Correlation Between Gene Expression Patterns and Clinical Origin of *Leishmania Infantum* Infectious Promastigote Forms

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Abstract: Differential expression of genes between cutaneous and visceral *L. infantum* parasites is hypothesized to be among the mechanisms potentially involved in the interplay the produce clinical variability in *Leishmania infantum* parasites.A variant of differential display technique was used to select differentially expressed genes in peanut-agglutinin negatively (PNA) selected promastigote forms of 2 visceral and 2 cutaneous *Leishmania infantum* parasites. Five cDNAs encoding for a nuclear transport factor 2-like and 4 ribosomal proteins were selected this way and their expression was analysed in these infectious forms of the 4 isolates. Quantitative real-time PCR (qRT-PCR) analysis of the transcripts highlighted variable gene expression among all the parasites. However, significant fold variations were obtained that defined relative expression phenotypes and patterns. Remarkably, 2 expression patterns were consistently observed with the 2 CL parasites when compared to one or the other of the VL isolates. In one particular case, coordinated expression of *NTF2-like, 40S RPS6*, *RPS26* and *60S RPL6* genes is highlighted. The results provide preliminary evidence on occurrence of correlated gene expression patterns to clinical origin of *L. infantum* parasites. [New York Science Journal .2010;3(3):57-66]. (ISSN: 1554-0200).

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1. Introduction

The leishmaniases correspond to a range diverse neglected disease caused by protozoan parasites of the genus *Leishmania* that are transmitted by female sandflies. World Health Organization (WHO) estimates that 350 million people are at risk of these diseases which affect more than 12 million people worldwide; two million cases are reported every year (Herwald,1999).

In the Mediterranean basin, *L. infantum* is the causative agent of visceral (VL) (Chappuis *et al*., 2007) and cutaneaous (CL) leishmaniasis (Ben Said *et al.*, 2006). Clinical variability is likely the result of a complex interaction between different factors including host genetic background and immune status, vector factors and parasite factors such as differentially expressed or secreted virulence determinants, pathoantigenic cytosolic proteins and others molecules (Rivas *et al*., 2004). Differential expression of genes between cutaneous and visceral *L. infantum* parasites is hypothesized to be among the mechanisms potentially involved in this interplay. Therefore, identification of differentially expressed transcripts in infectious stages of cutaneous and visceral isolates of *L. infantum* constitutes a fine approach for better understanding molecular mechanisms associated to or in preparation of virulence have been identified using cDNA libraries (Zhang and Matlashewski, 1997) or cDNA comparisons using for instance differential display reaction (DD) (Ben Achour *et al.*, 2002).

The differential display technique (Liang *et al*., 1992) was used in studies aiming at identifying for instance developmentally regulated genes in *Leishmania* species like *L. mexicana* and *L. braziliensis* (Liu *et al.*, 2000; Gamboa *et al.*, 2007), and differentially expressed transcripts in promastigotes or amastigotes of *L. major* isolates having contrasted virulence, respectively (Ben Achour *et al*., 2002 ; Ouakad *et al*., 2007). Its application to *L. chagasi* (synonymous species to *L. infantum*) parasites from cutaneous and visceral leishmaniasis in Honduras did not uncover transcript differences among them but with Mediterranean isolates causing VL in Tunisia and CL in France. This similarity of the profiles in Honduras is correlated with their genetic similarity. The observation of differences with parasites from other geographical origin, underscored the importance to approach the identification of *L. infantum* parasite factors within the same transmission region (Noyes *et al.*, 1997). The purpose of identifying differentially expressed genes an infectious promastigote stages of *L. infantum* isolates in Tunisia we used a variant of differential display that adapted a commercial kit to include anchored *Leishmania* mini-exon primers (in addition to the randomly defined primers) for isolation and identification of

differentially displayed *Leishmania* cDNAs (Turki-Mannoubi *et al.*, 2008). The addition of anchored mini-exon primers to the reaction is justified by the fact that trans-splicing where mini-exon is added to 5'-terminus of all known protein-encoding RNAs is part of pre-mRNA maturation process in *Leishmania* (Haile *et al.*, 2007). It also presented the advantage of directly screening differentially expressed coding sequences (Turki-Mannoubi *et al.*, 2008). Differential agglutination to PNA constitutes a mean to separate infectious (PNA^-) from non infectious (PNA^+) promastigotes (Louassini *et al.*, 1998 Alcolea *et al.*, 2009) exploiting differences due to D-galactose residues exposure/non-exposure in lipophosphoglycan, the major component of the promastigote coat (Soares *et al.*,2002).

In this study, we report on selection and characterization of 5 differentially displayed transcripts in *in vitro* PNA- promastigotes in 2 VL and 2 CL Tunisian *L. infantum* isolates. Quantification by qRT-PCR of the transcripts defined expression phenotypes and patterns that allowed correlations with clinical origin or genetic background of the parasites.

2. Materials and methods

Parasites

Four *L. infantum* isolates, obtained from human cutaneous lesions (CL) (MHOM/TN/96/DREP05 and MHOM/TN/96/DREP14) and from bone marrow of visceral leishmaniasis patients (VL) (MHOM/TN/94/LV49 and MHOM/TN/94/LV50) belonging to different zymodemes: MON-01 (DREP05 and LV50) and MON-24 (DREP14 and LV49), and cryopreserved very early after their isolation were thawn in coagulated rabbit serum and maintained at 26°C. Dividing promastigotes were progressively adapted to Schneider's insect medium (Sigma) containing 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 10% heat–inactivated fetal calf serum (GibcoBRL). For transcript expression analysis, PNA^- promastigotes were negatively selected by PNA as previously described (Louassini *et al.*, 1998). Parasite cultures initiated at $3x10^6$ PNA^+ cells/ml were collected at early stationary phase (day 4), time point where PNA^- parasites were shown to peak for all isolates (82.7, 79.1, 53.6 and 47.6% for LV50, LV49, DREP14 and DREP05 parasites, respectively). Promastigotes were resuspended at a density of $2x10^8$/mL in SIM-FCS containing 50 µg/mL PNA and incubated for 30 min at room temperature. The PNA^- cells were re-suspended in Trizol reagent (Invitrogen).

RNA extraction and cDNA synthesis

Total RNA was extracted from $2x10^8$ PNA^- parasites in Trizol reagent according to the manufacturer's instructions and was treated with 40 U DNase I- RNase free (Boehringer Mannheim) for 1h at 37°C. RNA concentration was determined through spectrophotometric absorbance measuremen. The quality of purified total RNA was examined on agarose gel, where 18S, 24Sα and 24Sβ ribosomal bands indicated good quality. Absence of DNA contamination was confirmed by no PCR amplification of an 800 bp fragment of the α-tubulin gene, constitutively expressed in promastigotes (*LinJ13_V3.0330*) (Purdy *et al.*, 2005 ; Srividya *et al.*, 2007), using the primers α-tub_up (5'-ATGCGTGAGGCTATCTGCATC-3') and α-tub_low (5'-GTCAGCACGAAGTGGATGCGC-3'). Synthesis of cDNA was performed as previously described (Turki-Mannoubi *et al.*, 2008). Quality of synthesized cDNA was assessed through the positive amplification of the α-tubulin gene.

DD reactions

DD analysis was done applying a variant of the technique using the Delta™ differential Display kit components (Clontech) and anchored mini-exon primers (Turki-Mannoubi *et al.*, 2008). The cDNA was PCR amplified using a set of 4 primers: 2 arbitrary primers (P3/5'ATTAACCCTCACTAAATG CTGGTGG3'andP9/5'ATTAACCCTCACTAAATGT GGCAGG-3') and two anchored mini-exon primers (ME4/5'GTATCAGTTTCTGTACTTTATTGGA3'an dME5/5'GTATCAGTTTCTGTACTTTATTGGC-3') that we designed. DD profiles was obtained with the 2 combinaisons (P3/ME5) and (P9/ME4) The PCR reactions were performed in a 20 µL mix containing 1 µL a or b cDNA dilution (a and b, corresponding respectively to 1/10 and 1/40 cDNA dilutions), 20 µM P primer, 20 µM ME primer, 1X PCR buffer, 50 µM dNTPs, 10 µCi [α-^{35}S] dATP (1000 Ci/mmole, Amersham-Pharmacia), 1X Advantage KlenTaq DNA polymerase mix (Clontech). Reactions consisted of 1 cycle at 94°C -5 min, 40°C-5 min, and 68°C-5 min, 2 cycles at 94°C-30 s, 40°C-30 s, and 68°C-5 min, 23 cycles at 94°C-20 s, 47°C (for ME4) or 49°C (for ME5) for 30 s, and 68°C-2 min and 1 cycle at 68°C-7 min. PCR products were resolved on a 6% denaturing polyacrylamide gel. The gels were dried onto Whatman 3 MM paper and exposed to X-ray Biomax MR Film (Amersham-Pharmacia) at –70 °C. Differentially displayed bands were cut from the gels, eluted, re-amplified by PCR, sub-cloned into the pMOSBlue blunt ended vector (Amersham-Pharmacia) and sequenced on an ABI 377 sequencer.

Sequence analysis

Sequences of the bands were analysed by homology searches performed on a locally installed version of the BLAST program (ftp://ftp.ncbi.nih.gov/blast/). Thus, the sequences of interest were blasted against version 3 of *Leishmania infantum* annotated genome obtained from gene DB (ftp://ftp.sanger.ac.uk/pub/pathogens/*Leishmania/infantum*). BLAST results were then used as entries in the Artemis Comparison Tool ("ACT"http://www.sanger.ac.uk/software/ACT) to localize and annotate the sequences according to their corresponding hits on the *L. infantum* genome (Carver *et al.*,2005).

Gene expression analysis by quantitative real-time PCR

The differential expression of the bands was confirmed by a relative quantitative real-time PCR (qRT-PCR) using the SYBR Green I chemistry (Heid et al. 1996). Specific primers were designed for each gene within the coding sequence, including an endogenous control (*GAPDH*) that has been chosen among genes described as constitutively expressed in *L. infantum* (Gagnon *et al.*, 2006), using the primer express software (version 1.5 PE Applied Biosystem) (Table 1). When the genes were present in more than one copy, the primers targeted the 2 copies. All qRT-PCR experiments were performed in 25 µl using SYBR Green Universal PCR Master Mix (PE Applied Biosystems) for amplification and detection with the ABI PRISM 7700 sequence detection system (with version 1.9.1 software, PE Applied Biosystems). Amplification was performed at 40 cycles as follows: 95°C-15 sec, 60°C-1 min, 60°C -1 min. The relative amount of PCR products generated from each primer set was determined based on the threshold cycle (C*t*) value and amplification efficiencies, and data were normalized by referring to the expression of the endogenous control. No template controls (NTC) were included in each run to confirm absence of primers-dimers and absence of cross contaminations in the reaction.

Statistical analysis

Statistical significance of the relative mRNA expression of each gene, calculated according to the $2^{-2\Delta Ct}$ model (Livak and Schmittgen, 2001), was achieved by paired t-test where the null hypothesis states that the expression fold is equal to 1. *P-values* less than 0.05 were considered statistically significant.

3. Result

DD identification of 5 transcripts in PNA$^-$ forms of 2 CL and 2 VL *L. infantum* isolates

cDNAs of PNA$^-$ promastigotes of the 4 isolates were studied in DD experiments. DD profiles obtained with the 2 primes pairs (P3/ME5) and (P9/ME4) showed that ninety percent of the bands observed were shared between the 4 isolates; the remaining bands had different intensities among isolates or were absent or present. Five bands differentially displayed were selected having different intensities among the different lanes (11-4; 11-9; 12-7) or being only present in the lanes of the 2 CL parasites (12-5; 12-11) (Fig. 1). The display of each band selected is illustrated on figure 1.

Sequences of the rescued bands were analysed using bioinformatics programs which included homology search and annotation exploiting *L. infantum* genome resources. In 3 cases, the sequenced end corresponded to the mini-exon- bearing 5' end of the cDNA. Significant hits were obtained for all fragments with similarity percent with *L. infantum* genome ranging between 96 and 100% (data not shown). The 5 bands matched a total of 7 different chromosomes (10, 15, 21, 28, 33, 35 and 36), with hits of each of the bands 11-4, 12-5 and 12-7 located on two different chromosomes overlapping genes encoding for the same protein, respectively; based on the score and *e-value*, 11-4 mapped on chromosome 35, 12-5 on chromosome 28, and 12-7 on chromosome 15 (Table 2). The five cDNAs overlapped five genes encoding for known proteins implicated in biological processes like protein import into nucleus (band 11-9), translation (bands 12-5, 12-7, 11-4), or regulation of cell growth and cell proliferation (band 11-4). The cDNA band 11-9 encodes for a putative nuclear transport factor 2- like (NTF2-like) (*LinJ10_V3.0900*), the remaining bands 11-4, 12-5, 12-7 and 12-11 correspond to genes coding for 4 putative ribosomal proteins (RP): 40S RPS6 (*LinJ35_V3.2000*), RPS26 (*LinJ28_V3.0570*), 60S RPL6 (*LinJ15_V3.1060*) and ribosomal P protein AGP2 beta-1 (*LinJ30_V3.3800*), respectively (Table 2).

Gene expression analysis of selected genes using quantitative real-time PCR

To assess expression levels of the 5 genes identified through DD experiments, we used qRT-PCR. Analysis of qRT-PCR results revealed that values of efficiencies were significant in case of four genes and excluded *ribosomal P protein AGP2 beta-1* (*LinJ30_V3.3800*) gene from the analysis. For each of the 4 other genes, results were expressed as a relative fold expression in the 6 possible pair-wise parasite comparisons by calculating $2^{-2\Delta Ct}$. Differences were

statistically validated which allowed assigning 3 expression phenotypes to the expression levels corresponding to steady (-), higher (up) or lower (down) transcript amounts.

Significant differences in transcript levels were observed in all comparisons (Fig. 2A), partly validating the observations made on the differential display gels. Significant fold variations were modest and ranged from 0.4 to 3.59. Transcripts of *40S RPS6* gene were more abundant in PNA^- forms of VL isolate LV49 than in any of the parasites tested independently of their clinical origins or zymodeme assignment. Three genes had transcript levels that were significantly different in the CL isolates as compared to each one of the 2 VL isolates: *RPL6* transcripts were less abundant than in LV49 while those of *60S RPS26* were more abundant than in LV50. Levels of *NTF2-like* transcripts were also shown more important in each of the CL parasites than in LV49. Two transcripts (*NTF2-like* (*LinJ10_V3.0900*), *RPS26* (*LinJ28_V3.0570*/*LinJ30_V3.3240*)) were shown more elevated in one of the CL isolates (DREP14) as compared to the other (DREP05); in case of VL parasites, *40S RPS6* transcripts were more abundant in isolate LV49 than in LV50.

Identification of consistent patterns and trends according to clinical origin of the parasites

The expression phenotypes of the 4 genes in all pair-wise comparisons were used to define expression patterns. Four patterns (A, B, C and D) were identified with 2 observed twice (A and B) (Fig.2A). The first one (pattern A) was obtained comparing anyone of the 2 CL isolates to LV49; it was characterized by over-expression of *NTF2-like* and down regulation of *40S RPS6* and *60S RPL6* in CL parasites. The second (pattern B) was observed while comparing these parasites to LV50 and highlighted their overexpression of *RPS26.* Two other expression patterns were obtained comparing CL (pattern C) or VL (pattern D) isolates suggesting variable gene expression even among parasites from similar clinical presentations.

Expression patterns of CL/VL comparisons were different within each of the zymodemes considered: MON-01 (DREP05/LV50) or MON-24 (DREP14/LV49), or across zymodemes (MON-01/MON-24 or MON-24/MON-01). In order to seek for consistent trends between isolate categories, comparisons were made lumping isolates according to clinical origin (all CL against all VL) or to zymodeme assignment (all MON-01 against all MON-24) (Fig. 2B). It was interesting to see a significant transcript overexpression of *60S RPS26* in MON-24 parasites while *NTF2-like* transcript levels seemed to be more abundant in CL isolates with a *p-value* at the limit of significance (Fig. 2B).

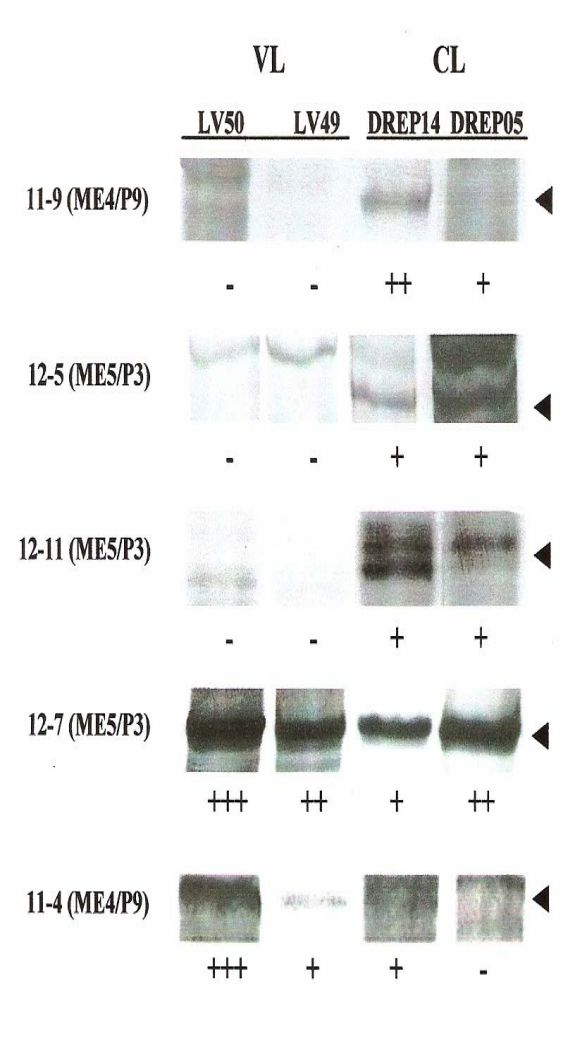

Figure 1. Display of cDNAs selected from cutaneous (DREP 14 and DREP05) and visceral (LV49 and LV50) *L. infantum* isolates. The panels were mounted to represent results obtained with 1/40 (dilution b) dilution of the cDNAs with arrowheads indicating the bands that were selected. The legend on the left side of the panels indicates band codes and primer pairs that were used in the DD. +++, ++, + and - : correspond to signal intensity levels that were assigned to the bands selected.

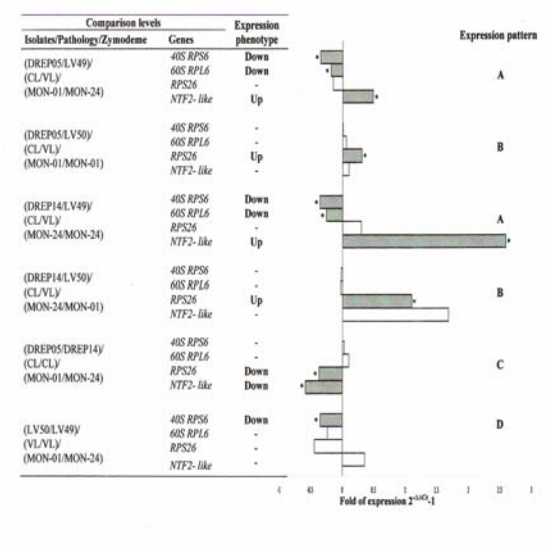

Figure 2. Expression analysis of genes identified by differential display using real-time PCR in the 4 *L. infantum* isolates. Panel A illustrates the six possible pair-wise comparisons of the 4 isolates which also allows seeking correlation to clinical origin (CL/VL, CL/CL, VL/VL) or genetic background (MON-01/MON-24, MON-01/MON-01, MON-24/MON-24). In panel B, (**Figure 3**) comparisons were made lumping the isolates according to clinical origin (all CL versus all VL) or according to genetic background (all MON-01 versus all MON-24). Grey bars represent fold of expression values which are statistically valid with a *$p < 0.05$; ** $p \leq 0.05$. "Up", "Down" or "-" indicate over-, under-, or steady expression respectively.

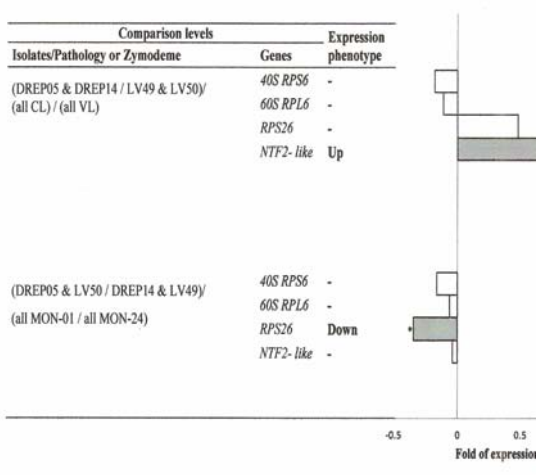

Figure 3: Comparative analysis of the lumping of isolates according to clinical origin (all CL versus all VL) and genetic background (all MON-01 versus all MON-24).

Table 1: List of primer sequences used in qRT-PCR analysis

Primer code	Forward primer (5'-3')	Reverse primer (5'-3')
11-4	AAGGATGGTTTCCCGATGGT	AAGGTGTTGAAGCCGATTGC
12-7	CATCACCCCCGAGGTGTTC	CTGTTTGTCGCCCATGAAGTC
12-5	CGATGCCGAAGCTGTACATG	CGGTGCGGCTGTGGATAG
11-9	GACTGCCAGCCCTCAATGA	GTGATCCTCACCCCTCAACTTC
12-11	CGCGGTCGAGGTCTTCTC	GGAAGGATACCTTGCTGGGTTT
GAPDH	CAGGAGATAGACAAGGCCATCAA	CGGTGAAGCCGAGAATGC

Table 2: Homology searches and annotation of the cDNA sequences

Band code[a]	Selected from [b]	Primer combination used[c]	Size of sequenced fragment[d]	GeneDB Id[e]	Chromosome[f]	Gene products[g]	Score (E-value)[h]
11-4	LV50	ME4/P9	673	*LinJ35_V3.2000**	35	40S ribosomal protein S6	880 (0.0)*
				LinJ21_V3.2150	21		862 (0.0)

12-7	LV49	ME5/P3	374	*LinJ15_V3.1060**	15	60S ribosomal protein L6	654 (0.0)*
				LinJ33_V3.0770	33		502 (E-142)
12-5	DREP14	ME5/P3	697	*LinJ28_V3.0570**	28	Ribosomal protein S26	714 (0.0)*
				LinJ30_V3.3240	30		519 (E-147)
11-9	DREP14	ME4/P9	493	*LinJ10_V3.0900*	10	Nuclear transport factor 2, NTF2- like	944 (0.0)
12-11	DREP14	ME5/P3	137	*LinJ30_V3.3800*	30	Ribosomal P protein AGP2 beta-1	188 (6E-48)

[a] indicates the code of the cDNA bands selected on DD gels.

[b] indicates the isolate lane from which the band was rescued and analysed.

[c] indicates the primer combination in DD reactions that generated the band.

[d] indicates the sequenced size of the fragments (bp).

[e] indicates gene annotation of the BLAST hits according to geneDB on version 3 of *L. infantum* genome (http://www.genedb.org).

[f] chromosome where is located the BLAST hit.

[g] shows putative functions encoded by the genes.

[h] statistical parameters that indicate significance of the BLAST hits; biggest scores and smallest *e-values* correspond to most significant hits.

[*] indicates the gene that most likely corresponds to the cDNA analysed according to the score and the *e-value*.

4. Discussion

In *Leishmania*, differentiation of the promastigotes is a preparatory and adaptive process to invasion and survival in the host cell (Bates, 2008). In *L. infantum*, PNA^- forms are more infective than PNA^+ forms (Louassini *et al.*, 1998 ; Alcolea *et al.*, 2009) and over-express important genes for infectivity (Alcolea *et al*., 2009). Indeed, differences observed in PNA^- promastigotes may relate to mechanisms associated to infection process or its following early stages. It is interesting that recurrent patterns were observed for 2 CL *L. infantum* when compared to 2 VL ones involving genes coding for ribosomal proteins, relevant to translation, and a protein putatively involved in the protein import in the nucleus. These genes/proteins were highlighted in different previous studies (Leifso *et al.*, 2007 ; Saxena *et al.*, 2003 ; Chenik *et al.*, 2006 ; Ubeda *et al.*, 2008 ; McNicoll *et al.*, 2006 ; Rosenzweig *et al.*, 2008 ; Cohen-Freue *et al.*, 2007)..

The *LinJ10_V3.0900* gene encodes for an NTF2-like protein which has a function yet unknown. A recent study, assessing the role of this protein and its homologue encoded by *LinJ06_V3.1210* demonstrated that this latter is a functional NTF2 protein involved in the nuclear transport of RanGDP. It was further

demonstrated that impairment of this transport by silencing the *NTF2* gene constitutes a sufficient intrinsic factor for triggering programmed cell death of the parasites (Casanova *et al.*, 2008). However, a comparative genome analysis of the 3 *Leishmania* species, *L. major*, *L. infantum* and *L. braziliensis* listed *NTF2-like* (*LinJ10_V3.0900*) among the genes under positive selection likely due to immunological pressure (Peacock *et al.*, 2007) suggesting a role in the interaction with the vertebrate host.

In *Leishmania* species, ribosomal proteins such as eukaryotic initiation factors, acidic ribosomal phosphoprotein PO or ribosomal protein P2a participating in different biological processes are considered as virulence factors (Chang and McGwire, 2002). In general in addition to the conventional role in translation, ribosomal proteins can be implicated in various extra ribosomal functions such as replication, DNA repair, apoptosis, resistance to drugs, cellular proliferation and inflammation (Wool, 1996). Multiple studies on differential gene expression in *Leishmania* provide convergent evidence that expression of ribosomal proteins is submitted to a complex and fine regulation depending on parasite stages, isolates and/or species. In *L. major*, it was noted that 6.3% of the total number of non-redundant stage-regulated genes encoded for ribosomal proteins with differences in the number and classes of ribosomal proteins differentially expressed in the different developmental stages (Almeida *et al.*, 2004).

Indeed, substantial changes in ribosomal transcripts abundance were observed during promastigote to amastigote differentiation in *L. major* transcriptomic analyses (Almeida *et al*., 2004 ; Leifso *et al.*, 2007). Particularly, *60S RPL6* transcripts from the two genes, *LmjF15.1000* and *LmjF33.0720* were found up-regulated in metacyclic stage of *L. major* (Saxena *et al.*, 2003), *LmjF33.0720* gene was even further considered as metacyclic-specific (Almeida *et al*., 2004). The protein 60S RPL6 was listed among excreted/secreted proteins of a highly virulent *L. major* parasite that was submitted to temperature and pH stress mimicking those in the host cell (Chenik *et al.*, 2006). Furthermore, transcripts of the *RPS26* genes (*LmjF28.0540*, *LmjF30.3200*) were also found upregulated in a study comparing a methotrexate resistant *L. major* to its sensitive parental line (Ubeda *et al.*, 2008). Interestingly, *L. infantum* proteomics studies comparing *in vitro* promastigotes to axenic amastigotes highlighted RP S26 (*LinJ28_V3.0570*) as overexpressed in promastigotes (Leifso *et al.*, 2007) and unique polypeptide spots corresponding to 40S RPS6 were also observed in promastigotes (McNicoll *et al.*, 2006).

Furthermore, proteome of *L. donovani*, another viscerotropic parasite, was quantitatively measured through an *in vitro* late- promastigote to late- amastigote differentiation model showing that amastigote proteins of the translation machinery are down-regulated in a coordinated manner with similar expression profiles (Rosenzweig *et al.*, 2008). Particularly, this study informed on the quantitative temporal profiles of the 4 proteins NTF2-like, 40S RPS6, 60S RPL6 and RPS26 which appeared correlated and down-regulated in the mature amastigote (Rosenzweig *et al.*, 2008). It is important to note that fold variations of this selection of genes/proteins in the different studies including this one were modest and in the similar range. It is also now well accepted that in *Leishmania* parasites there is little correlation between RNA and protein levels (McNicoll *et al.*, 2006 ; Cohen-Freue *et al.*, 2007). Remarkably, our study illustrated in *L. infantum* association of expression patterns of the genes encoding for these proteins in the two CL *L. infantum* isolates with concomitant down-regulation of *40S RPS6* and *60S RPL6* transcripts and over-expression of *NTF2-like* when compared to the MON-24 VL parasite (LV49). However, when the 2 CL *L. infantum* isolates were compared to the MON-01 VL (LV50), an over-expression of the *RPS26* transcripts was observed. This suggests that *L. infantum* parasites having contrasted virulence, causing different clinical forms, may be under different levels of regulation, may involve different regulatory pathways or could have evolved different virulence networks.

Recently a large scale study demonstrated clear differences in gene expression in PNA^+ vs PNA^- promastigotes of an *L. infantum* canine isolate with activation in PNA^+ forms of genes involved in ribosome biogenesis and assembly, regulation of translational elongation and developmental processes while genes up regulated in PNA^- forms relate more to infectivity (Alcolea *et al.*, 2009). However our selection of genes has not been highlighted through this study. Such discrepancy could be due to differences in the approaches or the scale of the studies, or to the fact that different parasites have been studied. So far, studies focused on only one parasite isolate while here we analysed expression in 4 parasites. The variations in the levels of expression observed are modest which may suggest ability of the *Leishmania* parasites to fine tune its biological processes and regulatory mechanisms.

This study brings preliminary evidence on the occurrence of consistent gene expression patterns in PNA^- forms of 4 *Leishmania infantum* isolates that are correlated to clinical origin.

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