INTERLABORATORY COMPARISON of ***Leishmania*-PCR METHODS:** towards a common protocol to be used within the **RAPSODI** consortium (European Commission **FP7**)

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RAPSODI (www.fp7_rapsodi.eu) is an EC granted research project integrated by partners from *Leishmania*-endemic countries in four continents. The aim of the project is to develop a human vaccine candidate against leishmaniasis and all the associated procedures required for the subsequent clinical trials. The use of common protocols will allow the comparison of results among the different partners. A common PCR method would be desirable to assess: i) the parasitic burden on asymptomatic and symptomatic individuals, ii) the changes in the parasitic load defining the infection outcome evolution, iii) the monitoring of the vaccine efficacy at the parasitological level, iv) the causative species of leishmaniasis in different settings. And this in the different scenarios of the project: i) Peru, cutaneous (CL) and mucosal leishmaniasis (ML), ii) Tunis, CL and visceral leishmaniasis (VL), iii) India, PKDL and VL, and iv) France and Spain, VL. For this purpose, we settled up a workshop on which a panel of samples including DNA from different *Leishmania* species, *Trypanosoma cruzi*, and DNA from human specimens (patients with different clinical conditions -CL, ML, VL-, and healthy individuals) was assessed.

WORKSHOP SAMPLES (DNA)

VISCERAL LEISHMANIASIS

N=37 samples PERIPHERAL BLOOD / BONE MARROW

COUNTRY	N° OF SAMPLES
INDIA	6
TUNIS	7
SPAIN	24

TEGUMENTARY LEISHMANIASIS

N=27 samples MUCOSAL / SKIN

COUNTRY	N° OF SAMPLES
PERU	9
TUNIS	18

HEALTHY SUBJECTS

N=13 samples PERIPHERAL BLOOD

COUNTRY	N° OF SAMPLES
INDIA	2
TUNIS	11

PARASITE STRAINS

N=9 samples CULTURE

PARASITE	N° OF SAMPLES
Leishmania species	8
Trypanosoma cruzi	1

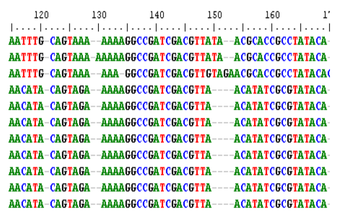
TOTAL N=86 samples

PREPARATORY PHASE (STEPS)

1st- DRIED AND SENT TO REFERENCE LABORATORY

2nd- ELUTION ACCORDING TO PREVIOUS DNA EXTRACTION

3rd- ITS-1 REGION AMPLIFICATION AND **SEQUENCING**



4th- SPECIES IDENTIFICATION AND ASSIGNMENT OF **GENOTYPES**

5th- POOLING ACCORDING TO GENOTYPE

21 DNA POOLS

4 ALIQUOTS EACH

BLIND CODED, DRIED

DISTRIBUTED TO EACH PARTNER

TEST SAMPLES (POOLS)

VISCERAL LEISHMANIASIS **(6 POOLS)**

4 POOLS SPANISH PATIENTS	***L. infantum***
1 POOL TUNISIAN PATIENTS	***L. infantum***
1 POOL INDIAN PATIENTS	***L. donovani***

TEGUMENTARY LEISHMANIASIS **(3 POOLS)**

1 POOL PERUVIAN PATIENTS	***L. guyanensis***
1 POOL TUNISIAN PATIENTS	***L. major***
1 POOL TUNISIAN PATIENTS	***L. infantum***

HEALTHY SUBJECTS **(3 POOLS)**

2 POOLS TUNISIAN SUBJECTS	**PCR negative**
1 POOL INDIAN SUBJECTS	**PCR negative**

PARASITE STRAINS **(9 POOLS)**

L. Infantum	**MHOM/FR/78/LEM-75**
L. donovani	**MHOM/IN/80/DD8**
L. major	**MHOM/SU/73/5-AsKH**
L. tropica	**MHOM/SU/74/K27**
L. aethiopica	**MHOM/ET/72/L100**
L. amazonensis	**IFLA/BR/67/PH8**
L. braziliensis	**MHOM/BR/75/M2904**
Trypanosoma cruzi	**CL2**

PCR METHODS TESTED

	CONVENTIONAL	REAL-TIME
GROUP 1	**SSUrRNA nested♣**	**SSUrRNA♣ SYBRGreenI**
	ITS-1§	**kDNA (120 bp)* SYBRGreenI**
	kDNA (120 bp)*	
GROUP 2		**kDNA (120bp)* SYBRGreenI**
		kDNA (120bp)* TaqMan
GROUP 3	**ITS-1§**	**kDNA (120bp)* SYBRGreenI**
GROUP 4	**ITS-1§**	**kDNA (70bp)****
	HSP70 PCR-RFLP¥	
	CPB PCR-RFLP¥	
	MPI PCR¥	

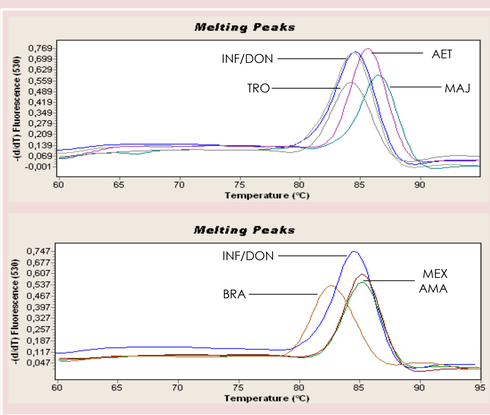
* Mary *et al J Clin Microbiol* 2004
** López *et al Am J Trop Med Hyg* 1993
§ Schönian *et al Diagn Microbiol Infect Dis* 2003
¥ Boggild *et al Clin Infect Dis* 2010
♣ Cruz *et al Trans R Soc Trop Med Hyg* 2002

RESULTS SPECIES IDENTIFICATION

The three groups applying **ITS-1 PCR + RFLP** were able to properly identify the culture samples and all but one clinical samples. The pool containing DNA from CL lesions due to *L. infantum* (P21) was positive and identified by one group only. Cause: Low DNA amount

***SSUrRNA* nested PCR** detected all positive samples but P21. DNA sequencing of the PCR products allowed identificatioin to the complex level: i) *L. donovani* complex, ii) *L. tropica* + *L. aethiopica* + *L. major*, iii) *L. mexicana* complex, and iv) *Viannia* subgenus.

Combination of **MPI**, **CPB** and **HSP70** targets analysis was critical to discriminate within the *Viannia* subgenus.



Melting Peak Analysis of **SSUrRNA** RT-PCR (**SYBRGreenI**) helps, but do not fully resolve, in species identification

RESULTS SENSITIVITY (on five 10-fold dilutions of each test sample)

Amplification of the kDNA (120 bp)* target in Real Time PCR format (either *SYBRGreen I* or *TaqMan*) returned the highest sensitivity to detect the *L. donovani* complex, 1-2 logs for each pool.

For the other species in the *Leishmania* subgenus, there were no major differences in the level of detection for kDNA (120 bp)*, *SSUrRNA* and ITS-1 PCRs in conventional formats *versus* SSUrRNA and kDNA (120 bp)* in any of the Real Time formats (though generally in favour of Real Time and nested formats, ± 1 log).

For the *Viannia* subgenus, kDNA (70 bp)** target in Real Time PCR format (*SYBRGreen I*) presented the highest sensitivity, 2 logs more sensitive in clinical specimens. Though MPI, CPB and HSP70 PCR methods are highly resolutive for species identification, their sensitivity is generally 1-2 logs lower than that of ITS-1 PCR

CONCLUSION

- We take the methodology employed for sample preparation, pre-analysis and shipment as a proof of concept for further inter-laboratorys studies.
- Though this study is partially biased to samples from the *Leishmania donovani* complex. It can be concluded that kDNA is a highly sensitive target to be used in Real Time PCR applications. However, care must be taken in the choice of primers, as they could affect the sensitivity to detect different species.