# CHAPTER VI
# DISCUSSION

The proteomes of *Leptospira* spp. were studied using the 2DE-based method. The 2DE-based proteomics has some limitations, *i.e.,* the 2DE is not suitable for studying components in highly complex protein mixtures or membrane proteins of low solubility (Washburn *et al*., 2001; Delahunty and Yates, 2005). Nevertheless, the *Leptospira* components were readily revealed by the proteomic conditions used in this study. In order to solubilize as much as possible the *Leptospira* spp. proteins especially the outer membrane lipoproteins, the lysis buffer containing both thiourea and urea at high molarity, *i.e.* 2 M and 7 M, have used in the study. The buffer was found to solubilize most if not all of the bacterial proteins as there were only minute amounts of the sediments after high speed centrifugation of the *Leptospira* spp. homogenates in the lysis buffer.

In the first part of this study, 2D-DIGE system was used to determine the difference of proteins expressed by non-pathogenic and pathogenic *Leptospira* spp. which are currently used as antigens for MAT compared with the primary clinical isolated *L. interrogans* serogroup Pomona, serovar Pomona.

The proteins common to pathogenic *Leptospira* spp. from the mass spectrometry analysis were 1) proteins involved in transcription and translation *i.e.* DNA polymerase III, beta subunit **(spot no.1, Figure 32)** and elongation factor Ts **(spot no. 9, Figure 32)**; 2) proteins fumcting as enzymes for metabolisms and nutrient acquisition *i.e.* succinate dehydrogenase **(spot no. 6, Figure 32), t**ransaldolase **(spot no. 8, Figure 32);** 3) protein/enzymes necessary for energy and electron transfer *i.e.* electron transport/transfer flavoprotein beta subunit **(spot no. 7, figure 32)**; 4) proetin used for host immune evasion *i.e.* 2-cys thioredoxin peroxidase peroxiredoxin **(spot no. 10, Figure 32;** 5) cell structure *i.e.* rod shape-determining protein mreB **(spot no. 2, figure 32)**, periplasmic flagella, endoflagellar filament core protein, flagellin protein and flagellar filament sheath protein **(spot no. 3, Figure 32)**, possible hook-associated protein, flagellin family **(spot no. 6, Figure 32);** 6) outer membrane

protein **(spot no. 5, Figure 32)** and 7) hypothetical protein of spot no 11. (LA1044, LBJ_0284, LBL_2792, and LIC12621), LIC10483 **(spot no. 2, Figure 32),** LIC11848 **(spot no. 5, Figure 32)**.In the second part of this study, we produced two specific hybridomas secreting MAbs that bound to antigens with different molecular masses of all pathogenic *Leptospira* spp. tested but did not bind to a homogenate of the non-pathogenic *L. biflexa* serovar Patoc. The MAbs reacted to only some components of *L. biflexa* serovar Andamana; this strain behaved as saprophyte, as even though it can infect hosts, it does not cause disease and seems to have some physiological characteristics in between saprophytes and pathogens (Fuzi and Csoka 1961; Kmety et al., 1966; Johnson and Harris, 1967). Thus, it can be concluded that the MAbs are specific to only pathogenic leptospires. Our finding that both MAbs neutralized the *in vitro Leptospira* mediated-hemolysis and rescued the infected animals from severe morbidity and mortality, prompted us to search for the proteins of the pathogenic *Leptospira* spp. that contained the epitopes of these MAbs, as these proteins could be broad spectrum vaccine candidates.

By using gel based-proteomics, 2DE-immunomics and database searching, *Leptospira* orthologous proteins containing peptides matched with those that were bound by the two MAbs were identified. Although the MAbLPF1 did not bind to proteins in spots no. 1, 2 and 21 **(Figure 44B)** which are orthologous to UDP-glucose 4-epimerase, ABC transporter- ATP-binding protein, GrpE, flagellin protein, and hypothetical protein LIC12326 **(Table 11)**, it bound to other proteins with similar functions, for examples that of spots no. 10, 15 and 16. The MAbLPF1 on the same weight basis was equally effective as the MAbLPF2 in the inhibition of *Leptospira*-mediated hemolysis *in vitro* and in rescuing the infected hamsters from lethality caused by heterologous *Leptospira* spp. (data not shown). The protein orthologs of the two MAb epitopes were found to be pivotal for several biologic, chemical and physiological functions of pathogenic *Leptospira* spp. It is likely, therefore, that the *in vivo* protective/therapeutic effects of the MAbs observed in this study were mediated by several mechanisms depending on the functions of the target proteins that the antibodies bound to. Possible protective mechanisms include: inhibition of protein synthesis; inhibition of motility by binding to flagella proteins and also causing *Leptospira* agglutination by binding to surface lipoprotein components, thus,

immobilizing the bacteria and facilitating ingestion and intracellular killing by host phagocytes; protection via complement mediated bacterial lysis; interference in the functions of bacterial enzymes and proteins necessary for various metabolic and biosynthetic pathways; interference with bacterial enzymes and proteins required for the acquisition of food and nutrients; blockade of electron, energy and protein transport; impairing cellular integrity; and abrogation of the bacterial ability to evade the host immunity. Further experiments are needed to demonstrate these speculated activities of the MAbs.

Although the data from this study show that several *Leptospira* proteins contain epitopes of the MAbLPF1 and MAbLPF2 and that they have high potential as broad spectrum leptospirosis vaccine candidates, the exact amino acid sequences of the epitopes have not been revealed. We have tried to match the peptide sequences from the proteins of all spots generated by mass spectrometry in order to see if there was any common amino acid sequence but did not find any. It is possible that the peptide sequences of the epitopes were destroyed during the proteomic processes such as tryptic digestion and ionization. It is unlikely that they were conformational epitopes because their antigen binding activity was not affected by the protein separation by both SDS-PAGE and 2DE during which the *Leptospira* proteins were exposed to several reducing agents, *i.e.* SDS, mercaptoethanol and dithiothreitol. Possible approaches to identify the sequences of the MAb epitopes include an *in silico* search with the B cell epitope database, epitope mapping using peptide segments of the MAb bound-proteins, and mimotope searching using a peptide phage display library (Ramasoota et al., 2006). The sequences may be linked with a suitable T cell epitope derived from either *Leptospira* or non-*Leptospira* protein to create an immunogenic non-culture based, broad spectrum leptospirosis vaccine component. The so-constructed molecule in a suitable formulation (with adjuvant) and with an appropriate immunization procedure should bring about immune effector functions that exert protection by interfering with several vital activities as well as cellular integrity of the infecting leptospires. In this part, the study shows that using monoclonal antibodies and the knowledge about their target proteins could lead to a more efficient and safer vaccine. Additionally these proteins could be used as targets

for an antigen based diagnostic kit for the rapid bed-side diagnosis of human leptospirosis.

For the third and the fourth part of research, two dimensional electrophoresis (2DE)-based-proteomics, 2DE-immunomics and bioinformatics was used to study proteomes, immunomes, proteins unique to pathogenic *Leptospira* spp. and in vivo expressed antigens of the bacteria.

The immune mouse serum had satisfactorily high titer after immunization with the *L. interrogans*, serogroup Icterohaemorrhagiae, serovar Icterohaemorrhagiae using the indicated routes, doses and schedule. The antibodies in the immune serum could recognize many immunogenic proteins in the homogenates of the heterologous species, serogroups and serovars, *i.e. L. interrogans,* serogroup Icterohaemorrhagiae serovar Copenhageni and heterologous serogroup and serovar, *i.e. L. borpetersenii*, serogroup Tarassovi, serovar Tarassovi. Unfortunately, the immunome of the *L. interrogans*, serogroup Icterohaemorrhagiae, serovar Icterohaemorrhagiae which is the homologous system was not done in this study because of the inadequate supply of the bacterial cells at the time of study.

Because the complete genome sequences of at least three *Leptospira* serovars, *i.e.* Copenhageni, Lai and *L. borgpetersenii* were established and the information are available in the database (http://www.matrixscience.com, http://www.ebi.ac.uk, and http://www.brenda.uni_koeln.de), thus, identification of the most immunogenic proteins of the two studied *Leptospira* spp. were possible. Nevertheless, there are still few immunogenic proteins, *i.e.* hypothetical proteins that are needed to be elucidated.

The *in vivo* expressed expressed antigens of pathogenic *Leptospira* spp. obtained from immunomes of convalescence phase sera of patient with leptospirosis, protein reactive to murine monoclonal antibodies (MAb, LPF1 & LPF2) specific to pathogenic *Leptospira* spp. (epitopes unique to pathogenic *Leptospira* spp.) and mouse immune serum (*Leptospira* immunome) are the genes candidates for leptospirosis DNA vaccine For leptospirosis, antibodies are believed to be the immune correlate of protection against and recovery from the infection/disease (Midwinter *et al*., 1994 Sonrier *et al*., and 2000 Guerreiro *et al*., 2001). However, the *Leptospira* spp. grows slowly in the *in vitro* culture with the doubling time of 12-14 hours, (Levett, 2001)

rendering inadequate supply of the vaccine raw materials, *i.e.* the whole cells and the native outer membrane proteins. Moreover, the vaccines confer immunity limited only to the homologous or closely related *Leptospira* infection (Koizumi and Watanabe, 2005). As such, identification of the immunogenic proteins common among the pathogenic *Leptospira* spp. is necessary as they are not only the potential candidates of the vaccine that are likely to protect across serogroups and serovars of *Leptospira* spp. (broad protective spectrum vaccine) but also their coding DNA as well as their recombinant protein counterparts could be more readily prepared than the conventional whole cells and native membrane components.

Among gram negative bacteria, several transmembrane/membrane associated proteins and their functions are known (http://en.wikipedia.org/wiki/Transmembrane _proteins). These are for examples: the light absorption-driven transporters (rhodopsin-like protein, light harvesting complex), the oxidoreduction-driven transporters (*e.g*. succinate dehydrogenase, transmembrane cytochrome B-like proteins, coenzymes–cytochrome C reductase, formate dehydrogenase, cytochrome C oxidases), the electrochemical potential-driven transporters (proton and sodium translocating ATPases), the P-P-bond hydrolysis-driven transporters (calcium ATPase, general secretory pathway translocon, ABC transporter, drug transporter), the porters (uniporters, symporters, antiporters), siderophores, and other α-helical and β-barrels transmembrane proteins (http://en.wikipedia.org/wiki/Transmembrane_ proteins). For the pathogenic *Leptospira* spp., the proteins known to be constitutively expressed on the bacterial surface are, for examples, LipL41, LipL32 (Hap1) and trimeric OmpL1 porin proteins. These proteins prepared from the *Leptospira* spp. grown *in vitro* have been tested as the vaccine candidates (Haake *et al*., 1999 and Branger *et al*., 2005). While the results are encouraging, the inadequate supply of the components limits the vaccine accessibility by the population at the leptospirosis risk. Other integral transmembrane-, membrane associated-and secreted/recreted- proteins and enzymes also have potential as vaccine candidates provided that they are immunogenic in mammalian hosts.

The results on the proteomes and immunomes of the pathogenic *Leptospira* spp. should be, more or less, useful information for designing a vaccine, either aiming at

inducing humoral and/or cell-mediated immunity, and development of both antibody based- and antigen-based diagnostic assays.

The proteomes of two *Leptospira* species, *i.e. L. interrogans,* serogroup Icterohaemorrhagiae, serovar Copenhageni, Icterohaemorrhagiae and *L. borgpetersenii,* serogroup Tarassovi, serovar Tarassovi were studied by using two dimensional-gel electrophoresis (2DE), LC/MS-MS and database search. The antigenic components (immunomes and *in vivo* expressed antigens) of the *Leptospira* spp. were determined by probing the 2DE-blots with immune serum of a mouse immunized with pathogenic *Leptospira* spp. homogenate and pateints' sera with acute and convalescence phase. It was found that there are many antigenic components shared by both pathogenic *Leptospira* serovars and they are likely to be broad spectrum vaccine candidates.

Nevertheless, we have chosen to study the immunogenicity and protective efficacy of a DNA vaccine made up of the gene encoding the outer membrane protein, *i.e.* OmpL1, for the reasons that *ompL1* DNA vaccine has not been studied previously. Besides, the protein has been found to be a transmembrane porin of pathogenic *Leptospira*; therefore it is likely that a portion of the protein might be exposed on the bacterial surface which is accessible by the host immune apparatus.

Information so-gained together with the data on the complete genomes of pathogenic *Leptospira* spp. deposited in the database were used to select a *ompL1* gene of *Leptospira* as DNA vaccine candidate. The Genomic DNA of *Leptospira interrogans*, serogroup Icterohaemorrhagiae, serovar Copenhageni was used as a template to amplify the selected gene by PCR. The recombinant plasmid was used to intramuscularly immunize a group of four week old hamsters. The booster dose were given 14 days intervals, three times. Hamsters of three control groups received injections of empty plasmids and phosphate buffered saline as placebos. All treated mice were then challenged with 10 LD50 of heterologous *Leptospira interrogans*, serogroup Pomona, serovar Pomona which is a human clinical primary isolate (heterologous challenge). It was found that all hamsters injected with empty plasmids and PBS died from the lethal *Leptospira* challenge. The *Leptospira* plasmid DNA vaccine conferred 33% protection to the immunized hamsters.

Even though most OMPs are present in small amount but they play several important roles in the bacterial survival and pathogenesis. OMPs were found to function as bacterial adhesin (Isberg and Falkow, 1985; Bessen and Gotschich, 1986), porins (Elkins and Sparling, 1990) and receptors for soluble molecules, such as siderophores (Stoebner and Payne, 1998). Because they are more or less exposed to the extracellular milieu they should be accessible by the host immunological factors such as antibody. Infact, OMP was shown to be a target of bactericidal antibody (Elkins and Sparling, 1990; Jeanteur *et al*., 1991; Saukkonen *et al*., 1987). They could be bound *in situ* by the host complement proteins (Hoffman *et al*., 1992) rendering the bacterial more approachable by the host phagocyte; thus enhances phagocytosis.

OmpL1 is an immunogenic porin protein found in pathogenic *Leptospira* spp (Shang *et al*., 1995). OmpL1 and LipL41 proteins exhibited synergistic immunoprotection against leptospirosis (Haake *et al*., 1999). In this study the *ompL1*-plasmid DNA, constructed by using genomic DNA of *L. interrogans*, serogroup Icterohaemorrhagiae, serovar Copenhageni and a mammalian expression plasmid vector, was tested for the protective efficacy in the leptospirosis susceptible animal model, *i.e.* hamsters after three consecutive intramuscular doses at two weeks intervals. The vaccine was found to be well tolerated by the immunized animals and confer immunity that protected some immunized hamsters against the heterologous lethal challenge by *L. borgpetersenii*, serogroup Tarassovi, serovar Tarassovi isolated from patient with leptospirosis. The vaccine was shown to confer the delay in the death time, morbidity in other vaccinated animals when compared to animals immunized with the plasmid alone or PBS. Further experiments are needed, however, to optimize the immunization schedule, dosages as well as the vaccine formulation in order to maximize the protective efficacy.