CHAPTER III
LITERATURE REVIEW

1. Leptospirosis

Human leptospirosis is a zoonotic disease caused by bacteria of the family leptospiraceae, order Spirochaetales, genus *Leptospira.* The organisms in this genus have a typical shape that are fine spiral. Leptospires are highly motile, obligate aerobic spirochetes that share features of both Gram-positive and Gram-negative bacteria. Leptospires are about 0.25 × 6-25 μm in size and can pass through 0.45 μm filters. Dark-field or phase contrast microscopy or wet preparations is required for direct visualization of leptospires, since the bacteria stain poorly. The appearance and motility of leptospires varies with the nature of the medium in which they are grown.. In liqiud media, cells appear bent or hooked at one or both ends, although straight mutants do exist. Leptospires are cultivated in artificial media containing rabbit or bovine serum plus long chain fatty acids at pH 6.8-7.4. Optimum growth temperature is between 28°C and 30°C. A commonly used medium is Ellinghausen-McCullough-Johnson-Harris (EMJH) medium which contain 1% bovine serum albumin and Tween 80 (source of long-chain fatty acids); commercial formulation are available. Serum-containing liquid or semisolid media include Korthof's (peptone, NaCl, $NaHCO_3$, KCL, CaCl, KH_2PO_4, Na_2HPO_4) and Fletcher's (peptone, beef extract, NaCl, and agar).

Bacteria in the genus *Leptospira* have been traditionally classified into two species, *i.e. L. interrogans* which included all pathogenic strains of both animals and humans, and *L. biflexa* which are non-pathogenic, free-living saprophytes. The two species are different also in their growth at different temperatures. There are more than 200 different serovars of *Leptospira* spp. based on different agglutinating antigens. The antigenically related serovars have been arbitrarily allocated into the same serogroups. Recently, molecular techniques, such as 16S rRNA analysis and multi-locus sequence typing, based on sequence alignment of genes encoding house-keeping enzymes, have been used for re-delineation of members of *Leptospira* spp.

into genomospecies. *Leptospira* spp. are divided into genomospecies which do not correlate to the *L. interrogans sensu lato* and *L. biflexa sensu lato.* These genomospecies include: *L. alexanderi, L. biflexa, L. borgpetersenii, L. fainei, L. inadai, L. interrogans, L. kirschneri, L. meyeri, L. noguchii, L. parva, L. santoirasai, L. weilii, and L. wolbachii* and few other unnamed genomospecies. Members of *L. biflexa, L. parva* and *L. wolbachii* are non-pathogenic; strains of *L. inadai* and *L. meyeri* may be either pathogenic or non-pathogenic, while strains belonging to the remaining genomospecies are pathogenic. Several antigenically related strains previously allocated in the same serogroup/serovar are now found in more than one genomospecies. For example, strains of serogroup Bataviae may belong to either *L. interrogans* or *L. santarosai*; serogroup Hardjo may be found in *L. borgpetersenii, L. interrogans* and *L. meyeri* genomospecies.

Human gets infection through wounded, abraded, and macerated skin, or mucous membranes (*e.g.* oral, conjunctival mucosae), either directly by contact with infected/reservoir animals or their contaminated specimens, or indirectly by exposure to damp soil, mud, vegetation or fresh water seeded with the urine or carcass of the infected/reservoir animals. Human-to-human transmission is relatively rare. Human leptospirosis used to be recognized as an occupational disease with high incidence among veterinarians, abattoir workers, sewer workers and farmers. However, a number of cases were found among travelers to the disease endemic areas and individuals after various re-creational activities such as canoeing, swimming, hiking, and rafting.

1.1 Epidemiology

In developing countries, leptospirosis is a significant health burden for poor rural populations (Levett, 2001; Bharti *et al*., 2003). Subsistence farmers who are exposed to environments contaminated with the urine of domestic and sylvatic animal reservoirs are risk groups. However leptospirosis has become an urban problem as the rural poor have moved to cities (Vinetz *et al*., 1996; Ko *et al*., 1999; Kariv *et al*., 2001; Sarkar *et al*., 2002; Karande *et al*., 2003; Romero *et al*., 2003; Johnson *et al*., 2004; Tassinari *et al*., 2004; Karande *et al*., 2005; LaRocque *et al*., 2005). One billion people reside in urban slums where the lack of basic sanitation has produced the ecological conditions for rodent-borne transmission (Ko *et al*., 1999; United Nations,

2003; Johnson *et al*., 2004). In Brazil, outbreaks occur each year in poor urban communities (Favelas) during the same seasonal period of heavy rainfall (Ko *et al*., 1999; Sarkar *et al*., 2002; Romero *et al*., 2003; Tassinari *et al*., 2004). More than 10,000 cases of severe leptospirosis are reported annually due to cyclic rainfall-associated urban epidemics in Brazil alone. The situation of urban leptospirosis is expected to become more urgent as the world's urban slum population doubles in the next few decades (United Nations, 2003). Of note, this problem is not limited to 'tropical' settings for the importance of urban leptospirosis was first recognized among US inner-city populations (Vinetz *et al*., 1996). These epidemiological patterns sharply contrast with the trend that has evolved in developed countries. Leptospirosis, traditionally an occupational disease (Levett, 2001) is now identified among the affluent who engage in recreational activities (Nardone *et al*., 2004), sporting events (Morgan *et al*., 2002), travel and adventure tourism (Sejvar *et al*., 2003; Haake *et al*., 2002).

Investigations from Asia provide evidence that leptospirosis has become an important public health problem (Phraisuwan *et al*., 2002; Karande *et al*., 2003; Jena *et al*., 2004; Murdoch *et al*., 2004; Karande *et al*., 2005; LaRocque *et al*., 2005) and Latin America (Ko *et al*., 1999; Sarkar *et al*., 2002; Romero *et al*., 2003; Johnson *et al*., 2004; Tassinari *et al*., 2004; Segura *et al*., 2005). Yet it continues to be under-recognized by policy makers due to the poor quality of surveillance data. Information on the global burden has not been compiled since 1999 (Smythe, 1999); and therefore it is critical that health ministries report cases to the web-based *Leptonet* surveillance system (website: http://www.leptonet.net) developed by the International Leptospirosis Society. Moreover, surveillance significantly under estimates the impact of leptospirosis: reliance on classic severe manifestations does not identify the majority of cases which present with mild disease; few laboratories obtain paired serum samples and perform the standard diagnostic test, the microagglutination test (MAT); and little population based information is available on disease burden. Syndrome-based protocols have been effectively used in outbreaks (Karande *et al*., 2003; Russell *et al*., 2003; Karande *et al*., 2005; LaRocque *et al*., 2005) and surveillance (Laras *et al*., 2002; Murdoch *et al.,* 2004) and found that a large proportion of leptospirosis cases would have otherwise gone unrecognized or been

attributed to other diseases. In a multicenter study from four countries in Southeast Asia, leptospirosis was found to be the cause of disease in 17 and 13% of the patients with non-viral hepatitis jaundice and non-malarial fever, respectively (Laras *et al*., 2002). Establishing similar sentinel syndrome-based surveillance should be a priority to obtain more accurate information on disease burden.

An important epidemiological question concerns why certain exposed individuals develop mild symptomatic infections while others progress to develop severe disease forms such as Weil's disease (the triad of jaundice, acute renal failure and bleeding) and severe pulmonary hemorrhage symdrome (SPHS). During the 1995 Nicaragua epidemic (Ashford *et al*., 2000) and an outbreak in Thailand (Phraisuwan *et al*., 2002), asymptomatic infections occurred in 60–70% of all serologically identified infections. In contrast, in the 1998 Lake Springfield outbreak (Morgan *et al*., 2002), almost all triathletes with serologic evidence for infection developed symptomatic disease. The differences in disease risk may relate to several factors including the size of the infecting inoculum dose and the backgroud immunity of the host. Population-based studies have found that adult males had increased risk for severe leptospirosis (Perrocheau and Perolat, 1997; Ko *et al*., 1999; Everard *et al*., 1995). A longitudinal study in Peru found that 12% of 195 urban slum residents had serologic evidence for a new infection during the flood season (Johnson *et al*., 2004).

At present there are few effective prevention measures for leptospirosis. An outbreak investigation in Thailand found that the use of protective clothing and presence of skin wounds was associated with decreased and increased, respectively, risk for infection (Phraisuwan *et al*., 2002). These findings confirm the general belief that breaks in the skin facilitate entry of leptospires. Unfortunately, the use of protective clothing, including boots, will not be an option in most situations. Doxycycline (100 mg orally per week) is used for chemoprophylaxis but, given that doxycycline's half-life is 18 h, the dosage may need to be reconsidered (Haake *et al*., 2002). Although chemoprophylaxis may be feasible in target groups such as travellers, it will be impractical in large at-risk populations. Apart from vaccination of domestic animals, sustained control of reservoirs is difficult to implement. Rural leptospirosis may be an intractable problem given the complex ecological interactions

involving domestic and wild reservoirs and environmental transmission sources (Johnson *et al*., 2004).

1.2 Pathophysiology and clinical management

The major burden of leptospirosis is due to severe disease forms, Weil's disease and severe pulmonary hemorrhage symdrome (SPHS). Case fatality is over 10% for Weil's disease (Daher *et al*., 1995; Ko *et al*., 1999; Dupont *et al*., 1997; Panaphut *et al*., 2002; Covic *et al*., 2003; Esen *et al*., 2004; Lopes *et al*., 2004) and over 50% for SPHS (Marotto *et al*., 1999; Vieira and Brauner, 2002; Segura *et al*., 2005) in most settings. Prompt triage of high-risk patients is critical since complications require aggressive treatment and monitoring. Older adults (over 30–40 years of age) have an increased risk for death (Daher *et al*., 1999; Ko *et al*., 1999; Vieira and Brauner, 2002; Lopes *et al*., 2004). Independent prognostic factors for mortality are acute renal failure (oliguria, hyperkalemia, serum creatinine ≥3.0 or 4.0 mg/dl), respiratory insufficiency (dyspnea, pulmonary rales, radiological evidence for infiltrates), hypotension, arrhythmias and altered mental status (Dupont *et al*., 1997; Daher *et al*., 1999; Ko *et al*., 1999; Panaphut *et al*., 2002; Esen *et al*., 2004). Altered mental status was found to be the strongest predictor of death in two studies (Ko *et al*., 1999; Esen *et al*., 2004) yet a specific central nervous system process has not been identified which can explain this finding. Nevertheless, the basis for reducing mortality relies on antibiotic therapy and management of acute renal failure, respiratory insufficiency and shock (McBride *et al*., 2005).

Leptospirosis causes a unique non-oliguric hypokalemic form of acute renal insufficiency (Seguro *et al*., 1990). Its hallmark features are impaired proximal sodium reabsorption, increased distal sodium delivery and potassium wasting (Seguro *et al*., 1990; Abdulkader *et al*., 1996). When identified during this initial phase of renal insufficiency, patients have a better overall prognosis (Ko *et al*., 1999; Marotto *et al*., 1999; Panaphut *et al*., 2002; Esen *et al*., 2004) and can be treated with potassium and volume repletion (Abdulkader *et al*., 1996). With continued sodium and volume loss, however, patients will develop oliguric renal insufficiency including acute tubular necrosis (Covic *et al*., 2003). For these patients, dialysis is the critical intervention for preventing mortality (WHO, 2003). Peritoneal dialysis, although commonly used in resource limited settings, may not efficiently correct the

hypercatabolic disturbances in systemic leptospirosis. The use of continuous hemofiltration, which has been shown to be more effective than peritoneal dialysis in treating infection-associated acute renal failure (Phu *et al*., 2002). Furthermore, all efforts should be made to prevent delays in starting dialysis. Among SPHS patients with acute renal failure at an infectious disease hospital in São Paulo, Brazil, reducing the time between hospitalization and initiation of dialysis was responsible in part for a decrease in mortality from 55 (Marotto *et al*., 1999) to 18% over a 10-year period (McBride *et al*., 2005).

Magaldi and colleagues have identified possible mechanisms for this peculiar form of acute renal failure (Magaldi *et al*., 1992). The lesion site is believed to be the proximal tubule since infected guinea pigs have intact thick ascending limb function. Studies found that *L. santarosai* serovar *shermani* infected patients from Taiwan had defective responses to furosemide, however, suggesting that the lesion may involve the thick ascending limb. The target may be the sodium–potassium–chloride co-transporter since *Leptospira* outer membrane extracts inhibit transporter activity *in vitro* and downregulate Na(+)-K(+)-Cl(−) co-transporter (*NKCC2*) mRNA transcription (Wu *et al*., 2004). Alternatively, Burth and colleagues have proposed that *Leptospira*-derived unsaturated fatty acids act as toxins that inhibit kidney sodium–potassium ATPase (Burth *et al*., 2004). Disease severity in patients was correlated with the serum oleic and linoleic acid : albumin ratio. Moreover, sera from healthy individuals and albumin reversed oleic acid-mediated inhibition of sodium–potassium ATPase activity *in vitro*, whereas patient sera failed to do so. These results suggest that albumin is a "serum protection factor" against the toxic effects of unsaturated fatty acids released from *Leptospira* (Burth *et al*., 2004).

Leptospirosis-associated SPHS is now recognized as a widespread public health problem (Trevejo *et al*., 1995; Marotto *et al*., 1999; Panaphut *et al*., 2002; Vieira *et al*., 2002; Segura *et al*., 2005). Frank hemoptysis, the characteristic sign of SPHS, may not be evident until patients are intubated.

SPHS patients appear to have a high (approximately 1,000,000 bacteria/mg of lung tissue) leptospiral burden in the lungs (Segura *et al*., 2005). Yet few intact leptospires are observed in autopsy (Nicodemo *et al*., 1997) and experimental animal tissues (Nally *et al*., 2004; Pereira *et al*., 2005), suggesting a possible role for an

immune-mediated process. Damage to the pulmonary endothelium occurs without evidence for disseminated intravascular coagulation (Nicodemo *et al*., 1997; Nally *et al*., 2004; Pereira *et al*., 2005). Nally and colleagues have found that in infected guinea pigs, immunoglobulin and C3 are deposited along the alveolar basement membrane in a similar pattern to that seen in Goodpasture's syndrome (Nally *et al*., 2004). A major advance has been the trials from Thailand (Panaphut *et al*., 2003; Suputtamongkol *et al*., 2004), which showed that treatment with ceftriaxone, cefotaxime and doxycycline had equivalent efficacy to penicillin therapy.

1.3 Clinical and laboratory diagnosis

Timely diagnosis is essential since antibiotic therapy provides greatest benefit when initiated early in the course of illness (Faine *et al*., 1999; WHO, 2003). Although severe late-phase disease can be recognized by its classic manifestations, identification of early-phase leptospirosis is hampered by its non-specific presentation. Recent findings emphasize that leptospirosis is a frequent cause of undifferentiated febrile illness in developing countries (Karande *et al*., 2003; Russell *et al*., 2003; Murdoch *et al*., 2004; Segura *et al*., 2005).

Misdiagnosis has become an evermore critical issue in regions where dengue and other infectious diseases with overlapping presentations are endemic (Ko *et al*., 1999; Karande *et al*., 2000; Karande *et al*., 2002; LaRocque *et al*., 2005; Murdoch *et al*., 2004). Co-infection with diseases such as scrub typhus and malaria have been reported (Karande *et al*., 2002; Wongsrichanalai *et al*., 2003) and presents another source of confusion in tropical settings. Identification of leptospirosis will therefore depend on a high index of suspicion among clinicians and the availability of an accurate "point-of-care" test (Vinetz, 2001; Murdoch *et al*., 2004).

The lack of an adequate laboratory test, however, remains the major barrier for diagnosis and epidemiologic surveillance. Reliance on the MAT is a significant cause of underreporting since paired serum samples need to be tested in order to reliably interpret results (Faine *et al*., 1999; WHO, 2003). Furthermore, quality control measures are rarely used to ensure the integrity of live reference strain panels used in agglutination reactions. The International Leptospirosis Society launched an initiative to establish MAT proficiency testing (Chappel *et al*., 2004). Evaluations of a serum panel in 60 laboratories from 37 countries found a false-negative result rate of 13%.

This rate decreased from 15 to 5% among laboratories who participated in sequential 2002 and 2003 evaluations, respectively, highlighting the importance of proficiency testing as a measure to improve the reference laboratory performance.

Major emphasis has focused on developing improved serologic tests that use whole *Leptospira* antigen preparations. Commercially available whole *Leptospira*-based assays are available in rapid formats amenable for "point-of-care" use. Field evaluations indicate that these assays have essentially similar performance characteristics with low sensitivities (39–72%) during acute-phase illness **(Table 1)** (Smits *et al*., 2001a; Smits *et al*., 2001b; Effler *et al*., 2002; Levett and Branch, 2002; Vijayachari *et al*., 2002; Bajani *et al*., 2003; Sehgal *et al*., 2003). Moreover, the sensitivity may be under 25% during the first week of illness (Effler *et al*., 2002). As a caveat, sensitivity increases significantly between the first and second weeks of illness **(Table 1)**; testing of a late acute-phase sample (past 10th day of illness) is therefore recommended. Anti-whole *Leptospira* antibodies may persist for years after exposure (Cumberland *et al*., 2001). In Peru and Vietnam, over 25% of healthy control individuals had positive IgM enzyme-linked immunosorbent assay (ELISA) reactions (Johnson *et al*., 2004; Wagenaar *et al*., 2004). In addition to their low sensitivity during acute-phase illness, whole *Leptospira*-based assays may therefore have low specificity in regions of high endemic transmission.

Polymerase chain reaction (PCR) based diagnosis remains restricted to reference laboratories and in-house kits, many of which have not been thoroughly evaluated. It is unlikely that PCR will be widely applied in developing countries where prompt diagnosis is most needed. PCR has, however, provided the opportunity to address important research questions. Measurement of leptospiral loads in patients could not be accurately determined prior to development of real-time PCR methods (Smythe *et al*., 2002; Levett *et al*., 2005b; Palaniappan *et al*., 2005). Segura *et al.* used these methods to determine that leptospiraemia is 10,000 or more bacteria per milliliter of blood or milligram of tissue in SPHS patients (Segura *et al*., 2005). Their findings confirmed those of a previous semi-quantitative PCR study (Truccolo *et al*., 2001). The critical threshold appears to be 10,000 or more bacteria per millilitre for developing severe outcomes such as SPHS and death. Rapid antigen detection assays

Table 1 Evaluation trials of whole *Leptospira*-based serologic tests.

Source: McBride *et al*., 2005.

		Proportion (total number tested)		
		Sensitivity		
Evaluation site	Test format (source)[a]	First sample	Second sample	Specificity[b]
Multicentre[c] [37]	ELISA (KIT)	57% (135)	84% (133)	96–99% (389)
	Lateral flow (KIT)	66% (135)	81% (133)	93–96% (389)
	MAT[d]	70% (135)	94% (133)	99–99% (389)
Multicentre[c] [36]	DriDot (KIT)	72% (171)	88% (171)	94–90% (489)
	ELISA (KIT)	60% (171)	89% (171)	96–99% (489)
	MAT[d]	66% (171)	92% (171)	99–99% (489)
Barbados[e] [39]	ELISA (Integrated Diagnostics)	69% (48)	90% (48)	96–93% (110)
	ELISA (PanBio)	70% (48)	88% (48)	96–96% (110)
	ELISA (in-house)	73% (48)	ND	ND
	MAT[d]	29% (48)	ND	ND
India[f] [40]	DriDot (KIT)	68% (74)	86% (74)	66–80% (100)
	ELISA (in-house)	49% (74)	89% (74)	78–84% (100)
India[g] [42]	Dipstick	49% (70)	88% (57)	85–85% (94)
	ELISA (in-house)	50% (70)	88% (57)	79–87% (94)
	Lateral flow (KIT)	53% (70)	86% (57)	94–89% (94)
Multicentre[h] [41]	Dipstick (KIT)	53% (148)	84% (128)	90% (642)
	Dipstick (Integrated Diagnostics)	50% (148)	84% (128)	99% (642)
	ELISA (PanBio)	49% (148)	75% (128)	97% (642)
	IHA (MRL Diagnostics)	39% (148)	67% (128)	96% (642)
	MAT[d]	49% (148)	94% (128)	97% (642)
USA[i] [38]	Dipstick (Organon-Teknika)	28% (88)	ND	96% (291)
	Dot ELISA (PanBio)	51% (88)	ND	95% (291)
	ELISA (Bios GmbH Labordiagnostik)	50% (88)	ND	90% (291)
	ELISA (PanBio)	35% (88)	ND	98% (291)
	ELISA (Virion/Serion)	43% (88)	ND	98% (291)
	IFA (Bios GmbH Labordiagnostik)	38% (88)	ND	85% (291)
	IHA (MRL Diagnostics)	26% (88)	ND	100% (291)
	Latex (Bios GmbH Labordiagnostik)	90% (88)	ND	10% (291)

Information was extracted from selected evaluation trials in which patient groups had culture or microagglutination test evidence for leptospirosis. Only those patients who fulfilled these criteria are shown in the table. ELISA, IgM enzyme-linked immunosorbent assay; MAT, microagglutination test; DriDot, card agglutination test; IHA, indirect hemagglutination assay; ND, no data; IFA, IgM indirect fluorescent antibody assay; Latex (latex agglutination test).

[a]KIT, Amsterdam, The Netherlands; Integrated Diagnostics, Baltimore, Maryland, USA; PanBio, Brisbane, Queensland, Australia; MRL Diagnostics, Cypress, California, USA; Organon-Teknika, Amsterdam, The Netherlands; Bios GmbH Labordiagnostik, Gräfelfing, Germany; Virion/Serion, Würzburg, Germany.

[b]The range of specificities is shown when more than one control group was evaluated.

[c]First and second serum samples were obtained from 0–10 days and >10 days after onset of illness, respectively.

[d]The criteria for a positive MAT titre were either ≥1:200 or ≥1:800 depending on the trial.

[e]First and second serum samples were obtained on first and on fourth day of hospitalization, respectively.

[f]First and second serum samples were obtained from 0–7 days and >7 days after onset of illness, respectively.

[g]First and second serum samples were obtained during the first and second to fourth week after onset of illness, respectively.

[h]First and second serum samples were obtained from 0–14 and >14 days after onset of illness, respectively.

[i]Serum samples were obtained from 0–6 weeks after the onset of symptoms.

have not yet been developed. Saengjaruk *et al.* produced a monoclonal antibody that detected a 35–36 kDa *Leptospira* antigen in clinical samples (Saengjaruk *et al*., 2002). These results are promising yet it is unclear whether current rapid format technology will achieve sufficient sensitivity to detect antigen at levels (100–10,000,000 leptospires/ml) found in the PCR studies (Truccolo *et al*., 2001; Segura *et al*., 2005).

The use of recombinant proteins has been successfully used to develop serologic tests for another spirochaetal infection, Lyme disease. Leptospirosis patients mount robust antibody responses to *Leptospira* proteins early in infection (Guerreiro *et al*., 2001). The performance of recombinant LipL32 and GroEL-based assays have not been entirely encouraging (Flannery *et al*., 2001, Boonyod *et al*., 2005). A promising candidate, however, is leptospiral immunoglobulin-like (Lig) protein (Palaniappan *et al*., 2002; Matsunaga *et al*., 2003; Koizumi and Watanabe, 2004; Palaniappan *et al*., 2004). McBride *et al*. found that a recombinant Lig-based immunoblot assay had improved sensitivity and specificity (>90%) over whole *Leptospira*-based tests (McBride *et al*., 2005).

1.4 Serologic and molecular typing

Strain typing is a useful epidemiologic tool because establishing the causative serogroup or serovar is the first step towards identifying reservoirs and generating control strategies. Most laboratories are unable to serotype due to the limited availability of typing reagents. Results from MAT testing of patients sera are used as a surrogate to infer the infecting serogroup. This approach may be unreliable (positive predictive value, 65–82%) (Katz *et al*., 2003; Levett, 2003). Hence, there is an urgent need for improved typing tools. Pulsed-field gel electrophoresis, 16S rRNA sequencing or PCR-based typing methods have not gained wide acceptance because of their limited discriminatory power, lack of adequate electronic databases of typing and sequence patterns or low reproducibility. A breakthrough for typing has been the discovery of seven polymorphic variable number tandem-repeat (VNTR) loci in the *L. interrogans* serovar Lai genome (Majed *et al*., 2005). PCR amplification of these markers was able to discriminate between 43 of 51 *L. interrogans* serovars. Since VNTR PCR can be easily performed, it is an ideal candidate to replace serotyping. Nevertheless, a major obstacle is the limited capacity of epidemiological services to perform culture isolation. Methods are needed which can directly type strains from

samples. Efforts are being made in Peru to adapt their real-time PCR method (Segura *et al*., 2005) to detect and quantify leptospiral serovars from environmental samples (McBride *et al*., 2005).

1.5 Genome and microbiology

As part of national research initiatives for leptospirosis, China and Brazil sequenced the genome of the causative agents of rural leptospirosis in China (*L. interrogans* serovar Lai strain 56601) and urban epidemics in Brazil (*L. interrogans* serovar Copenhageni strain Fiocruz L1-130) (Ko *et al*., 1999). The Lai and Copenhageni genomes were published in 2003 (Ren *et al*., 2003) and 2004 (Nascimento *et al*., 2004), respectively. A database, LeptoList, is available online for comparison of the two genomes (website: http://bioinfo.hku.hk/LeptoList/). Furthermore, information is available for the incomplete genomes sequences of *L. interrogans* serovar *pomona*, and *L. borgpetersenii* serovar Hardjo-bovis (McBride *et al*., 2005).

The genome comprises two circular chromosomes, CI (approximately 4.3 Mb) and CII (approximately 350 kb), and is highly conserved between the two serovars. This was not unexpected since they are members of the same serogroup and species. The two differences in the genome organization are a large inversion flanked by the insertion sequence IS*lin1* and a 54 kb insertion in the Lai CI chromosome. The Lai and Copenhageni genomes contain 4,768 and 3,728 predicted open reading frames (ORFs), respectively. This difference was due to the exclusion in the Copenhageni genome annotation of ORFs shorter than 150 bp, which lacked significant orthologs (Nascimento *et al*., 2004). Both genomes share 3,340 ORFs with an average DNA identity of 99% between homologs (Nascimento *et al*., 2004). Lai and Copenhageni contain 118 and 64 unique ORFs, respectively, most of which encode hypothetical proteins.

The high homology raises questions with respect to the genetic determinants for host reservoir specificities among the serovars. The hosts for serovar Lai and Copenhageni are the striped field mouse (*Apodemus agrarius*) (Fain *et al*., 1999) and the domestic rat (*Rattus norvegicus*), respectively. Lipopolysaccharide (LPS) is believed to influence reservoir specificity and is the basis for differences at the serovar level (Fain *et al*., 1999). Adler and colleagues identified a 40 kb *rfb* locus that

contains the genes for synthesis of the LPS O-antigen (de la Pena-Moctezuma *et al*., 1999). In the Lai and Copenhageni genomes, however, this locus is identical suggesting that serovar and reservoir specificity are due to genes outside the locus (Nascimento *et al*., 2004).

The Institute Pasteur group has made major advances in developing tools for genetic exchange, which can be used to elucidate *Leptospira* biology. They have developed a shuttle vector with an LE1 origin of replication and a homologous recombination gene-knockout system for saprophytic strains. Bauby and colleagues identified a second spectinomycin marker that, in addition to kanamycin, can be used to complement chromosomal mutations (Bauby *et al*. 2003). A series of elegant studies used this system to identify the function of putative metabolic pathways identified from the Lai genome. Unlike other spirochetes, *Leptospira* have a heme biosynthesis and uptake pathway (Guegan *et al*., 2003; Louvel *et al*., 2005), two functional methionine and one salvage pathway (Picardeau *et al*., 2003), and an alternative pyruvate pathway which is used to synthesize isoleucine (Xu *et al*., 2004). This knockout system, however, was unable to mutate pathogenic *Leptospira*. As an alternative approach Bourhy *et al.* have developed a mariner random mutagenesis technique to produce insertional mutations in the *L. interrogans* genome. This technique will facilitate identification of virulence determinants, for which selectable phenotypes can be identified (Bourhy *et al.*, 2005).

Leptospira, unlike other bacteria, have three toxin–antitoxin systems that may mediate global gene regulation during nutritional stress (Zhang *et al*., 2004). Moreover, *Leptospira* have over 70 genes with putative regulatory roles (Nascimento *et al*., 2004); this repertoire is more than twice the number seen in other spirochetes. Elucidation of these regulatory pathways will enable us to better understand how *Leptospira* persist for extended periods in the host and external environment.

1.6 Pathogenesis

Disease determinants for leptospirosis presumably relate to exposures that influence the inoculum size during infection, host factors and the pathogen's virulence characteristics. Epidemiological investigations have been useful in identifying the role of inoculum size effects through risk associations with proxies such as the

number of skin wounds (Phraisuwan *et al*., 2002) or the distance an urban slum resident lives from an open sewer (Sarkar *et al*., 2002).

1.6.1 Host factors

The identification of the first host genetic susceptibility factor for leptospirosis is an excellent example of how an outbreak investigation provided an insight into disease pathogenesis (Lingappa *et al*., 2004). Lingappa and colleagues found an association (OR, 2.8; P = 0.04) between the human leukocyte antigen (HLA)-DQ6 genotype and the risk of acquiring leptospirosis among triathletes during the 1998 Lake Springfield outbreak (Morgan *et al*., 2002). There was a synergistic interaction between HLA-DQ6 and swallowing water.

The clinical course and pathology of leptospirosis suggests an underlying immunopathogenic process (Fain *et al*., 1999). Jarisch-Herxheimer reactions are not an infrequent complication, when investigated. Plasma tumor necrosis factor (TNF)-α levels are a predictor of poor outcomes in patients (Tajiki and Salomao, 1996). Efforts have focused on delineating the proinflammatory responses *in vitro*. Whole *Leptospira* have been found to induce type 1 cytokines from whole blood of naïve individuals (de Fost *et al*., 2003). In a comprehensive study, Klimpel and colleagues showed that high and low numbers of *Leptospira* led to preferential expansion of γδ and αβ T cells, respectively, in naïve peripheral blood mononuclear cells (PBMCs). Stimulated γδ T cells appeared to secrete IFN-γ without a requirement for antigen processing. They also found that leptospirosis patients had increased proportions of γδ T cells. These findings indicate that γδ T cells may play an important role in the proinflammatory response and perhaps provide "a vital bridge" between the innate and adaptive responses during infection (Klimpel *et al*., 2003). Questions are raised concerning the moiety, which is inducing this response. *Leptospira* LPS has been shown to activate cells through Toll-like receptor-2 (Werts *et al*., 2001). This unusual finding may relate to the unique structure of *Leptospira* lipid A, which has a 1-methylphosphate group not found in other bacterial lipid A (Que-Gewirth *et al*., 2004). Diament and colleagues showed that *Leptospira* glycolipoprotein induces naïve PBMCs to secrete TNF-α and IL-10 and induces cell activation (Diament *et al*.,

2002). It may be possible that this extract has the moiety that stimulates γδ T cell responses.

1.6.2 Virulence

The unique feature of *Leptospira* pathogenesis is the ability of the pathogen to rapidly penetrate and disseminate during host infection and establish persistent colonization in the renal tubules. It is believed that *Leptospira* migrate through intercellular junctions. Barocchi and colleagues found that pathogenic *Leptospira* translocated efficiently across polarized Madin–Darby canine kidney monolayers without altering the transepithelial electrical resistance (Barocchi *et al*., 2002). Electron microscopy demonstrated organisms within the cytoplasm and not contained in a membrane compartment or the intercellular junction. *Leptospira* therefore appear to use a novel cell entry mechanism. Nally and colleagues established a rat model for renal persistence and found that LPS O-antigen content from *Leptospira* harvested from colonized rats was greater than the content from those harvested from acutely ill guinea pigs and had equal content to that found in cultured *Leptospira*. They proposed that O-antigen regulation may determine whether *Leptospira* cause acute disease or persistent infection (Nally *et al*., 2005).

Strategies to identify virulence determinants have focused on identifying surface-exposed proteins. A challenge has been determining which proteins are truly surface-exposed among the over 260 membrane-associated proteins that are predicted to exist on the basis of genome information (Nascimento *et al*., 2004). Matsunaga and colleagues have been the pioneers in developing approaches to identify such proteins (Matsunaga *et al*., 2003). Their methods include screening expression libraries with sera to identify host infection-expressed proteins (Matsunaga *et al*., 2003), evaluating differential expression of target genes with stimuli that mimic the host environment (Cullen *et al*., 2002) and confirming surface expression with immunofluorescence and electron microscopy, immunochemical analysis of outer membrane vesicles and ELISA with intact *Leptospira* (Haake and Matsunaga, 2002; Matsunaga *et al*., 2003; Matsunaga *et al*., 2005; Cullen *et al*., 2005). This strategy has identified six surface-exposed proteins (porin OmpL1; peripheral protein $P31_{LipL45}$; lipoproteins LipL41, LipL32, LipL21 and LipL48) and over 10 candidate proteins (Haake and Matsunaga, 2002; Cullen *et al*., 2003; Cullen *et al*., 2005). LipL32 and

LipL21 are of interest since they are expressed in all pathogenic *Leptospira* (Cullen *et al*., 2002; Cullen *et al*., 2005). Furthermore they have found that ompL1 is a mosaic gene that arose during intragenic horizontal transfer (Haake *et al*., 2004).

Proteomics has become a feasible strategy for identifying surface-exposed proteins now that the genome sequence is available. Cullen and colleagues were the first to apply this approach and identified eight candidates in detergent-extracted outer membrane preparations, of which one was later confirmed to be surface-exposed and named LipL21 (Cullen *et al*., 2003; Cullen *et al*., 2005). Nally and colleagues (2005) identified 15 novel candidates from outer membrane vesicles (Nally *et al*., 2005). A common concern has been contamination of preparations with non-outer membrane components. A "surfaceome" strategy has been developed which uses cell surface-biotinylation coupled with affinity capture (Cullen *et al*., 2005). It is expected that a combination of this approach with proteomics will provide a more efficient prediction of surface-exposed proteins and potential virulence determinants.

A major discovery has been the identification of the Lig proteins (Flannery *et al*., 2001; Palaniappan *et al*., 2002; Matsunaga *et al*., 2003). These proteins have 90-amino acid bacterial immunoglobulin-like (Big) repeat domains found in virulence factors such as intimin, invasin and BipA proteins. The *lig* genes are present in pathogenic but not saprophytic *Leptospira* species (Palaniappan *et al*., 2002; Matsunaga *et al*., 2003). Matsunaga and colleagues found that the *lig* gene family comprises two genes, *ligA* and *ligB*, which encode large lipoproteins (128 and 212 kDa, respectively), and a third pseudogene, *ligC*. As with Big virulence factors, Lig proteins are surface–expressed. The *lig* gene expression is significantly reduced or lost as virulent strains are attenuated during culture passage (Palaniappan *et al*., 2002). Recently, Lig protein expression has been found to be regulated by osmolarity (Matsunaga *et al*., 2005). Together these findings indicate that Lig proteins may play a role in virulence. Since Big virulence factors mediate host cell attachment and entry, current work focuses on evaluating whether Lig proteins have a similar function. Furthermore, these proteins have been shown to confer protective immunity (Koizumi *et al*., 2004).

1.7 Vaccines and immunity

Vaccines can conceivably be used as a prevention measure through immunization of humans or the reservoirs that transmit leptospirosis to humans. Bacterin vaccines have been used for years in the veterinary field. Cuba and China have developed bacterin and outer membrane envelope-based vaccines, respectively, for human use (Yan *et al*., 2003; Martinez *et al*., 2004). The Cuban and Chinese vaccines were reported to have an effectiveness of 78% (95% CI, 59–88%) and 75% (95% CI, 72–79%), respectively, in preventing clinical leptospirosis at 1-year follow-up. The trials did not report major adverse reactions, which have been attributed to bacterin vaccines. These evaluations are impressive for their scale (>70,000 participants) and findings but there still remain major concerns. The duration of immunity is likely to be short-term since veterinary bacterin vaccines require annual booster immunizations. These vaccines do not confer cross-protective immunity to serogroups that are not represented in the vaccine. The Cuban vaccine had to be reformulated in order to include serovar Ballum after a nationwide outbreak of this serovar in the 1990s (Rodriguez *et al*., 2003). To date, these vaccines have not been licensed outside of their respective countries (McBride *et al*., 2005).

Because of these concerns, efforts have focused on developing subunit vaccines. Haake and colleagues identified suface-exposed proteins that are conserved across pathogenic serovars and may elicit cross-protective immunity (Haake *et al*., 2004). Immunization with recombinant LipL41 and OmpL1 induced protective responses against lethal challenge in hamsters (Haake *et al*., 1996) while Branger *et al*., found that immunization with an adenovirus construct encoding for Hap1 [also known as LipL32 (Cullen *et al*. 2005)] conferred protection in gerbils. Although protection was partial, these findings demonstrated the feasibility of the subunit approach. Koizumi *et al.* found that recombinant Lig protein induced complete protection against lethal challenge in C3H/HeJ mice (Koizumi and Watanabe, 2004). The Chinese (Ren *et al*., 2003) and Brazilian (Nascimento *et al*., 2004) genome projects were performed as part of large national vaccine development initiatives. *L. interrogans* serovar Copenhageni genome has 264 ORFs that encode putative surface-associated proteins (Nascimento *et al*., 2004). High-throughput screening (Gamberini *et al*., 2005) and proteomic approaches (Cullen *et al*., 2002; Nally *et al*., 2005) are anticipated to

shortly identify additional candidates for preclinical evaluation. A clear understanding is needed on the mechanism for acquired immunity if effective vaccines are to be developed. Correlates have not been identified, however, for naturally-acquired immunity to re-infection in humans. Immunity has been believed to be antibody-mediated since *Leptospira* are extracellular pathogens and protection can be passively transferred in hamsters (Faine *et al*., 1999). Recent findings suggest that this paradigm may need to be reevaluated. Bacterin vaccine trials in cattle found that protective immunity was not associated with agglutinating antibody titers but was correlated with Th1 responses, characterized by $CD4^+$ and γδ T cell production of IFN-γ (Naiman *et al*., 2002; Brown *et al*., 2003). These findings were unexpected since Th1 responses are usually associated with protective responses to intracellular pathogens. T cells from healthy individuals were found to make similar responses when stimulated *in vitro* with *Leptospira* (de Fost *et al*., 2003; Klimpel *et al*., 2003).

2. Proteomic technology

By definition "Proteomics" is the simultaneous analysis of complex protein mixtures like cell lysates and tissue extracts to look for quantitative changes of expression levels. The scope of applications extends from drug discovery, to diagnostics, therapy, microbiology, biochemistry, and plant research. It had been substantially facilitated in the past decade because of developments in mass spectrometry and the availability of genomic information. Development in proteomics have produced in parallel: *e.g.*

The technology for high-resolution two-dimensional electrophoresis has been considerably improved, which make the method more reliable, and reproducible. The resolution has been further increased as well. Image analysis software of these complex spot patterns has been developed to such a degree that also non-computer experts can use it and get reliable results.

Novel ionization techniques and detectors for mass spectrometry have been invented, which allow the analysis of proteins and peptides with high sensitivity, accuracy and throughput. Online peptide fragmentation allows quick amino acid

sequence analysis of low amounts of peptides at low running cost. Also the analysis of post-translational modification can be addressed using this technology.

The development of high throughput DNA sequencing genomic database of many different organisms have been established in a short period of time. Genomic sequence data is growing with immense speed. Unfortunately for most genes, the function is unknown. A gene code, for more than one gene product, alternative splicing of mRNA can result in different proteins. Furthermore most proteins become modified by complex gene interactions, cellular events, and environmental influences that result in post-translational modifications. Knowledge of DNA sequence of organisms to be analysed is very important for protein identification and characterization with mass spectrometry.

Activities in the developing field of bioinformatics have been initiated to develop tools for combining and bundling the huge amount of data produced by new high throughput analysis methods. Only in this way it will be possible to draw meaningful conclusions from huge amount of data generated.

Additionally, a few developments in related fields are very helpful for proteomics analysis:

Biochemistry and biomedical research had focused on studying the structure and function of proteins, which have been proposed as key enzymes in related pathways. The technology for micro-characterization of proteins has been continuously with respect to sensitivity and accuracy. If antibodies were available, identification of a protein was no problem. With *de novo* sequencing of protein purified by HPLC or electrophoretic methods databases have been complied. Those could be used also for cross-species protein identification using amino acid composition and short sequence information. But the techniques applied are relatively slow and allow only low throughput. In most cases tedious and expensive *de novo* amino acid sequencing is no longer needed. With the available genomics databases it is sufficient to acquire short sequence information for protein by mass spectrometry to identify it, and to retrieve its complete amino acid sequence with the help of *in-silico* translation of the open reading frames.

Labeling techniques using fluorescent tags and stable isotopes have been made available for differential analyses of related samples. Quantitative changes in expression levels can thus be easily uncovered.

Developments of complementary technology: for the analysis of smaller protein subsets, the detection of proteins at very low expression levels, determination and assay of protein function, many novel methods are under development

The points listed above describe an approach, which is often also defined as "Expression proteomics" or "Classical proteomics". With most of the techniques employed, the proteins become denatured; their three-dimensional and quaternary stucture are destroyed, information on function and complexing of the proteins cannot be determined. For that purpose "Functional proteomics" methods are applied, which do not denature the proteins and keep complexes intact: *e.g.* affinity chromatography and electrospray ionization mass spectrometry (Westermeier and Naven, 2002).

2.1 Application of proteomics

The greatest expectation from proteomics come from pharmaceutical research for faster new drug protein targets identification in transformed cell lines or diseased tissues. Also the validation of the detected targets, *in- vitro* and *in- vivo* toxicology studies, and checks for side effects can be performed with this approach. Clinical researchers want to compare normal *versus* diseases samples, disease *versus* treated samples, find molecular markers in body fluids for diagnosis monitor diseases and their treatments, determined and characterize post-transaltional modificaton. In clinical chemistry it would be interesting to subtype individuals to predict response to therapy.

Biologist study basic cell functions and molecular organizations. Another big field is microbiology for various research areas. Proteomics is also applied for plant research for many different purposes, drought, and other resistances, increasing the yield of crop and many more. For all this research a combined strategy with genomics is employed (Westermeier and Naven, 2002).

2.2 Separation of the protein mixture

High-resolution two-dimentional electrophoresis is the mainly applied separation technique. The separation according to the two completely independent physico-chemical parameters of the proteins: isoelectric point and size offer the highest

resolution. Several thousand proteins can be separated, displayed and stored in one gel. The history of this technique goes back to a paper in German language by Stegemann (1970), combining isoelectric focusing (IEF) and SDS polyacrylamide gel electrophoresis. The resolution of 2D- electrophoresis was considerably increased by the introduction of denaturing condition during sample preparation and isoelectric focusing by O'Farrel in the year 1975. With this modification the method gained a wide acceptance. But the only with the application of immobilized pH gradients (Bjellqvist *et al*., 1982) for the first dimension the technique become reproducible enough for proteome analysis.

2.3 Detection

Ideally one protein expressed in a cell should be detectable. With the current state of technology this is completely impossible. With non-radioactive labeling and staining techniques (fluorescence or Silver staining) down to 100 proteins per cell can be visualized in a 2D- gel, when 10 mg total protein corresponding for instance to 108 cell equivalents of lymphoma cells-are loaded on the isoelectric focusing gel (Hoving *et al*., 2000). Mass spectrometry method are generally more sensitive than these staining method, however in practice 10 to 20 ng of protein in a spot is required for good signals in mass spectrometry.

2.4 Image analysis

The 2D- patterns are very complex, only with informatics tools it is possible to find expressed changes in a series of gels: like up and down regulated proteins, post-translational modifications. Image analysis has still been the bottle-neck in the proteomics procedure, because the spot detection parameters had to be adjusted and optimized manually. Only since a very short time develop software can perform fully automate and hands-off evaluation. The reliability of quantitative determinations of protein amounts in spots is strongly dependent on the protein detection technique applied. Protein spots of interest are then further analysed.

2.5 Identification of proteins

2.5.1 Peptide mass fingerprinting

Peptide mass fingerprinting (PMF) was introduced by four independent groups, including Pappin *et al*. (1993). The gel plug containing the protein of interest is cut out of the gel slab. The protein is digested inside the gel plug with a proteolytic

enzyme, mostly trypsin. The cleavage products, the peptides are eluted from the plug and submitted to mass spectrometry analysis. Mostly MALDI ToF instruments are employed, because they are easier to handle than electrospray systems. The mass spectrum with the accurately measured peptide masses is matched with theoretical peptide spectra in various databases using adequate bioinformatics tools. Since the cleavage sites of trypsin are known, theoretical tryptic peptide masses can be generated and compared with the experimentally determined masses. If a sufficient number of experimental peptide masses match with the theoretical peptides within a protein, then protein identification with high confidence can be achieved

2.5.2 Amino acid sequence analysis

Amino acid sequence analysis is highly discriminating information for unambiguous protein identification. During mass spectrometry analysis a peptide can be selected from the spectrum and fragment inside instrument, termed tandem mass spectrometry. The resultant fragment ion masses are indicative of amino acid sequence can be used to generate a sequence ladder. Amino acid sequence derived from mass spectrometry can be used to search not only the protein databases, but also the EST databases and used for *de novo* sequencing when necessary.

2.6 Characterization of proteins

Beside amino acid sequence information, also other structural data of protein can be determined with mass spectrometry: disulfide bonds, post-translational modification like phosphorylation, truncation, acylation and glycosylation. The identification of sites of disulfide bonds, phosphorylation and glycocylation can all be determined using tandem mass spectrometry, though quantitation of these modifications is considerably more difficult.

2.7 Functional proteomics

The following strategy is pursued: at first target proteins are identified, characterized and correlated with “protein families”. Once some structural informations are known, smaller subsets of proteins are analysed with milder separation and measuring techniques: for instance some proteins are fished out of cell lysate with affinity chromatography and the protein with intact tertiary structure, or protein-protein complexes are analysed after electrospray ionization (Lamond and Mann, 1997; Pandey and Mann, 2000).

2.8 Expression proteomics: Two-dimensional electrophoresis technology for proteomics

Only high-resolution 2D- electrophoresis, with both dimensions run under denaturing conditions, is used in proteomics. Native 2D- separations do not play a big role in proteome expression analysis. Two-dimensional gel electrophoresis (2DE) with immobilized pH gradients (IPGs) combined with protein identification by mass spectrometry (MS) is currently the workhorse for proteomics (Görg *et al*., 1988; Görg *et al*., 2000; Görg *et al*., 2004). In spite of promising alternative/ complementary technologies (*e.g.* multidimensional protein identification technology, stable isotope labeling, protein arrays) that have emerged recently (Figeys *et al*., 1999; Gygi *et al*., 1999; Link *et al*., 1999; Haynes *et al*., 2000; Aebersold and Mann, 2003; Ducret *et al*., 2003), 2DE is currently the only technique that can be routinely applied for parallel quantitative expression profiling of large sets of complex protein mixtures such as whole cell lysates. Whatever technology is used, proteome analysis is technically challenging, because the number of different proteins expressed at a given time under defined biological conditions is likely to be in the range of several thousands for simple prokaryotic organisms and up to at least 10,000 in eukaryotic cell extracts. Moreover, current proteomic studies have revealed that the majority of identified proteins are abundant housekeeping proteins that are present in numbers of 105 to 106 copies *per* cell, whereas proteins such as receptor molecules that are present in much lower concentrations (typically, 100 molecules *per* cell) are usually not detected. Consequently, improved methods for enrichment of low-abundance proteins are required, such as prefractionation procedures, as well as more sensitive detection and quantitation methods. 2DE couples isoelectric focusing (IEF) in the first dimension with SDS-PAGE in the second dimension, and enables the separation of complex mixtures of proteins according to isoelectric point (p*I*), molecular mass (M_r), solubility, and relative abundance. Depending on the gel size and pH gradient used, 2DE can resolve more than 5,000 proteins simultaneously (~2,000 proteins routinely), and can detect ~1 ng of protein *per* spot. Furthermore, it delivers a map of intact proteins, which reflects changes in protein expression level, isoforms or post-translational modification (PTM). This is in contrast to liquid chromatography tandem mass spectrometry (LC-MS/MS) based methods, which perform analysis on

peptides, where M_r and p*I* information is lost, and where stable isotope labelling is required for quantitative analysis. One of the greatest strengths of 2DE is its capability to study proteins that have undergone some form of PTM (such as phosphorylation, glycosylation or limited proteolysis) and which can, in many instances, be readily located in 2DE gels as they appear as distinct spot trains in the horizontal and/or vertical axis of the 2DE gel. In addition, 2DE not only provides information on protein modifications and/or changes in their expression levels, but also permits the isolation of proteins in mg amounts for further structural analyses by Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) MS, Electrospray Ionization MS (ESI-MS) or Edman microsequencing (Celis and Gromov, 1999; Fey and Mose Larsen, 2001; Ong and Pandey, 2001; Graves and Haystead, 2002; Lilley *et al*., 2002; Rabilloud, 2002). The former limitations of carrier ampholyte (CA) based 2DE (O'Farrell, 1975; Scheele, 1975; Klose, 1975) with respect to reproducibility, resolution, separation of very acidic and/or very basic proteins, and sample loading capacity have been largely overcome by the introduction of IPGs for the first dimension of 2DE (Görg *et al*., 1988). Narrow-overlapping pH gradients provide increased resolution (D*p*I = 0.001) (Görg *et al*., 1985; Görg *et al*., 1988) and detection of low abundance proteins (Westbrook *et al*., 2001; Wildgruber, 2001), whereas alkaline proteins up to pH 12 have been separated under steady-state conditions (Görg *et al*., 1997; Görg *et al*., 1998; Görg, 1999; Görg *et al*., 1999; Wildgruber *et al*., 2002; Drews *et al*., 2004). The major steps of the 2DE-MS workflow include: (i) sample preparation and protein solubilization; (ii) protein separation by 2DE; (iii) protein detection and quantitation; (iv) computer assisted analysis of 2DE patterns; (v) protein identification and characterization; (vi) 2D protein database construction (Görg *et al*., 2004).