Leptospirosis

Alan J.A. McBride[a], Daniel A. Athanazio[a], Mitermayer G. Reis[a] and Albert I. Ko[a,b]

Purpose of review

Leptospirosis, a spirochaetal zoonotic disease, has been recognized as an important emerging infectious disease in the last 10 years. This review addresses the issues in the epidemiology, diagnosis and clinical management which confront public health responses, and highlights the progress made towards understanding the *Leptospira* genome, biology and pathogenesis.

Recent findings

Leptospirosis has spread from its traditional rural base to become the cause of epidemics in poor urban slum communities in developing countries. Mortality from severe disease forms, Weil's disease and severe pulmonary haemorrhage syndrome, is high (>10% and >50%, respectively) even when optimal treatment is provided. Moreover, the overall disease burden is underestimated, since leptospirosis is a significant cause of undifferentiated fever and frequently not recognized. Barriers to addressing this problem have been the lack of an adequate diagnostic test and effective control measures. China and Brazil, countries in which leptospirosis is a major health problem, have completed the sequence of the *Leptospira interrogans* genome. Together with new genetic tools and proteomics, new insights have been made into the biology of *Leptospira* and the mechanisms used to adapt to host and external environments. Surface-exposed proteins and putative virulence determinants have been identified which may serve as sub-unit vaccine candidates.

Summary

Major progress has been made in the basic research of leptospirosis. Future challenges will be to translate these advances into public health measures for developing countries. Yet the most effective responses may be interventions that directly address the determinants of poverty, such as poor sanitation, which are often responsible for transmission.

Keywords

diagnosis, epidemiology, genome, *Leptospira*, leptospirosis, treatment, vaccine

Curr Opin Infect Dis 18:376–386. © 2005 Lippincott Williams & Wilkins.

[a]Gonçalo Moniz Research Centre, Oswaldo Cruz Foundation, Ministry of Health, Salvador, Brazil and [b]Division of International Medicine and Infectious Disease, Weill Medical College of Cornell University, New York, USA

Correspondence to Dr Albert Ko, Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz/Ministério da Saúde, Rua Waldemar Falcão, 121, 40295-001 Salvador, Bahia, Brazil
Tel: +55 71 3356 4320; fax: +55 71 3356 2155;
e-mail: aik2001@med.cornell.edu

Sponsorship: The work of our group was supported by the Oswaldo Cruz Foundation, Brazilian Ministry of Health (09224-7, PDTIS), Brazilian National Research Council (300.861/96-6) and the National Institutes of Health, USA (AI052473 and TW00919)

Current Opinion in Infectious Diseases 2005, 18:376–386

Abbreviations

Big	bacterial immunoglobulin-like
HLA	human leukocyte antigen
Lig	leptospiral immunoglobulin-like
LPS	lipopolysaccharide
MAT	microagglutination test
ORF	open reading frame
PCR	polymerase chain reaction
SPHS	severe pulmonary haemorrhage syndrome

0951-7375

Introduction

World attention has focused on leptospirosis as an emerging infectious disease due to the 1995 Nicaragua epidemic of severe pulmonary haemorrhage syndrome (SPHS) [1], identification of disease among US inner-city homeless population [2] and the 1998 Lake Springfield Triathlon [3] and 2000 Borneo Eco-Challenge [4] outbreaks. Yet more importantly, the new interest in leptospirosis has led to the realization that the disease has been an underrecognized problem with major health impacts in developing countries [5,6].

Since the last review in 2001 [7], major progress has been made towards understanding the biology and pathogenesis of leptospirosis. The large burden of leptospirosis in China [8] and Brazil [9••] has motivated these nations to complete the first genome sequences for *Leptospira*. There is a striking disparity, however, between these advances and the limited progress made towards implementing effective public health responses. Challenges with respect to diagnosis, clinical management and prevention are the same as when leptospirosis was first reviewed in this journal in 1997. This review will focus on the recent advances as well as highlight the issues involved in translating these advances into interventions.

Epidemiology

Leptospirosis is a paradigm of an infectious disease for which globalization and social inequality have produced divergent epidemiological patterns for the poor and wealthy. In developing countries, it is a significant health burden for poor rural populations [5,6]. Subsistence farmers are risk groups who are exposed to environments contaminated with the urine of domestic and sylvatic animal reservoirs. Leptospirosis has become an urban

problem [2,10–14,15•,16,17,18•], however, as the rural poor have moved to cities. One billion people reside in urban slums [19] where the lack of basic sanitation has produced the ecological conditions for rodent-borne transmission [10,15•]. In Brazil, outbreaks occur each year in poor urban communities (*favelas*) during the same seasonal period of heavy rainfall [10,12,14,16]. 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 25 years [19]. Of note, this problem is not limited to 'tropical' settings for the importance of urban leptospirosis was first recognized among US inner-city populations [2]. These epidemiological patterns sharply contrast with the trend that has evolved in developed countries. Leptospirosis, traditionally an occupational disease [5], is now identified among the affluent who engage in recreational activities [20], sporting events [3], travel and adventure tourism [4,21].

Investigations from Asia [13,17,18•,23–25] and Latin America [10,12,14,15•,16,26•] provide evidence that leptospirosis has become an important public health problem. Yet it continues to be underrecognized by policy makers due to the poor quality of surveillance data. Information on the global burden has not been compiled since 1999 [27]; 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 underestimates 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 [13,17,18•,28] and surveillance [22,25] and found that a large proportion of leptospirosis cases would have otherwise gone unrecognized or been attributed to other diseases. In a multicentre study [22] 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. 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 SPHS. During the 1995 Nicaragua epidemic [29] and an outbreak in Thailand [23], asymptomatic infections occurred in 60–70% of all serologically identified infections. In contrast, in the 1998 Lake Springfield outbreak [3], 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. Population-based studies have found that adult males had increased risk for severe leptospirosis [10,30,31]. A longitudinal study in Peru found that 12% of 195 urban slum residents had serologic evidence for a new infection during the flood season [15•]. In this setting, women and men had similar risk for acquiring infection. Such prospective investigations will be useful in determining the infection : disease ratio and identifying risk factors for the development of disease. Yet the immediate priority should focus on enhancing the capacity of epidemiological services in developing countries to conduct outbreak investigations and active surveillance and thus target interventions to appropriate risk groups.

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 [23]. 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 [21]. 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 [15•]. Prevention of leptospirosis may need to wait until a vaccine is available. In urban slums, there are definable infrastructural deficiencies, such as open sewers, which contribute the transmission of leptospirosis during epidemics [10,12,16]. The public health response for urban leptospirosis must therefore address these deficiencies by providing proper sanitation in urban slum communities.

Clinical and laboratory diagnosis

Timely diagnosis is essential since antibiotic therapy provides greatest benefit when initiated early in the course of illness [32,33]. 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 [13,25,26•,28].

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

		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.

Misdiagnosis has become an evermore critical issue in regions where dengue and other infectious diseases with overlapping presentations are endemic [10,13,17,18•,25]. Co-infection with diseases such as scrub typhus and malaria have been reported [17,34] and presents another source of confusion in tropical settings. Identification of leptospirosis will therefore depend on a high index of suspicion among clinicians [7,25] and the availability of an accurate 'point-of-care' test.

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 [32,33]. 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 [35•]. 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)

[36–42]. Moreover, the sensitivity may be under 25% during the first week of illness [38]. 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 [43]. In Peru and Vietnam, over 25% of healthy control individuals had positive IgM enzyme-linked immunosorbent assay (ELISA) reactions [15•,44•]. 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 [45–47]. Segura *et al*. [48] used these methods to determine that leptospiraemia is 10 000 or more bacteria per millilitre of blood or milligram of tissue in SPHS patients [26•]. Their findings confirmed those of a previous semi-quantitative PCR study [48]. 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 have not yet been developed. Saengjaruk *et al*. [49] produced a monoclonal antibody that detected a 35–36 kDa *Leptospira* antigen in clinical samples. 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 [26•,48].

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 [50]. The performance of recombinant LipL32 and GroEL-based assays have not been entirely encouraging [51]. A promising candidate, however, is leptospiral immunoglobulin-like (Lig) protein [52,53,54•,55]. Our studies found that a recombinant Lig-based immunoblot assay had improved sensitivity and specificity (>90%) over whole *Leptospira*-based tests (J. Croda *et al*., unpublished data). The Brazilian Ministry of Health is using this protein to develop a prototype rapid test that will be shortly evaluated in field trials.

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 patient sera are used as a surrogate to infer the infecting serogroup. This approach may be unreliable (positive predictive value, 65–82%) [56,57]. 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 [58•]. 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 [26•] to detect and quantify leptospiral serovars from environmental samples (J.M. Vinetz, personal communication). If validated, such a tool will enhance the capacity to identify transmission sources and implement targeted control measures.

Pathophysiology and clinical management

The major burden of leptospirosis is due to severe disease forms, Weil's disease and SPHS. Case fatality is over 10% for Weil's disease [10,59–62,63•,64] and over 50% for SPHS [26•,65,66] 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 [10,60, 64,66]. Independent prognostic factors for mortality are acute renal failure (oliguria, hyperkalemia, serum creatinine $\geq$3.0 or 4.0 mg/dl), respiratory insufficiency (dyspnea, pulmonary rales, radiological evidence for infiltrates), hypotension, arrhythmias and altered mental status [10,59–61,63•]. Altered mental status was found to be the strongest predictor of death in two studies [10,63•] 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.

Leptospirosis causes a unique non-oliguric hypokalemic form of acute renal insufficiency [67]. Its hallmark features are impaired proximal sodium reabsorption, increased distal sodium delivery and potassium wasting [67,68]. When identified during this initial phase of renal insufficiency, patients have a better overall prognosis [10,61,63•,65] and can be treated with potassium and

volume repletion [68]. With continued sodium and volume loss, however, patients will develop oliguric renal insufficiency including acute tubular necrosis [62]. For these patients, dialysis is the critical intervention for preventing mortality [33]. Peritoneal dialysis, although commonly used in resource limited settings, may not efficiently correct the hypercatabolic disturbances in systemic leptospirosis. We recommend the use of continuous haemofiltration, which has been shown to be more effective than peritoneal dialysis in treating infection-associated acute renal failure [69]. 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 [65] to 18% over a 10-year period. (A.C. Seguro and L. Andrade, personal communication).

Recent studies have identified possible mechanisms for this peculiar form of acute renal failure. The lesion site is believed to be the proximal tubule since infected guinea pigs have intact thick ascending limb function [70]. 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 *mNKCC2* transcription [71•]. Alternatively, Burth and colleagues [72•] have proposed that *Leptospira*-derived unsaturated fatty acids act as toxins that inhibit kidney sodium–potassium ATPase. 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*. Further elucidation of the mechanism for acute renal failure and evaluation of intravenous albumin therapy may provide new treatment options for this life-threatening complication.

Leptospirosis-associated SPHS is now recognized as a widespread public health problem [1,26•,61,65,66]. Frank haemoptysis, the characteristic sign of SPHS, may not be evident until patients are intubated. Clinicians should therefore suspect SPHS in patients with signs of respiratory distress, regardless of whether or not they have haemoptysis. It is important to note that SPHS patients have physiologic [26•,61,65,73] and pathologic [74] evidence for acute respiratory distress syndrome. Protective ventilation strategies should be used which are based on low tidal volumes (<6 ml/kg) and high post-expiratory end-pressures. This approach has been shown to improve outcomes in a trial that included SPHS patients [73]. Nevertheless, mortality from SPHS is high even when optimal treatment is provided.

SPHS patients appear to have a high (approximately 1 000 000 bacteria/mg) leptospiral burden in the lungs [26•]. Yet few intact leptospires are observed in autopsy [74] and experimental animal [75••,76•] tissues, suggesting a possible role for an immune-mediated process. Damage to the pulmonary endothelium occurs without evidence for disseminated intravascular coagulation [74,75••,76•]. Nally and colleagues [75••] 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. This finding needs to be confirmed for human leptospirosis but suggests an underlying autoimmune process for SPHS. Furthermore, it suggests a possible rationale for the use of immunosuppression or plasmaphoresis. Now that a primate model for SPHS has been developed [76•], preclinical evaluations can be performed. Ultimately, multicentre trials will be needed to evaluate treatments, such as desmopressin and steroids, which have only been evaluated in uncontrolled case series.

We strongly recommend the use of antibiotic therapy for severe leptospirosis. This issue has aroused a debate due to the lack of evidence demonstrating that antibiotics provide a benefit against mortality [77,78]. The rationale for the use of antibiotics has been extensively covered by Vinetz and we refer readers to these comments [79]. More importantly, the priority should focus on ensuring that antibiotic therapy is delivered without delay and at the appropriate dosage. A major advance has been the trials from Thailand [80,81•], which showed that treatment with ceftriaxone, cefotaxime and doxycycline had equivalent efficacy to penicillin therapy. In the setting of overburdened hospital services in developing countries, health care professionals are often unable to deliver intravenous penicillin six times a day. The worldwide fall in the price of third generation cephalosporins has now made treatment with these antibiotics an option.

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 [10]). The Lai and Copenhageni genomes were published in 2003 [8] (website: http://www.chgc.sh.cn/lep/) and 2004 [9••] (website: http://aeg.lbi.ic.unicamp.br/world/lic/), respectively. A database, *LeptoList*, is available online for comparison of the two genomes (website: http://bioinfo.hku.hk/LeptoList/). Furthermore, information

will soon be available for the genomes of *L. interrogans* serovar Pomona, *L. borgpetersenii* serovar Hardjobovis and *L. borgpetersenii* serovar Hardjo.

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 [9••]. Both genomes share 3 340 ORFs with an average DNA identity of 99% between homologs [9••]. 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*) [32] 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 [32]. Adler and colleagues [9••] identified a 40 kb *rfb* locus that contains the genes for synthesis of the LPS O-antigen. In the Lai and Copenhageni genomes, however, this locus is identical suggesting that serovar and reservoir specificity are due to genes outside the locus [9••].

The Institut 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 *et al.* [82] identified a second spec-tinomycin marker that, in addition to kanamycin, can be used to complement chromosomal mutations. A series of elegant studies used this system to identify the function of putative metabolic pathways identified from the Lai genome. Unlike other spirochaetes, *Leptospira* have a haem biosynthesis and uptake pathway [83,84], two functional methionine and one salvage pathway [85], and an alternative pyruvate pathway which is used to synthesize isoleucine [86•]. This knockout system, however, was unable to mutate pathogenic *Leptospira*. As an alternative approach Bourhy *et al.* [87•] 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.

Leptospira, unlike other bacteria, have three toxin–antitoxin systems that may mediate global gene regulation during nutritional stress [88]. Moreover, *Leptospira* have over 70 genes with putative regulatory roles [9••]; this repertoire is more than twice the number seen in other spirochaetes. Elucidation of these regulatory pathways will enable us to better understand how *Leptospira* persist for extended periods in the host and external environment.

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 [23] or the distance an urban slum resident lives from an open sewer [12]. This section will focus specifically on host factors and virulence.

Host factors

The identification of the first host genetic susceptibility factor for leptospirosis [89••] is an excellent example of how an outbreak investigation provided an insight into disease pathogenesis. Lingappa and colleagues [89••] 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 [3]. There was a synergistic interaction between HLA-DQ6 and swallowing water. This association was the first gene–environment interaction identified for an infectious disease. Swallowing lake water may have been a proxy for an inoculum size effect. The authors hypothesize a role for superantigens since polymorphisms at the HLA-peptide binding pocket were not associated with acquiring leptospirosis. This hypothesis needs to be evaluated in other settings; if correct, however, it may significantly change current thinking on *Leptospira* pathogenesis and approaches to therapy.

The clinical course and pathology of leptospirosis suggests an underlying immunopathogenic process [32]. Jarisch-Herxheimer reactions are not an infrequent complication, when investigated. Plasma tumour necrosis factor (TNF)-α levels are a predictor of poor outcomes in patients [90]. 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 [91]. In a comprehensive study, Klimpel and colleagues showed that high and low numbers of *Leptospira* led to preferential expansion of γ-δ

and α-β T cells, respectively, in naïve peripheral blood mononuclear cells (PBMCs). Stimulated γ-δ T cells appeared to secrete IFN-γ without a requirement for antigen processing. They also found that leptospirosis patients had increased proportions of γ-δ T cells. These findings indicate that γ-δ 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 [92]. Questions are raised concerning the moiety which is inducing this response. *Leptospira* LPS has been shown to activate cells through Toll-like receptor-2 [93]. 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 [94••]. Diament and colleagues [95] showed that *Leptospira* glycolipoprotein induce naïve PBMCs to secrete TNF-α and IL-10 and induce cell activation [95]. It may be possible that this extract has the moiety that stimulates γ-δ T cell responses.

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 [96] found that pathogenic *Leptospira* translocated efficiently across polarized Madin–Darby canine kidney monolayers without altering the transepithelial electrical resistance. 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 [97•] 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. Now that these models have been established, they will be useful in elucidating determinants for dissemination and persistence phenotypes.

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 [9••]. Haake and colleagues have been the pioneers in developing approaches to identify such proteins. Their methods include screening expression libraries with sera to identify host infection-expressed proteins [53], evaluating differential expression of target genes with stimuli that mimic the host environment [98] and confirming surface expression with immunofluorescence and electron microscopy, immunochemical analysis of outer membrane vesicles and ELISA with intact *Leptospira* [53,99,100,101•]. 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 [99,100,102]. LipL32 and LipL21 are of interest since they are expressed in all pathogenic *Leptospira* [98,99]. Furthermore they have found that *ompL1* is a mosaic gene that arose during intragenic horizontal transfer [103••].

Proteomics has become a feasible strategy for identifying surface-exposed proteins now that the genome sequence is available. Cullen and colleagues [98,102] 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. Nally and colleagues [104•] identified 15 novel candidates from outer membrane vesicles. 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 [99]. 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 [51–53]. These proteins have 90-amino acid bacterial immunoglobulin-like (Big) repeat domains found in virulence factors such as intimin, invasin and BipA proteins. *lig* genes are present in pathogenic but not saprophytic *Leptospira* species [52,53]. 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. *lig* gene expression is significantly reduced or lost as virulent strains are attenuated during culture passage [52]. Recently, Lig protein expression has been found to be regulated by osmolarity [101•]. 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 [54•] and serve as serodiagnostic markers for infection (J. Croda *et al*., unpublished data).

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 [105,106]. 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 remains 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 [107] after a nationwide outbreak of this serovar in the 1990s. To date, these vaccines have not been licensed outside of their respective countries.

Because of these concerns, efforts have focused on developing sub-unit vaccines. Haake and colleagues [103••] identified surface-exposed proteins that are conserved across pathogenic serovars and may elicit cross-protective immunity. Immunization with recombinant LipL41 and OmpL1 induced protective responses against lethal challenge in hamsters [108] while Branger *et al.* [109] found that immunization with an adenovirus construct encoding for Hap1 (also known as LipL32 [99]) conferred protection in gerbils. Although protection was partial, these findings demonstrated the feasibility of the sub-unit approach. Koizumi *et al.* [54•] found that recombinant Lig protein induced complete protection against lethal challenge in C3H/HeJ mice. The mouse is not an ideal model since it requires large challenge doses to induce disease. Nevertheless, Lig proteins will be a promising candidate for preclinical and clinical trials, if these findings are reproduced in more appropriate models. The Chinese [8] and Brazilian [9••] 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 [9••]. High-throughput screening [110] and proteomic approaches [98,104•] 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 [32]. Recent findings suggest that this paradigm may need to be re-evaluated. Bacterin vaccine trials in cattle found that protective immunity was not associated with agglutinating antibody titres but was correlated with Th1 responses, characterized by $CD4^+$ and γ-δ T cell production of IFN-γ [111,112]. 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* [91,92]. The potential role of Th1 responses in acquired immunity has important implications for screening of vaccine candidates and the use of adjuvants. Findings obtained from vaccine immunity in cattle, however, may not be generalizable to humans since cattle develop chronic carriage rather than acute disease. The role of cell-mediated responses in protective immunity will therefore need to be evaluated in animal models of acute disease.

Conclusion

Recent globalization and worsening social inequality have changed the epidemiology of leptospirosis. It is now an important cause of disease among urban slum dwellers as well as poor subsistence farmers. The barriers that prevent public health responses to this emerging infectious disease continue to be the lack of accurate disease burden information, an adequate diagnostic test and effective treatment and prevention. In contrast, significant advances have been made in our understanding of the *Leptospira* genome, biology and pathogenesis. Together with progress achieved in applying new technologies such as genetic transformation, proteomics and microarrays, it is anticipated that virulence factors, improved diagnostic markers and vaccine candidates will be shortly identified. Certainly, a priority for the field will be to translate these research advances into tangible benefits in developing countries where interventions are needed most. Yet more importantly policy makers need to be convinced that the most effective gains may be made by addressing the underlying conditions of poverty, such as poor sanitation, which have led to the emergence of leptospirosis.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1 Trevejo RT, Rigau-Perez JG, Ashford DA, *et al.* Epidemic leptospirosis associated with pulmonary hemorrhage-Nicaragua, 1995. J Infect Dis 1998; 178:1457–1463.

2 Vinetz JM, Glass GE, Flexner CE, *et al.* Sporadic urban leptospirosis. Ann Intern Med 1996; 125:794–798.

3 Morgan J, Bornstein SL, Karpati AM, *et al.* Outbreak of leptospirosis among triathlon participants and community residents in Springfield, Illinois, 1998. Clin Infect Dis 2002; 34:1593–1599.

4 Sejvar J, Bancroft E, Winthrop K, *et al.* Leptospirosis in ''Eco-Challenge'' athletes, Malaysian Borneo, 2000. Emerg Infect Dis 2003; 9:702–707.

5 Levett PN. Leptospirosis. Clin Microbiol Rev 2001; 14:296–326.

6 Bharti AR, Nally JE, Ricaldi JN, *et al.* Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis 2003; 3:757–771.

7 Vinetz JM. Leptospirosis. Curr Opin Infect Dis 2001; 14:527–538.

8 Ren SX, Fu G, Jiang XG, *et al.* Unique physiological and pathogenic features of *Leptospira* interrogans revealed by whole-genome sequencing. Nature 2003; 422:888–893.

9 •• Nascimento AL, Ko AI, Martins EA, *et al.* Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. J Bacteriol 2004; 186:2164–2172.

The second report of the *L. interrogans* genome from a Brazilian initiative provides new information on the biology and pathogenesis through comprehensive annotation of the genome sequence of serovar Copenhageni, the causative agent of urban leptospirosis, and comparison with the *L. interrogans* serovar Lai sequence reported by the Chinese consortium [8].

10 Ko AI, Galvao Reis M, Ribeiro Dourado CM, *et al.* Urban epidemic of severe leptospirosis in Brazil. Salvador Leptospirosis Study Group. Lancet 1999; 354:820–825.

11 Kariv R, Klempfner R, Barnea A, *et al.* The changing epidemiology of leptospirosis in Israel. Emerg Infect Dis 2001; 7:990–992.

12 Sarkar U, Nascimento SF, Barbosa R, *et al.* Population-based case-control investigation of risk factors for leptospirosis during an urban epidemic. Am J Trop Med Hyg 2002; 66:605–610.

13 Karande S, Bhatt M, Kelkar A, *et al.* An observational study to detect leptospirosis in Mumbai, India, 2000. Arch Dis Child 2003; 88:1070–1075.

14 Romero EC, Bernardo CC, Yasuda PH. Human leptospirosis: a twenty-nine-year serological study in Sao Paulo, Brazil. Rev Inst Med Trop Sao Paulo 2003; 45:245–248.

15 • Johnson MA, Smith H, Joseph P, *et al.* Environmental exposure and leptospirosis, Peru. Emerg Infect Dis 2004; 10:1016–1022.

This study was a thorough investigation of the epidemiology of leptospirosis in different ecological settings in Peru. In addition to new information on seroprevalence, this report provides prospective evidence for high infection rates among peri-urban shantytown residents.

16 Tassinari WS., Pellegrini DCP, Sabroza PC, Carvalho MS. Spatial distribution of leptospirosis in the city of Rio de Janeiro, Brazil, 1996–1999 [in Portuguese]. Cad Saude Publica 2004; 20:1721–1729.

17 Karande S, Gandhi D, Kulkarni M, *et al.* Concurrent outbreak of leptospirosis and dengue in Mumbai, India, 2002. J Trop Pediatr 2005; 51:174–181.

18 • LaRocque RC, Breiman RF, Ari MD, *et al.* Leptospirosis during dengue outbreak, Bangladesh. Emerg Infect Dis 2005; 11:766–769.

This is a good example of a surveillance system designed to detect inapparent cases of leptospirosis during a dengue epidemic and applied real-time PCR in the diagnostic protocol during surveillance.

19 United Nations. The challenge of slums: global report on human settlements. Nairobi: UN-Habitat; 2003.

20 Nardone A, Capek I, Baranton G, *et al.* Risk factors for leptospirosis in metropolitan France: results of a national case-control study, 1999–2000. Clin Infect Dis 2004; 39:751–753.

21 Haake DA, Dundoo M, Cader R, *et al.* Leptospirosis, water sports, and chemoprophylaxis. Clin Infect Dis 2002; 34:e40–e43.

22 Laras K, Cao BV, Bounlu K, *et al.* The importance of leptospirosis in Southeast Asia. Am J Trop Med Hyg 2002; 67:278–286.

23 Phraisuwan P, Whitney EA, Tharmaphornpilas P, *et al.* Leptospirosis: skin wounds and control strategies, Thailand, 1999. Emerg Infect Dis 2002; 8:1455–1459.

24 Jena AB, Mohanty KC, Devadasan N. An outbreak of leptospirosis in Orissa, India: the importance of surveillance. Trop Med Int Health 2004; 9:1016–1021.

25 Murdoch DR, Woods CW, Zimmerman MD, *et al.* The etiology of febrile illness in adults presenting to Patan hospital in Kathmandu, Nepal. Am J Trop Med Hyg 2004; 70:670–675.

26 • Segura ER, Ganoza CA, Campos K, *et al.* Clinical spectrum of pulmonary involvement in leptospirosis in a region of endemicity, with quantification of leptospiral burden. Clin Infect Dis 2005; 40:343–351.

This study alerted clinicians to the importance of SPHS and its clinical presentation and used real-time PCR to determine a prognostic threshold level of leptospiraemia associated with this severe disease form.

27 Smythe L. Leptospirosis worldwide. Wkly Epidemiol Rec 1999; 74:237–242.

28 Russell KL, Montiel GMA, Watts DM, *et al.* An outbreak of leptospirosis among Peruvian military recruits. Am J Trop Med Hyg 2003; 69:53–57.

29 Ashford DA, Kaiser RM, Spiegel RA, *et al.* Asymptomatic infection and risk factors for leptospirosis in Nicaragua. Am J Trop Med Hyg 2000; 63:249–254.

30 Everard COR, Edwards CN, Everard JD, Carrington DG. A twelve-year study of leptospirosis on Barbados. Eur J Epidemiol 1995; 11:311–320.

31 Perrocheau A, Perolat P. Epidemiology of leptospirosis in New Caledonia (South Pacific): a one-year survey. Eur J Epidemiol 1997; 13:161–167.

32 Faine SB, Adler B, Bolin C, Perolat P. *Leptospira* and leptospirosis. 2nd ed. Melbourne: MediSci; 1999.

33 WHO. Human leptospirosis: guidance for diagnosis, surveillance and control. Malta: World Health Organization; 2003.

34 Wongsrichanalai C, Murray CK, Gray M, *et al.* Co-infection with malaria and leptospirosis. Am J Trop Med Hyg 2003; 68:583–585.

35 • Chappel RJ, Goris M, Palmer MF, Hartskeerl RA. Impact of proficiency testing on results of the microscopic agglutination test for diagnosis of leptospirosis. J Clin Microbiol 2004; 42:5484–5488.

This report is an advance with respect to implementing quality control measures for MAT testing among references laboratories.

36 Smits HL, Chee HD, Eapen CK, *et al.* Latex based, rapid and easy assay for human leptospirosis in a single test format. Trop Med Int Health 2001; 6:114–118.

37 Smits HL, Eapen CK, Sugathan S, *et al.* Lateral-flow assay for rapid serodiagnosis of human leptospirosis. Clin Diagn Lab Immunol 2001; 8:166–169.

38 Effler PV, Bogard AK, Domen HY, *et al.* Evaluation of eight rapid screening tests for acute leptospirosis in Hawaii. J Clin Microbiol 2002; 40:1464–1469.

39 Levett PN, Branch SL. Evaluation of two enzyme-linked immunosorbent assay methods for detection of immunoglobulin M antibodies in acute leptospirosis. Am J Trop Med Hyg 2002; 66:745–748.

40 Vijayachari P, Sugunan AP, Sehgal SC. Evaluation of Lepto Dri Dot as a rapid test for the diagnosis of leptospirosis. Epidemiol Infect 2002; 129:617–621.

41 Bajani MD, Ashford DA, Bragg SL, *et al.* Evaluation of four commercially available rapid serologic tests for diagnosis of leptospirosis. J Clin Microbiol 2003; 41:803–809.

42 Sehgal SC, Vijayachari P, Sugunan AP, Umapathi T. Field application of Lepto lateral flow for rapid diagnosis of leptospirosis. J Med Microbiol 2003; 52 (Pt 10):897–901.

43 Cumberland P, Everard COR, Wheeler JG, Levett PN. Persistence of anti-leptospiral IgM, IgG and agglutinating antibodies in patients presenting with acute febrile illness in Barbados 1979–1989. Eur J Epidemiol 2001; 17:601–608.

44 • Wagenaar JF, Falke TH, Nam NV, *et al.* Rapid serological assays for leptospirosis are of limited value in southern Vietnam. Ann Trop Med Parasitol 2004; 98:843–850.

This is a report of the issues facing the use of current serologic tests in regions with high endemic transmission of leptospirosis.

45 Smythe LD, Smith IL, Smith GA, *et al.* A quantitative PCR (TaqMan) assay for pathogenic *Leptospira* spp. BMC Infect Dis 2002; 2:13.

46 Levett PN, Morey RE, Galloway RL, *et al.* Detection of pathogenic leptospires by real-time quantitative PCR. J Med Microbiol 2005; 54 (Pt 1):45–49.

47 Palaniappan RU, Chang YF, Chang CF, *et al.* Evaluation of *lig*-based conventional and real time PCR for the detection of pathogenic leptospires. Mol Cell Probes 2005; 19:111–117.

48 Truccolo J, Serais O, Merien F, Perolat P. Following the course of human leptospirosis: Evidence of a critical threshold for the vital prognosis using a quantitative PCR assay. FEMS Microbiol Lett 2001; 204:317–321.

49 Saengjaruk P, Chaicumpa W, Watt G, *et al.* Diagnosis of human leptospirosis by monoclonal antibody-based antigen detection in urine. J Clin Microbiol 2002; 40:480–489.

50 Guerreiro H, Croda J, Flannery B, *et al.* Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. Infect Immun 2001; 69:4958–4968.

51 Flannery B, Costa D, Carvalho FP, *et al.* Evaluation of recombinant *Leptospira* antigen-based enzyme-linked immunosorbent assays for the serodiagnosis of leptospirosis. J Clin Microbiol 2001; 39:3303–3310.

52 Palaniappan RU, Chang YF, Jusuf SS, *et al.* Cloning and molecular characterization of an immunogenic LigA protein of *Leptospira* interrogans. Infect Immun 2002; 70:5924–5930.

53 Matsunaga J, Barocchi MA, Croda J, *et al.* Pathogenic *Leptospira* species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily. Mol Microbiol 2003; 49:929–945.

54 • Koizumi N, Watanabe H. Leptospiral immunoglobulin-like proteins elicit protective immunity. Vaccine 2004; 22:1545–1552.

This report provides the first experimental evidence that the Lig proteins protect against lethal challenge. Although the experimental model used is not ideal, the findings indicate that Lig proteins are a promising vaccine candidate.

55 Palaniappan RU, Chang YF, Hassan F, *et al.* Expression of leptospiral immunoglobulin-like protein by *Leptospira interrogans* and evaluation of its diagnostic potential in a kinetic ELISA. J Med Microbiol 2004; 53:975–984.

56 Katz AR, Effler PV, Ansdell VE. Comparison of serology and isolates for the identification of infecting leptospiral serogroups in Hawaii, 1979–1998. Trop Med Int Health 2003; 8:639–642.

57 Levett PN. Usefulness of serologic analysis as a predictor of the infecting serovar in patients with severe leptospirosis. Clin Infect Dis 2003; 36:447–452.

58 • Majed Z, Bellenger E, Postic D, *et al.* Identification of variable-number tandem-repeat loci in *Leptospira interrogans* sensu stricto. J Clin Microbiol 2005; 43:539–545.
This report of a polymorphic loci in the *Leptospira* genome is a breakthrough with respect to developing a more effective molecular typing method.

59 Dupont H, Dupont-Perdrizet D, Perie JL, *et al.* Leptospirosis: Prognostic factors associated with mortality. Clin Infect Dis 1997; 25:720–724.

60 Daher E, Trevisan Zanetta DM, Cavalcante MB, Abdulkader RCRM. Risk factors for death and changing patterns in leptospirosis acute renal failure. Am J Trop Med Hyg 1999; 61:630–634.

61 Panaphut T, Domrongkitchaiporn S, Thinkamrop B. Prognostic factors of death in leptospirosis: A prospective cohort study in Khon Kaen, Thailand. Int J Infect Dis 2002; 6:52–59.

62 Covic A, Goldsmith DJ, Gusbeth-Tatomir P, *et al.* A retrospective 5-year study in Moldova of acute renal failure due to leptospirosis: 58 cases and a review of the literature. Nephrol Dial Transplant 2003; 18:1128–1134.

63 • Esen S, Sunbul M, Leblebicioglu H, *et al.* Impact of clinical and laboratory findings on prognosis in leptospirosis. Swiss Med Wkly 2004; 134:347–352.
This describes a thorough investigation of the clinical presentation of severe leptospirosis cases in Turkey. The study used multivariate analysis to identify prognostic factors for mortality.

64 Lopes AA, Costa E, Costa YA, *et al.* Comparative study of the in-hospital case-fatality rate of leptospirosis between pediatric and adult patients of different age groups. Rev Inst Med Trop Sao Paulo 2004; 46:19–24.

65 Marotto PC, Nascimento CM, Eluf-Neto J, *et al.* Acute lung injury in leptospirosis: clinical and laboratory features, outcome, and factors associated with mortality. Clin Infect Dis 1999; 29:1561–1563.

66 Vieira SR, Brauner JS. Leptospirosis as a cause of acute respiratory failure: clinical features and outcome in 35 critical care patients. Braz J Infect Dis 2002; 6:135–139.

67 Seguro AC, Lomar AV, Rocha AS. Acute renal failure of leptospirosis: nonoliguric and hypokalemic forms. Nephron 1990; 55:146–151.

68 Abdulkader RCRM, Seguro AC, Malheiro PS, *et al.* Peculiar electrolytic and hormonal abnormalities in acute renal failure due to leptospirosis. Am J Trop Med Hyg 1996; 54:1–6.

69 Phu NH, Hien TT, Mai NT, *et al.* Hemofiltration and peritoneal dialysis in infection-associated acute renal failure in Vietnam. N Engl J Med 2002; 347:895–902.

70 Magaldi AJ, Yasuda PN, Kudo LH, *et al.* Renal involvement in leptospirosis: a pathophysiologic study. Nephron 1992; 62:332–339.

71 • Wu MS, Yang CW, Pan MJ, *et al.* Reduced renal Na^+-K^+-Cl^- co-transporter activity and inhibited NKCC2 mRNA expression by *Leptospira shermani*: from bed-side to bench. Nephrol Dial Transplant 2004; 19:2472–2479.
This report provides evidence suggesting that Na^+-K^+-Cl^- transporter in the thick ascending limb may be the lesion in leptospirosis-associated non-oliguric hypokalemic acute renal insufficiency.

72 • Burth P, Younes-Ibrahim M, Santos MC, *et al.* Role of nonesterified unsaturated fatty acids in the pathophysiological processes of leptospiral infection. J Infect Dis 2005; 191:51–57.
This report provides clinical evidence for the role of *Leptospira*-derived unsaturated fatty acids in the pathogenesis of leptospirosis and for albumin to be a serum protection factor against their toxic actions.

73 Amato MB, Barbas CS, Medeiros DM, *et al.* Effect of a protective-ventilation strategy on mortality in the acute respiratory distress syndrome. N Engl J Med 1998; 338:347–354.

74 Nicodemo AC, Duarte MI, Alves VA, *et al.* Lung lesions in human leptospirosis: microscopic, immunohistochemical, and ultrastructural features related to thrombocytopenia. Am J Trop Med Hyg 1997; 56:181–187.

75 •• Nally JE, Chantranuwat C, Wu XY, *et al.* Alveolar septal deposition of immunoglobulin and complement parallels pulmonary hemorrhage in a guinea pig model of severe pulmonary leptospirosis. Am J Pathol 2004; 164:1115–1127.
This study provided evidence that an autoimmune process may be responsible for the pathology of SPHS in the guinea pig model.

76 • Pereira MM, Da Silva JJ, Pinto MA, *et al.* Experimental leptospirosis in marmoset monkeys (*Callithrix jacchus*): a new model for studies of severe pulmonary leptospirosis. Am J Trop Med Hyg 2005; 72:13–20.
This reports established a much-needed primate model for SHPS which now can be used for preclinical evaluation of new therapies.

77 Daher EF, Nogueira CB. Evaluation of penicillin therapy in patients with leptospirosis and acute renal failure. Rev Inst Med Trop Sao Paulo 2000; 42:327–332.

78 Costa E, Lopes AA, Sacramento E, *et al.* Penicillin at the late stage of leptospirosis: a randomized controlled trial. Rev Inst Med Trop Sao Paulo 2003; 45:141–145.

79 Vinetz JM. A mountain out of a molehill: do we treat acute leptospirosis, and if so, with what? Clin Infect Dis 2003; 36:1514–1515.

80 Panaphut T, Domrongkitchaiporn S, Vibhagool A, *et al.* Ceftriaxone compared with sodium penicillin g for treatment of severe leptospirosis. Clin Infect Dis 2003; 36:1507–1513.

81 • Suputtamongkol Y, Niwattayakul K, Suttinont C, *et al.* An open, randomized, controlled trial of penicillin, doxycycline, and cefotaxime for patients with severe leptospirosis. Clin Infect Dis 2004; 39:1417–1424.
This equivalency trial demonstrated that the use of cefotaxime and doxycycline has equivalent efficacy to penicillin therapy for leptospirosis and supported the findings from Panaphut *et al.* for the use of alternative antibiotic regimens in the treatment of severe leptospirosis.

82 Bauby H, Saint GI, Picardeau M. Construction and complementation of the first auxotrophic mutant in the spirochaete *Leptospira meyeri*. Microbiology 2003; 149 (Pt 3):689–693.

83 Guegan R, Camadro JM, Saint GI, Picardeau M. *Leptospira spp*. possess a complete haem biosynthetic pathway and are able to use exogenous haem sources. Mol Microbiol 2003; 49:745–754.

84 Louvel H, Saint Girons I, Picardeau M. Isolation and characterization of FecA- and FeoB-mediated iron acquisition systems of the spirochete *Leptospira biflexa* by random insertional mutagenesis. J Bacteriol 2005; 187:3249–3254.

85 Picardeau M, Bauby H, Saint GI. Genetic evidence for the existence of two pathways for the biosynthesis of methionine in the *Leptospira spp*. FEMS Microbiol Lett 2003; 225:257–262.

86 • Xu H, Zhang Y, Guo X, *et al.* Isoleucine biosynthesis in *Leptospira interrogans* serotype Lai strain 56601 proceeds via a threonine-independent pathway. J Bacteriol 2004; 186:5400–5409.
This is a good example of how information from the *Leptospira* genome and genetic tools led to the functional characterization of an amino acid synthesis pathway.

87 • Bourhy P, Louvel H, Saint Girons I, Picardeau M. Random insertional mutagenesis of *Leptospira interrogans*, the agent of leptospirosis, using a mariner transposon. J Bacteriol 2005; 187:3255–3228.
This is the first report of a method to disrupt genes in pathogenic *Leptospira*.

88 Zhang YX, Li J, Guo XK, *et al.* Characterization of a novel toxin-antitoxin module, VapBC, encoded by *Leptospira interrogans* chromosome. Cell Res 2004; 14:208–216.

89 •• Lingappa J, Kuffner T, Tappero J, *et al.* HLA-DQ6 and ingestion of contaminated water: possible gene-environment interaction in an outbreak of Leptospirosis. Genes Immun 2004; 5:197–202.
This is the report of a major finding with respect to the identification of the first host genetic susceptibility determinant in leptospirosis and the first gene–environment interaction for an infectious disease.

90 Tajiki MH, Salomao R. Association of plasma levels of tumor necrosis factor alpha with severity of disease and mortality among patients with leptospirosis. Clin Infect Dis 1996; 23 (5):1177–1178.

91 de Fost M, Hartskeerl RA, Groenendijk MR, van der Poll T. Interleukin 12 in part regulates gamma interferon release in human whole blood stimulated with *Leptospira interrogans*. Clin Diagn Lab Immunol 2003; 10:332–335.

92 Klimpel GR, Matthias MA, Vinetz JM. *Leptospira interrogans* activation of human peripheral blood mononuclear cells: preferential expansion of TCR gamma delta+ T cells vs TCR alpha beta+ T cells. J Immunol 2003; 171:1447–1455.

93 Werts C, Tapping RI, Mathison JC, *et al.* Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. Nature Immunol 2001; 2:346–352.

94 •• Que-Gewirth NL, Ribeiro AA, Kalb SR, *et al.* A methylated phosphate group and four amide-linked acyl chains in *Leptospira interrogans* lipid A. The membrane anchor of an unusual lipopolysaccharide that activates TLR2. J Biol Chem 2004; 279:25420–25429.
This report demonstrates that *Leptospira* lipid A have a unique structure unlike that found in other bacterial lipid A and may provide an explanation for the unique biological characteristics associated with *Leptospira* LPS.

95 Diament D, Brunialti MKC, Romero EC, *et al.* Peripheral blood mononuclear cell activation induced by Leptospira *interrogans* glycolipoprotein. Infect Immun 2002; 70:1677–1683.

96 Barocchi MA, Ko AI, Reis MG, *et al.* Rapid translocation of polarized MDCK cell monolayers by *Leptospira* interrogans, an invasive but nonintracellular pathogen. Infect Immun 2002; 70:6926–6932.

97 • Nally JE, Chow E, Fishbein MC, *et al.* Changes in lipopolysaccharide O-antigen distinguish acute versus chronic *Leptospira interrogans* infections. Infect Immun 2005; 73:3251–3260.
This report establishes animal models to study persistent infection and provides evidence suggesting that O-antigen may play a role in regulating whether *Leptospira* produce an acute versus persistent infection.

98 Cullen PA, Cordwell SJ, Bulach DM, *et al.* Global analysis of outer membrane proteins from *Leptospira interrogans* serovar Lai. Infect Immun 2002; 70:2311–2318.

99 Cullen PA, Xu X, Matsunaga J, *et al.* The Surfaceome of *Leptospira*. Infect Immun (in press).

100 Haake DA, Matsunaga J. Characterization of the leptospiral outer membrane and description of three novel leptospiral membrane proteins. Infect Immun 2002; 70:4936–4945.

101 • Matsunaga J, Sanchez Y, Xu X, Haake DA, Osmolarity. a key environmental signal controlling expression of leptospiral proteins LigA and LigB and the extracellular release of LigA. Infect Immun 2005; 73:70–78.
This report shows that osmolarity is the environmental signal that regulates *lig* gene expression. It also found that LigA is released in culture supernatants, a finding documented only previously with haemolysins.

102 Cullen PA, Haake DA, Bulach DM, *et al.* LipL21 is a novel surface-exposed lipoprotein of pathogenic *Leptospira* species. Infect Immun 2003; 71:2414–2421.

103 •• Haake DA, Suchard MA, Kelley MM, *et al.* Molecular evolution and mosaicism of leptospiral outer membrane proteins involves horizontal DNA transfer. J Bacteriol 2004; 186:2818–2828.
This report provides evidence that horizontal transfer has produced a mosaic *ompl1* gene. It is an excellent example of how detailed sequence analysis and modelling can provide insights into the evolution of *Leptospira*.

104 • Nally JE, Whitelegge JP, Aguilera R, *et al.* Purification and proteomic analysis of outer membrane vesicles from a clinical isolate of *Leptospira interrogans* serovar Copenhageni. Proteomics 2005; 5:144–152.
This study used 2D gel electrophoresis/mass spectrometry and liquid chromatography/mass spectrometry to identify novel candidate surface-associated proteins.

105 Yan Y, Chen Y, Liou W, *et al.* An evaluation of the serological and epidemiological effects of the outer envelope vaccine to *Leptospira*. J Chin Med Assoc 2003; 66:224–230.

106 Martinez R, Perez A, Quinones Mdel C, *et al.* Efficacy and safety of a vaccine against human leptospirosis in Cuba [in Spanish]. Rev Panam Salud Publica 2004; 15:249–255.

107 Rodriguez AG, Jimenez YR, Santiesteban NB, *et al.* Microbiological characterization of candidate vaccine strains of Ballum serogroup *Leptospira interrogans* [in Spanish]. Rev Cubana Med Trop 2003; 55:146–152.

108 Haake DA, Mazel MK, McCoy AM, *et al.* Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. Infect Immun 1999; 67:6572–6582.

109 Branger C, Sonrier C, Chatrenet B, *et al.* Identification of the hemolysis-associated protein 1 as a cross-protective immunogen of *Leptospira interrogans* by adenovirus-mediated vaccination. Infect Immun 2001; 69:6831–6838.

110 Gamberini M, Gomez RM, Atzingen MV, *et al.* Whole-genome analysis of *Leptospira interrogans* to identify potential vaccine candidates against leptospirosis. FEMS Microbiol Lett 2005; 244:305–313.

111 Naiman BM, Blumerman S, Alt D, *et al.* Evaluation of type 1 immune response in naive and vaccinated animals following challenge with *Leptospira borgpetersenii* serovar Hardjo: involvement of WC1(+) gammadelta and CD4 T cells. Infect Immun 2002; 70:6147–6157.

112 Brown RA, Blumerman S, Gay C, *et al.* Comparison of three different leptospiral vaccines for induction of a type 1 immune response to *Leptospira borgpetersenii* serovar Hardjo. Vaccine 2003; 21:4448–4458.