

The power of water has been highlighted over the past year, following the Boxing Day tsunami and the devastation wreaked on New Orleans by hurricane Katrina. However, water can represent a danger in other ways; for example, in the transmission of leptospirosis, as Richard A Collins explains.

# Leptospirosis

**Leptospirosis is an emerging zoonotic disease caused by pathogenic bacteria of the genus *Leptospira*. Transmission to humans occurs through contact with domestic or wild animal reservoirs or an environment contaminated by their urine. Leptospirosis refers to the relatively mild form of the disease that is readily treatable with antibiotics. The more severe form of the disease is known as Weil's disease.**

## History

Leptospirosis was first recognised as an occupational disease of sewer workers in 1883. In 1886, German physician Adolf Weil described the clinical manifestations in four men who had severe jaundice, fever and haemorrhage with renal involvement. In 1915, Ryukichi Inada and co-workers in Japan detected both spirochaete bacteria and specific antibodies in the blood of Japanese miners with infectious jaundice.<sup>1</sup> They named the new organism *Spirochaeta icterohaemorrhagiae*. However, in 1907, Arthur M Stimson described the same organism in silver-stained kidney tubules from a patient who reportedly died of yellow fever.<sup>2</sup> The spirochaetes had hooked ends, and Stimson named them *Spirochaeta interrogans* because of their resemblance to a question mark (Fig 1). The genus was renamed *Leptospira* in 1917.

The main occupational groups at risk today include farm and agricultural workers, veterinary surgeons, pet shop workers, plumbers, abattoir workers and meat handlers, coal miners, workers in the fishing industry, sewer workers and the military. Other groups at high risk of contracting leptospirosis include the survivors of natural disasters (eg flooding) and the increasing number of people engaging in recreational water sports.

## Bacterial morphology

*Leptospira* are spirochaetes – Gram-negative, thin, coiled, motile, obligate, slow-growing aerobes 5–25 µm in length and 0.1–0.3 µm in diameter (Fig 1). They possess characteristic

internal flagellae, which allow them to burrow into tissue. *Leptospira* are endemic to feral and domestic animals, with rats and other rodents recognised as the most important reservoirs.

## Classification

The family Leptospiraceae contains only three genera: *Leptonema*, *Turneria* and *Leptospira*. The genus *Leptospira* comprises 10 genomospecies, of which the most important are the pathogenic *L. interrogans* and the non-pathogenic *L. biflexa*. Each genomospecies is subdivided into about 23 serogroups, into which are placed individual serovars (Fig 2). To date, about 240 serovars have been characterised.

Precise classification of individual species is very difficult because there is almost no visible difference between serovars. Prior to the development of DNA analysis, classification was by serological cross-testing (using serum antibodies to identify similar and different types of bacteria). The current practice, adopted by the Spirochaete Group at the Institut Pasteur in Paris, is that pathogenic strains are assumed to be serovars of *L. interrogans*, while non-pathogenic strains are assigned to *L. biflexa*.

Lipopolysaccharide (LPS) is the major antigen involved in serological classification. Structural heterogeneity in the carbohydrate component of LPS moieties derived from differences in the genes involved in LPS biosynthesis appears to be the basis for the large degree of antigenic variation observed among serovars.<sup>3</sup>

## Genome

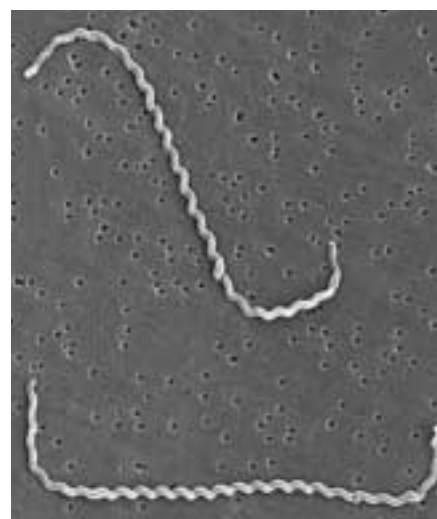
The *Leptospira* genome consists of two circular chromosomes totalling 4.63 million base pairs (bp). Chromosome I has 4.28 million bp while

the much smaller chromosome II has just 350,000 bp. Chromosome I encodes 3454 known or putative genes, while chromosome II encodes 274 genes.<sup>4</sup>

## Incidence

The annual reported incidence in England and Wales ranged between 29 and 48 cases during 1990–92.<sup>5</sup> Hawaii has the highest annual incidence of leptospirosis in the United States. From 1974 to 1998, the mean annual incidence rate was 1.29 per 100,000.<sup>6</sup> Data from the Czech Republic over the period 1963–2003 indicate that the incidence rate of leptospirosis was 0.3% per 100,000 population. However, during periods of

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**Fig 1.** Scanning electron micrograph of *Leptospira interrogans* bound to a 0.2-µm filter. Size range is 5–25 µm (average 10 µm) in length and 0.1–0.3 µm in diameter.

intense flooding (1997–2002) this increased to 0.9% per 100,000.<sup>7</sup> Fortunately, neither the New Orleans flood of August 2005 nor the Asian tsunami disaster of December 2004 appeared to increase the number of cases of leptospirosis, according to information posted on ProMed-Mail – the official website of the International Society for Infectious Diseases (www.promedmail.org).

### Disease

Leptospira infection produces a wide spectrum of clinical manifestations. The incubation period is usually seven to 12 days (range two to 20 days). Typically, the course of leptospirosis is biphasic, with an acute septicaemic phase followed by the immune phase (Fig 3).

### Septicaemic phase

The septicaemic phase, which lasts about four to seven days, is characterised by abrupt onset of fever, severe headache, muscle pain, and nausea. The bacterium may be isolated from blood culture, cerebrospinal fluid (CSF) and most tissues. About 90% of patients have a mild anicteric (ie without jaundice) form of the disease, while 5–10% experience a more severe form with jaundice, renal failure and haemorrhagic manifestations, otherwise known as Weil's disease.

### Interphase

During the one- to three-day period of improvement that follows the first stage, body temperature drops and the patient may become afebrile and relatively asymptomatic. The fever then recurs, indicating the onset of the second stage.

### Immune phase

The immune phase occurs as a consequence of the body's immune response to infection, and lasts up to 30 days or more. It is manifested by a fever of shorter duration and central nervous system involvement (meningitis). Circulating IgM antileptospiral antibodies may be detected or the bacterium isolated from urine. Bacteria may no longer be recoverable from blood or CSF.

Immunity to leptospirosis is primarily humoral (ie mediated by the antibody-producing branch of the immune system, the result of B-cell and Th2 T-helper (CD4) cell stimulation). Cell-mediated immunity (involving T lymphocytes, phagocytes and natural killer cells) does not appear to be important, but it may be responsible for some of the late manifestations of the disease.

Immunity to leptospirosis is serovar-specific and may persist for years. Immune serum has been used to treat human leptospirosis and passively protects experimental animals from the disease. The survival of leptospires in the convoluted tubules of the kidney may be related to the ineffectiveness of the antibody-complement system at this site.

Laboratory diagnosis of leptospirosis is based primarily on either isolation of the

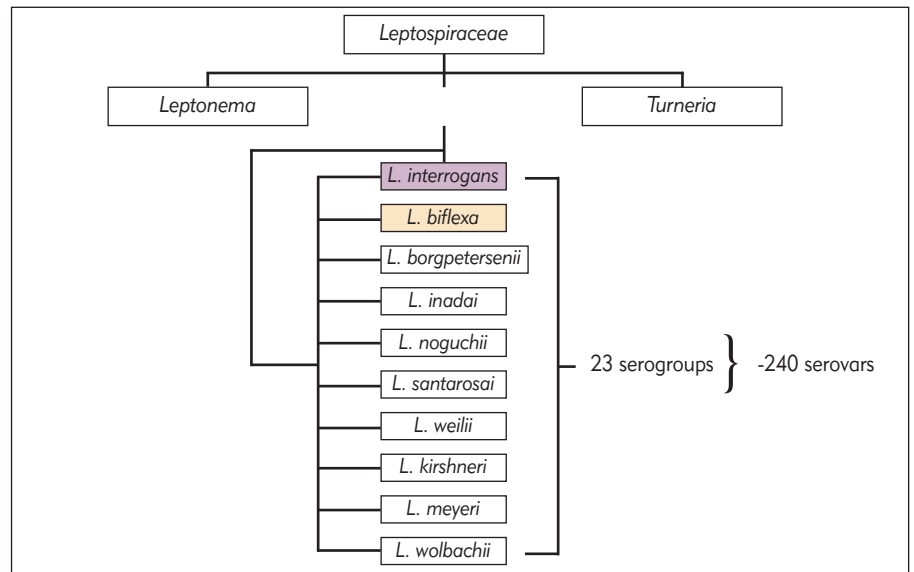


Fig 2. Classification of the Leptospiraceae.

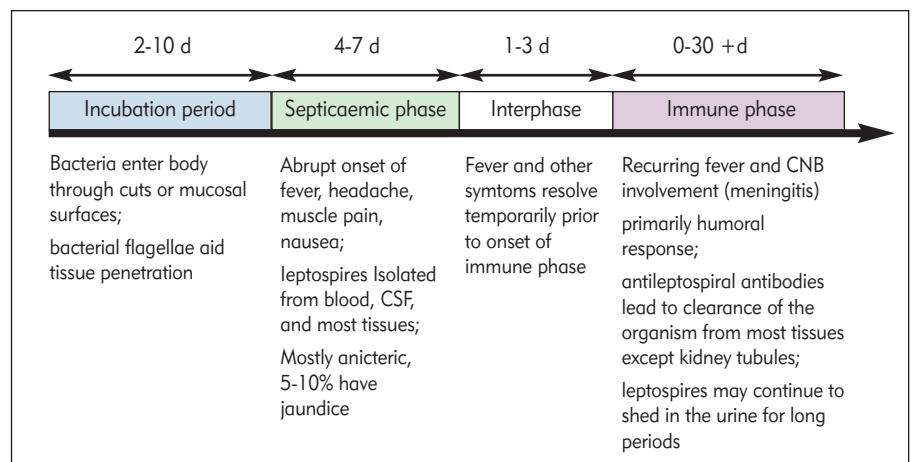


Fig 3. Typical course of leptospirosis.

pathogen from the specimen or demonstration of a rise in serum antibodies.

### Microscopy

Darkfield microscopy is the most common method used to examine the growth of leptospires in culture. To evaluate its diagnostic accuracy when used directly on body fluids, darkfield microscopy was compared with established tests including culture, microscopic agglutination test (MAT), IgM enzyme-linked immunosorbent assay (ELISA) and immobilised antigen dipstick on 170 clinical samples suspected of containing leptospirosis.<sup>8</sup> The gold standard for diagnostic comparison was positive blood culture, seroconversion or a four-fold rise in MAT titre.

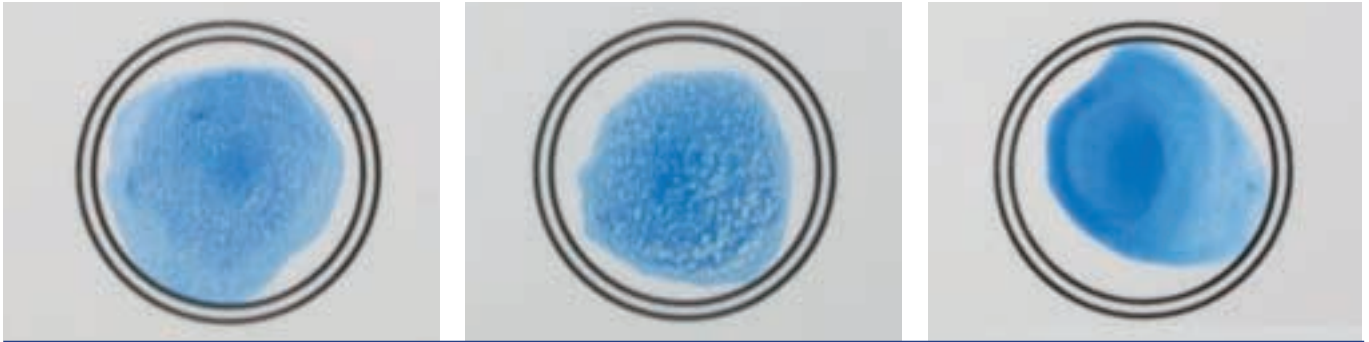
Plasma and serum were used for darkfield microscopy following centrifugation at 1000  $\times g$  and 3000  $\times g$ , respectively. Darkfield microscopy had low sensitivity (40.2%), low specificity (61.5%), poor positive (55.2%) and negative (46.6%) predictive value compared with the gold standard and was not

recommended as a sole diagnostic procedure for early diagnosis of leptospirosis.<sup>8</sup>

### Culture

Leptospires are the most readily cultivated of the pathogenic spirochaetes. They have relatively simple nutritional requirements, with long-chain fatty acids and vitamins B<sub>1</sub> and B<sub>12</sub> being the only organic compounds known to be necessary for growth. When cultivated in media at 30°C and pH 7.4, average generation time is about 12 hours, with aeration required for maximal growth. They can be cultivated in plates containing soft (1%) agar medium, in which they form primarily subsurface colonies.

In the acute phase, leptospires may be cultured from blood or CSF. Leptospires remain viable in anticoagulated blood for up to 11 days. This allows specimens to be mailed to a reference laboratory for culture. Leptospires may not be detected in the blood until four days after the onset of symptoms (seven to 12 days after exposure). Once the immune system is activated, blood cultures



**Fig 4.** Card agglutination test for diagnosis of leptospirosis. A: weak positive; B: strong positive; C: negative.

may become negative. Hence, blood culture may be negative if drawn too early or too late.

Leptospire may be isolated from urine for several weeks after the initial infection. In some patients, however, urine cultures remain positive for months or years after the onset of illness. Positive urine cultures may take up to eight weeks to grow. Thus, leptospire culture from urine is too slow for early diagnosis.

## Serology

### Microscopic agglutination test

The MAT is the reference method (gold standard) against which other techniques have to be compared to evaluate their diagnostic sensitivity, specificity, and accuracy. Generally, it is performed by reference laboratories, due to the inherent safety risks of handling cultures of live leptospiral organisms, the high cost of commercial media and the need for ongoing maintenance of representative serovars or serogroups. These factors limit the wider use of MAT in the developing world, where leptospirosis occurs more extensively.

The MAT has been used to diagnose leptospirosis since at least 1954.<sup>9</sup> A standard antigen preparation (a live bacterial culture with a transmittance of 60–70% in a spectrophotometer with a 400-nm filter or a reading of 25 nephelometer units) is added to multiple wells of a microtitre plate. To these are added an equal volume of the test or control serum to a final dilution of 1 in 100. After incubation (two to four hours at 29 + 1 °C), the wells are examined under darkfield microscopy at about x45 magnification and scored for the degree of agglutination, as follows: 0 (no agglutination), 1+ (<50% agglutination), 2+ (>50% agglutination), 3+ (>75% agglutination), 4+ (100% agglutination). The end point is the highest dilution showing a 2+ reaction. For screening purposes, any serum that has a 2+ reaction at a 1 in 100 dilution is titrated to an end point using doubling dilutions of serum, starting at a final dilution of 1 in 100, through to 1 in 12,800 or higher. The end-point titre is the reciprocal of the highest dilution with a 2+ or greater reaction.

A specific antibody response detectable by

## ‘Rats and other rodents are the most important reservoirs of *Leptospira* organisms’

MAT generally occurs around eight to 10 days after onset of illness. Diagnosis of leptospirosis by MAT often requires paired serum samples, which delays the diagnosis. Moreover, false-negative results occur frequently when the causative leptospire serovar is not included in the panel of typing organisms.

### Other agglutination assays

Apart from the MAT, several other diagnostic tests for leptospirosis, based on agglutination of antigen and antibody, have been developed. These have increased the speed and convenience of diagnosis considerably.

The card agglutination test consists of blue latex particles activated with a broadly reactive *Leptospira* antigen dried on an agglutination card. The assay is based on the binding of *Leptospira*-specific antibodies, present in the serum sample, to the *Leptospira* antigen, producing a fine granular agglutination that tends to settle at the edge of the droplet (positive result; Fig 4).

The broadly reactive antigen allows the detection of *Leptospira* infections caused by a wide range of strains of different serovars. When no specific antibodies are present, the blue suspension remains homogeneous (negative result). Agglutination due to the interaction of serum antibodies and labelled antigen can be observed two to five minutes after the test components are mixed.

### Other serological assays

Several other types of antibody detection assay have been developed for early diagnosis of leptospirosis. These include the haemolytic test,<sup>10</sup> indirect haemagglutination assay,<sup>11</sup> indirect immunofluorescence,<sup>12</sup> indirect IgM ELISA,<sup>13</sup> IgM dot-ELISA,<sup>14</sup> immobilised antigen dipstick<sup>15</sup> and lateral flow assay.<sup>16</sup>

While these methods are much simpler than MAT, they still need a lag period after infection before antibodies become detectable.

An immunoblot for the detection of leptospirosis was developed.<sup>17</sup> Antigen prepared from *L. interrogans* serovar Bataviae was dotted onto nitrocellulose paper and blocked with skim milk. Test and control sera diluted 1 in 20 were applied to the dot, incubated and washed. Anti-human IgM colloidal gold conjugate was added and the dots were washed. A positive reaction was shown by the development of a pink dot against a white background. There was 100% concordance between positive results detected by the gold immunoblot method and MAT ( $n=62$ ). The results confirmed previous findings that most antibodies present in leptospirosis patients are of the IgM type. The gold immunoblot can be completed in 30 minutes and the test blot can be kept as a permanent record.

The ELISA tests have been the most readily applicable for the rapid detection and diagnosis of leptospirosis. Several published studies have compared and evaluated a range of commercially available assays.<sup>18–21</sup> In a 2002 study, eight such rapid screening tests were evaluated, including indirect haemagglutination, IgM immunofluorescence, IgM ELISA, dipstick and latex agglutination.<sup>18</sup> In 379 serum samples from 236 patients, the test specificity was 85–100% for all tests except the latex agglutination test, which had significantly lower specificity at 10%. Sensitivity was very low (<25%) for all tests on specimens collected during the first week of illness. The authors concluded that IgM detection tests have limited utility for diagnosing leptospirosis during the initial evaluation of patients – a time when important therapeutic decisions are made.<sup>18</sup>

In a comparison of two commercially available IgM ELISA assays on 103 suspected leptospirosis cases, 48 were confirmed.<sup>19</sup> In 33 cases, both assays were positive on the first sample taken at admission (a mean of 6.7 days after onset of symptoms) and seroconversion was detected in a further nine cases. Both assays were negative in five cases and a single case gave discordant results in the two assays. False-positive

## Weil's disease is the more severe form of leptospirosis'

results were detected in four patients without leptospirosis. The sensitivity of the two assays was 89.6–97.5% and the specificity 92.7–96.4%, respectively. The positive predictive values were 87.8–95.5% and the negative predictive value 90% with each assay.<sup>19</sup>

In a 2004 study, four rapid tests for serological diagnosis of leptospirosis were evaluated.<sup>20</sup> The four tests included a microplate IgM ELISA, indirect haemagglutination (IHA), IgM dipstick and IgM dot ELISA dipstick. A panel of 276 sera from 133 cases of leptospirosis and 642 control sera (from normal individuals or from individuals with other infections or autoimmune diseases) were examined. Specificity for all assays ranged from 89.6% to 98.8%. However, IHA had the lowest sensitivity (79.0%) compared with the other three tests, the sensitivities of which ranged from 86.5% to 93.2%. The IgM dipstick had the highest rate of false-positive results, cross-reacting to sera from individuals with Epstein-Barr virus, human immunodeficiency virus (HIV) and periodontal disease.<sup>20</sup>

A smaller study in Italy confirmed the usefulness of IgM ELISA. The commercially available assay accurately detected the presence of IgM antibodies in 19 serum samples from nine patients with confirmed leptospirosis.<sup>21</sup> Controls included 23 serum samples from blood donors and 29 serum samples from patients with other infectious diseases. Sensitivity and specificity were 100% and 95.9%, respectively.<sup>21</sup>

### Nucleic acid assays

The polymerase chain reaction (PCR) has been used to detect a large number of microorganisms, including those of clinical significance. The sensitivity of PCR often precludes the need for isolation and culture, thus making it ideal for the rapid detection of organisms involved in acute infection. The first PCR assay to detect leptospires in cattle urine was developed in 1989.<sup>22</sup> This was not serovar-specific but could produce a visible band on agarose gel from 10–20 leptospires. In 1992, the first application of PCR to detect leptospirosis in clinical samples was reported.<sup>23</sup> The PCR product was a 1631-bp 5' region of the 16S rDNA. As little as 10<sup>1</sup> pg of purified DNA and as few as 10<sup>1</sup> leptospires could be detected.

Several recommendations for the optimal PCR amplification of leptospiral DNA from human urine have been proposed.<sup>24</sup> These include adjusting the sample to pH 7.6 immediately after collection, with phosphate-buffered saline (PBS) being superior to Tris or NaOH as the buffer. Elimination of epithelial cells, leucocytes and crystals by centrifugation (3000 rpm for 10 minutes at

room temperature) increased sensitivity.

Freezing and thawing the urine sample prior to centrifugation prevented amplification of leptospiral DNA, presumably due to release of PCR inhibitors and degradative enzymes from ruptured cells.

Use of a washing step after centrifugation and prior to release of DNA by boiling and the addition of 0.1% (w/v) bovine serum albumin in place of gelatin in the PCR mix minimised interference from inhibitory compounds. The reported lower limit of detection was 10<sup>4</sup> leptospires/mL urine, corresponding to about 20 genome copies per PCR reaction.

Using real-time PCR, it is possible to quantify the amount of template and therefore the number of target organisms. A real-time PCR (TaqMan) assay to detect leptospires in clinical and environmental samples was reported in 2002.<sup>25</sup> The limit of detection of leptospires was 10 cells in urine and two cells in serum. The reason for the lower sensitivity in urine samples is not clear. It may be that a component in the urine sample was extracted with the DNA and interfered with the PCR, or that extraction of DNA from cells in urine is not as efficient as that from serum. As real-time PCR enables the quantitative monitoring of leptospiral cells, it is possible to monitor treatment efficacy, in particular the use of chemotherapeutics. For example, patients in intensive care management, where ineffective treatment is life threatening, would benefit greatly from direct measurement of infectious organisms.

A rapid and quantitative assay for leptospires in human clinical samples using LightCycler PCR technology has been reported.<sup>26</sup> LightCycler PCR was able to distinguish leptospiral species using melting curves, which provides an approach for identification with a specific melting temperature assigned to a single species or set of species. Assay sensitivity was approximately 50 leptospires/mL, corresponding to 1–2 genome copies in a PCR mixture.

### Treatment

Mild leptospirosis is treated with oral antibiotics such as doxycycline (100 mg, twice a day), ampicillin (500–750 mg, four times a day) or amoxicillin (500 mg, four times a day). For severe leptospirosis, the primary therapy is penicillin G (20–24 million units a day, intramuscularly in divided doses every four to six hours). Alternative regimens include erythromycin (500 mg intravenously, four times a day). Several other antibiotics, including cephalosporins, may be useful, but clinical experience with these is limited.

### Prevention

Environmental control measures are difficult to implement because of the long-term survival of pathogenic leptospires in soil and water, and the abundance of wild and domestic animal reservoirs. The risk of acquiring leptospirosis can be reduced considerably by not swimming or wading in water that might be contaminated with animal urine. Protective clothing or footwear should be worn by those exposed to contaminated water or soil through their job or recreational activities.

### Vaccine

There are no human vaccines licensed for use in Europe or North America. Commercial human vaccines have been produced in China (since 1978) and Cuba (since 1998). The Chinese vaccine uses the outer envelope of *L. interrogans* serogroup Icterohaemorrhagiae as antigen.<sup>27</sup> The Cuban vaccine is a whole-cell suspension of three leptospiral serogroups, inactivated with formaldehyde.<sup>28</sup> Both vaccines induce immune responses against leptospiral LPS. However, such vaccines do not induce long-term protection against infection and do not provide cross-protective immunity against heterologous leptospiral serovars.

Protein antigens conserved among pathogenic serovars may contribute to overcoming the limitations of the vaccines currently available. Genes related to toxins, lipoproteins and several surface-exposed proteins may facilitate a better understanding of *Leptospira* pathogenesis and may serve as potential candidates for future vaccines. ■

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