REVIEW

INTEGRATION OF OMICS RESEARCH IN DISCOVERY OF BIOMARKERS FOR LEPTOSPIROSIS DIAGNOSIS AND VACCINE DEVELOPMENT

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Abstract. Leptospirosis, a life-threatening disease caused by *Leptospira* spp, is a serious global public health issue, especially in humid tropical and subtropical countries. Humans commonly are infected through occupational, recreational or domestic contact with urine of carrier animals. Despite numerous tests having been developed, availability of sensitive and specific biomarkers for diagnostic and vaccination purposes remains an issue, mainly due to the existence of more than 250 *Leptospira* serovars and limited knowledge on *Leptospira* pathogenesis. Bacterial virulence factors are often targeted and developed as biomarkers to leptospirosis detection and vaccination development. Here, we review studies using genomics, transcriptomics and proteomics approaches on pathogenic, intermediate pathogenic and saprophytic *Leptospira*. In addition, the challenges facing biomarker discovery and suggestions to improve the overall output of the biomarker discovery are addressed.

Keywords: biomarker, genomics, leptospirosis, proteomics, transcriptomics

INTRODUCTION

Leptospirosis is a worldwide zoonotic disease caused by *Leptospira* spp. Humans are usually infected through contact with water contaminated with bacteria from rodents or other reservoir hosts (Bharti *et al*, 2003). Bacteria enter the human body through abrasions, and circulate and reproduce in the blood stream for up to seven days (Adler and de la Piňa Moctezuma, 2010). The estimated number of human leptospirosis cases averages over 500,000 per year, with an annual prevalence of 10-100 per 100,000 population and a mortality rate of up to 25% (Bharti *et al*, 2003; Victoriano *et al*, 2009; WHO, 2010).

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OCCURRENCE AND PATHOLOGY

Leptospirosis is an endemic disease

in Malaysia, with a prevalence of 27.2 per 100,000 populations (Ministry of Health Malaysia, 2015). In Malaysia, individuals of 18-45 years of age are at greater likelihood of being infected by *Leptospira* as generally they are more mobile and, thereby, at higher risk of exposure compared to school children or the elderly (Benacer *et al,* 2016). Although no significant association has been found between average rainfall and number of reported leptospirosis cases in the country (Benacer *et al,* 2016), however several leptospirosis outbreaks have occurred following floods (Levett, 2001; Bharti *et al*, 2003; Thayaparan *et al*, 2013). In addition, multiple leptospirosis outbreaks also have been related to National Service training, as trainees undergo outdoor activities, which increases their exposure to leptospiral urine-contaminated soil and water (Mohamed-Hassan *et al*, 2012).

Leptospira belongs to order Spirochaetales, family Leptospiraceae, genus *Leptospira* (Faine *et al*, 1999). Leptospires are spirochetes of 0.1 μm in diameter and 6-20 μm in length (Adler and de la Piňa Moctezuma, 2010) and have hooks at one or both ends (Evangelista and Coburn, 2010). Infectious groups (pathogenic and intermediate pathogenic) are classified into over 250 distinct serotypes, while non-infectious group is referred to as saprophytic (Brenner *et al*, 1999; Slack *et al*, 2009). The nine pathogenic species cause disease of varying severity, ranging from subclinical to lethal infection; the five intermediate pathogenic species cause mild, self-resolving illnesses without fatal complications (Schmid *et al*, 1986; Brenner *et al*, 1999; Petersen *et al*, 2001; Levett *et al*, 2006; Matthias *et al*, 2008). Approximately half of all pathogenic and saprophytic (6 species) serovars are identified as *L. interrogans* and *L. biflexa,* respectively (Picardeau *et al*, 2008; Adler *et al*, 2011).

Leptospirosis is considered an occupational disease, where those exposed to activities such as mining, sewer maintenance, livestock farming and meat processing, veterinary medicine and military training are at high risk of contracting the disease (Bharti *et al*, 2003). Infected patients present with fever, headache, diarrhea, vomiting, and abdominal and muscle pain (Fernandes *et al*, 2012), Typical presentation of leptospirosis is divided into two phases: an initial acute phase with septicemia characterized by bacteremia, which normally lasts about seven days (Bharti *et al*, 2003); followed by a second icterohemorrhagic phase, with appearance of Weil syndrome normally lasting 4-30 days, accompanied by renal and hepatic failure, pulmonary distress and ultimately death (Adler and de la Piňa Moctezuma, 2010; Seguro and Andrade, 2013).

DIAGNOSIS: CURRENT STATUS

Leptospirosis can be diagnosed by bacterial culture, molecular and serological methods (WHO, 2003). Bacterial culture is the golden standard of diagnosis and is sensitive prior to initiation of antibiotic treatment (WHO, 2003). However, *Leptospira* growth is very fastidious, with a number of strains unable to thrive in selective media containing multiple antibiotics (Ridzlan *et al,* 2010) and may require four to six months to form visible colonies (Khaki, 2016). Molecular methods, such as PCR, quantitative PCR, restriction fragment length polymorphism PCR and pulsed-field gel-electrophoresis are much more rapid and sensitive, enabling detection even when serological and culture results are negative (WHO, 2003; Khaki, 2016). However, many laboratories cannot

afford to apply these DNA-based methods because they are costly requiring special equipments and reagents, in addition to standardized procedures and separate laboratory space. Serological methods, such as microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA) are easier to conduct. MAT is the serologic gold standard due to its high specificity; nevertheless, this method is tedious and time-consuming due to the need for regular subculture and quality control check for purity and maintenance of several reference leptospiral serovars (Khaki, 2016). ELISA is more sensitive than MAT because it can detect IgM in the first week of infection; however, current ELISA kits are unable to detect local and infective serovars as the majority of commercial ELISA kits only use a nonpathogenic *L. biflexa* (Patoc I strain) as whole cell antigen (Ahmad *et al*, 2005).

The majority of tests require follow-up samples for diagnostic proof of recent infection (WHO, 2003); however, follow-up samples are difficult to obtain in many hospitals (Levett, 2001). Thus, the first diagnosis from a suspected infected subject should be sufficiently specific, rapid and sensitive. In addition, progress in identifying the appropriate *Leptospira* virulent antigen for use in molecular and serologic diagnostic assays remains limited and no single antigen has been identified as sufficiently accurate, sensitive and/or specific for routine use (Champagne *et al*, 1991; Faine *et al*, 1999; Levett, 2001; Rajapakse *et al*, 2015). The tests results are usually negative when patients have mild symptoms or have already received antibiotics (Zeng *et al*, 2017).

As currently available vaccines do not provide long-term and cross-protective immunity against many *Leptospira* serovars (Adler and de la Piňa Moctezuma, 2010), it becomes important to discover biomarkers for the development of rapid and accurate diagnosis and of universal leptospirosis vaccines against heterologous *Leptospira* infections. Omics approaches, such as genomics, transcriptomics and proteomics should be able to assist in the identification of biomarkers beneficial for appropriate and specific therapy of leptospirosis patients with severe forms and allergy to certain drugs.

STRATEGIES FOR DISCOVERY OF LEPTOSPIROSIS BIOMARKERS

Biomarkers are biological measurements, which can be used to enable early disease detection, improve treatment selection and monitor the outcome of therapeutic interventions (Simon, 2011). In the context of leptospirosis, commonly many samples, such as serum, tissues and urine, are collected and used in leptospiral biomarker discovery. In the present review, we discuss three main omics technologies, namely, genomics, transcriptomics and proteomics used in biomarker discovery studies, together with new omic technologies that can enhance quality of output from these three omic technologies (Fig 1).

Genomics studies of pathogenic, intermediate pathogenic and saprophytic *Leptospira*

Numerous studies have been conducted to understand the mechanisms and virulent factors that underlie the pathogenesis of *Leptospira* spp through genomics studies. One of the most important strategies in genomics technology is whole genome sequencing. For instance, whole genome sequencing has been performed for pathogenic *L. interrogans* sv Lai strain 56601 (Ren *et al*, 2003), *L. interrogans* sv Copenhageni strain Fiocruz L1-130 (Nascimento *et al*, 2004), *L. borgpetersenii*

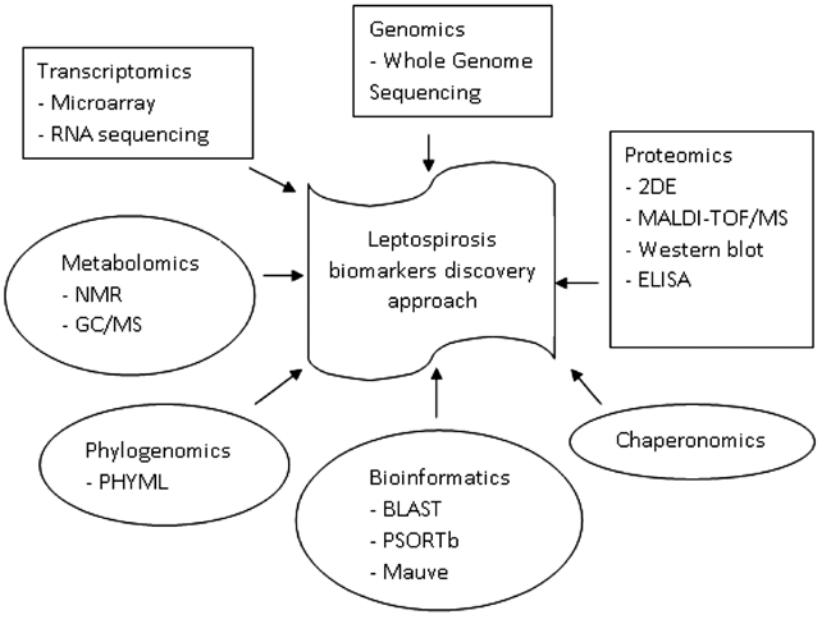

Fig 1-Omic technologies employed in leptospirosis biomarkers discovery. Squares indicate omics commonly used; and oval indicates new omics able to improve quality of output from current technologies.

sv Hardjo (Bulach *et al*, 2006), *L. interrogans* sv Hardjo (Llanes *et al*, 2016), *L. santarosai* sv Shermani (Chou *et al*, 2012), *L. alexanderi*, *L. alstoni*, *L. kirschneri*, *L. noguchii*, *L. santarosai* and *L. weilii* (Xu *et al*, 2016); the intermediate pathogenic *L. licerasiae* sv Varillal strain VAR010T (Ricaldi *et al*, 2012) and the saprophytic *L. biflexa* sv Patoc strain Patoc 1 (Picardeau *et al*, 2008). The genome sequence comparisons not only allowed the taxonomy of Leptospiraceae to be reviewed; it can also identify genomic variability among different *Leptospira* spp (Smythe *et al,* 2013).

Pathogenic *L. interrogans* has the largest genome (4.77 Mb) compared to other *Leptospira* spp genomes, such as *L. biflexa* (3.6 Mb) and *L. borgpetersenii* (3.9 Mb) (Xu *et al,* 2016). *L. borgpetersenii* and *L. interrogans* genome contains ~2,800 and 3,400 predicted open reading frames (ORFs), respectively, 656 of which are not present in *L. biflexa* genome (Ren *et al*, 2003; Bulach *et al*, 2006; Picardeau *et al*, 2008). In addition, *L. borgpetersenii* and *L. interrogans* have two chromosomes, while *L. biflexa* contains a 74 kb plasmid (Picardeau *et al*, 2008).

Pathogenic and intermediate pathogenic *Leptospira* spp have diverged from saprophytic *Leptospira* spp (Fouts *et al*, 2016), with *L. licerasiae* sharing ~48% of genes common to saprophytic *Leptospira* spp; but more than 50% similarity with pathogenic strains (Ricaldi *et al*, 2012). This suggests the intermediate pathogenic group is more closely related to the pathogenic group than to the saprophytic group (Azali *et al*, 2016).

Pan-genome analysis predicts the structural characteristics of *Leptospira* pan-genome contain many protein clusters (Fouts *et al,* 2016). The same study also shows pathogenic *Leptospira* spp

contain two alternative sigma (σ) 54 regulatory networks, namely LepA-σ54 and LepB-σ54, while saprophytic *Leptospira* spp only carry LepA-σ54 (Fouts *et al,* 2016), suggesting the latter might be important for pathogenicity of *Leptospira* spp in the host.

Selective gene loss and gain in different pathogenic species may have contributed to the ability of *Leptospira* to retain virulence in different conditions. Compared to *L. interrogans*, *L. borgpetersenii* has fewer signal transduction systems, transcriptional regulatory factors and metabolic and solute transport functions. Bulach *et al* (2006) proposed these characteristics should enable long survival of *L. interrogans* in the environment and ready adaptation to hosts compared with *L. borgpetersenii* that is always restricted to direct contact for transmission. Although *L. borgpetersenii* has impaired capacity for acquiring nutrients and surviving in the environment (Evangelista and Coburn, 2010); however it carries more transcription genes, transposases and pseudogenes compared to *L. biflexa* and *L. interrogans* (Picardeau *et al*, 2008). The fewer transposable elements in *L. biflexa* genome makes its genetic background more stable than that of pathogenic *Leptospira* spp (Picardeau *et al*, 2008).

Insertion sequence (IS)-mediated rearrangements affect both *L. borgpetersenii* and *L. interrogans* genomes (Bulach *et al*, 2006). For example, *L. borgpetersenii* has 8 copies of IS*1501* and 94 copies of IS*1533*, while *L. interrogans* has 8 copies of IS*Lin2* and 37 copies of IS*Lin1* (Xu *et al*, 2016). Furthermore, the clustered regularly interspaced short palindromic repeats (CRISPR) elements, which have propagated via horizontal gene transfer during bacteriophage infection, have been detected in *L. interrogans* and pathogenic *L. santarosai* but not in *L. borgpetersenii* and saprophytic *L. biflexa* (Chou *et al,* 2014). Table 1 lists several mobile DNA elements that are diverse among *Leptospira* spp.

Both pathogenic and saprophytic *Leptospira* spp have 2,052 core genes involved in DNA and RNA metabolism, protein processing and secretion, cell structure, cellular processes, and energy and intermediary metabolism (Picardeau *et al*, 2008). However, 900 of those genes are found only in pathogenic *Leptospira* spp and absent from saprophytic *Leptospira* spp, such as genes encoding *Leptospira* immunoglobulin-like (Lig) proteins, lipoproteins (Lip) and *Leptospira* endostatin-like (Len) proteins, all related to virulence-associated proteins (Adler *et al*, 2011). Interestingly, *L. interrogans* also possesses *ctsA* that is involved in peptide transport, expanding the range of substrates, and in assisting the cell to escape starvation of carbon source (Tenor *et al*, 2004; Zhong *et al*, 2011).

Combined genomic and phylogeny analyses show more genes have been lost than gained before the separation of pathogenic and intermediate pathogenic strains into separate groups (Xu *et al*, 2016). However, more genes have been gained than lost in the evolution of each pathogenic *Leptospira* sp, with genes lost including those encoding carbohydrate and energy metabolism. Furthermore, there are gene duplications, *eg* leucine-rich repeat protein family, PF13855, expansion in pathogenic but not intermediate pathogenic *Leptospira* spp (Xu *et al*, 2016). The pathogenic and intermediate pathogenic *Leptospira* spp also possess at least two copies of vitamin B_{12} (cobalamin) riboswitch gene that is not present in the saprophytic group (Ricaldi *et al*, 2012). These data suggest the pathogenic and intermediate pathogenic *Leptospira* spp

can respond to nutrient-limited niches and produce metabolites from simpler molecules. In addition, the pathogenic and intermediate pathogenic CRISPR *Leptospira* spp can escape phage / plasmid intrusion using CRISPR-Cas (CRISPR-associated protein) systems which able to defend them from the exogenous nucleic acids (Fouts *et al*, 2016).

Moreover, pathogenic *L. interrogans* and *L. kmetyi* are the only *Leptospira* spp possessing *dapA-E* required to convert L-aspartate-4-semialdehyde to LL-2,6,-diaminopimelate for peptidoglycan and lysine biosynthesis (Fouts *et al*, 2016). Both of these *Leptospira* spp also have a complete set of genes of the folate (vitamin B_9) biosynthesis pathway.

In addition, lipopolysaccharides (LPS) of pathogenic *L. interrogans* contain more sugar and fatty acid components than those of intermediate pathogenic *L. licerasiae* (Patra *et al*, 2015). On the other hand, LPS biosynthesis *rfb* encoding O antigen is located in the same genomic position in both pathogenic and intermediate pathogenic *Leptospira* spp, thereby allowing exchange of genetic material with non-invasive environmental *Leptospira* spp (Ricaldi *et al*, 2012; Fouts *et al*, 2016). However, *rfb* loci of these two *Leptospira* spp have greatly different complexities, *eg* *L. interrogans* has 95 *rfb* loci (Nascimento *et al*, 2004) while *L. licerasiae* 6 *rfb* loci (Ricaldi *et al*, 2012).

L. interrogans contains genes encoding sphingomyelinases while non-pathogenic *L. biflexa* does not (Picardeau *et al*, 2008). These genes might be involved in vascular damage (Louvel *et al*, 2006; Picardeau *et al*, 2008) and hemolytic anemia leptospirosis (Bernheimer and Bey, 1986). The number of sphingomyelinase genes are different among serovars, *eg* *L. interrogans* sv Lai, Copenhageni, Manilae, and Pomona each have five sphingomyelinase genes (*sph1*, *sph2*, *sph3*, *sph4*, and *sphH*) while *L. borgpetersenii* strains only have three sphingomyelinase genes (*sphA*, *sphB* and *sph4*) (Bulach *et al*, 2006).

Transcriptomics studies of pathogenic, intermediate pathogenic and saprophytic *Leptospira*

For biomarkers in gene expression study, microarray is the most common tool for measuring simultaneous expression patterns of thousands of genes and for monitoring gene expression differences between case and control samples (Debouck and Goodfellow, 1999). By investigating bacterial genome expression and polymorphism profiles from the host, microarrays detect and characterize microbial pathogens, monitor microbial infection and determine antimicrobial resistance gene profiles (Miller and Tang, 2009). The transcriptional response of pathogenic *Leptospira* spp to temperature, serum, physiological osmolality, iron depletion and host immune cells have been investigated using the microarray approach (Table 2).

Recent development in RNA sequencing technologies have overcome some of the limitations of microarrays, namely, requirements for probes, prior knowledge of gene targets and low sensitivity (Zhao *et al*, 2014). In addition, very small quantities of mRNA are sufficient to allow RNA sequencing to be performed to identify not only the genes transcribed but also the relative levels of expression (Adler *et al*, 2011, Filiatrault, 2011). RNA sequencing has been performed on a number of species, *eg* pathogenic *L. interrogans* serovar Copenhageni (Caimano *et al*, 2014), *L. interrogans* serovar Manilae (Zhukova *et al*, 2017) and saprophytic *L. biflexa* serovar Patoc (Iraola *et al*, 2016).

Table 1
Bioinformatics softwares employed in comparative genomic analysis and identification of mobile DNA elements.

Leptospira spp	*Leptospira* spp used for comparison	Analysis software	Mobile DNA element	Reference
L. alexanderi sv Manhao, *L. alstoni* sv Pingchang, *L. borgpetersenii* sv Javanica, *L. interrogans* sv Copenhageni, *L. kirschneri* sv Cynopteri, *L. kmetyi* sv Malaysia, *L. noguchii* sv Panama, *L. weilii*, *L. santarosai* sv Shermani (pathogenic)	*L. broomii* sv Hurstbridge, *L. fainei* sv Hurstbridge, *L. inadai* sv Lyme, *L. licerasiae* sv Varillal, *L. wolffii* sv Khorat (intermediate pathogenic), *L. biflexa* sv Patoc, *L. meyeri* sv Hardjo, *L. terpstrae* sv Hualin, *L. vanthielii* sv Holland, *L. wolbachii* sv Codice, *L. yanagawae* sv Saopaulo (saprophytic)	Celera Assembler, Phage_ Finder, NUCmer, HMMER3, Belvu, phylipFasta, Clustal Omega, MLST, ClustalW, MEGA, PhyML, CLC Main Workbench, trimAl, raxmlHPC, PanOCT, COBRApy toolbox, Prokaryotic Sequence homology Analysis Tool	O-antigen	Fouts *et al* (2016)
L. santarosai sv Shermani strain LT821 (pathogenic)	*L. interrogans* sv Copenhageni/strain Fiocruz L1-130/strain 56601/ strain IPAV (pathogenic), *L. borgpetersenii* sv Hardjo-bovis strain L550/ strain JB197 (pathogenic), *L. biflexa* sv Patoc strain Ames (saprophytic)	MAUVE, BLASTP, CGView Comparison Tool, IslandPick, IslandPath-DIMOB, SIGI-HMM, Island Viewer web (http://www.pathogenomics.sfu.ca/islandviewer)	CRISPRs, genomic island (GI), transposase, group II intron	Chou *et al* (2014)
L. interrogans sv Copenhageni/ Lai (pathogenic)	*L. interrogans* sv Bratislava/Canicola/ Hebdomadis (pathogenic) *L. kirschneri* sv Cynopteri/ Grippotyphosa (pathogenic)	SYSTAT	IS*1500*, IS*lin1*, IS*1533*	Eribo *et al* (2012)

Table 1 (Continued)

Leptospira spp	*Leptospira* spp used for comparison	Analysis software	Mobile DNA element	Reference
L. licerasiae sv Varillal strain VAR010T/strain MMD0835 (intermediate pathogenic)	*L. interrogans*, *L. borgpetersenii* (pathogenic), *L. biflexa* (saprophyte)	Glimmer 3, BLASTP, BLAST-Extend-Repraze, Protein Naming Utility, tRNAscan-SE, ISsaga, IslandViewer, PROmer, MUMmerplot, Gnuplot 4.0, QuartetS, HMMER3, Phage_Finder	Prophage vB-LliZ_VAR010-LE1 and vB-LliZ_MMD0835, LE1, O antigen, type II toxin–antitoxin systems, IS, GI	Ricaldi *et al* (2012)
L. interrogans sv Lai strain IPAV (attenuated pathogenic)	*L. interrogans* sv Lai strain 56601 (pathogenic)	PeptideSieve, MEGA 4	IS*1500*, IS*lin1*	Zhong *et al* (2011)
L. interrogans sv Lai strain 56601 (pathogenic)	*L. interrogans* sv Lai strain IPAV (attenuated pathogenic)	BLASTp, NCBI Conserved Domains, Pfam Domain, Softberry, OligoRep, GeneSpring 4.0	Prophages, GI (LA0186–LA0219)	Qin *et al* (2008)
L. interrogans sv Lai strain Lai (pathogenic)	*L. interrogans* sv Copenhageni strain Fiocruz L1-130 (pathogenic)	BLASTn, CLUSTER, TreeView, GenePix Pro 4.0	Transposase, GI (LA0702–LA0717 and LA1747–LA1851), IS*lin1*, IS*3*, IS*1501*, O antigen	He *et al* (2007)
L. biflexa sv Patoc strain Paris/Ames (saprophytic)	*L. borgpetersenii*, *L. interrogans* (pathogenic)	AMIGene, MaGe	IS*Lbi1*, toxin–antitoxin systems, GI (LaiGI-1)	Picardeau *et al* (2008)

Table 1 (Continued)

Leptospira spp	*Leptospira* spp used for comparison	Analysis software	Mobile DNA element	Reference
L. interrogans sv Lai, *L. kirschneri*, *L. noguchii* sv. Panama, *L. borgpetersenii* sv Ballum/Tarassovi (pathogenic)	*L. interrogans* sv Copenhageni/ Canicola/ Bataviae/Hardjobovis/ Hebdomadis/ Icterohaemorrhagiae/ Pyrogenes (pathogenic), *L. biflexa* (saprophytic)	MaGe, AMIGene	GI I (LaiGI I) (LA1768–LA1847), IS*4* (LA1848 and LA1849)	Bourhy *et al* (2007)
L. borgpetersenii sv Hardjo strain L550/JB197 (pathogenic)	*L. interrogans* sv. Lai (pathogenic)	GeneMarkS, Glimmer, Artemis, BLASTp, RPS-BLAST, PSORT, PSORTb, CELLO, LipoP, SpLip, SignalP, TMHMM, TMpred, BOMP, BLASTp	IS*1533*, IS*Lbp1*–IS*Lbp*10, IS*Lin1*, IS*1501*, IS*1501a*, IS*1477*-like, YhgA-like, IS*L2*, group II introns	Bulach *et al* (2006)
L. interrogans sv Pomona strain RZ11 (pathogenic)	*L. interrogans* sv Pomona strain RZ11 (mutated pathogenic)	Sequencher 4.1, Clone Manager 7, Primer Designer 5, BLAST, BPROM	IS*1501*	Zuerner and Trueba (2005)
L. interrogans sv Copenhageni strain Fiocruz L1-130 (pathogenic)	*L. interrogans* sv Lai (pathogenic)	MUMmer, TMHMM, PSORT, signalP, BLASTp, BLASTn	IS*1500*, IS*1501*, IS*1502*, IS*1533*, IS*lin1*	Nascimento *et al* (2004)
L. borgpetersenii sv Hardjo subtype Hardjobovis strain L171 (pathogenic)	*L. interrogans* sv Copenhageni strain L45, *L. interrogans* sv Hardjo subtype Hardjoprajitno strain L375 (pathogenic)	Sequencher 3.0, Australian National Genomic Information Service	IS*5*	Pena-Moctezuma *et al* (1999)

Recent *in vivo* studies have applied RNA sequencing to study gene expression by pathogenic leptospires within a mammalian host-adapted state showing the majority of the 110 and 56 genes upregulated and downregulated, respectively in the mammalian cells (compared to *in vitro*-grown bacteria) are absent in saprophytic *Leptospira* sp (Caimano *et al,* 2014). More recent RNA sequencing experiments revealed genes regulating genes motility, sugar/lipid metabolism, iron scavenging and outer membrane formation are upregulated, while those of DNA replication and cell division are downregulated in *L. biflexa* forming biofilm (Iraola *et al,* 2016).

A combination of RNA sequencing and the genomic analysis demonstrated expression of several lipoprotein genes, including *LipL32*, *LipL21* and *LipL36*, are more highly upregulated in pathogenic *L. interrogans* sv Copenhageni compared to pathogenic *L. santarosai* sv Shermani strain LT821 during infection in HK-2 cells (Chou *et al,* 2014). Compared to normal culture temperature of 30°C, upregulated expression levels of *ligA* and *ligB* mRNA at 37°C increase protein expression 20- and 14-folds, respectively in *L. interrogans* (Matsunaga *et al,* 2013). This characteristic enables pathogens to exploit the temperature shift in warm-blooded mammalian hosts for their successful infection (Matsunaga *et al,* 2013).

RNA sequencing also allows detection of small non-coding RNAs (sRNAs), which can inhibit or activate protein translation by binding adjacent to the translation start site of target mRNAs (Ahmed *et al,* 2016). There are 11 sRNAs in pathogenic *L. interrogans* sv Copenhageni, confirmed by RT-quantitative PCR (Caimano *et al,* 2014). However, more than 200 sRNAs are expressed in pathogenic *L. interrogans* sv Manilae, among which LIC2nc10 (targeting cobalamin riboswitch), LICnc60 (targeting RNaseP mRNA), LICnc10 (targeting tmRNA) and LIC2nc40 are also present in *L. interrogans* sv Copenhageni (Zhukova *et al,* 2017).

Recently, a global transcriptional start site (TSS) map of *L. interrogans* was generated and predicting 2,865 primary (p)TSSs, sites of transcriptional intitiation overlapping with the start codon and hence generating leaderless transcripts were situated within the first ten nucleotides upstream of the translational initiation site (Cortes *et al*, 2013; Li *et al,* 2015; Zhukova *et al*, 2017). Comparative differential RNA sequencing analysis has suggested that the translation efficiency can be indirectly affected by the large number of pTSSs (Cortes *et al,* 2013; Zhukova *et al*, 2017).

Proteomics studies of pathogenic, intermediate pathogenic and saprophytic *Leptospira*

Proteomics is an analysis of gene expression at the protein level (Chevalier, 2010). Many strategies have been applied in proteomics studies of *Leptospira*; however, the most common strategies used in the detection of *Leptospira* biomarkers are two-dimensional gel-electrophoresis (2DE), matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS), liquid chromatography MS (LC/ MS), western blotting and enzyme-linked immunosorbent assay (ELISA) (Nally *et al,* 2011; Srikram *et al,* 2011; Srivastava *et al,* 2012; Tan *et al,* 2017).

Proteomes of pathogenic *L. interrogans* sv Canicola (Humphryes *et al*, 2014), *L. interrogans* sv Copenhageni (Eshghi *et al*, 2009; Malmström *et al*, 2009), *L. interrogans* sv Pomona (Vieira *et al*, 2009), *L. interrogans* sv Lai (Cao *et al*, 2010; Zhong *et al*, 2011; Zeng *et al*, 2013), *L. interrogans*

sv Australis, Bratislava and Autumnalis, and Icterohaemorrhagiae and saprophytic *L. biflexa* sv Patoc (Thongboonkerd *et al,* 2009) have been reported. The outer membrane protein OmpL1; lipoproteins LipL32, LipL36, LipL41, and LipL48; leptospiral OmpA-like protein Loa22; and leptospiral immunoglobulin-like protein LigA and LigB in many pathogenic *Leptospira* spp. (Cullen *et al*, 2002; Haake and Matsunaga, 2002; Nally *et al*, 2005; Nally *et al*, 2007; Monahan *et al*, 2008; Eshghi *et al*, 2009; Nally *et al*, 2011; Srikram *et al*, 2011; Shokri and Aghaiypour, 2016) have a greater composition and structural complexity than those of intermediate and non-pathogenic species (Murray *et al*, 2010; Patra *et al*, 2015). A number of these proteins bind to extracellular matrix components, such as collagen, fibronectin, laminin, and plasminogen for adhesion, penetration, colonization and pathogenesis (Oliveira *et al*, 2011).

Pathogenic *Leptospira* spp have many immunogenic proteins that have the potential as candidates for diagnosis and vaccine development. The common immunogenic proteins, such as glutamine synthetase, succinyl-CoA synthetase beta subunit, LipL41, LipL45, DNA polymerase III beta subunit, elongation factor Ts, flagellin, electron transport flavoprotein beta subunit and proteolytic subunit of ATP-dependent Clp protease are found in proteomes of *L. interrogans* sv Canicola, Copenhageni and Pomona and in that of *L. borgpetersenii* sv Tarassovi (Sakolvaree *et al,* 2007; Humphryres *et al*, 2014).

Recently, a proteomic map depicting soluble and membrane-associated proteins of saprophytic *L. biflexa* during its exponential growth and stationary phases was drawn up, indicating *L. biflexa* has many post-translational modification systems which can also be found in pathogenic *L. interrogans* (Stewart *et al*, 2016). The post-translational modifications are methylation and acetylation of membrane-associated proteins, while phosphorylation occurs mainly among soluble proteins. Not unexpected, genomes of both pathogenic and saprophytic *Leptospira* spp have the homologs of kinases, methyltransferases, acyltransferases and GCN5-related N-acetyltransferase which can catalyze the abovementioned processes. Hence, these modification systems might play key physiological roles.

Saprophytic *Leptospira* spp are considered to have many important survival mechanisms as they have to survive in aquatic environment as well as in the mammalian host (Haake and Matsunaga, 2010). Louvel *et al* (2006) demonstrated both *L. biflexa* and *L. interrogans* have three putative TonB proteins to transport iron across the outer membrane. Eshghi *et al* (2015) observed presence of 274/325 exoproteins (proteins transported to the extracellular space), involved in metabolic and energy generation functions in *L. biflexa*; however, no orthologous putative ATP-binding cassette (ABC) transporter and hemolysin secretion protein D were detected in *L. interrogans*.

Sialic acid cluster was detected in most pathogenic *Leptospira* spp, but not in intermediate pathogenic and saprophytic *Leptospira* spp (Fouts *et al,* 2016). In addition, pathogenic *Leptospira* contains leucine-rich repeat (LRR) domains not found in saprophytes (Miras *et al,* 2015). LRR can act as a negative modulator of host inflammatory responses and induce a strong host transcriptional response to the infecting pathogens (Ng and Xavier, 2011). Combined genomic and proteomic analysis of extracellular proteome demonstrated *L. interrogans* has a complete type I

and type II secretion system to transport proteins into the extracellular environment (Zeng *et al,* 2013).

CHALLENGES TO THE DISCOVERY OF *LEPTOSPIRA* VIRULENCE FACTORS

Currently, many leptospiral virulence factors have been discovered but their functions remain unknown. An examination of the pathogenicity of virulence proteins from every serovar remains very challenging because there are >250 *Leptospira* serovars. Furthermore, discovery efforts become complicated when transfer of genes or mobile gene elements occurs between different *Leptospira* serovars or with other bacteria. For example, *ligA* and *ligB* have immunoglobulin-like regions that are homologous to *Escherichia coli* intimin-binding protein, *Clostridium acetobutylicum* cell adhesion domain and *Yersinia pseudotuberculosis* invasin (Palaniappan *et al*, 2002). In addition, leptospiral *lsa66* and *loa22* resemble *E. coli* OmpA protein C-terminal domain (Ristow *et al*, 2007; Oliveira *et al*, 2011).

The optimum environment for experimentation has to be maintained as gene regulations can vary with environmental conditions, such as osmolality, temperature and iron availability (Table 2). The expression level of each gene can also differ when leptospires are cultured under various *in vitro* conditions. Leptospiral virulence genes, *eg ligA*, *ligB* and *ompL37*, are downregulated when RNA is extracted under *in vitro* culture conditions (Palaniappan *et al*, 2002; Adler *et al*, 2011; Matsui *et al*, 2012). This is one of the key limitations of genomics technology in biomarker discovery study. However, expression of other genes would be expected to be different between *in vivo* and *in vivo*. For example, *lipL32* from *in vivo* culture is downregulated to avoid recognition by the host immune system (Matsui *et al*, 2012).

Genes are expressed at altered levels in different animal models. For example, *flaB* is downregulated upon interaction with mice but not with human and hamster cells (Xue *et al*, 2010; Matsui *et al*, 2012). However, determining biomarkers in human infection is very important for establishing which bacterial protein is virulent. Pathogenicity of a serovar in humans can only be established after it has been isolated from a patient. On the other hand, it is necessary to ensure that the leptospiral organisms will express the virulence protein in mammalian or human cells but not the environmental organisms during inoculation (Guerreiro *et al*, 2001). Hence, the virulence of an antigen has to be carefully validated.

Although *Leptospira* virulence proteins, such as LipL41 (Haake *et al*, 1991), LipL45 and GroEL (Matsunaga *et al*, 2003) can be detected in both high- and low-passage cultures, high numbers of passaging might result in loss of virulence, such as attenuation in *L. interrogans* Lai strain 56601 after 400 passages (Lehmann *et al,* 2016). Haake *et al* (1991) reported a large, high-passage inoculum ($\geq 10^7$ cells) fails to produce lethal infection in hamsters. LigA and LigB levels are significantly produced only in low-passage isolates. In high-passage *L. kirschneri* isolates attenuation in virulence is attributed to an inability of host antibodies to detect Lig proteins (Matsunaga *et al*, 2003), suggesting high numbers of passages could result in deleterious mutations in these virulence genes impairing growth in host (Lehmann *et al,* 2016).

The growth phase of the culture can

Table 2
Leptospira interrogans genes differentially expressed under experimental conditions using microarray approach.

Locus tag of *L. interrogans* sv Lai or Copenhageni	Gene regulated and experimental condition								
	Gene	COG[a]	Function of the gene	Physio temp *vs* Environ temp[b]	Temp upshift from 30 to 37°C[c]	Serum[d]	Osmolality[e]	Iron[f]	Interaction with phagocytic cells[g]
LA0502 (LIC13053)	*desA*	I	enoyl-CoA hydratase	+		+			+
LA0594 (LIC12982)		P	Cation transport ATPase, possibly copper	+	+	+			
LA0802 (LIC12818)		N, U	Pilus assembly protein	+	+		+		
LA0816 (LIC12807)		T	Receiver component of a two-component response regulator	+	+	-			
LA1402 (LIC12339)			Conserved hypothetical protein				+		-
LA1456 (LIC12297)	*radC*	L	DNA repair protein	+	+		+		
LA1457		G, M	Membrane protein of ABC transporter complex	+	0			-	
LA1538 (LIC12228)			Conserved hypothetical protein				-		-
LA1539 (LIC12227)			Orotate phosphoribosy ltransferase				-	-	-
LA1681	*phoH*	T	Phosphate starvation-inducible protein	-				-	
LA1859	*katE*	P	Catalase	+					+

Table 2 (Continued)

Locus tag of *L. interrogans* sv Lai or Copenhageni	Gene regulated and experimental condition								
	Gene	COG[a]	Function of the gene	Physio temp *vs* Environ temp[b]	Temp upshift from 30 to 37°C[c]	Serum[d]	Osmolality[e]	Iron[f]	Interaction with phagocytic cells[g]
LA1879 (LIC12017)	*clpA*	O	Endopeptidase Clp, ATP-dependent proteolytic subunit	+	+	-			
LA2014 (LIC11893)		V	CreD-like protein	-	-		-		
LA2117 (LIC11801)		T	Anti-sigma factor antagonist	+	+		+		
LA2200 (LIC11728)		M	Amidase			+	+		
LA2824			Anonymous protein					-	+
LA3234 (LIC10900)		T	Adenylate/guanylate cyclase				+	+	
LA3778 (LIC10464)	*ligB*	N	LigB lipoprotein	-	+	+	+		
LA4127 (LIC13289)			Sensor histidine kinase of a two- component response regulator			+	+		
LA4299 (LIC13442)	*btuE*	O	Glutathione peroxidase	+	+	-			
LB102 (LIC20079)		G	Sugar phosphatase	+	+		-		
LB139		T, K	Sigma subunit regulator	-				+	
LB186 (LIC20148)		P	Heme oxygenase				+	+	

Table 2 (Continued)

Locus tag of *L. interrogans* sv Lai or Copenhageni	Gene regulated and experimental condition								
	Gene	COG[a]	Function of the gene	Physio temp *vs* Environ temp[b]	Temp upshift from 30 to 37°C[c]	Serum[d]	Osmolality[e]	Iron[f]	Interaction with phagocytic cells[g]
LB187 (LIC20149)	G	Permease			+	+			

[a]Cluster of orthologous groups (Tatusov *et al*, 1997). [b]Leptospires grown at physiological (37°C and 39°C) versus environmental (20°C and 30°C) temperatures (Lo *et al*, 2006). [c]Leptospires grown at 30°C then shifted to 37°C overnight (Lo *et al*, 2006). [d]Leptospires in serum response (Patarakul *et al*, 2010). [e]Physiological osmolality upshift on leptospires growth (Matsunaga *et al*, 2007). [f]Leptospires grown in iron-limited medium (Lo *et al*, 2010). [g]Leptospires in response to macrophage-derived cells (Xue *et al*, 2010). +, upregulated; -, downregulated; 0, no significant difference in gene expression.

also affect gene expression level. Abundant *lipL36* expression in the early log phase of leptospiral growth begins to decline early in the mid-log phase (Haake *et al*, 1998), and extracellular protein is produced at high levels only in early-mid phase of *Leptospira* cultures (Zeng *et al*, 2013). In addition, posttranslational modifications of membrane-associated proteins vary according to the growth phase of *L. biflexa* (Stewart *et al*, 2016).

Compositions of culturing media can affect the gene expression. Different batches of reagents and bovine serum albumin in Ellinghausen-McCullough-Johnson-Harris (EMJH) growth medium cause different alterations in gene expression thereby producing variations in *Leptospira* growth in culture (Stewart *et al*, 2016).

The amounts of samples required can be an issue. A relatively large amount of sample is required for proteomics (in 2DE step) compared with genomics studies (Hanash, 2003). Genomics assays can utilize PCR to amplify a limited sample while no such options exist in proteomics (Srinivas *et al*, 2002). Despite Cullen *et al* (2005) reported that their leptospiral surfaceomic study was able to profile surface protein expression using a small amount of sample ($<10^7$ cells); however, proteomic assays usually require a purification step to enrich a sample (Koomen *et al*, 2008).

Compared to proteins, genetic material is more stable under various conditions. MicroRNA (miRNA) remains stable even after being subjected to severe conditions, *viz*. boiling, very low or high pH levels, RNase A treatment, different storage

conditions and up to ten freeze-thaw cycles (Chen *et al*, 2008; Grasedieck *et al*, 2012). On the other hand, relatively mild chemical and physical treatments can readily lead to protein denaturation, aggregation and precipitation (Jacob *et al*, 2006). Thus, precautions are required to maintain protein stability through appropriate choice of buffers, surfactants, anionic polymers, cyclodextrins, metal ions, salts, and lyophilization and freezing procedures (Manning *et al*, 2010).

Another challenge in biomarker discovery is that differential mRNA expression does not always correlate with the protein expression (Gygi *et al*, 1999; Adler *et al*, 2011). LipL36 level is reduced under iron depletion conditions, but not *lipL36* transcription (Cullen *et al*, 2002). The discrepancy might be attributed to post-translational processing and regulation events not related to transcript level (Haake *et al*, 1998; Adler *et al*, 2011).

Expression of a virulence gene might depend on the type of tissue infected owing host-pathogens interactions specific to the host cell types. For example, a dialysis membrane chamber used to study the response of leptospires to host-derived signals was insufficient to describe the interactions in lung (Caimano *et al*, 2014). A novel leptospiral adenylate/guanylate cyclase responsive to cAMP-stimulating activity is upregulated only in a tissue-specific manner (Lehmann *et al*, 2014). Thus, care should be taken when interpreting data obtained from a cell line; if possible a number of cell lines from different lineages should be evaluated in host-pathogen interaction experiments.

Numerous studies have applied 2DE in their leptospiral proteomics research (Cullen *et al*, 2002; Nally *et al*, 2005, Nally *et al* 2007; Hoke *et al*, 2008; Eshghi *et al*, 2009; Srikram *et al*, 2011; Tan *et al*, 2017). 2DE can resolve different protein isoforms (Sickmann *et al*, 2001) and is useful in studies of post-translational modifications (Chandramouli and Qian, 2009). However, this gel-based method is sequential, labor-intensive and difficult to automate. 2DE also has limitations in identification of proteins with extreme isoelectric properties (beyond the pH range of pH gradient) and unusually large or small sizes (Minden, 2007; Chevalier, 2010). In addition, it is also difficult to detect membrane proteins and peptides because the strong detergents used in membrane protein extraction are not compatible with the isoelectric focusing procedure (Schwartz *et al*, 2001). In order to overcome these limitations, an increasing number of recent studies have resorted to adopting gel-free methods, such as isobaric tags (iTRAQ) for relative and absolute quantitation, liquid chromatography-mass spectrometry (LC-MS) and protein array, all of which are more accurate, simple, fast, sensitive and reproducible (Sakolvaree *et al*, 2007; Eshghi *et al*, 2009; Nally *et al*, 2011; Tan *et al*, 2017).

Moreover, proteomics research is always complicated by alternate splicing of transcripts and post-translational modifications of proteins (Griffiths *et al*, 2010). Analysis of proteomics results is time-consuming, as proteomes differ due to epistasis and environmental influences on gene expression and protein composition and modification (Barth *et al*, 2013).

IMPROVEMENTS TO OMICS TECHNOLOGIES

Integration of other applications could improve the quality of output from current omics technologies. In transcriptional profiling, single-nucleotide poly-

morphism or polymorphism genotyping in general can be integrated with mass spectrometry or protein arrays to improve their output. Other improvements, *viz*. higher-speed arraying, humidified arraying chambers, generation of stable and long-lasting antibodies for proteomics, larger scale cell-free protein synthesis, higher-throughput and rate of sequencing or arraying with online detection, clearer imaging, and integration of simpler analysis tools, are also expected to improve the overall output.

Phylogenetic analysis is important for gaining insight into the evolutionary process ranging from *L. biflexa* to *L. interrogans*. It can also provide information on gene gain, loss, transfer and duplication or on abundance of expansion of specific virulence-related protein families in *Leptospira* spp (Xu *et al*, 2016). The combination of phylogenetic and omics technologies can help to identify new serovars and their origins without the need for serotyping (Lehmann *et al,* 2014).

Metabolomics can provide information on the possible roles of metabolites of interest in leptospirosis pathogenesis and effects of *Leptospira* on host metabolism (Fiehn, 2001). The study of metabolites plays an important role in revealing interactions between host and pathogen, as metabolites are required for all pathogens to survive and multiply (Li *et al*, 2013). Many host metabolites have already been suggested as important in pathogenesis as well as indicators of leptospirosis severity, *viz*. apolipoprotein AI, interleukin 6, serum amyloid A, nitric oxide, serum creatinine phosphokinase, alanine transaminase, aspartate transaminase, and alkaline phosphatase (Kalugalage *et al*, 2013; Soares *et al*, 2017; Tan *et al*, 2017). These findings upon integrating with metabolomics technology should be useful in the treatment and care of *Leptospira*-infected patients.

Chaperonomics is an emerging omics technology with which to study chaperone genes, transcripts, proteins, and their interaction networks (Vinaiphat and Thongboonkerd, 2018). Several chaperone/heat shock proteins have been found in pathogenic *Leptospira* but absent in the saprophytic species, *eg* Qlp42 (Nally *et al,* 2001) and Hsp15 (Guerreiro *et al,* 2001; Cullen *et al,* 2002). Response of leptospiral heat shock proteins towards temperature shift has been reported (Fayet *et al,* 1989; Nally *et al,* 2001; Lo *et al,* 2009; King *et al,* 2014). Hence, an integration of this new approach with other existing omics should enable further development of chaperone/ heat shock protein-based diagnostics and vaccine development for leptospirosis (Vinaiphat and Thongboonkerd, 2018).

Currently, computational or bioinformatics approach is of use in assisting omics technologies to study *Leptospira* pathogenicity. It has shown more sensitivity than high-throughput laboratory approaches for resolving some of the limitations of experimental work, such as in the detection of low-abundance proteins and cross-contamination of cellular compartments during sample preparation (Rey *et al*, 2005). Computational methods can also assist in predicting bacterial protein subcellular localization, membrane proteins and lipoproteins; hence, this tool can provide clues to the understanding of protein function and disease pathogenicity, as well as assisting in novel drug development. Several computational programs have been developed, *viz*. SpLip for predicting lipoproteins (Setubal *et al*, 2006); ProtCompB (Viratyosin *et al*, 2008), Proteome Analyst (Amineni *et al*, 2010), P-CLASSIFIER and PSORT/PSORTb for

predicting protein cellular localization (Oliveira *et al*, 2011; Xu *et al*, 2016); SignaP for predicting presence and location of signal peptide cleavage sites in export/ membrane proteins (Nascimento *et al*, 2004; Gamberini *et al*, 2005); TMHMM for predicting protein transmembrane helices (Nascimento *et al*, 2004; Gamberini *et al*, 2005); and BLAST for searching putative proteins homologous to previously characterized surface proteins (Gamberini *et al*, 2005) to assist in meeting these needs. However, these approaches are insufficiently specific because they are dependent on data from previously identified proteins in related organisms (Krogh *et al*, 2001; Bendtsen *et al*, 2004; Kall *et al*, 2004; Emanuelsson *et al*, 2007).

CONCLUSION

Leptospirosis is one of the most common and widespread zoonotic disease worldwide. Discovery of virulent factors and mechanism of pathogenesis are very important for efficient diagnosis and vaccine development. However, the utilization of multiple omics approaches is encouraged as it can assist in the selection of appropriate antigens for the development of diagnostic tests, therapeutic drugs and vaccines in combating leptospirosis.

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