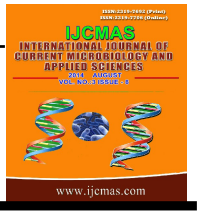
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 8 (2014) pp. 426-435**
http://www.ijcmas.com



Original Research Article

Expression microarray of leptospiral strains recovered from the patients with pulmonary haemorrhages – a special reference to fatal complications

Ratchagadasse Vimal Raj, K Vinod Kumar, Chandan Lall, Itta Krishna Chaaithanya, Kumaresan Vedhagiri, Ramanathan Thamizhmani and Paluru Vijayachari*

Regional Medical Research Centre (ICMR), WHO Collaborating Centre for Diagnosis, Reference, Research and Training in Leptospirosis, Port Blair -744101, Andaman and Nicobar Islands, India
**Corresponding author*

A B S T R A C T

Leptospirosis is extremely variable in its clinical manifestations, ranging from mild flu-like illness to fatal forms involving multi-organ failure, associated with high case fatality ratio range of 5-25%. In the recent past the pulmonary haemorrhages as a complications of leptospirosis was observed globally and associated with high morbidity and mortality. In fatal cases the disease progresses rapidly and the deaths were reported within 24 hours to 48 hours of hospitalization. Despite its severity and global importance, the molecular pathogenesis and genes involved in the fatal manifestations of the disease remains poorly understood. In the present study, efforts were made to find out whether, there were any virulent genes associated, differentially expressed and involved in the pathogenesis of fatal strain compared with that of strain isolated from a patient with pulmonary haemorrhages, but responded to the treatment and recovered. Both the strains were recovered from the patients associated with the pulmonary haemorrhages in form of haemoptysis during an outbreak in North Andaman. Among 11,168 genes were studied, Sixty eight genes were found to be differentially expressed, of which 44 (64.7%) were hypothetical genes and remaining 24 (35.2%) of genes were known functions. Out of 68 genes 28 hypothetical genes and 15 known functions genes were up-regulated, 15 hypothetical genes and three genes of known functions were down-regulated in strain which could responsible for the fatal complications respectively. Interestingly the genes LIC11226, LIC20202 (hypothetical) and LA 1021, LA 2008, LA 3980 (known function) were differentially up-regulated. The hypothetical genes LA 3440 and LA 0280 were differentially down regulated in strain responsible for the fatal complications. To conclude, the transcriptional analysis of the fatal strain associated with pulmonary complications resulted in distinct pattern of gene expression compared with genes found to be regulated by non-fatal strain. Genes of unknown function warrant further investigation to gain a better insight into their role in the pathogenesis of leptospirosis. This information could help to understand the pathogenesis of leptospirosis and help in adopting treatment strategies.

Keywords

Leptospirosis, Pulmonary, Haemorrhages, Microarray, Gene expression.

Introduction

Leptospirosis is an emerging zoonosis of worldwide distribution, characterized by haemorrhages, diarrhoea, jaundice, severe renal impairment, and aseptic meningitis, etc. In the recent past leptospirosis has emerged as a significant public health

problem throughout the developing countries due to its increased morbidity and mortality. Leptospirosis is extremely variable in its clinical manifestations, ranging from mild flu to severe fatal forms involving multi-organ failure, with death occurring in 5-25% of severe cases (Bharti et al., 2003; McBride et al., 2005). Leptospirosis is known to be endemic in Andaman Islands since early 20th century (Taylor and Goyle, 1931). Leptospirosis a mysterious illness with the majority of cases presenting as pulmonary involvement and severe pulmonary haemorrhage was shown for the first time as a complication of leptospirosis from India with high case fatality rates ranging from 10 to 50% (Sehgal et al., 1995).

Haemorrhages in general, and pulmonary haemorrhage in particular has been increasingly recognized worldwide and have a major impact on disease prognosis. Despite its severity and global importance, the molecular pathogenesis of leptospirosis remains poorly understood (Ko et al., 2009). The only virulence factor genetically defined to date is the surface lipoprotein Loa22 (Ristow et al., 2007), but mechanisms by which it contributes to disease pathogenesis remain unknown. Other virulence-associated genes includes hemeoxygenase (Miranda et al., 2006), LPS (Murray et al., 2010), clpB (Lourdault et al., 2011), and flagellar components (Lambert et al., 2012; Liao et al., 2009) were also documented. Although a few putative leptospiral virulence related genes were identified using random transposon mutagenesis (Bourhy et al., 2005; Murray et al., 2009), further progress has been hindered by the lack of efficient gene-targeted mutagenesis techniques in pathogenic *Leptospira* (Ko et al., 2009).

However, pathogenesis of leptospirosis is remains unclear and little is known about pathogenesis mechanism or transcriptional regulation in *Leptospira* spp. A pathogenic strain within the host simultaneously encounters multiple signals that are different from the *in vitro* condition and from environment conditions. However, leptospiral genes differentially expressed during infective stage which results in several clinical outcomes of disease particularly pulmonary haemorrhagiae and hepato-renal complication in a small proportion of people but the case fatality rate is much higher in pulmonary haemorrhagic complication (Singh et al., 1999; Vijayachari et al., 2008). The expression of proteins and genes plays role in pulmonary haemorrhagic complication remains unclear. Previous studies demonstrated the *in vivo* expression of several outer membrane proteins, based on the presence of antibodies against several proteins in acute and convalescent immune sera (Natarajaseenivasan et al., 2004). The responses of leptospires at transcriptional and translational levels to changes in various environmental factors such as temperature, osmolarity, and iron availability have been reported previously (Matsunaga et al., 2007; Miranda et al., 2006). Published reports on microarray with single stimulus such as temperature, osmolarity and iron has shown up regulation of various protein Qlp42, Hsp15, LigA, LigB, Sph2, and Lsa21 (Matsunaga et al., 2007). In contrast, LipL36 is down-regulated at 37°C and during mammalian infection (Miranda et al., 2006).

In the present study, we employed gene expression microarray approach to compare the gene expression profiles between the virulent strains recovered from the patients associated with pulmonary haemorrhages complication of fatal and non-fatal cases. The gene expression profile comparison between strains of fatal and non-fatal pulmonary haemorrhages complication has

not been characterized. And these differential genes expression among the strains may give a clue that the genes which enable pathogenic *Leptospira* which could results in severe pulmonary haemorrhages.

Materials and Methods

Leptospiral Strains

Two leptospiral strains used in this study were recovered from the patients during an epidemic occurred in north Andaman Island. Both the patients had the pulmonary haemorrhages, one responded with treatment and recovered other patient was died. The details of the strains are shown in table 1. The strains were coded DS 15 and DS 18 were periodically sub cultured and maintained in 5 ml of Ellinghausen-McCullough-Johnson-Harris liquid medium (EMJH) (Diffco – USA) until use.

Oligonucleotide Probe designing

The transcripts were retrieved from the available genome sequences of four strains of *Leptospira* (*L. interrogans* serovar Lai strain 56601 and serovar Copenhageni str. Fiocruz L1-130, *L. borgpetersenii* serovar Hardjo-bovis strain JB197 of pathogenic *Leptospira*, and saprophytic *L. biflexa* serovar Patoc strain Patoc I Paris/Ames). About 14016 transcripts were retrieved from NCBI database and compared with the NGS genome sequences of our strains CH11, CH31, DS 15 and DS 18 (data not shown). The unique sequences matching with the NGS consensus sequence were considered for probe design. Probes were designed with 45-60mer oligonucleotides in agilent platform in the sense direction. For each of the unique transcript sequences an average of one probe was designed and a total of 11168 probes were included in the array. The probes were categorized as specific and cross hybridizing on the basis of BLAST result (Megablast). The criteria for the specific probe are single hit against the target; alignment length of 60-31 bp length; allowed mismatches less than 3bp; gaps less than 2 bp and having the minimum word length of 28 bases.

RNA purification

Leptospiral strains were grown at 28-30°C to a density of 1-2 X 10^8 cells/ml before harvesting for RNA purification. Cell count was determined using Petroff - Hausser chamber (Electron Microscopy Sciences, Hot field). Leptospiral total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) as per manufacturers instruction and then treated with DNase (20 U for 30 min at 37°C). RNA was further purified using RNeasy mini columns (QIAGEN, Inc., Valencia, CA) with on-column DNase treatment according to the manufacturer's protocol. RNA concentration and purity were evaluated using the Nanodrop Spectrophotometer (Thermo Scientific; 1000). The integrity of the extracted RNA were analysed on the Bioanalyzer (Agilent; 2100). We considered RNA to be of good quality based on the 260/280 values (Nanodrop), rRNA 28S/18S ratios and RNA integrity number (RIN-Bioanalyzer).

Labelling and microarray hybridization

The sample Labelling was performed using Quick-Amp Labelling Kit, One Color (Agilent Technologies, Part Number: 5190-0442). About 500ng of total RNA was denatured along with appropriate diluted Spike in controls; Spike a Mix (Agilent Technologies, RNA Spike In Kit, One Color, Part Number 5188-5282) and Random hexamer primer tagged to T7 promoter region at 65°C for 10 min

followed by snap chilling on ice for 5 minutes. The cDNA master mix was added to the denatured RNA sample and incubated at 40°C for 2 hours for double stranded cDNA synthesis. The cDNA sample was denatured at 65°C for 15 min. The *in vitro* transcription master mix was added to the cDNA sample and incubated at 40°C for 2.30 hours. The cDNA was *in vitro* transcribed into cRNA in presence of Cyanine 3-CTPdye. The Cyanine 3-CTP labeled cRNA sample was purified using QiagenRNeasy column (Qiagen, Cat No: 74106). The concentration of cRNA and dye incorporation were determined using Nanodrop-1000 (JH Bio) and the specific activity was estimated using the formula

$$\text{pmol Cy3 per µgcRNA} = \frac{\text{Concentration of Cyanine 3 – CTP x 1000}}{\text{Concentration of cRNA}}$$

The labelled Cyanine 3-CTP cRNAs (2000ng for 8 array formats) were fragmented to an average size of approximately 50 to 100 nucleotides with fragmentation buffer at 60°C for 30 minutes and the reaction was stopped by adding 2X GE HI-RPM hybridization buffer (Agilent Technologies, In situ Hybridization kit, Part Number 5190-0404). The samples were pipetted onto the gasket slide and hybridized onto array. The hybridization was carried out at 65°C for 16 hours. After hybridization, the slides were washed using Gene Expression Wash Buffer1 (Agilent Technologies, Part Number 5188-5325) at room temperature for 1 minute and Gene Expression Wash Buffer 2 (Agilent Technologies, Part Number 5188-5326) at 37°C for 1 minute. The slides were then washed with Acetonitrile for 30 seconds. The microarray slide was scanned using Agilent Scanner (Agilent Technologies, Part Number G2565CA).

Microarray Data Analysis

Images were quantified using Feature Extraction Software (Version-10.7, Agilent). Feature extracted raw data was analysed using GeneSpring GX Version 12.0 software from Agilent. Normalization of the data was done in GeneSpring GX v12.0 using the 75th percentile shift (Percentile shift normalization is a global normalization, where the locations of all the spot intensities in an array are adjusted. This normalization takes each column in an experiment independently, and computes the n^{th} percentile of the expression values for this array, across all spots (where n=75 is the median). It subtracts this value from the expression value of each entity). The probes with fold >1 and with fold < -1 were considered as significant high and low expressed.

Results and Discussion

Among 11,168 genes were studied, 1265 genes were commonly up-regulated and 518 genes were down regulated among the strains (Data not shown). The gene expression pattern of strains responsible for non-fatal and fatal complications revealed that, about 68 genes were differentially expressed (table 2&3). Of which 44 (64.7%) were hypothetical genes and remaining 24 (35.2%) of genes were known functions.

Of these 68 genes 13 (19.1%) hypothetical genes and 3 (4.4%) genes of known functions were up-regulated in strain which could responsible for the non-fatal complication. Twenty eight (41.2%) hypothetical genes and 17 (25%) genes of known functions were down regulated in strain which could responsible for the non-fatal complication. Strain responsible for the fatal complications expressed 28 (41.2%) hypothetical and 15 (22%) genes of known functions were up-regulated. Fourteen

hypothetical genes (20.6%) and 3 (4.4%) genes of known functions were down regulated. Seven genes of strain responsible of causing non-fatal and eight genes in fatal strain were differentially expressed.

A total of 37 (54.4%) hypothetical and known genes were up regulated in fatal strain and all the strain were down regulated in non-fatal strain respectively. Interestingly the out these genes, LIC11226, LIC20202 (Hypothetical) and LA 1021 (transcription termination factor Rho), LA 2008 (dihyrolipoamideacetyltransferase), LA 3980 (glycerophospho-diesterphospho-diesterase) genes of known function were differentially up-regulated respectively. Whereas LA 3440 (hypothetical) gene and LA 0280 (cAMP-binding protein) of known function were differentially down regulated only in strain which could responsible for the fatal complications but not expressed in non-fatal strains.

The differentially expressed genes were classified into functional categories based on clusters of orthologous groups (COGs) as described elsewhere (Miranda et al., 2006; Patarakul et al., 2010; Tatusov et al., 1997). The majority of differentially expressed genes were poorly characterized are of unknown function 28 (81.2%) & 13 (62.2%) of up and down-regulated genes, respectively in the strain responsible for non-fatal complication (Table 4). Whereas, in strain responsible for fatal complications were 28 (65.1%) and 14 (82.3%) up and down regulated respectively (fig. 1). In contrast, studies have shown 45% and 65% of hypothetical genes were up-down regulated in different stimuli such as osmolality temperature and serum exposure in *Leptospira interrogans*, serovar Lai and Copenhageni (Miranda et al., 2006; Patarakul et al., 2010). The present study reports, higher up regulation and lower down regulation in strain causes fatal pulmonary haemorrhagic complications.

Major genes involved in cellular processing and signaling followed by information storage and processing, then genes involved in metabolism. The cellular processing and signaling genes were up regulated in fatal strain in comparison with non- fatal. But these genes were down regulated in non-fatal strain. Recent reports have shown that in response to serum exposure, predominance of genes were involve in metabolism (Patarakul et al., 2010) which may be due to regulation of genes required for coping with other environments. The present studies suggests the majority of gene involved in cellular processing and signaling in fatal strain, which may be crucial for virulence and pathogenicity. Little is known about pathogenesis mechanisms or transcriptional regulation in *Leptospira* spp.

Table.1 Leptospiral strains used for expression microarray analysis

Sl.No.	Strains	Serogroup	Serovar	Species	Clinical manifestations
1	DS 15	Grippo-typhosa	Valbuzzi	*L. interrogans*	Fever, Headache, Bodyache, Haemoptysis (Expired)
2	DS 18	Grippo-typhosa	Valbuzzi	*L. interrogans*	Fever, Headache, Bodyache, Haemoptysis (Recovered)

Table.2 Differentially expressed genes of hypothetical proteins of the strains responsible for non-fatal and fatal complication

S. No	Systematic Name	Gene Name	Description	Accessions	Regulations of genes	
					Non -Fatal	**Fatal**
1	LA_0030	LA_0030	hypothetical protein	ref|NC_004342	↑	↓
2	LA_0117	LA_0117	hypothetical protein	ref|NC_004342	↑	↓
3	LA_0198	LA_0198	hypothetical protein	ref|NC_004342	↑	↓
4	LA_0203	LA_0203	hypothetical protein	ref|NC_004342	↑	↓
5	LA_0471	LA_0471	hypothetical protein	ref|NC_004342	↓	↑
6	LA_0564	LA_0564	hypothetical protein	ref|NC_004342	↓	↑
7	LA_0570	LA_0570	hypothetical protein	ref|NC_004342	↑	↓
8	LA_0920	LA_0920	hypothetical protein	ref|NC_004342	↑	↓
9	LA_1000	LA_1000	hypothetical protein	ref|NC_004342	↑	↓
10	LA_1188	LA_1188	hypothetical protein	ref|NC_004342	↓	↑
11	LA_1359	LA_1359	hypothetical protein	ref|NC_004342	↓	↑
12	LA_1530	LA_1530	hypothetical protein	ref|NC_004342	↓	↑
13	LA_1538	LA_1538	hypothetical protein	ref|NC_004342	↓	↑
14	LA_1813a	LA_1813a	hypothetical protein	ref|NC_004342	↑	↓
15	LA_1910	LA_1910	hypothetical protein	ref|NC_004342	↓	↑
16	LA_2259	LA_2259	hypothetical protein	ref|NC_004342	↓	↑
17	LA_2519	LA_2519	hypothetical protein	ref|NC_004342	↓	↑
18	LA_2798	LA_2798	hypothetical protein	ref|NC_004342	↓	↑
19	LA_3145	LA_3145	hypothetical protein	ref|NC_004342	↑	↓
20	LA_3440	LA_3440	hypothetical protein	ref|NC_004342		↓
21	LA_3522	LA_3522	hypothetical protein	ref|NC_004342	↓	↑
22	LA_3524	LA_3524	hypothetical protein	ref|NC_004342	↓	↑
23	LA_3532	LA_3532	hypothetical protein	ref|NC_004342	↓	↑
24	LA_3533	LA_3533	hypothetical protein	ref|NC_004342	↓	↑
25	LA_3534	LA_3534	hypothetical protein	ref|NC_004342	↓	↑
26	LA_3688	LA_3688	hypothetical protein	ref|NC_004342	↑	↓
27	LA_3779	LA_3779	hypothetical protein	ref|NC_004342	↑	↓
28	LA_3911	LA_3911	hypothetical protein	ref|NC_004342	↓	↑
29	LA_3982	LA_3982	hypothetical protein	ref|NC_004342	↓	↑
30	LA_4135	LA_4135	hypothetical protein	ref|NC_004342	↑	↓
31	LA_4293	LA_4293	hypothetical protein	ref|NC_004342	↓	↑
32	LB_070	LB_070	hypothetical protein	ref|NC_004343	↓	↑
33	LBF_0241	LBF_0241	hypothetical protein	ref|NC_010842	↓	
34	LBF_2051	LBF_2051	hypothetical protein	ref|NC_010842	↓	
35	LIC10411	LIC10411	hypothetical protein	ref|NC_005823	↓	↑
36	LIC10476	LIC10476	hypothetical protein	ref|NC_005823	↑	↓
37	LIC11226	LIC11226	hypothetical protein	ref|NC_005823		↑
38	LIC11677	LIC11677	hypothetical protein	ref|NC_005823	↓	↑
39	LIC12228	LIC12228	hypothetical protein	ref|NC_005823	↓	↑
40	LIC12503	LIC12503	hypothetical protein	ref|NC_005823	↓	↑
41	LIC12926	LIC12926	hypothetical protein	ref|NC_005823	↓	↑
42	LIC13071	LIC13071	hypothetical protein	ref|NC_005823	↓	↑
43	LIC14004	LIC14004	hypothetical protein	ref|NC_005823	↓	↑
44	LIC20202	LIC20202	hypothetical protein	ref|NC_005824		↑

↓ down regulated , ↑ up-regulated

Table.3 Differentially expressed genes of known proteins of the strains responsible for non-fatal and fatal complications

S. No	Systematic Name	Gene Name	Description	Accessions	Regulations of genes	
					Non - Fatal	**Fatal**
1	LA_0280	crp	cAMP-binding protein	ref|NC_004342		↓
2	LA_0314	trkG	potassium uptake transporter	ref|NC_004342	↑	↓
3	LA_0587	LA_0587	lipase	ref|NC_004342	↓	↑
4	LA_0894	nuoB	NADH dehydrogenase subunit B	ref|NC_004342	↓	↑
5	LA_1007	btuE	glutathione peroxidase	ref|NC_004342	↓	
6	LA_1021	rho	transcription termination factor Rho	ref|NC_004342		↑
7	LA_1397	LA_1397	export protein	ref|NC_004342	↑	↓
8	LA_1685	LA_1685	recombinational DNA repair protein	ref|NC_004342	↑	
9	LA_2008	aceF	dihydrolipoamideacetyltransferase	ref|NC_004342		↑
10	LA_2623	gst	glutathione transferase	ref|NC_004342	↓	
11	LA_3242	LA_3242	TonB-dependent outer membrane receptor	ref|NC_004342	↓	↑
12	LA_3244	tolQ	biopolymer transport protein TolQ	ref|NC_004342	↓	↑
13	LA_3245	LA_3245	ExbD-like biopolymer transport protein	ref|NC_004342	↓	↑
14	LA_3246	exbD	ExbD-like biopolymer transport protein	ref|NC_004342	↓	↑
15	LA_3531	arsR	transcriptional regulator	ref|NC_004342	↓	↑
16	LA_3536	LA_3536	activator of Hsp90 ATPase 1 family protein	ref|NC_004342	↓	↑
17	LA_3673	msrA	peptide methionine sulfoxidereductase	ref|NC_004342	↓	
18	LA_3839	LA_3839	phospholipid binding protein	ref|NC_004342	↓	↑
19	LA_3980	ugpQ	glycerophosphodiesterphosphodiester ase	ref|NC_004342		↑
20	LA_4102	vicR	response regulator	ref|NC_004342	↓	↑
21	LBF_2735	rrl	23S ribosomal RNA	ref|NC_010842	↓	↑
22	LBJ_1770	LBJ_1770	putative peptidoglycan peptidase	ref|NC_008510	↓	
23	LBJ_2282	LBJ_2282	Alpha-galactosidase	ref|NC_008510	↓	
24	LIC11363	LIC11363	glutathione-S-transferase	ref|NC_005823	↓	↑

↓ down regulated , ↑ up-regulated

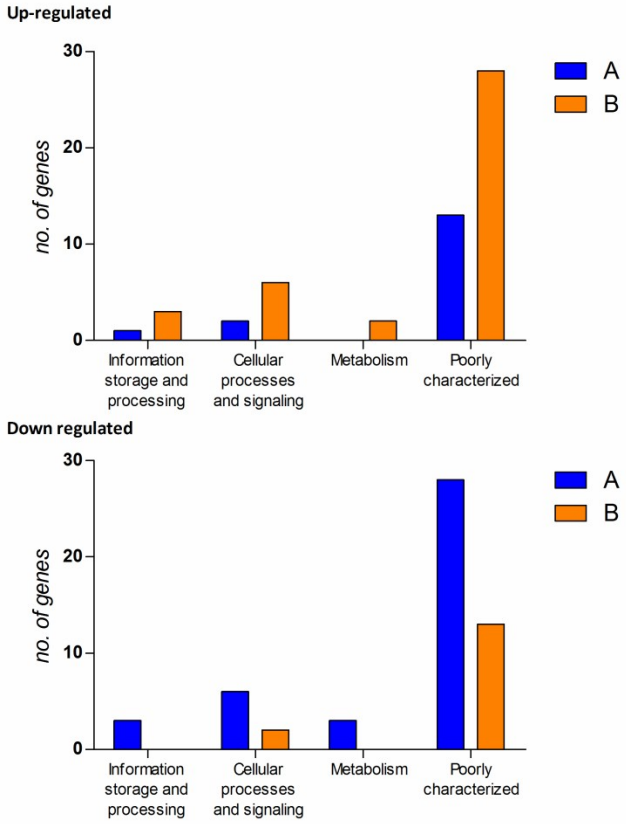
Table.4 Number of leptospiral genes differentially expressed in strains responsible for non fatal and fatal complications

Genes	No. of genes					
	A			**B**		
	Down-regulated (%[a])	**Up-regulated (%[a])**	**Total (%[b])**	**Down-regulated (%[a])**	**Up-regulated (%[a])**	**Total (%[b])**
Known or predicted function	17 (37.7)	3 (18.7)	20 (32.7)	3(17.6)	15 (34.8)	18 (30)
Un known or poorly characterized function	28 (62.2)	13 (81.2)	41 (67.2)	14 (76.4)	28 (65.1)	42 (70)
Total	45	16	61	17	43	60

A: strain responsible for the non-fatal complications, B: strain responsible for fatal complications

[a] percentage of genes per total number of genes in up-regulated or down regulated group, [b] percentage of genes per total number of differentially expressed genes

Fig.1 Expression of up and down regulated genes of general COGs grouping A. strain responsible for non-fatal complication B. strains responsible for fatal complications



To conclude, the transcriptional analysis of the fatal strain associated with pulmonary complications resulted in distinct pattern of gene expression compared with genes found to be regulated by non-fatal strain. However, our results serve to understanding of the genes which may involve in pathogenesis of leptospirosis. Genes of unknown function that are differentially regulated in fatal strain warrant further investigation to gain a better insight into their role in the pathogenesis of leptospirosis. This information could help to understand the pathogenesis of leptospirosis and to adopt treatment strategies.

Acknowledgments

The authors would like to thank Indian Council of Medical Research, New Delhi, India for providing grant for the study. The authors would also like to thank Genotypic Technology Pvt. Ltd., Bangalore, for the Microarray analysis.

Conflict of Interest

The authors do not have any commercial or other associations that may pose a conflict of interest.

References

Bharti, A.R., Nally, J.E., Ricaldi, J.N., Matthias, M.A., Diaz, M.M., Lovett, M.A., Levett, P.N., Gilman, R.H., Willig, M.R., Gotuzzo, E. Vinetz, J.M. 2003. Leptospirosis: a zoonotic disease of global importance. Lancet infect dis. 3, 757-771.

Bourhy, P., Louvel, H., Saint Girons, I. Picardeau, M. 2005. Random insertional mutagenesis of *Leptospira interrogans*, the agent of leptospirosis, using a mariner transposon. J bacteriol. 187, 3255-3258.

Ko, A., Goarant, C. Picardeau, M. 2009. *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. Nature rev Microbiol. 7, 736-747.

Lambert, A., Picardeau, M., Haake, D.A., Sermswan, R.W., Srikram, A., Adler, B. Murray, G.A. 2012. FlaA proteins in *Leptospira interrogans* are essential for motility and virulence but are not required for formation of the flagellum sheath. Infect Immun. 80, 2019-2025.

Liao, S., Sun, A., Ojcius, D.M., Wu, S., Zhao, J. Yan, J. 2009. Inactivation of the fliY gene encoding a flagellar motor switch protein attenuates mobility and virulence of *Leptospira interrogans* strain Lai. BMC Microbiol. 9, 253.

Lourdault, K., Cerqueira, G.M., Wunder, E.A., Jr. Picardeau, M. 2011. Inactivation of clpB in the pathogen *Leptospira interrogans* reduces virulence and resistance to stress conditions. Infect Immun. 79, 3711-3717.

Matsunaga, J., Lo, M., Bulach, D.M., Zuerner, R.L., Adler, B. Haake, D.A. 2007. Response of *Leptospira interrogans* to physiologic osmolarity: relevance in signaling the environment to host transition. Infect Immun. 75, 5848–5859.

McBride, A.J., Athanazio, D.A., Reis, M.G. Ko, A.I. 2005. Leptospirosis. Curr Opin Infect Dis. 18, 376-386.

Miranda, L., Dieter, M.B., David, R.P., David, A.H., James, M., Michael, L.P., Richard, L.Z. Ben, A. 2006. Effects of temperature on gene expression patterns in *Leptospira interrogans* serovar Lai as assessed

by whole-genome microarrays. Infect Immun. 74, 5848-5859.

Murray, G.L., Morel, V., Cerqueira, G.M., Croda, J., Srikram, A., Henry, R., Ko, A.I., Dellagostin, O.A., Bulach, D.M., Sermswan, R.W., Adler, B. Picardeau, M. 2009. Genome-wide transposon mutagenesis in pathogenic *Leptospira* species. Infect Immun. 77, 810-816.

Murray, G.L., Srikram, A., Henry, R., Hartskeerl, R.A., Sermswan, R.W. Adler, B. 2010. Mutations affecting *Leptospira interrogans* lipopolysaccharide attenuate virulence. Mol Microbiol. 78, 701-709.

Natarajaseenivasan, K., Vijayachari, P., Sugunan, A.P., Sharma, S. Sehgal, S.C. 2004. Leptospiral proteins expressed during acute & convalescent phases of human leptospirosis. Indian J Med Res. 120, 151-159.

Patarakul, K., Miranda, L. Adler, B. 2010. Global transcriptomic response of *Leptospira interrogans* serovar Copenhageni upon exposure to serum. BMC Microbiol. 10, 31.

Ristow, P., Bourhy, P., da Cruz McBride, F.W., Figueira, C.P., Huerre, M., Ave, P., Girons, I.S., Ko, A.I. Picardeau, M. 2007. The OmpA-like protein Loa22 is essential for leptospiral virulence. PLoS Pathogens. 3, e97.

Sehgal, S.C., Murhekar, M.V. Sugunan, A.P. 1995. Outbreak of leptospirosis with pulmonary involvement in north Andaman. Indian J Med Res. 102, 9-12.

Singh, S.S., Vijayachari, P., Sinha, A., Sugunan, A.P., Rasheed, M.A. Sehgal, S.C. 1999. Clinico-epidemiological study of hospitalized cases of severe leptospirosis. Indian J Med Res. 109, 94-99.

Tatusov, R.L., Koonin, E.V. Lipman, D.J., 1997. A genomic perspective on protein families. Science. 278, 631-637.

Taylor, R.J. Goyle, A.N., 1931. Leptospirosis in the Andamans. Supplement to Indian Journal of Medical Research, Memoir no. 20. Indian Research Fund Association. Thacker, Spink & Co., Calcutta.

Vijayachari, P., Sugunan, A.P., Sharma, S., Roy, S., Natarajaseenivasan, K. Sehgal, S.C., 2008. Leptospirosis in the Andaman Islands, India. Trans R Soc Trop Med Hyg. 102, 117-122.