Heat shock protein-70 polymorphism characterizes *Leishmania donovani* complex strains from Mediterranean area.

T DI MUCCIO[1], D TONANZI[2], G LA ROSA[2], M GRAMICCIA[1].

[1]Vector-Borne Diseases Unit, Infectious Diseases Department, Istituto Superiore di Sanità, Rome, Italy. [2]European Union Reference Laboratory for Parasites, Infectious Diseases Department, Istituto Superiore di Sanità, Rome, Italy.

INTRODUCTION. *Leishmania donovani* complex (cx) includes *L.donovani* sp. and *L.infantum* sp., the aetiological agents of visceral (VL) and cutaneous (CL) leishmaniasis, species very close but characterized by different ecology and clinic. *L.infantum* is the main species causing leishmaniasis in Mediterranean area where it shows wide genetic polymorphism. Over the past few decades, several molecular targets have been proposed for *Leishmania* diagnosis and species typing, but there are not international guidelines so far. We analysed a large and geographically representative set of *L.donovani* cx strains by Hsp-70 gene, according to suggestion of LeishMan European consortium strategy for validation of this locus for typing (van der Awera et al, 2016 Euro Surveill. 21:49).

MATERIALS AND METHODS. Eighty-nine *L.infantum* strains (15 zymodemes, ZMONs) from Mediterranean area and five *L.donovani* strains (3 ZMONs) from Africa and India were submitted to Hsp-70 PCR-RFLP (*Mlu*I) assay and sequencing and the results were compared with those obtained by ITS and cpB assays already widely recognized as molecular tools for species identification.

RESULTS AND DISCUSSION. The results obtained by Hsp-70, ITS and cpB assays were in agreement in distinguishing the *L.infantum* from *L.donovani* spp. However, 12 out 25 *L.infantum* MON24 strains showed an unexpected polymorphism at positions shown in Fig.1. The G:A polymorphism switched on/off a diagnostic restriction *Mlu*I site useful in discriminating between *L.donovani* and *L.infantum* spp.

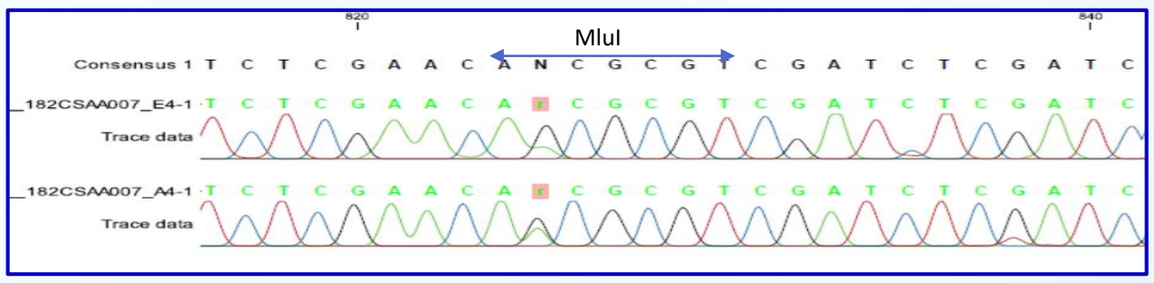

Fig.1. Electropherogram analysis. *L.infantum* MON24 CL Hsp-70 sequences showing the species-specific polimorphic site.

Consequently, the strains carrying this polymorphism showed an unusual *Mlu*I restriction pattern composed of 4 fragments common to *L.infantum* and *L.donovani* spp. The "mixed" pattern is shown in Fig.2.

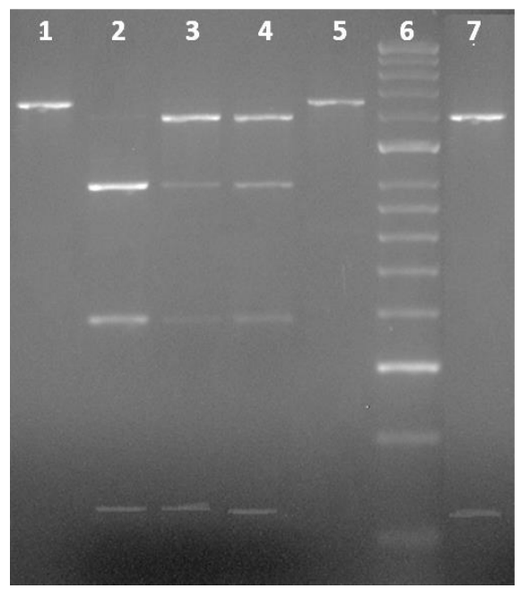

Fig 2. Hsp-70 PCR-RFLP (*Mlu*I) of *L.infantum* ZMON24 strains and *L.donovani* sp. Fragments size on the right.
Lane 1: *L.infantum* ZMON24 VL (ISS815) undigested
Lane 2: *L.infantum* ZMON24 VL (ISS815) pattern
Lanes 3,4: *L.infantum* ZMON24 CL (ISS416, 417) 'mixed' pattern
Lane 5: *L.infantum* ZMON24 CL (ISS416) undigested
Lane 6: 100 bp marker;
Lane 7: *L.donovani* sp. pattern

The Hsp-70 analysis grouped *L. infantum* ZMON24 strains in two clusters which mainly correlated with clinical features rather than geographical origin (Tab.1). *L.infantum* ZMON24 VL strains were similar to *L.infantum sensu stricto*, whereas ZMON24 CL strains showed hybrid molecular traits *L.infantum/L.donovani*.

This result was supported by cpB analysis, that showed in these "hybrid" strains both cpB gene copies, cpBE (*L.infantum* specific, 703 bp) and cpBF (*L.donovani* specific, 741 bp) (Fig.3 and Tab.1).

Tab.1 *L.infantum* ZMON24 strains analyzed in this study.

ISS code	ZMON	ITS Genotype	Hsp-70 PCR-RFLP (*Mlu*I)	*cpb*	Clinical	Origin
ISS176	24	A	4 bands	E F	CL	CZ, Calabria, Italy
ISS835	24	A	4 bands	E F	CL	SS, Sardinia, Italy
ISS319	24	B	4 bands	E F	CL	SS, Sardinia, Italy
ISS100	24	A	4 bands	E F	CL	PE, Abruzzo, Italy
ISS79	24	A	4 bands	E F	CL	PE, Abruzzo, Italy
ISS98	24	B	4 bands	E F	CL	PE, Abruzzo, Italy
ISS99	24	A	4 bands	E F	CL	TE, Abruzzo, Italy
ISS101	24	A	4 bands	E F	CL	TE, Abruzzo, Italy
ISS320	24	A	4 bands	E F	CL	TE, Abruzzo, Italy
ISS417	24	A	4 bands	E F	CL, VL, HIV	MC, Marche, Italy
ISS182	24	B	4 bands	E F	CL	Algeria, Africa
ISS416	24	A	4 bands	E F	CL	Tunis, Tunisia, Africa
ISS493	24	A	3 bands	E	CL	CA, Sardinia, Italy
ISS2492	24	A	3 bands	E	CL	Sicily, Italy
ISS913	24	Lombardi	3 bands	E	CL	Spain
ISS815	24	A	3 bands	E	VL, HIV	CS, Calabria, Italy
ISS1889	24	A	3 bands	E	VL, HIV	CA, Sardinia, Italy
ISS1740	24	A	3 bands	E	VL, HIV	CT, Sicily, Italy
ISS1739	24	A	3 bands	E	VL, HIV	CT, Sicily, Italy
ISS860	24	A	3 bands	E	VL, HIV	Tuscany, Italy
ISS976	24	A	3 bands	E	VL, HIV	Granada- Spain
ISS267	24	A	3 bands	E	VL	Tunisia, Africa
ISS1028	24	A	3 bands	E	VL, HIV	Malaga, Spain
ISS965	24	Lombardi	3 bands	E	VL, HIV	Granada, Spain
ISS966	24	Lombardi	3 bands	E	VL	Granada, Spain

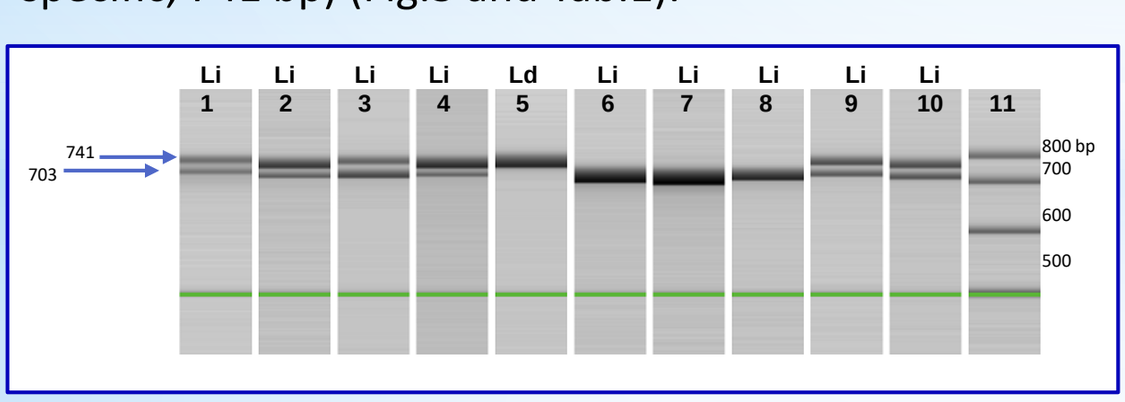

Fig.3. PCR of cpB locus of *L.infantum* (Li) and *L.donovani* (Ld) strains. Lane 1: MON24 CL (ISS176); lane 2: MON24 CL (ISS835); lane 3: MON24 CL (ISS417); lane 4*:* MON24 CL (ISS100); lane 5: *L.donovani*; lane 6: MON24 VL (ISS815); lane 7: MON24 VL (2492); lane 8: MON24 VL (913); lane 9: MON24 CL (ISS416); lane 10: MON24 CL (ISS182); lane 11 marker 100bp.

In conclusion, the current results support the validity of the Hsp70 assay for species typing. Futhermore, we propose for *L.infantum* ZMON24 CL strains the use of a Hsp70 PCR-RFLP (*Mlu*I) method to identify those strains with *L.infantum/L.donovani* molecular traits. A putative hybrid event between *L.infantum* and *L.donovani* spp populations, spread from Africa throughout the Mediterranean area, could be discussed.