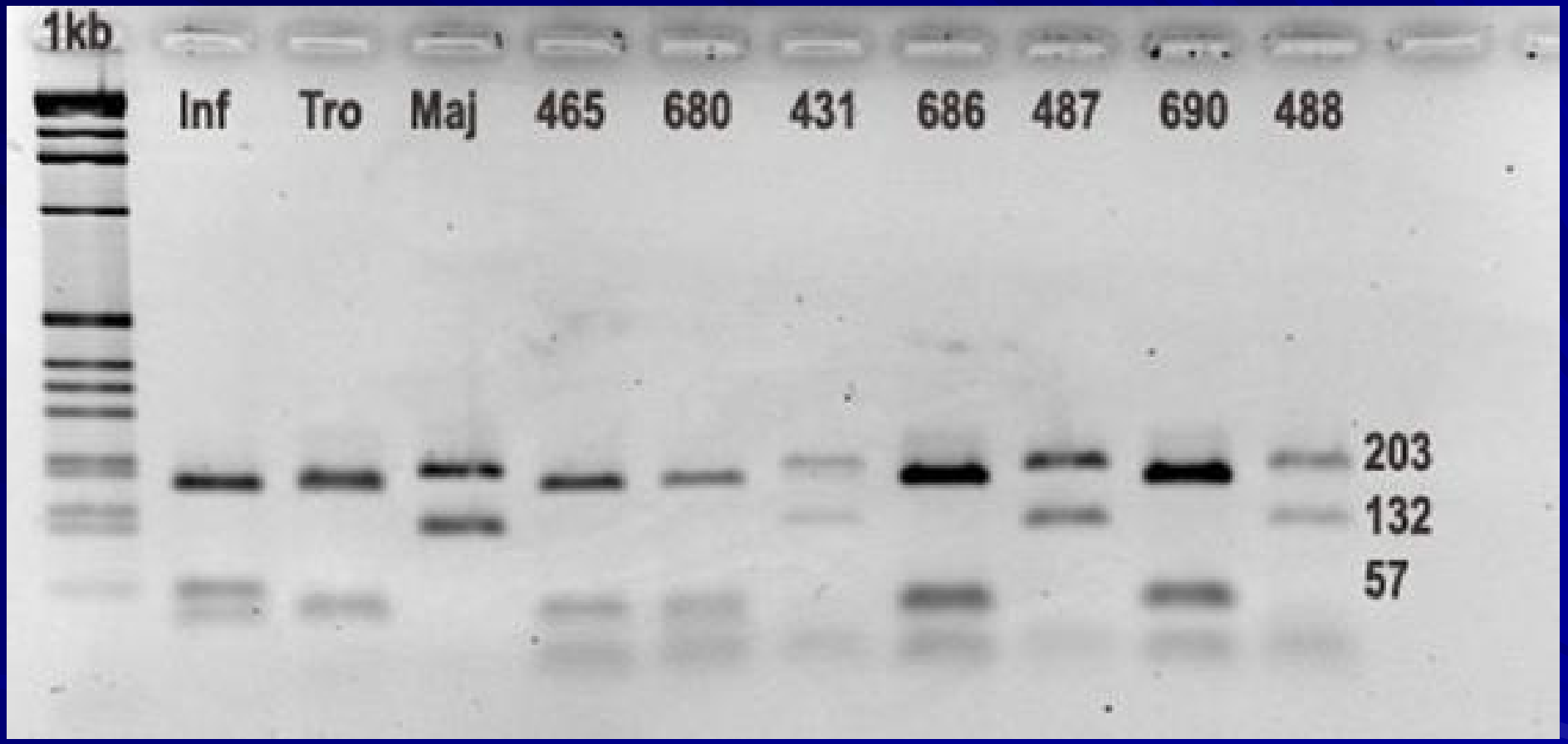
rRNA Gene : 16S-23S / Internal Transcribed Spacers

r RNA locus contains between the small sub-unit 18S and the large subunit, 2 variable sequences ITS 1 and ITS 2

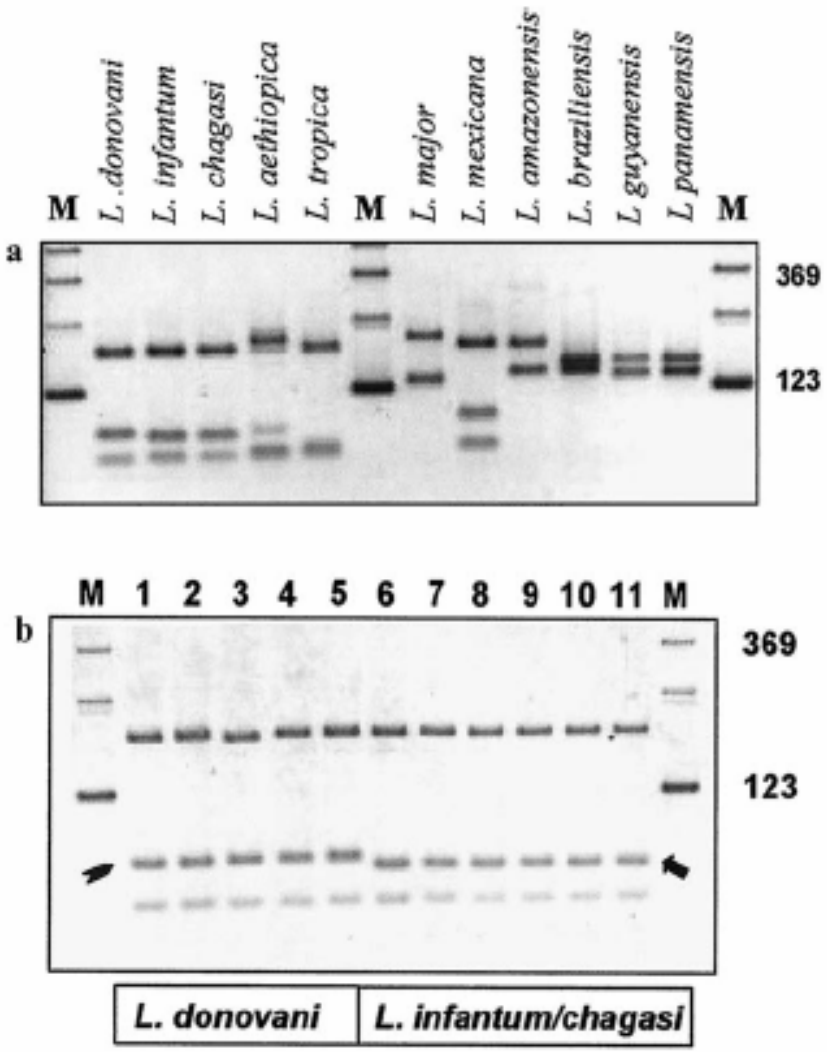


It was possible to amplify this region with 2 primers located between the Ssu and the Lsu

PCR-RFLP (ITS1)



Using 1 restriction enzyme, *Hae*III



ITS1 region of SSU rRNA genes

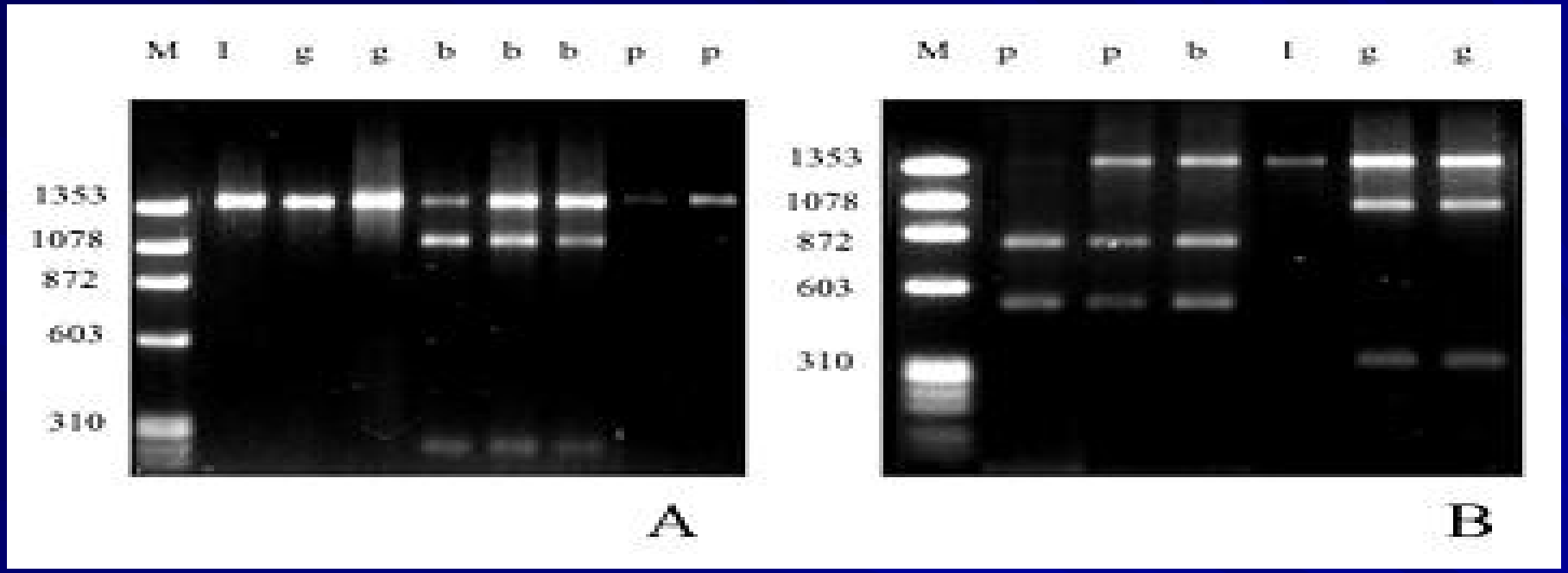
- RFLP using enzyme *Hae*III

* *Schonian G, Nasereddin A, Dinse N, Schweynoch C, Schallig HDFH, Presber W, et al. Diagn Microbiol Infect Dis. 2003;47:349-58.*

Gp63

- Promastigote surface protease (GP63 or leishmanolysin), one of *Leishmania* virulence factors
- During differentiation process, expression of certain genes occur that preadapt the parasites to enter and survive in macrophage phagolysosome.

PCR-RFPL profile of Gp63



- 2 restriction enzymes using *Sal*I (A) and *Bgl*I (B)

Leishmania (subgenus Viannia)

l = L. (V.) lainsoni; g = L. (V.) guyanensis; b = L. (V.) braziliensis;

p = L. (V.) peruviana M= Molecular Marker

Real-Time PCR

- *Compare the traditional leishmania diagnostic methods (culture and microscopy) versus RT-PCR in cutaneous leishmaniasis

Sensitivities

- RT-PCR 95% (95% CI; 90-98%)
- Culture 78% (95% CI; 99-86%, P=0.001)
- Microscopy 73% (95% CI; 63-81% P <0.0005)

Wortmann G et al., Am J Trop Med Hyg 2007; 76 (5): 906-908

Molecular Diagnosis for Parasitic Diseases

Diagnostic technique	Identification of the parasite: PCR	Culture	Direct search	Serology	
Simple	+	-	++	+/-	
Sensitive	+++	++	-	++	Accuracy in diagnosis
Specific	+++	+++	+++	++	
Fast	+++	-	++	+++	Early diagnosis
Cost efficient	+/-	-	+++	+/-	applicability

Leishmaniasis, Chagas disease, onchocercosis, toxoplasmosis, assymptomatic malaria

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Ministry of Health, Rio de Janeiro, Brasil

Comparison of 3 PCR assays for Diagnosis of Cutaneous Leishmaniasis

-kDNA gene (the highest sensitivity)	98.7% (77/78)
- ITS1of SSu r RNA gene	91.0% (71/78)
- Miniexon gene	53.8% (42/78)
- Microscopy	83.3% (65/78)

Bensoussan E. et al., J Clin Microbiol 2006; 44: 1435-1439.

Species identification

- Multilocus enzyme electrophoresis (Isoenzyme) analysis is the gold standard

Disadvantages

- requires parasite isolation and cultivation
- Time-consuming
- Require technical expertise

Species identification

- When parasite isolation and cultivation could not be available, a simplified and specific method for species diagnosis and identification of *Leishmania* is needed

- Using PCR, a minute amount of DNA is enough to amplify well-known marker genes by this technique.

Species identification

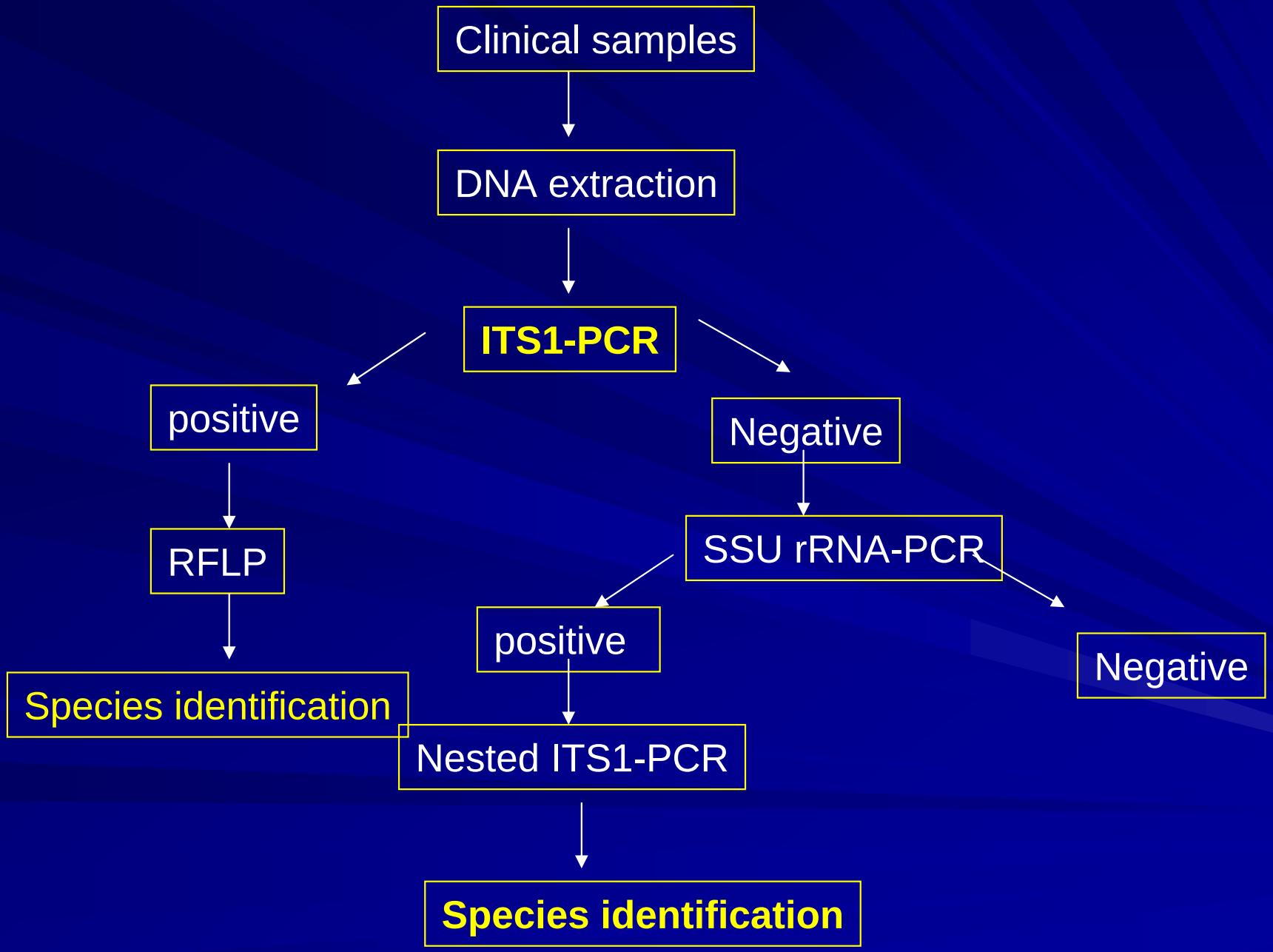
- **PCR-RFLP**
  - ITS1 of SSU rRNA gene; restriction enzyme *Hae III*
  - Mini-exon gene; restriction enzyme, *Eae I*
- **DNA sequencing of PCR products**

Rotureau B, etal., J Clin Microbiol. 2006;44:459–67.

Marfurt J. et al.,Diagn Microbiol Infect Dis. 2003;46:115-24.

Species identification (ITS1*)

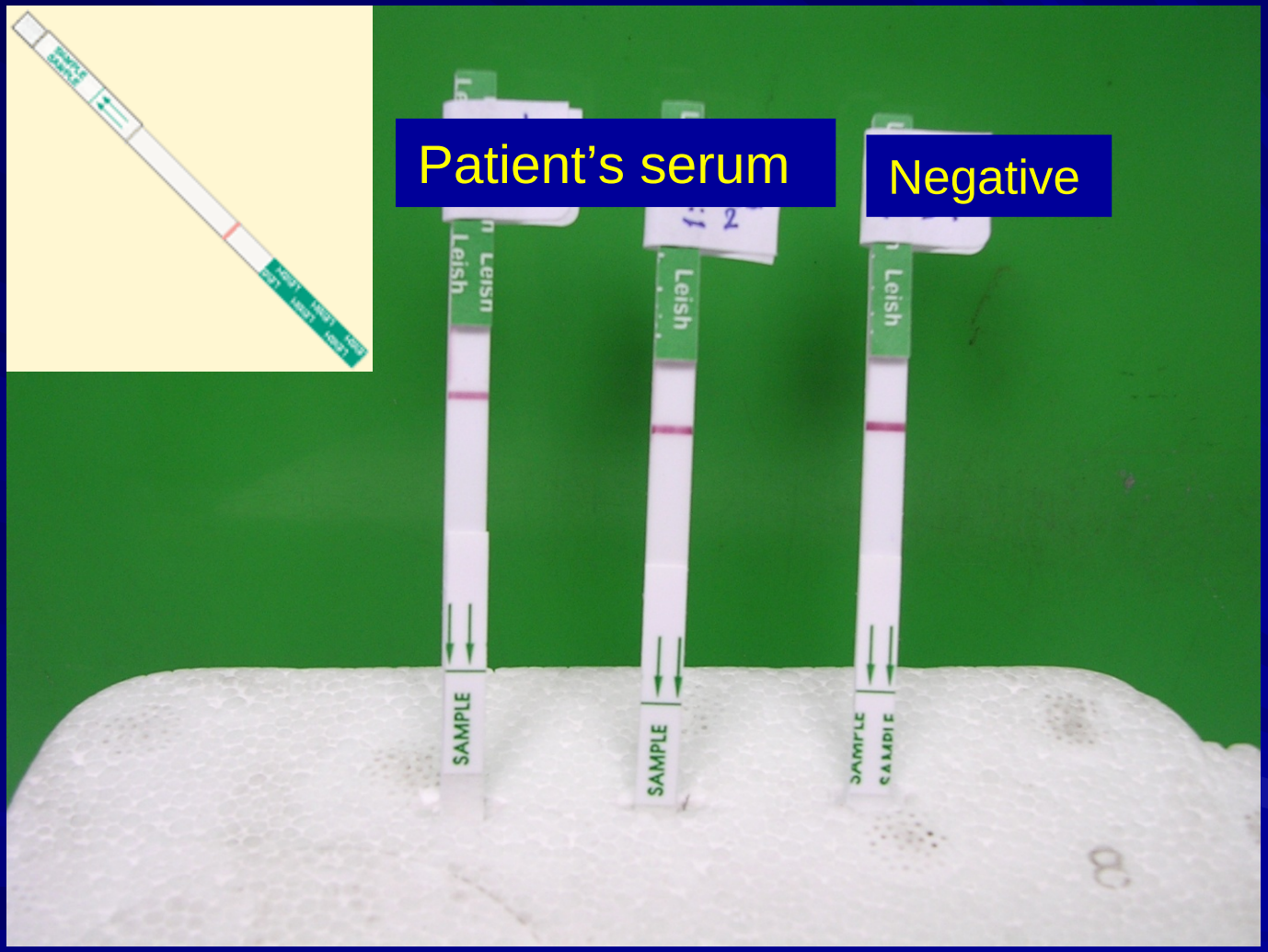
by Schonian G et al., Diag Microbiol Inf Dis 2003; 47: 349-358



An autochthonous Visceral leishmaniasis, Phangnga province

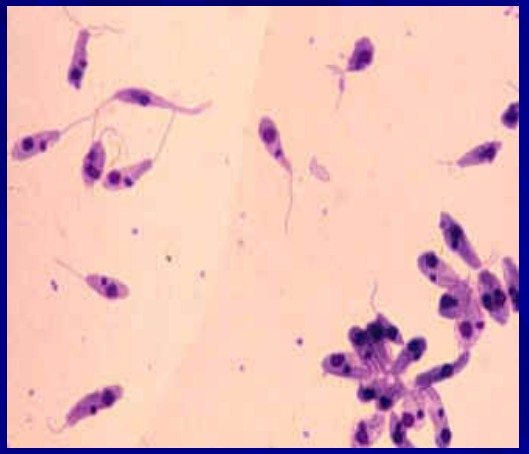
- No VL cases were previously reported in this province.
- Bone marrow smear : positive amastigotes
- DAT : positive, titer 1:200
- rk39 : negative

rk39 Strip Test

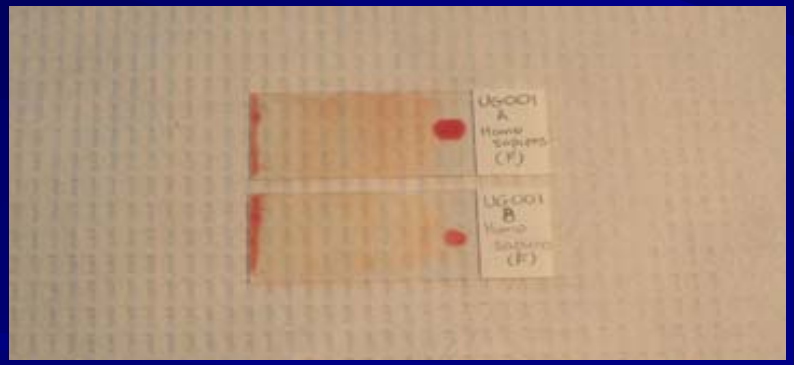


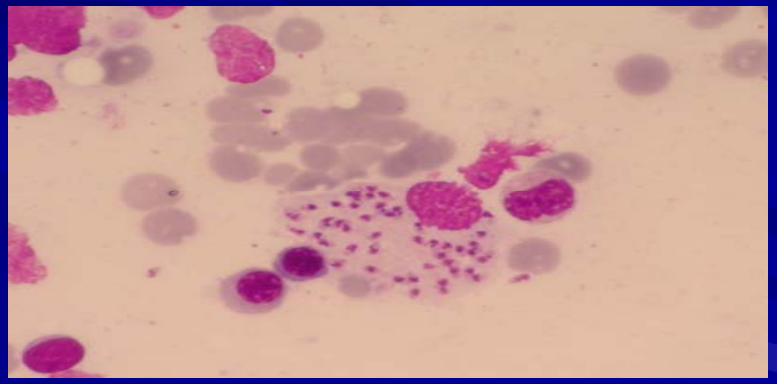
Samples

- Promastigotes from cultivation



- Giemsa-Stained bone marrow smears on glass slides

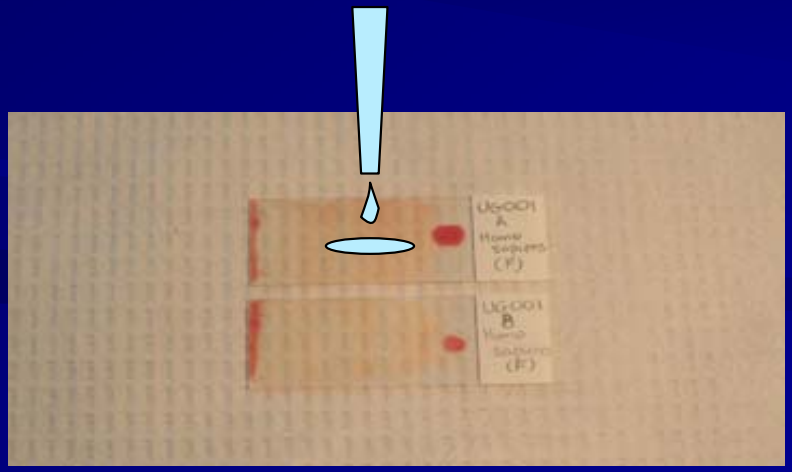


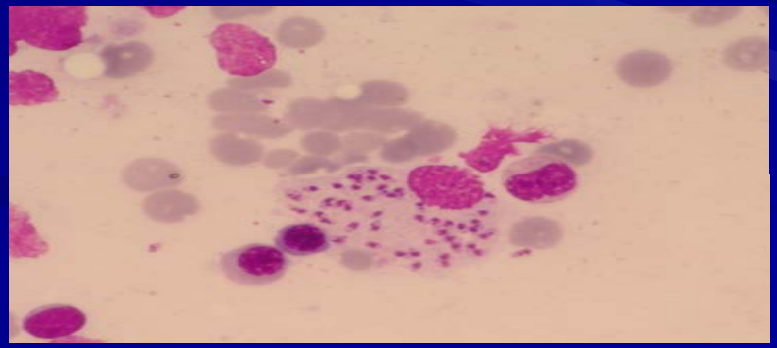


MATERIALS AND METHODS

1. Samples

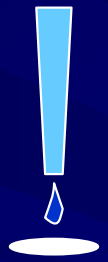
   - Giemsa-Stained bone marrow smears on glass slides





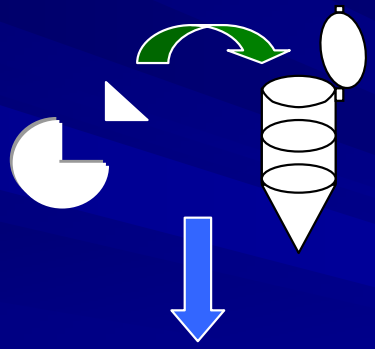
MATERIALS AND METHODS

2. DNA Extraction



14 μl of specimen was applied on to the FTA disk

Air dried

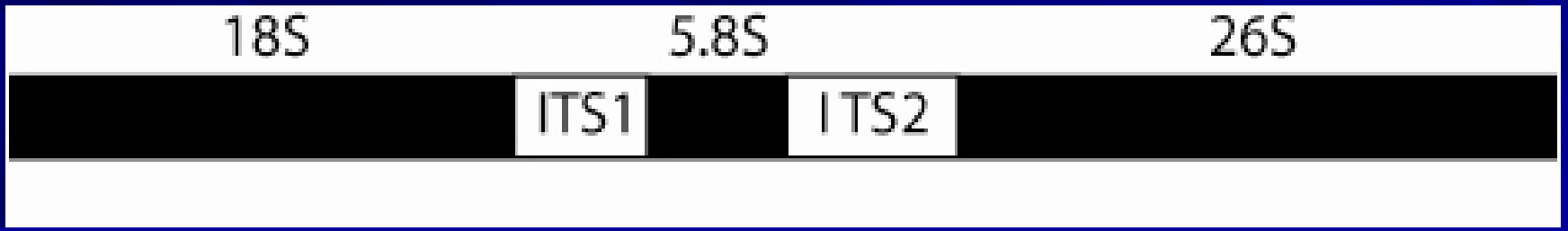


Washed twice with FTA purification reagent and washed twice again with TE buffer

Dried at 80°C for 2 hr.

MATERIALS AND METHODS

3. Amplification of SSU-rRNA / ITS1 region



SSU rRNA gene, primers

S4 : 5′-GAT CAA GCT GCA GGT TCA CC-3′

S12 : 5′-GGT TGA TTC CGT CAA CGG AC-3′

ITS1, primers

LITSR : 5′-CTG GAT CAT TTT CCG ATG -3′

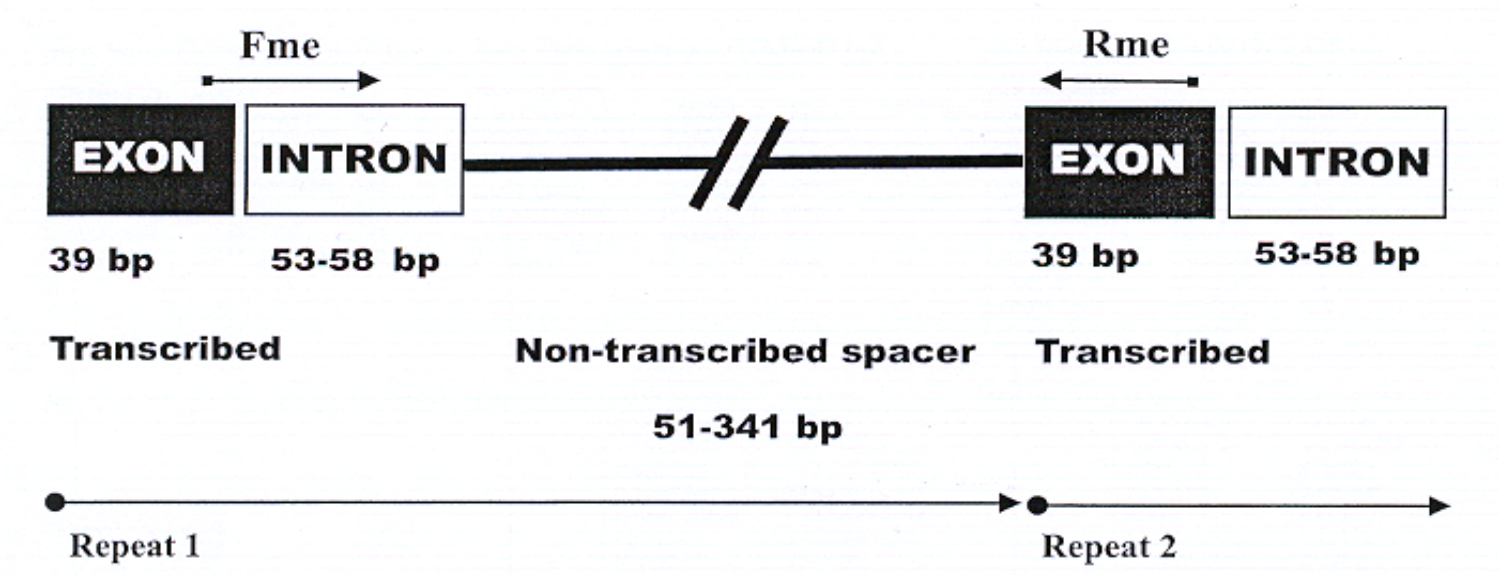
L5.8S : 5′-TGA TAC CAC TTA G TCG CAC TT-3′

MATERIALS AND METHODS

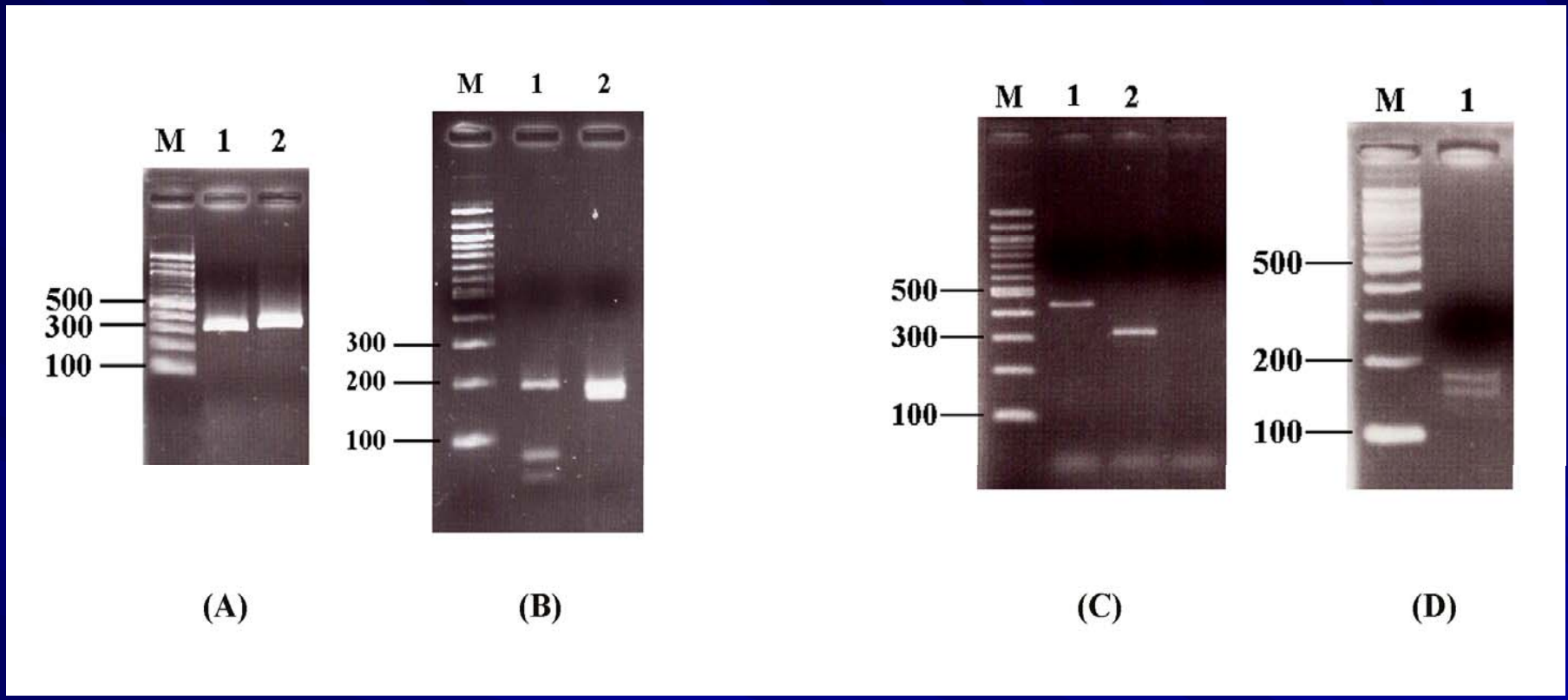
- Mini-exon gene amplification, primers

Fme : 5′-TAT TGG TAT GCG AAA CTT CCG -3′

Rme : 5′-ACA GAA ACT GAT ACT TAT ATA GCG -3′



Marfurt J, et al., J Clin Microbiol. 2003;41:3147-53.



1 = positive control *L. donovani*;

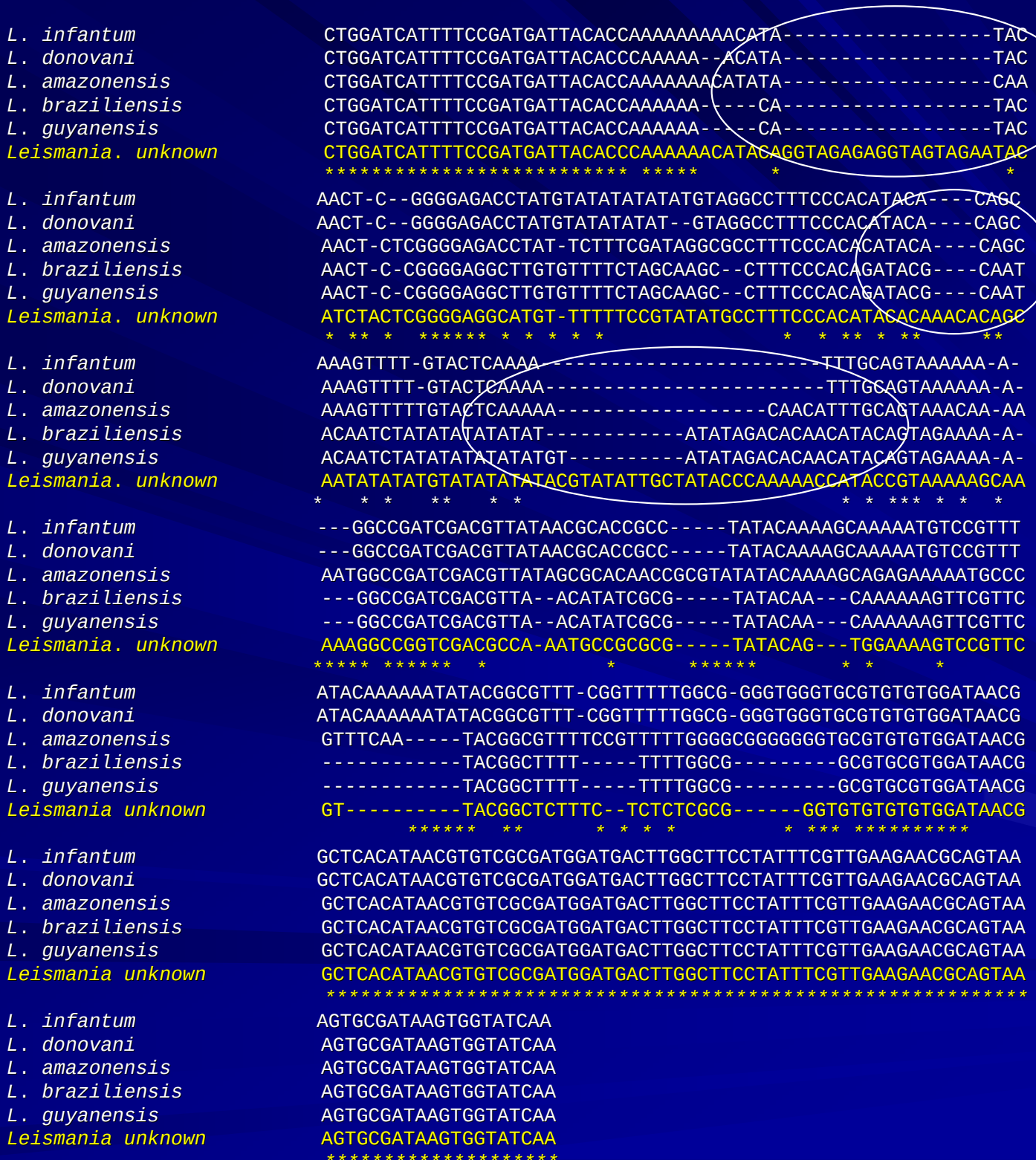
2 = *Leishmania* unknown

A) PCR amplification of ITS1 region of SSU-RNA gene

B) RFLP patterns of ITS1 region of SSU-RNA gene digest with *Hae*III

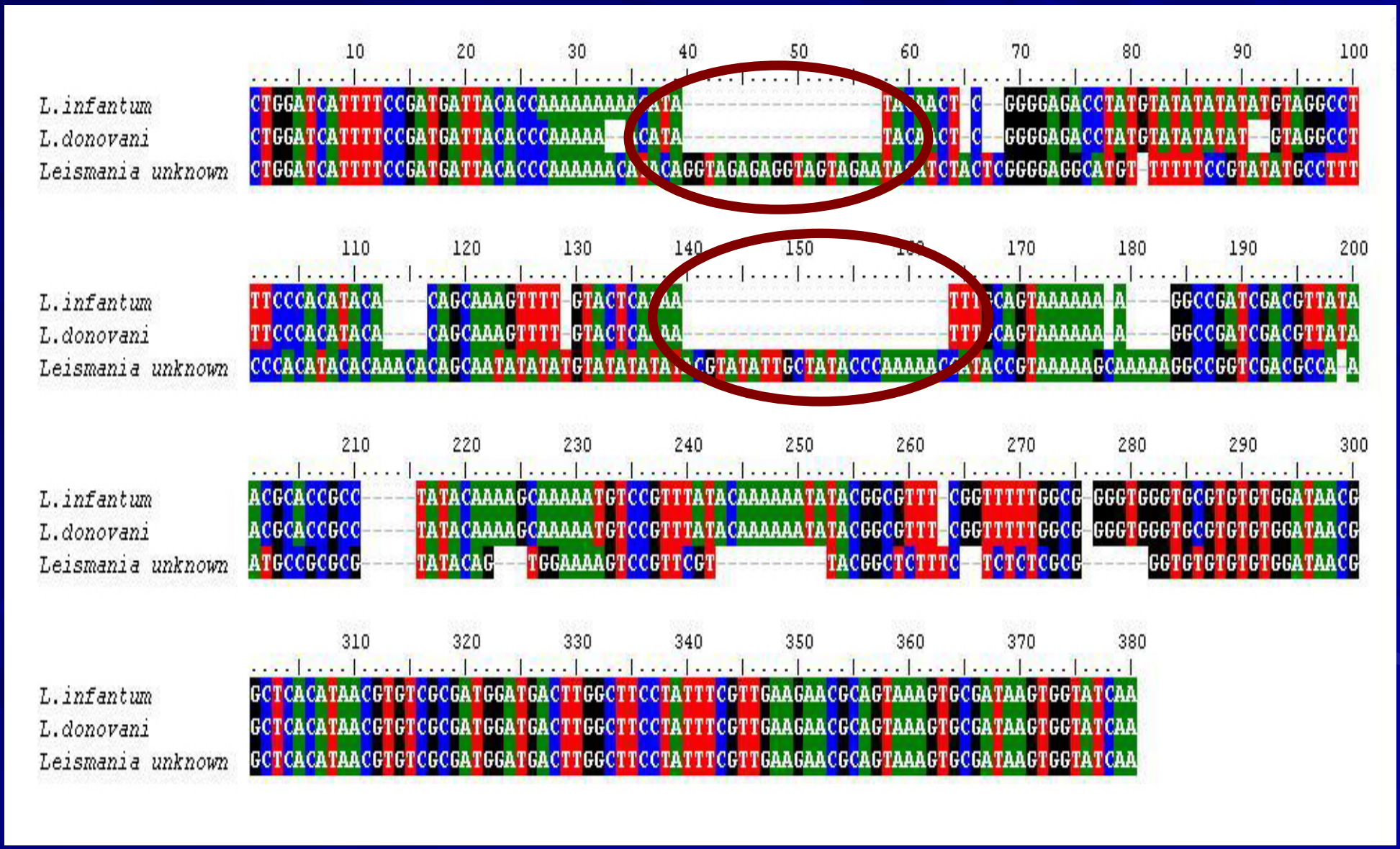
C) PCR amplification of mini-exon region of SSU-RNA gene

D) RFLP patterns of mini-exon gene of *Leishmania* unknown with *Eae*I.

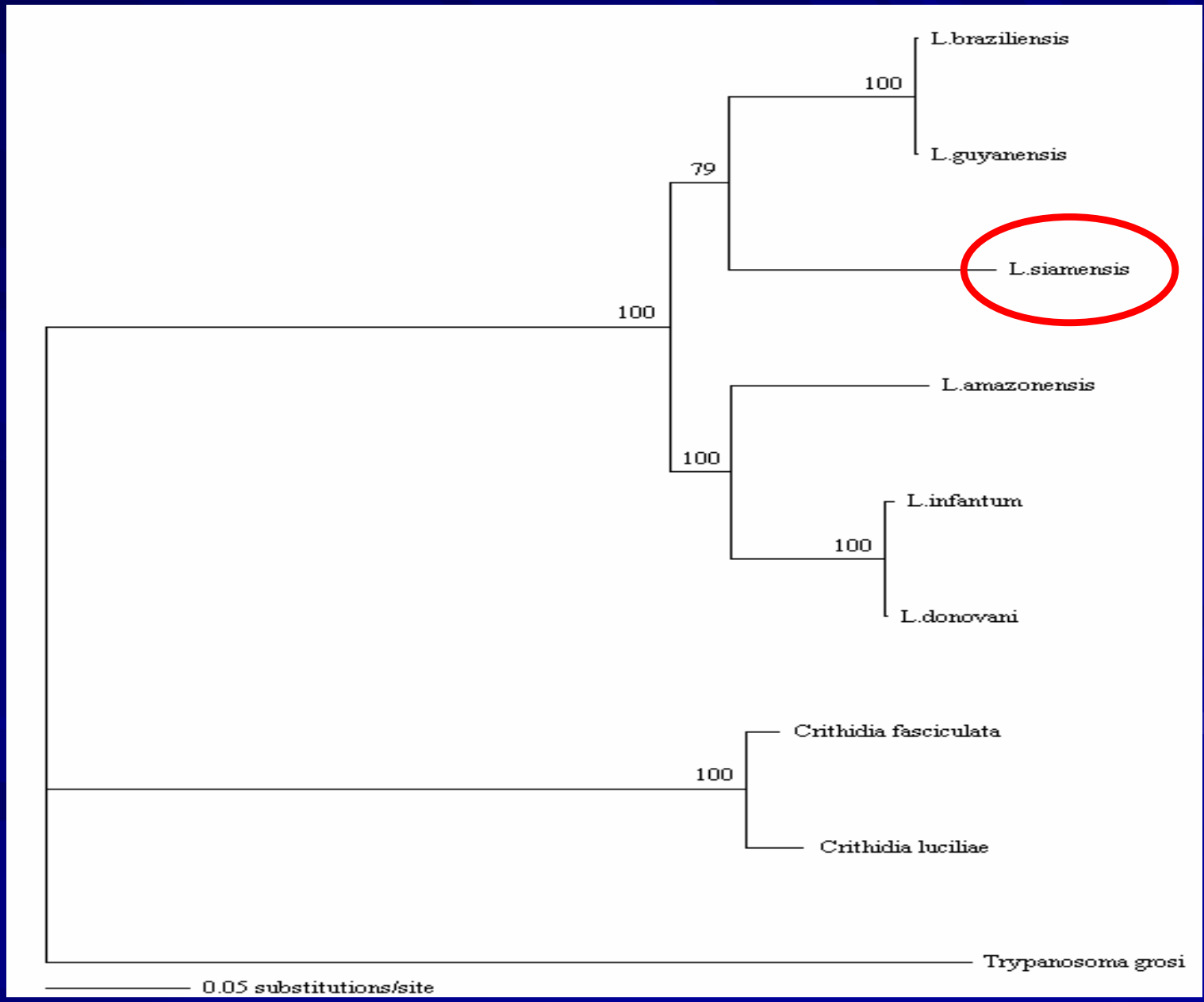


Neighbor-joining based on ITS1 region sequence analysis containing 6 species.

Alignment of ITS1 region



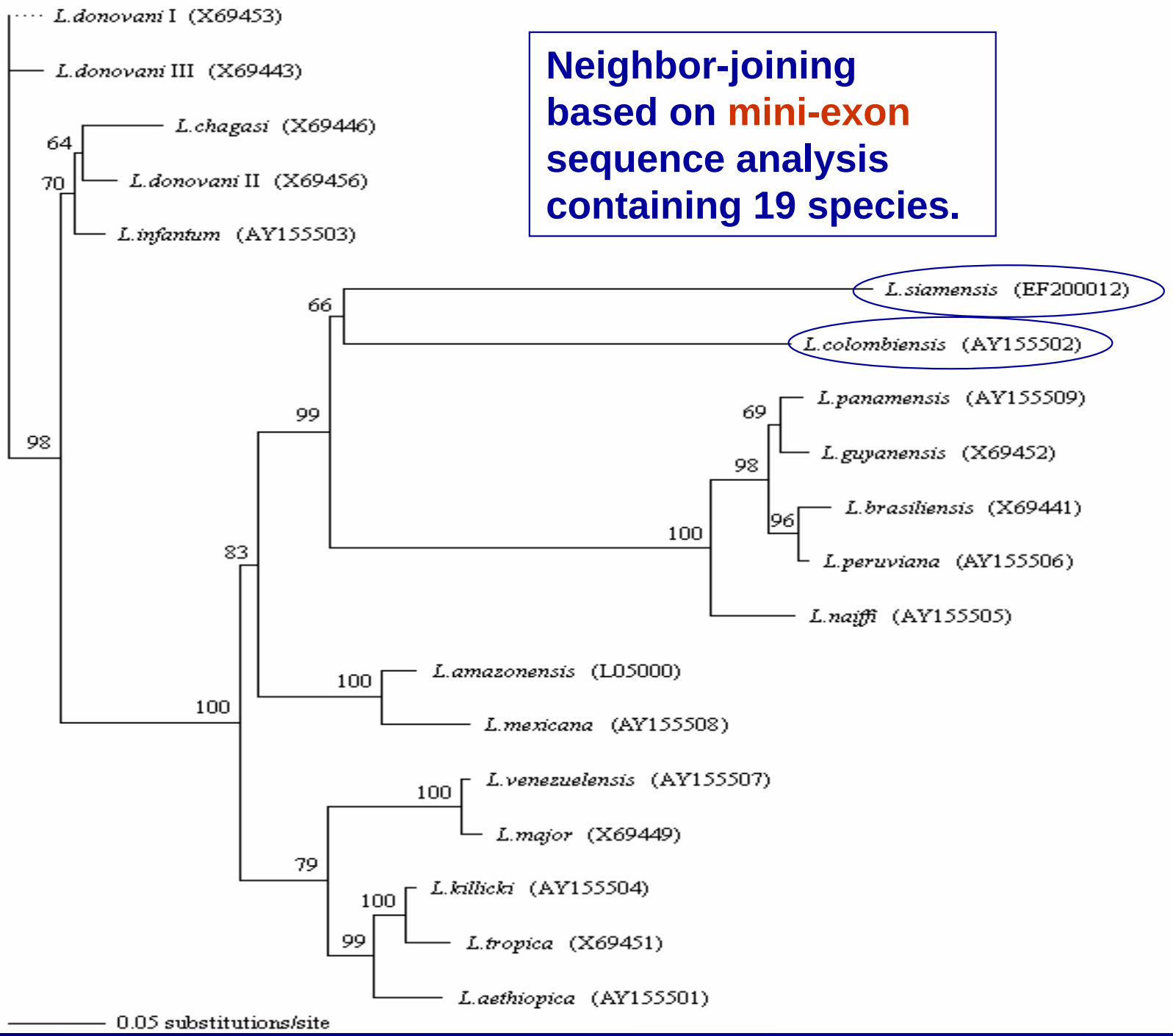
Phylogenetic tree, ITS1



Percent identity of ITS1 sequences comparison between unknown *Leismania*, and other species

Pair-wise comparison	% Identity of ITS1
L. braziliensis vs. *Leismania* unknown	83
L. guyanensis vs. *Leismania* unknown	84
L. amazonensis vs. *Leismania* unknown	74
L. infantum vs. *Leismania* unknown	77
L. donovani vs. *Leismania* unknown	78
L. braziliensis vs. *L. guyanensis*	100
L. braziliensis vs. *L. amazonensis*	79
L. braziliensis vs. *L. infantum*	82
L. braziliensis vs. *L. donovani*	81
L. guyanensis vs. *L. amazonensis*	79
L. guyanensis vs *L. infantum*	81
L. guyanensis vs *L. donovani*	81
L. amazonensis vs. *L. infantum*	90
L. amazonensis vs. *L. donovani*	90
L. infantum vs. *L. donovani*	99

Neighbor-joining based on mini-exon sequence analysis containing 19 species.



Alignment of the 320 basepairs from the sequenced PCR products of mini-exon gene of *L. siamensis* (EF200012) and *L. colombiensis* (AY155502).

```
L.colombiensis      TTTTGGAAGCGCGCAAGCGCTACATTTTTTTTTTTTG---TCATGTGCGGG------TGCG
Leishmania Unknown  TTTTGG-AGCGCGCCAGCGCTCTCTTTTTTTTTTTTG-GTGC-TGCGCGTG------TGCA
                    ****** ******* ******   ************    * ** *** *      ***

L.colombiensis      CGCCGCCCCTCCTCCACTGGGGGGGGCTTCCCACACGCGCTGTGGTCCCTTTTCCCCTTG
Leishmania Unknown  CGCCCACGCGCCGGCCCCCTCCACGGCGGGTGCGGCGCCCGGCTGCTGCTCGACGGC---
                    ****  * * **  *    *       ***     *** *        **   *  *

L.colombiensis      ----CTGCTGGGAGGGGGG-GGGGCGCGCCGCTGCGGGTGGTTTCCTCCTGTCGGCGTCC
Leishmania Unknown  ----------CGCCGCT----------------------GCCCCTCACGCCCGGCG-CC
                             *  *                          *       ***** **

L.colombiensis      CCTCGGGCCTGCCCGGCAT-CCTTGCTGGGGGCTCTGCCGGCGCGATTCCGGCACCCCAT
Leishmania Unknown  CGTCCGGCGGG-CACGCGCCGCCC-CAGCCG-----GCCCCCG-----GCAGTCCGCCAT
                    * ******  * *  **    *   * *  *     ***  **      * *  * ****

L.colombiensis      GCCCAGTGGCCGGGACCCCCCCCTCTGGTCCCCCCCCATGATCCGGCGCACGCCTGACAC
Leishmania Unknown  GACGCGCG-CCTCGGCGCAGCCAC---------------------------------C
                    * *  * * **  * * *  **

*
L.colombiensis      GCGGTTAGTGCA-TGCCAAATGAGCCACCC
Leishmania Unknown  ACCGCCACCCCCCAAACG-GCGCGCCGGCT
                     *    *   *    *    * ***  *
```

54% consensus identity.

*A novel species of *Leishmania*, ***Leishmania siamensis, sp. nov.***

Conclusion

- DAT and rK39 dipstick show similar diagnostic performance for serological studies in the field.
- The high sensitivity and ease of performance make both DAT and rK39 dipstick very suitable for surveillance surveys. However it is essential to evaluate any rapid diagnostic tests in the region in which it will be used.
- PCR-based protocols have increased the speed and sensitivities of species-specific leishmaniasis diagnosis.
- A novel species of *Leishmania* caused visceral leishmaniasis was identified in 1 autochthonous Thai patient, southern Thailand.
- Efforts should be made PCR platforms more user-friendly and cost-effective for epidemiological studies.

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