Differential in vivo gene expression of major Leptospira proteins in resistant or susceptible animal models

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Transcripts of *Leptospira* 16SrRNA, FlaB, LigB, LipL21, LipL32, LipL36, LipL41, OmpL37 were quantified in the blood of susceptible (hamsters) and resistant (mice) animal models of leptospirosis. We first validated adequate reference genes, then evaluated expression patterns *in vivo* compared to *in vitro* cultures. LipL32 expression was downregulated *in vivo* and differentially regulated in resistant and susceptible animals. FlaB expression was also repressed in mice while not in hamsters. In contrast, LigB and OmpL37 were upregulated *in vivo*. Thus, we demonstrated that a virulent strain of *Leptospira* differentially adapts its gene expression in the blood of infected animals.
Leptospirosis is a neglected tropical zoonosis caused by pathogenic spirochetes of the genus *Leptospira* with a high incidence in developing countries (11). Infection occurs by direct contact with reservoir animals or exposure to an environment contaminated with their urine (10). The survival of leptospires upon transition into host conditions and leptospirosis pathogenesis include the modulation of bacterial gene expression (1). Microarrays studies evidenced that shifts from environmental to mammalian host-simulating conditions (temperature or osmolarity) lead to major transcriptional modifications in *L. interrogans* (12, 14), especially for genes involved in chemotaxis, motility or signal transduction. Exposure to guinea pig serum or mammalian macrophages also influenced genome-wide *Leptospira* gene expression (17, 21). It was notably noticed that expression of several outer membrane proteins (OMPs) was down-regulated. These results emphasized the transcriptomic regulation of pathogenic *Leptospira* upon shift to host-simulating physiological conditions. However, the regulation of the leptospiral transcriptome has only been evaluated *in vitro* with host-simulating parameters that poorly reflect real *in vivo* conditions. Thus, its regulation in infected animal remains to be analyzed.

In a previous study (13) we evidenced significant differences in the expression of major immune mediators between a susceptible hamster and a resistant mouse model of leptospirosis. These animals presented distinct dissemination profiles of bacteria in the blood. Indeed, the early clearance of *Leptospira* was evidenced in mice while a continuous and dramatic increase of the leptospiremia was observed in hamsters (13). Because the onset of infectious diseases involves a complex interplay between the host and the pathogen, we here evaluated the *in vivo* gene expression of several OMPs in the blood of these contrasting animal models infected with the virulent *L. interrogans* serovar Icterohaemorrhagiae strain Verdun (LiVv). Using the same blood specimens collected at early time-points (13), we
quantified the transcripts of OMPs and extracellular matrix (ECM)-interacting proteins but also the 16S rRNA, flaB using Reverse Transcription-quantitative PCR (RT-qPCR).

Outbred OF1 mice and golden Syrian hamsters initially obtained from Charles River Laboratories (Charles Rivers Wiga GmbH, Sulzfeld, Germany) were infected with LiVv and studied as previously described (13). Animal experiments were conducted according to the appropriate ethical guidelines as mentioned before (13), following the Animal Care and Use Committees of the Institut Pasteur and the European Recommendation 2007/526/EC. Briefly, animals were injected intra-peritoneally with 2 x 10^8 bacteria. Blood was collected by cardiac puncture within the first 24h after the infection (3, 6 and 24h) and conserved in PAXgene blood RNA tubes (PreAnalytix; Qiagen, Australia). Total RNA was extracted using the PAXgene blood RNA system from PreAnalytix (Qiagen, Australia). *Leptospira* from in vitro cultures (4 x 10^8 bacteria) were also processed after overnight incubation at 30°C or 37°C in EMJH medium. Bacterial total RNA was extracted using the High Pure RNA Isolation kit (Roche Applied Science, New Zealand) following the manufacturer’s recommendations. RNA samples were treated with DNAse (Turbo DNA Free-kit; Ambion, Applied Biosystems). Then, 1 µg of total RNA was reverse transcribed using the Transcriptor First Strand cDNA synthesis kit (Roche Applied Science, New Zealand) on a GeneAmp PCR system 9700 instrument (Applied Biosystems) with the following program: 10min at 25°C; 30min at 55°C; 5min at 85°C.

After cDNA synthesis, qPCR assays were performed using primers (Table 1) purchased from Eurogentec (Seraing, Belgium) specific for the gene coding for the 16S rRNA, flaB an Ig/Cadherin-like protein (LIC10501), ligB, lipl21, lipl32, lipl36, lipl41, lp95, lsa24, ompl37 and a thermolysin-like protein (LIC13321). Amplifications were carried out on a LightCycler 480 II instrument using the LightCycler 480 software (v 1.5.0) and the
LightCycler 480 SYBR green I master kit (Roche Applied Science, New Zealand) according to the provided instructions. The amplification programs are detailed in Table 1. Results were validated only when threshold cycle (Ct) values were under the limit value of 40 cycles and with an acceptable reproducibility between qPCR replicates (less than 5% of variation). Gene expression studies using RT-qPCR require the selection of internal controls for normalization and potential reference genes (also known as housekeeping genes or HKG) were evaluated using the commonly employed softwares, geNorm and NormFinder (2, 20). We analysed the expression stability of the genes (Table 2) in 33 samples (3 replicate LiVv cultures in vitro at both 30°C or 37°C; 11 and 16 blood samples of hamsters and mice, respectively). After the selection of the best reference genes, the normalization of gene expression was processed on the qbasePLUS software (Biogazelle, Belgium). The relative normalized expression ratio of target gene was then calculated as the ratio of the expression level in vivo to the expression level in vitro at 30°C, used as calibrator.

To our knowledge, only one paper analysed the stability of potential HKG in Leptospira (3) and evaluated the effect of the culture temperature on the expression of three genes frequently employed for normalization: lipl41, coding for an abundantly expressed OMP; flaB, coding for the flagellin subunit B; and the highly conserved 16S rRNA that was found to be the most stable gene for normalization in this study. In our in vivo study however, 16S rRNA is not a suitable HKG as evidenced by its stability rank position (5th; Table 2) and high geNorm M and NormFinder stability values (1.742 and 1.203, respectively). The two algorithms evidenced lipl21, lipl36 and lipl41 as the most stable of the genes studied in our experimental conditions. Previous studies evidenced that lipl36 and lipl41 were regulated in vitro depending on culture conditions (8, 21). Our contrasting results highlight the importance of evaluating expression stability in the experimental conditions of each own study. As the minimal use of the three most stable genes is recommended for normalization (20), lipl21,
lipl36 and lipl41 were selected as the best set of internal controls to normalize gene expression using the qbase\textsuperscript{PLUS} software (Biogazelle, Belgium). However, our set of internal control genes has higher than optimal stability values and thus, selection and validation of HKG for \textit{in vivo} expression quantification could still be improved.

Our results revealed that 16S rRNA expression is not stable but is rather down-regulated in mouse blood (expression ratio at 0.17) compared to \textit{in vitro} cultures (Figure 1A). We previously quantified the 16S rRNA transcript level to assess the bacterial burden in tissues from infected animals and showed that this level presented an upward kinetic (13). However, because studying a bacterial burden, the normalization was based on host HKG. Herein, we aimed to evaluate the transcriptomic function in the pathogen by quantifying the regulation of the 16S rRNA expression by calculating the ratio to formerly validated bacterial HKG. The decrease of the 16S rRNA expression \textit{in vivo} is concordant with previous results showing that this gene, as other ribosomal proteins, was down-regulated in \textit{Leptospira} co-cultured with mammalian macrophages (21). Oppositely, the mean expression ratio calculated during the first day post-infection was not different in the hamster blood compared to cultures. However, the expression ratio of 16S rRNA at 24h presented a significant down-regulation compared to cultures ($P = 0.024$; data not shown). The decrease in 16S rRNA expression in mouse and hamster blood at 24h cannot be explained but could reflect different phenomena in these two models: it can be speculated that it reflects a loss of fitness of the leptospires paralleling their rapid clearance in mouse blood (13) and rather a physiological and transcriptional shift in hamster blood as hypothesized in guinea pig serum (17).

Colonisation of host tissues might involve motility of \textit{Leptospira} that possess periplasmic flagellar filaments (5). Interestingly, we evidenced that the expression of \textit{flaB}, a major flagellin subunit, was significantly down-regulated in mouse but not in hamster blood compared to \textit{in vitro} culture (Figure 1B). Although \textit{flaB} expression is not regulated \textit{in vitro}
upon temperature changes [Figure 1B; (16)], it was down-regulated upon interaction with murine but not human macrophages (21). Moreover, the amounts of flagellin decreased in leptospires excreted in urine from resistant reservoir hosts, suggesting a motility loss after tissue colonization (16). The differential regulation of flaB expression between mouse and hamster may reflect a disparity in invasivity partly accounting for the fatal issue in hamsters (13).

Direct adhesion to host cells or ECM proteins might also contribute to the dissemination of leptospires through the infected organism. Unfortunately, the transcripts of lsa24, lp95, LIC13321 and LIC10501 were either not or inconsistently detected, most probably reflecting a very low expression level. The leptospiral immunoglobulin-like protein LigB is known to attach to host cells by binding several ECM proteins (6). Its expression is highly upregulated in the blood of both infected animals with relative expression ratios higher than 150 compared to in vitro cultured bacteria (Figure 1C). However, no significant difference in ligB expression was observed between our animal models. An upregulation of ligB was also observed in vitro upon temperature or osmolarity changes from environmental to host conditions (6, 12).

LipL32 is a major leptospiral OMP highly conserved among pathogenic Leptospira species (7) and was defined as an ECM-interacting protein (4). We evidenced that lipL32 expression is dramatically down-regulated in vivo compared to culture conditions (Figure 1D) correlating with microarray results upon interaction with mammalian macrophages (21). An interesting finding was a differential gene expression of lipL32 between our two animal models with an expression ratio of 0.03 in mouse compared to 0.12 in hamster. It was evidenced that LipL32 was not required for the development of acute or non lethal infection using a mutant L. interrogans (15). Because LipL32 also increases the permeability and apoptosis of human umbilical vein endothelial cells (19), it can be hypothesized that LipL32
is only required for invasion in the early stages of the infection through endothelial cells but is not required and therefore repressed once *Leptospira* disseminate into the systemic circulation. Moreover, LipL32 was shown to interact with Toll-Like Receptor 2 (TLR-2) leading to the induction of inflammatory chemokine IL-8 (9). Thus, a decrease in *lipl32* expression could reflect a dissimulation strategy to avoid recognition from host immunity.

OmpL37 was identified as another ECM-interacting OMP exhibiting strong and dose-dependent binding activity to elastin (18). Quantification of the expression levels revealed that *ompL37* is highly upregulated *in vivo* with ratios of up to 225.2 and 147.5 compared to *in vitro* for hamster and mouse samples, respectively (Figure 1E). However, it was not regulated in *Leptospira* co-cultured with mammalian macrophages (21) and we evidenced that it is neither influenced by culture temperature (Figure 1E). As OmpL37 has a high affinity for skin and vascular elastin, it might play a role in the early invasion process by mediating attachment to skin and vascular tissues (18).

Surface-associated proteins like OMPs and ECM-interacting proteins, as a result of their location, are likely to be important in host-pathogen interactions, hence their potential to promote several activities, including motility, adhesion or in promoting an effective immune response from the host. Our results highlight the need for global *in vivo* evaluation of *Leptospira* gene expression in both susceptible and resistant models, to better understand leptospirosis pathophysiology.

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References


data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034.

Figure 1: *in vivo* regulation of *Leptospira* gene expression. Relative expression of the target genes was studied as previously described in Matsui et al., 2011 by quantifying transcripts from the blood of hamsters (susceptible; n = 11 animals) or mice (resistant; n = 16 animals) infected with the strain LiVv. The *in vivo* results represent the expression levels in samples collected at 3, 6 and 24 h post-infection (3, 6, 24) and the combination of all time-points (M). Transcripts from *in vitro* cultures were obtained after incubation at 30°C (30; n = 3) or 37°C (37; n = 3). Values are means ± SEMs. Significant difference in gene expression between conditions (M and 30°C) was evaluated using a Mann-Whitney’s nonparametric t-test. *P < 0.05, **P < 0.01, ***P < 0.001, ns: non significant.
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Locus tag</th>
<th>Sequence (5’-3’)</th>
<th>Efficiency</th>
<th>Size</th>
<th>Tm</th>
<th>Prog.</th>
</tr>
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<tr>
<td>16S rRNA</td>
<td></td>
<td>(F)GGCGGCGCGGTCTTAAAACATG (R)CTTAATGCTGCTCCTCCGTA</td>
<td>1.932 ± 0.010</td>
<td>309</td>
<td>86.2</td>
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<tr>
<td>flaB</td>
<td></td>
<td>(F)ACCGGACACACTTCTTCCGCCATA (R)ATGAAACGAGAGCCGATATGGA</td>
<td>1.948 ± 0.027</td>
<td>139</td>
<td>80.8</td>
<td>A</td>
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<td>Ig/Cad-like</td>
<td>LIC10501</td>
<td>(F)TAACGATCTTGTTGTTGGA (R)GTAAAGGTCGTTGATTAT</td>
<td>1.907 ± 0.009</td>
<td>154</td>
<td>85.6</td>
<td>B</td>
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<tr>
<td>ligB</td>
<td>LIC10464</td>
<td>(F)ATCCGAAGTGGCATAACTCTCC (R)ATCGCCGTTAGAGTGC</td>
<td>1.886 ± 0.014</td>
<td>263</td>
<td>81.3</td>
<td>A</td>
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<tr>
<td>lip21</td>
<td>LIC10011</td>
<td>(F)TGTTGAAGCTACTGCTCT (R)CACCTGGAAATTGGGCGG</td>
<td>1.903 ± 0.051</td>
<td>164</td>
<td>80.0</td>
<td>B</td>
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<tr>
<td>lip32</td>
<td>LIC11352</td>
<td>(F)TCGCGGTTTATGCGAGTG (R)GCTAATCCGCGACATTCC</td>
<td>1.850 ± 0.009</td>
<td>228</td>
<td>84.0</td>
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<tr>
<td>lip36</td>
<td>LIC13060</td>
<td>(F)GCCCTAAATGCGCGTGAGTG (R)GCATAAACCCTGTGTGAG</td>
<td>1.806 ± 0.017</td>
<td>188</td>
<td>80.8</td>
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<td>lip41</td>
<td></td>
<td>(F)TTTACCGTTCGATCATGAAACGCGGC (R)GCGGATCTGAGCGGATTCG</td>
<td>1.982 ± 0.035</td>
<td>140</td>
<td>80.9</td>
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<td>lp95</td>
<td>LIC12690</td>
<td>(F)CCCTTGTGTTGATGGTTC (R)GTACGCTTGGCCCTCTTG</td>
<td>1.883 ± 0.044</td>
<td>209</td>
<td>81.6</td>
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<td>lsa24</td>
<td>LIC12906</td>
<td>(F)CTGCCGCGTATCTTTCTTG (R)TGTTGACCGCTCCGACACTCA</td>
<td>1.921 ± 0.016</td>
<td>177</td>
<td>81.0</td>
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<td>ompl37</td>
<td>LIC12263</td>
<td>(F)GGCGACTCAAGCAACATA (R)CGTTTTATCACTCCGGTTTG</td>
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<td>Thermolysin-like</td>
<td>LIC13321</td>
<td>(F)CTCAAGCTATCCCGGAGTCA (R)TGTTGAGATCTATCGAGG</td>
<td>1.832 ± 0.020</td>
<td>238</td>
<td>77.5</td>
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\(^a\) Locus tag of corresponding gene sequence from *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 referenced in GenBank (NCBI) as NC_005823.1, and used for primer design.

\(^b\) (F) and (R), forward and reverse primer sequences, respectively.

\(^c\) Efficiency for PCR was calculated as previously described in Matsui *et al.*, 2011.

\(^d\) PCR product size (in base pairs).

\(^e\) \(T_m\), PCR product melting temperature (\(^\circ\)C).

\(^f\) Prog. for qPCR amplification program. A for 50 cycles with: 5sec at 95\(^\circ\)C; 5sec at 62\(^\circ\)C; 8sec at 72\(^\circ\)C; B for 50 cycles with: 5sec at 95\(^\circ\)C; 5sec at 60\(^\circ\)C; 8sec at 72\(^\circ\)C.

\(^g\) As mentioned in Matsui *et al.*, 2011 (13).

\(^h\) As described in Carrillo-Casas *et al.*, 2008 (3).
Table 2: Expression stability values of *Leptospira* genes using geNorm and NormFinder softwares.

<table>
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<tr>
<th>Gene name&lt;sup&gt;a&lt;/sup&gt;</th>
<th>geNorm&lt;sup&gt;b&lt;/sup&gt; M value</th>
<th>geNorm&lt;sup&gt;b&lt;/sup&gt; Rank position&lt;sup&gt;c&lt;/sup&gt;</th>
<th>NormFinder&lt;sup&gt;b&lt;/sup&gt; NF value</th>
<th>NormFinder&lt;sup&gt;b&lt;/sup&gt; Rank position&lt;sup&gt;c&lt;/sup&gt;</th>
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<td>lipl41&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.382</td>
<td>1</td>
<td>0.242</td>
<td>1</td>
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<td>lipl36&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.434</td>
<td>2</td>
<td>0.717</td>
<td>3</td>
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<td>lipl21&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.513</td>
<td>3</td>
<td>0.617</td>
<td>2</td>
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<td>flaB</td>
<td>1.651</td>
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<td>0.830</td>
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<td>16S rRNA</td>
<td>1.742</td>
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<td>6</td>
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<td>ligB</td>
<td>2.230</td>
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<td>omp137</td>
<td>2.569</td>
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<td>1.941</td>
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<sup>a</sup> Gene name are given as in the literature. See Table 1 for details.

<sup>b</sup> Average expression stability values were calculated using geNorm (M value) and NormFinder (NF value) softwares after RT-qPCR assays performed on 33 samples as described in the text.

<sup>c</sup> The rank position of the expression stability is given for each gene depending on software. The most stable genes (*) were selected for the normalization step prior to relative quantification.