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Leptospirosis

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INTRODUCTION

Leptospirosis is now identified as one of the emerging infectious diseases, exemplified by recent large outbreaks in Nicaragua (78, 100, 349, 507, 581), Brazil, India (645), southeast Asia, the United States (98, 102), and most recently in several countries as a result of the EcoChallenge Sabah 2000 competition in Malaysia (99, 126, 204). In the landmark Institute of Medicine report "Emerging Infections: Microbial Threats to Health in the United States," leptospirosis was used as an example of an infection which had in the past caused significant morbidity in military personnel deployed in tropical areas (340).

Much of the resurgent international interest in leptospirosis stems from several large clusters of cases which have occurred in Central and South America following flooding as a result of El Niño-related excess rainfall (201, 332, 436, 581, 664). However, the occurrence of large outbreaks of leptospirosis following severe floods is not a new phenomenon and is not restricted to tropical regions (226, 232, 425, 442, 526, 590).

In this review, the epidemiology and clinical features of leptospirosis are described, recent taxonomic changes affecting the genus *Leptospira* are discussed, and advances in the diag-

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nosis of leptospirosis by serological and molecular methods are analyzed.

HISTORICAL ASPECTS

Leptospirosis is a zoonosis of ubiquitous distribution, caused by infection with pathogenic *Leptospira* species. The spectrum of human disease caused by leptospires is extremely wide, ranging from subclinical infection to a severe syndrome of multiorgan infection with high mortality. This syndrome, icteric leptospirosis with renal failure, was first reported over 100 years ago by Adolf Weil in Heidelberg (624). However, an apparently identical syndrome occurring in sewer workers was described several years earlier (337, 338). Earlier descriptions of diseases that were probably leptospirosis were reviewed recently (207, 211). Leptospirosis was certainly recognized as an occupational hazard of rice harvesting in ancient China (211), and the Japanese name akiyami, or autumn fever, persists in modern medicine. With hindsight, clear descriptions of leptospiral jaundice can be recognized as having appeared earlier in the 19th century, some years before the description by Weil (211). It has been suggested that *Leptospira interrogans* serovar icterohaemorrhagiae was introduced to western Europe in the 18th century by westward extension of the range of of *Rattus norvegicus* from Eurasia (24).

The etiology of leptospirosis was demonstrated independently in 1915 in Japan and Germany (207). In Japan, Inada and Ido detected both spirochetes and specific antibodies in the blood of Japanese miners with infectious jaundice, and two groups of German physicians studied German soldiers afflicted by "French disease" in the trenches of northeast France. Uhlenhuth and Fromme (588) and Hubener and Reiter (289) detected spirochetes in the blood of guinea pigs inoculated with the blood of infected soldiers. Unfortunately, these two groups became so embroiled in arguments over priority that they overlooked the first publications in English (296) and German of papers by Inada's group, whose initial publications predated their own by 8 months (207). Confirmation of the occurrence of leptospirosis on both sides of the Western Front was obtained rapidly after the publication in Europe of Inada's work (131, 145, 543, 630).

Given the initial controversy over nomenclature, it is ironic that the organism had first been described almost 10 years before (542). Stimson demonstrated by silver staining the presence of clumps of spirochetes in the kidney tubules of a patient who reportedly died of yellow fever. The spirochetes had hooked ends, and Stimson named them *Spirochaeta interrogans* because of their resemblance to a question mark. Unfortunately, this sentinel observation was overlooked for many years (211).

The importance of occupation as a risk factor was recognized early. The role of the rat as a source of human infection was discovered in 1917 (293), while the potential for leptospiral disease in dogs was recognized, but clear distinction between canine infection with *L. interrogans* serovars icterohaemorrhagiae and canicola took several years (329). Leptospirosis in livestock was recognized some years later (24). Several monographs provide extensive information on the early development of knowledge on leptospirosis (24, 211, 213, 596, 634).

TABLE 1. Serogroups and some serovars of *L. interrogans* sensu lato

Serogroup	Serovar(s)
icterohaemorrhagiae,	Icterohaemorrhagiae, copenhageni, lai, zimbabwe
Hebdomadis	hebdomadis, jules, kremastos
Autumnalis	autumnalis, fortbragg, bim, weerasinghe
Pyrogenes	pyrogenes
Bataviae	bataviae
Grippotyphosa	grippotyphosa, canalzonae, ratnapura
Canicola	canicola
Australis	australis, bratislava, lora
Pomona	pomona
Javanica	javanica
Sejroe	sejroe, saxkoebing, hardjo
Panama	panama, mangus
Cynopteri	cynopteri
Djasiman	djasiman
Sarmin	sarmin
Mini	mini, georgia
Tarassovi	tarassovi
Ballum	ballum, aroborea
Celledoni	celledoni
Louisiana	louisiana, lanka
Ranarum	ranarum
Manhao	manhao
Shermani	shermani
Hurstbridge	hurstbridge

BACTERIOLOGY

Taxonomy and Classification

Serological classification. Prior to 1989, the genus *Leptospira* was divided into two species, *L. interrogans*, comprising all pathogenic strains, and *L. biflexa*, containing the saprophytic strains isolated from the environment (217, 309). *L. biflexa* was differentiated from *L. interrogans* by the growth of the former at 13°C and growth in the presence of 8-azaguanine (225 μg/ml) and by the failure of *L. biflexa* to form spherical cells in 1 M NaCl.

Both *L. interrogans* and *L. biflexa* are divided into numerous serovars defined by agglutination after cross-absorption with homologous antigen (162, 309, 330). If more than 10% of the homologous titer remains in at least one of the two antisera on repeated testing, two strains are said to belong to different serovars (297). Over 60 serovars of *L. biflexa* have been recorded (309). Within the species *L. interrogans* over 200 serovars are recognized; additional serovars have been isolated but have yet to be validly published. Serovars that are antigenically related have traditionally been grouped into serogroups (330). While serogroups have no taxonomic standing, they have proved useful for epidemiological understanding. The serogroups of *L. interrogans* and some common serovars are shown in Table 1.

Genotypic classification. The phenotypic classification of leptospires has been replaced by a genotypic one, in which a number of genomospecies include all serovars of both *L. interrogans* and *L. biflexa*. Genetic heterogeneity was demonstrated some time ago (80, 260), and DNA hybridization stud-

TABLE 2. Genomospecies of *Leptospira* and distribution of serogroups[a]

Species	Serogroups[b]
L. interrogans	Icterohaemorrhagiae, Canicola, Pomona, Australis, Autumnalis, Pyrogenes, Grippotyphosa, Djasiman, Hebdomadis, Sejroe, Bataviae, Ranarum, Louisiana, Mini, Sarmin
L. noguchii	Panama, Autumnalis, Pyrogenes, Louisiana, Bataviae, Tarassovi, Australis, Shermani, Djasiman, Pomona
L. santarosai	Shermani, Hebdomadis, Tarassovi, Pyrogenes, Autumnalis, Bataviae, Mini, Grippotyphosa, Sejroe, Pomona, Javanica, Sarmin, Cynopteri
L. meyeri	Ranarum, Semaranga, Sejroe, Mini, Javanica
L. wolbachii[c]	Codice
L. biflexa[c]	Semaranga, Andamana
L. fainei	Hurstbridge
L. borgpetersenii	Javanica, Ballum, Hebdomadis, Sejroe, Tarassovi, Mini, Celledoni, Pyrogenes, Bataviae, Australis, Autumnalis
L. kirschneri	Grippotyphosa, Autumnalis, Cynopteri, Hebdomadis, Australis, Pomona, Djasiman, Canicola, Icterohaemorrhagiae, Bataviae,
L. weilii	Celledoni, Icterohaemorrhagiae, Sarmin, Javanica, Mini, Tarassovi, Hebdomadis, Pyrogenes, Manhao, Sejroe
L. inadai	Lyme, Shermani, Icterohaemorrhagiae, Tarassovi, Manhao, Canicola, Panama, Javanica
L. parva[c]	Turneria
L. alexanderi	Manhao, Hebdomadis, Javanica, Mini

[a] Based on data reported by Brenner et al. (81) and Perolat et al. (450)
[b] Serogroups Semaranga, Andamana, Codice, and Turneria contain nonpathogenic leptospires.
[c] Currently only nonpathogenic strains of these species are known.

TABLE 3. Genomospecies associated with serogroups[a]

Serogroup	Genomospecies
Andamana	*L. biflexa*
Australis	*L. interrogans, L. noguchii, L. borgpetersenii, L. kirschneri*
Autumnalis	*L. interrogans, L. noguchii, L. santarosai, L. borgpetersenii, L. kirschneri*
Ballum	*L. borgpetersenii*
Bataviae	*L. interrogans, L. noguchii, L. santarosai, L. borgpetersenii, L. kirschneri*
Canicola	*L. interrogans, L. inadai, L. kirschneri*
Celledoni	*L. weilii, L. borgpetersenii*
Codice	*L. wolbachii*
Cynopteri	*L. santarosai, L. kirschneri*
Djasiman	*L. interrogans, L. noguchii, L. kirschneri*
Grippotyphosa	*L. interrogans, L. santarosai, L. kirschneri*
Hebdomadis	*L. interrogans, L. weilii, L. santarosai, L. borgpetersenii, L. kirschneri, L. alexanderi*
Hurstbridge	*L. fainei*
Icterohaemorrhagiae	*L. interrogans, L. weilii, L. inadai, L. kirschneri*
Javanica	*L. weilii, L. santarosai, L. borgpetersenii, L. meyeri, L. inadai, L. alexanderi*
Louisiana	*L. interrogans, L. noguchii*
Lyme	*L. inadai*
Manhao	*L. weilii, L. inadai, L. alexanderi*
Mini	*L. interrogans, L. weilii, L. santarosai, L. borgpetersenii, L. meyeri, L. alexanderi*
Panama	*L. noguchii, L. inadai*
Pomona	*L. interrogans, L. noguchii, L. santarosai, L. kirschneri*
Pyrogenes	*L. interrogans, L. noguchii, L. weilii, L. santarosai, L. borgpetersenii*
Ranarum	*L. interrogans, L. meyeri*
Sarmin	*L. interrogans, L. weilii, L. santarosai*
Sejroe	*L. interrogans, L. weilii, L. santarosai, L. borgpetersenii, L. meyeri*
Semaranga	*L. meyeri, L. biflexa*
Shermani	*L. noguchii, L. santarosai, L. inadai*
Tarassovi	*L. noguchii, L. weilli, L. santarosai, L. borgpetersenii, L. inadai*

[a] Based on data reported by Brenner et al. (81) and Perolat et al. (450).

ies led to the definition of 10 genomospecies of *Leptospira* (658). An additional genomospecies, *L. kirschneri*, was added later (475). After an extensive study of several hundred strains, workers at the Centers for Disease Control (CDC) more recently defined 16 genomospecies of *Leptospira* that included those described previously (475, 658) and adding five new genomospecies (81), one of which was named *L. alexanderi*. An additional species, *L. fainei*, has since been described, which contains a new serovar, hurstbridge (450). DNA hybridization studies have also confirmed the taxonomic status of the monospecific genus *Leptonema* (81, 474). The genotypic classification of leptospires is supported by multilocus enzyme electrophoresis data (348), but recent studies suggest that further taxonomic revisions are likely (348, 462).

The genomospecies of *Leptospira* do not correspond to the previous two species (*L. interrogans* and *L. biflexa*), and indeed, pathogenic and nonpathogenic serovars occur within the same species (Table 2). Thus, neither serogroup nor serovar reliably predicts the species of *Leptospira* (Table 3). Moreover, recent studies (81, 222) have included multiple strains of some serovars and demonstrated genetic heterogeneity within serovars (Table 4). In addition, the phenotypic characteristics formerly used to differentiate *L. interrogans* sensu lato from *L. biflexa* sensu lato do not differentiate the genomospecies (81, 658).

The reclassification of leptospires on genotypic grounds is taxonomically correct and provides a strong foundation for

TABLE 4. Leptospiral serovars found in multiple species[a]

Serovar	Species
bataviae	*L. interrogans, L. santarosai*
bulgarica	*L. interrogans, L. kirschneri*
grippotyphosa	*L. kirschneri, L. interrogans*
hardjo	*L. borgpetersenii, L. interrogans, L.meyeri*
icterohaemorrhagiae	*L. interrogans, L. inadai*
kremastos	*L. interrogans, L. santarosai*
mwogolo	*L. kirschneri, L. interrogans*
paidjan	*L. kirschneri, L. interrogans*
pomona	*L. interrogans, L. noguchii*
pyrogenes	*L. interrogans, L. santarosai*
szwajizak	*L. interrogans, L. santarosai*
valbuzzi	*L. interrogans, L. kirschneri*

[a] Based on data reported by Brenner et al. (81) and by Feresu et al. (223).

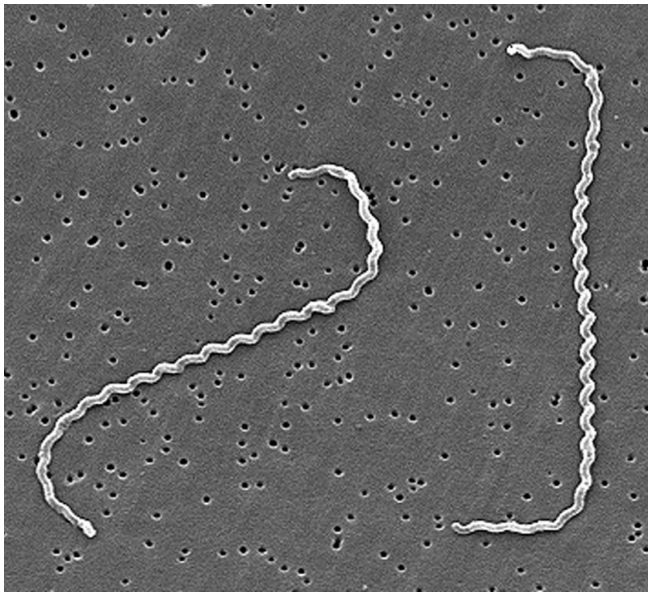

FIG. 1. Scanning electron micrograph of *L. interrogans* serovar icterohaemorrhagiae strain RGA bound to a 0.2-μm membrane filter. Reproduced from reference 625a with permission from the publisher.

future classifications. However, the molecular classification is problematic for the clinical microbiologist, because it is clearly incompatible with the system of serogroups which has served clinicians and epidemiologists well for many years. Until simpler DNA-based identification methods are developed and validated, it will be necessary for clinical laboratories to retain the serological classification of pathogenic leptospires for the foreseeable future. In addition, the retention of *L. interrogans* and *L. biflexa* as specific names in the genomic classification also allows nomenclatural confusion. In the following pages, specific names refer to the genomospecies, including *L. interrogans* sensu stricto and *L. biflexa* sensu stricto.

Biology of Leptospires

Leptospires are tightly coiled spirochetes, usually 0.1 μm by 6 to 0.1 by 20 μm, but occasional cultures may contain much longer cells. The helical amplitude is approximately 0.1 to 0.15 μm, and the wavelength is approximately 0.5 μm (213). The cells have pointed ends, either or both of which are usually bent into a distinctive hook (Fig. 1). Two axial filaments (periplasmic flagella) with polar insertions are located in the periplasmic space (550). The structure of the flagellar proteins is complex (583). Leptospires exhibit two distinct forms of movement, translational and nontranslational (60). Morphologically all leptospires are indistinguishable, but the morphology of individual isolates varies with subculture in vitro and can be restored by passage in hamsters (186). Leptospires have a typical double membrane structure in common with other spirochetes, in which the cytoplasmic membrane and peptidoglycan cell wall are closely associated and are overlain by an outer membrane (254). Leptospiral lipopolysaccharide has a composition similar to that of other gram-negative bacteria (603), but has lower endotoxic activity (519). Leptospires may be stained using carbol fuchsin counterstain (211).

Leptospires are obligate aerobes with an optimum growth temperature of 28 to 30°C. They produce both catalase and oxidase (530). They grow in simple media enriched with vitamins (vitamins B_2 and B_{12} are growth factors), long-chain fatty acids, and ammonium salts (309). Long-chain fatty acids are utilized as the sole carbon source and are metabolized by β-oxidation (530).

Culture Methods

Growth of leptospires in media containing either serum or albumin plus polysorbate and in protein-free synthetic media has been described (587). Several liquid media containing rabbit serum were described by Fletcher, Korthoff, Noguchi, and Stuart (587); recipes for these earlier media are found in several monographs (24, 213, 548, 634). The most widely used medium in current practice is based on the oleic acid-albumin medium EMJH (184, 310). This medium is available commercially from several manufacturers and contains Tween 80 and bovine serum albumin. Some strains are more fastidious and require the addition of either pyruvate (312) or rabbit serum (196) for initial isolation. Growth of contaminants from clinical specimens can be inhibited by the addition of 5-fluorouracil (311). Other antibiotics have been added to media for culture of veterinary specimens, in which contamination is more likely to occur (8, 413). Protein-free media have been developed for use in vaccine production (64, 504, 518, 541).

Growth of leptospires is often slow on primary isolation, and cultures are retained for up to 13 weeks before being discarded, but pure subcultures in liquid media usually grow within 10 to 14 days. Agar may be added at low concentrations (0.1 to 0.2%). In semisolid media, growth reaches a maximum density in a discrete zone beneath the surface of the medium, which becomes increasingly turbid as incubation proceeds. This growth is related to the optimum oxygen tension (213) and is known as a Dinger's ring or disk (164). Leptospiral cultures may be maintained by repeated subculture (608) or preferably by storage in semisolid agar containing hemoglobin (213). Long-term storage by lyophilization (31) or at −70°C (20, 432) is also used.

Growth on media solidified with agar has been reported (494, 587). Colonial morphology is dependent on agar concentration and serovar (582). Media can also be solidified using gellan gum (496). Solid media have been used for isolation of leptospires (572), to separate mixed cultures of leptospires, and for detection of hemolysin production (539).

Molecular Biology

Leptospires are phylogenetically related to other spirochetes (446). The leptospiral genome is approximately 5,000 kb in size (52, 669), although smaller estimates have been reported (558, 649). The genome is comprised of two sections, a 4,400-kb chromosome and a smaller 350-kb chromosome (669). Other plasmids have not been reported (125, 292). Physical maps have been constructed from serovars pomona subtype kennewicki (669) and icterohaemorrhagiae (74, 552). Leptospires contain two sets of 16S and 23S rRNA genes but only one 5S rRNA gene (230), and the rRNA genes are widely spaced (51, 231).

The study of leptospiral genetics has been slowed by the lack

of a transformation system (317, 677). Recently, a shuttle vector was developed using the temperate bacteriophage LE1 from *L. biflexa* (498). This advance offers the prospect of more rapid progress in the understanding of *Leptospira* at the molecular level.

Several repetitive elements have been identified (73, 317, 553, 641, 673), of which several are insertion sequences (IS) coding for transposases. IS*1533* has a single open reading frame (668), while IS*1500* has four (73). Both IS*1500* and IS*1533* are found in many serovars (73, 672), but the copy number varies widely between different serovars and among isolates of the same serovar (74). A role for these insertion sequences in transposition and genomic rearrangements has been identified (73, 74, 668, 677). Other evidence for horizontal transfer within the genus *Leptospira* has been reported (468).

A number of leptospiral genes have been cloned and analyzed, including several for amino acid synthesis (163, 486, 674), rRNA (228, 229), ribosomal proteins (676), RNA polymerase (483), DNA repair (540), heat shock proteins (47, 441), sphingomyelinase (508, 509), hemolysins (154, 343), outer membrane proteins (168, 255, 256, 515), flagellar proteins (354, 355, 398, 584, 640), and lipopolysaccharide (LPS) synthesis (88, 152, 317, 397).

Within serovar icterohaemorrhagiae, the genome appears to be conserved (281, 552). This conservation allowed the identification of at least one new serovar by recognition of distinct pulsed-field gel electrophoresis (PFGE) profiles (280). However, the recent demonstration of heterogeneity within serovars (81, 222) indicates the need for further study of multiple isolates of individual serovars.

EPIDEMIOLOGY

Leptospirosis is presumed to be the most widespread zoonosis in the world (646). The source of infection in humans is usually either direct or indirect contact with the urine of an infected animal. The incidence is significantly higher in warm-climate countries than in temperate regions (208, 479); this is due mainly to longer survival of leptospires in the environment in warm, humid conditions. However, most tropical countries are also developing countries, and there are greater opportunities for exposure of the human population to infected animals, whether livestock, domestic pets, or wild or feral animals. The disease is seasonal, with peak incidence occurring in summer or fall in temperate regions, where temperature is the limiting factor in survival of leptospires, and during rainy seasons in warm-climate regions, where rapid dessication would otherwise prevent survival.

The reported incidence of leptospirosis reflects the availability of laboratory diagnosis and the clinical index of suspicion as much as the incidence of the disease. Within the United States, the highest incidence is found in Hawaii (101). Leptospirosis ceased to be a notifiable infection within the United States after December 1994 (97).

The usual portal of entry is through abrasions or cuts in the skin or via the conjunctiva; infection may take place via intact skin after prolonged immersion in water, but this usually occurs when abrasions are likely to occur and is thus difficult to substantiate. Water-borne transmission has been documented; point contamination of water supplies has resulted in several outbreaks of leptospirosis (Table 5). Inhalation of water or aerosols also may result in infection via the mucous membranes of the respiratory tract. Rarely, infection may follow animal bites (55, 158, 244, 360, 525). Direct transmission between humans has been demonstrated rarely (see Other Complications, below). However, excretion of leptospires in human urine months after recovery has been recorded (46, 307). It is thought that the low pH of human urine limits survival of leptospires after excretion. Transmission by sexual intercourse during convalescence has been reported (167, 262).

Animals, including humans, can be divided into maintenance hosts and accidental (incidental) hosts. The disease is maintained in nature by chronic infection of the renal tubules of maintenance hosts (43). A maintenance host is defined as a species in which infection is endemic and is usually transferred from animal to animal by direct contact. Infection is usually acquired at an early age, and the prevalence of chronic excretion in the urine increases with the age of the animal. Other animals (such as humans) may become infected by indirect contact with the maintenance host. Animals may be maintenance hosts of some serovars but incidental hosts of others, infection with which may cause severe or fatal disease. The most important maintenance hosts are small mammals, which may transfer infection to domestic farm animals, dogs, and humans. The extent to which infection is transmitted depends on many factors, including climate, population density, and the degree of contact between maintenance and accidental hosts. Different rodent species may be reservoirs of distinct serovars, but rats are generally maintenance hosts for serovars of the serogroups lcterohaemorrhagiae and Ballum, and mice are the maintenance hosts for serogroup Ballum. Domestic animals are also maintenance hosts; dairy cattle may harbor serovars hardjo, pomona, and grippotyphosa; pigs may harbor pomona, tarassovi, or bratislava; sheep may harbor hardjo and pomona; and dogs may harbor canicola (69). Distinct variations in maintenance hosts and the serovars they carry occur throughout the world (266). A knowledge of the prevalent serovars and their maintenance hosts is essential to understanding the epidemiology of the disease in any region.

Human infections may be acquired through occupational, recreational, or avocational exposures. Occupation is a significant risk factor for humans (609). Direct contact with infected animals accounts for most infections in farmers, veterinarians, abattoir workers (95, 104, 570), meat inspectors (65), rodent control workers (155), and other occupations which require contact with animals (27, 357). Indirect contact is important for sewer workers, miners, soldiers (87, 314, 361), septic tank cleaners, fish farmers (241, 489), gamekeepers, canal workers (29), rice field workers (219, 430, 615), taro farmers (25), banana farmers (535), and sugar cane cutters (132).

Miners were the first occupational risk group to be recognized (86, 296). The occurrence of Weil's disease in sewer workers was first reported in the 1930s (23, 218, 308, 545). Serovar icterohaemorrhagiae was isolated by guinea pig inoculation from patients, from rats trapped in sewers (23, 308), and from the slime lining the sewers (23). In Glasgow, Scotland, a seroprevalence among sewer workers of 17% was reported (545). The recognition of this important risk activity led to the adoption of rodent control programs and the use of

TABLE 5. Documented outbreaks of leptospirosis associated with water

Place and yr	No. of cases	Exposure	Source of infection	Presumptive serogroup	Reference
Lisbon, Portugal, 1931	126	Drinking from water fountain	Contamination by rat urine	Unknown	315
Greece, 1931	31	Drinking water in a cafe	Contamination by rat urine	Unknown	457
Philadelphia, 1939	7	Swimming in a creek	Contamination by rat urine	Icterohaemorrhagiae; serovar icterohaemorrhagiae isolated from one case	272
Georgia, 1940	35	Swimming in a creek	Contamination by offal and a dead cow	Unknown	75
Wyoming, 1942	24	Swimming in a pool	Unknown	Canicola	120
Okinawa, 1949	16	Swimming in a pond	Unknown	Autumnalis	236
Alabama, 1950	50	Swimming in a creek	Suspected to be pigs	Pomona	503
Georgia, 1952	26	Swimming in a creek	Suspected to be dogs	Canicola	628
Russia, 1952	Not stated	Swimming in a lake	Suspected to be pigs and/or rats	Canicola	597
Japan, 1953	114	Swimming in a river	Suspected to be dogs	Canicola; serovar canicola isolated	396
Russia, 1954	62	Drinking and bathing in well water	Contamination by pigs	Serovar pomona isolated	68
South Dakota, 1956	3	Swimming in a river	Unknown	Pomona	304
Florida, 1958	9	Swimming in a stream	Contamination by cattle and/or pigs	Serovar pomona isolated from pigs	121
Iowa, 1959	40	Swimming in a stream	Contamination by cattle	Serovar pomona isolated from two cases and from cattle	79
Washington, 1964	61	Swimming in a canal	Suspected to be cattle	Pomona; serovar pomona isolated from cattle	414
Tennessee, 1975	7	Swimming in a creek	Unknown	Grippotyphosa	26
Buenos Aires, Argentina, 1976	10	Swimming in a drainage canal	Suspected to be pigs	Pomona; Pomona serogroup isolated from patients	93
Italy, 1984	35	Drinking from water fountain	Dead hedgehog in header tank	Australis	92
Missouri, 1985	4	Kayaking in creek during flooding	Unknown	Djasiman	306
Morón, Cuba, 1986	6	Swimming in a canal	Suspected to be dogs	Canicola	277
Okinawa, Japan, 1987	22	Swimming in a pool or jungle training	Unknown	Shermani	130
Kauai, Hawaii, 1987	8	Swimming in a river	Suspected to be cattle	Australis; serovars bangkok and bataviae isolated	320
São Paulo, Brazil, 1987	23	Swimming in a pool with river water	Unknown	Pomona	153
Illinois, 1991	5	Swimming in a pond	Unknown; several animal species seropositive	Grippotyphosa; serovar grippotyphosa isolated from patients and water	302
Kauai, Hawaii, 1992	8	Swimming in a waterfall	Unknown	Australis; serovar bangkok isolated	321
Costa Rica, 1996	9	White water rafting	Unknown	Unknown	482
Barbados, 1997	2	Swimming in a pond	Unknown	Serovar bim isolated from one case	542a
Illinois & Wisconsin, 1998	74	Swimming in a lake	Unknown	Unknown	98

protective clothing, resulting in a significant reduction in cases associated with this occupation. The presence in wastewater of detergents is also thought to have reduced the survival of leptospires in sewers (610), since leptospires are inhibited at low detergent concentrations (106).

Fish workers were another occupational group whose risk of contracting leptospirosis was recognized early. Between 1934 and 1948, 86% of all cases in the northeast of Scotland occurred in fish workers in Aberdeen (532). Recognition of risk factors and adoption of both preventive measures and rodent control have reduced the incidence of these occupational infections greatly. From 1933 to 1948 in the British Isles, there were 139 cases in coal miners, 79 in sewer workers, and 216 in fish workers. However, in the period from 1978 to 1983, there were nine cases in these three occupations combined (610). More recently, fish farmers have been shown to be at risk (489), particularly for infection with serovars of serogroup Icterohaemorrhagiae (241), presumed to be derived from rat infestation of premises. Because of the high mortality rate associated with Icterohaemorrhagiae infections, this was considered an important occupational risk group despite the very small absolute number of workers affected (240).

Livestock farming is a major occupational risk factor throughout the world. The highest risk is associated with dairy farming and is associated with serovar hardjo (66, 458, 500, 609), in particular with milking of dairy cattle (263, 352, 528). Human cases can be associated with clinical disease in cattle (263, 500), but are not invariably so (30, 138). Cattle are main-

TABLE 6. Documented outbreaks of leptospirosis associated with dogs

Place and yr	No. of cases	Source of infection	Presumptive serogroup	Infecting serovar	Reference
North Dakota, 1950	9	Infected family pet dog	Canicola	Not isolated	271
Texas, 1971	7	Infected pet dogs	Canicola	canicola	54
Portland, Oreg., 1972	9	Infected family pet dog	Autumnalis	fortbragg	225
St. Louis, Mo., 1972	5	Infected pet dogs, previously immunized	Icterohaemorrhagiae	icterohaemorrhagiae	221
Barbados, 1988	1	Infected guard dogs in kennels, immunized	Autumnalis	bim	206

tenance hosts of serovar hardjo (192), and infection with this serovar occurs throughout the world (45, 412, 466). Many animals are seronegative carriers (192, 267, 571). After infection, leptospires localize in the kidneys (249, 427, 465, 571, 626) and are excreted intermittently in the urine (189). Serovar hardjo causes outbreaks of mastitis (196) and abortion (190). Serovar hardjo is found in aborted fetuses and in premature calves (188, 194, 238, 268). In addition, hardjo has been isolated from normal fetuses (191), the genital tracts of pregnant cattle (191), vaginal discharge after calving (193), and the genital tract and urinary tract of >50% of cows (197, 198) and bulls (185). In Australia, both serovars hardjo and pomona were demonstrated in bovine abortions, but serological evidence suggested that the incidence of hardjo infection was much higher (182, 305, 529). In Scotland, 42% of cattle were seropositive for hardjo, representing 85% of all seropositive animals (187). In the United States, serovar hardjo is the most commonly isolated serovar in cattle (198), but pomona also occurs.

There is a significant risk associated with recreational exposures occurring in water sports (405), including swimming, canoeing (306, 517), white water rafting (482, 591, 627), fresh water fishing, and other sports where exposure is common, such as potholing and caving (611). The potential for exposure of large numbers of individuals occurs during competitive events (98, 99, 102, 126, 204). Several outbreaks of leptospirosis associated with water have been reported (Table 5). Many of these outbreaks have followed extended periods of hot, dry weather, when pathogenic leptospires presumably have multiplied in freshwater ponds or rivers. Cases of leptospirosis also follow extensive flooding (111, 153, 201, 226, 232, 425, 436, 442, 526, 590, 645).

Pathogenic serovars have been isolated from water in tropical regions (19) and in the United States, where serovars icterohaemorrhagiae, dakota, ballum, pomona, and grippotyphosa have been recovered (137, 161, 242). Survival of pathogenic leptospires in the environment is dependent on several factors, including pH, temperature, and the presence of inhibitory compounds. Most studies have used single serovars and quite different methodologies, but some broad conclusions may be drawn. Under laboratory conditions, leptospires in water at room temperature remain viable for several months at pH 7.2 to 8.0 (106, 246), but in river water survival is shorter and is prolonged at lower temperatures (106, 137). The presence of domestic sewage decreases the survival time to a matter of hours (106), but in an oxidation ditch filled with cattle slurry, viable leptospires were detected for several weeks (160). In acidic soil (pH 6.2) taken from canefields in Australia, serovar australis survived for up to 7 weeks, and in rainwater-flooded soil it survived for at least 3 weeks (531). When soil was contaminated with urine from infected rats or voles, leptospires survived for approximately 2 weeks (319, 531). In slightly different soil, serovar pomona survived for up to 7 weeks under conditions approximating the New Zealand winter (274).

Many sporadic cases of leptospirosis in tropical regions are acquired following avocational exposures that occur during the activities of daily life (205, 454). Many infections result from barefooted walking in damp conditions or gardening with bare hands (170). Dogs are a significant reservoir for human infection in many tropical countries (623) and may be an important source of outbreaks (Table 6). A number of outbreaks of leptospirosis have resulted from contamination of drinking water (Table 5) and from handling rodents (14).

Three epidemiological patterns of leptospirosis were defined by Faine (211). The first occurs in temperate climates where few serovars are involved and human infection almost invariably occurs by direct contact with infected animals though farming of cattle and pigs. Control by immunization of animals and/or humans is potentially possible. The second occurs in tropical wet areas, within which there are many more serovars infecting humans and animals and larger numbers of reservoir species, including rodents, farm animals, and dogs. Human exposure is not limited by occupation but results more often from the widespread environmental contamination, particularly during the rainy season. Control of rodent populations, drainage of wet areas, and occupational hygiene are all necessary for prevention of human leptospirosis. These are also the areas where large outbreaks of leptospirosis are most likely to occur following floods, hurricanes, or other disasters (111, 158, 201, 226, 232, 425, 436, 442, 526, 590). The third pattern comprises rodent-borne infection in the urban environment. While this is of lesser significance throughout most of the world, it is potentially more important when the urban infrastructure is disrupted by war or by natural disasters. This type of infection is now rarely seen in developed countries (157), but is exemplified by the recent rediscovery of urban leptospirosis in Baltimore (601) and by outbreaks occurring in slum areas in developing countries (332).

CLINICAL FEATURES OF LEPTOSPIROSIS

Leptospirosis has been described as a zoonosis of protean manifestations (456, 644). Indeed, this description has been so overused as to have become a cliché. The spectrum of symptoms is extremely broad; the classical syndrome of Weil's dis-

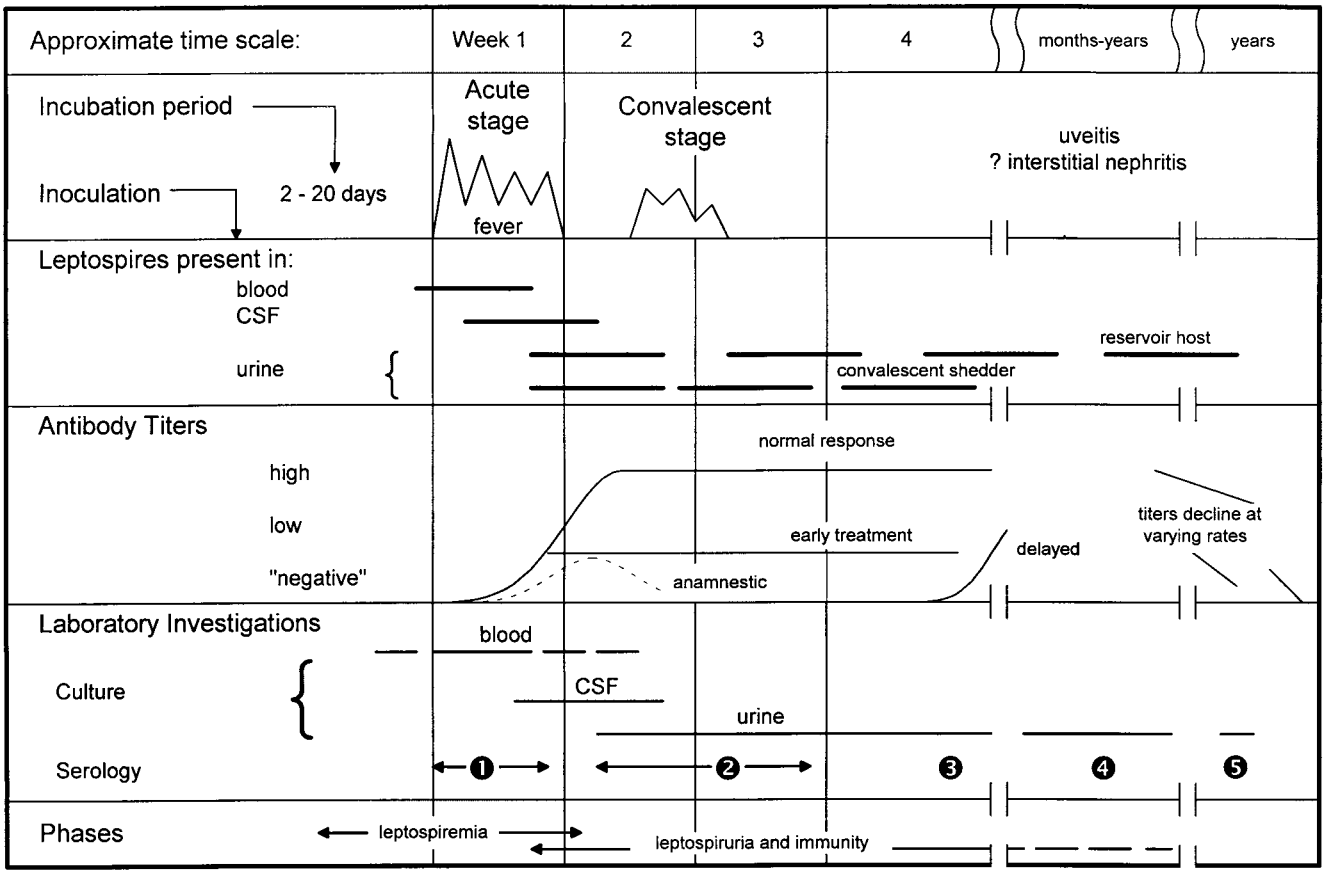

FIG. 2. Biphasic nature of leptospirosis and relevant investigations at different stages of disease. Specimens 1 and 2 for serology are acute-phase specimens, 3 is a convalescent-phase sample which may facilitate detection of a delayed immune response, and 4 and 5 are follow-up samples which can provide epidemiological information, such as the presumptive infecting serogroup. (Adapted from reference 586a with permission of the publisher.)

ease represents only the most severe presentation. Formerly it was considered that distinct clinical syndromes were associated with specific serogroups (596). However, this view was questioned by some authorities (18, 180, 220), and more intense study over the past 30 years has refuted this hypothesis. An explanation for many of the observed associations may be found in the ecology of the maintenance animal hosts in a geographic region. A region with a richly varied fauna will support a greater variety of serogroups than will a region with few animal hosts. In humans, severe leptospirosis is frequently but not invariably caused by serovars of the icterohaemorrhagiae serogroup. The specific serovars involved depend largely on the geographic location and the ecology of local maintenance hosts. Thus in Europe, serovars copenhageni and icterohaemorrhagiae, carried by rats, are usually responsible for infectious, while in Southeast Asia, serovar lai is common.

The clinical presentation of leptospirosis is biphasic (Fig. 2), with the acute or septicemic phase lasting about a week, followed by the immune phase, characterized by antibody production and excretion of leptospires in the urine (180, 325, 585). Most of the complications of leptospirosis are associated with localization of leptospires within the tissues during the immune phase and thus occur during the second week of the illness.

Anicteric Leptospirosis

The great majority of infections caused by leptospires are either subclinical or of very mild severity, and patients will probably not seek medical attention. A smaller proportion of infections, but the overwhelming majority of the recognized cases, present with a febrile illness of sudden onset. Other symptoms include chills, headache, myalgia, abdominal pain, conjunctival suffusion, and less often a skin rash (Table 7). If present, the rash is often transient, lasting less than 24 h. This anicteric syndrome usually lasts for about a week, and its resolution coincides with the appearance of antibodies. The fever may be biphasic and may recur after a remission of 3 to 4 days. The headache is often severe, resembling that occurring in dengue, with retro-orbital pain and photophobia. Myalgia affecting the lower back, thighs, and calves is often intense (18, 325).

Aseptic meningitis may be found in $\leq$25% of all leptospirosis cases and may account for a significant minority of all causes of aseptic meningitis (57, 236, 503). Patients with asep-

TABLE 7. Signs and symptoms on admission in patients with leptospirosis in large case series

Symptom	% of patients							
	China, 1955 (115), $n = 75$	Puerto Rico, 1963 (18), $n = 208$	China, 1965 (615), $n = 168$	Vietnam, 1973 (61) $n = 150$	Korea, 1987 (442), $n = 93$	Barbados, 1990 (177), $n = 88$	Seychelles, 1998 (660), $n = 75$	Brazil, 1999 (332), $n = 193$
Jaundice	72	49	0	1.5	16	95	27	93
Anorexia	92	—[a]	46	—	80	85	—	—
Headache	88.5	91	90	98	70	76	80	75
Conjunctival suffusion	97	99	57	42	58	54	—	28.5
Vomiting	51	69	18	33	32	50	40	—
Myalgia	100	97	64	79	40	49	63	94
Arthralgia	51	—	36	—	—	—	31	—
Abdominal pain	31	—	26	28	40	43	41	—
Nausea	56	75	29	41	46	37	—	—
Dehydration	—	—	—	—	—	37	—	—
Cough	55	24	57	20	45	32	39	—
Hemoptysis	37	9	51	—	40	—	13	20
Hepatomegaly	83	69	28	15	17	27	—	—
Lymphadenopathy	19	24	49	21	—	21	—	—
Diarrhea	30	27	20	29	36	14	11	—
Rash	0	6	—	7	—	2	—	—

[a] —, symptom not recorded.

tic meningitis have tended to be younger than those with icteric leptospirosis (57, 328, 522). In their series of 616 cases, Alston and Broom (24) noted that 62% of children ≤14 years old presented with aseptic meningitis, whereas only 31% of patients aged 15 to 29 years did so and only 10% of those over 30 years of age. Mortality is almost nil in anicteric leptospirosis (180), but death resulting from massive pulmonary hemorrhage occurred in 2.4% of the anicteric patients in a Chinese outbreak (615).

The differential diagnosis must include common viral infections, such as influenza (18), human immunodeficiency virus seroconversion (290), and, in the tropics, dengue (332, 350, 501), in addition to the bacterial causes of fever of unknown origin, such as typhoid. Turner (585) provided a comprehensive list of other conditions that may be mimicked by leptospirosis, including encephalitis, poliomyelitis, rickettsiosis, glandular fever (infectious mononucleosis), brucellosis, malaria, viral hepatitis, and pneumonitis. Hantavirus infections must also be considered in the differential diagnosis for patients with pulmonary involvement (32). Petechial or purpuric lesions may occur (18, 115), and recently, cases of leptospirosis resembling viral hemorrhagic fevers have been reported in travelers returning from Africa (278, 402).

Icteric Leptospirosis

Icteric leptospirosis is a much more severe disease in which the clinical course is often very rapidly progressive. Severe cases often present late in the course of the disease, and this contributes to the high mortality rate, which ranges between 5 and 15%. Between 5 and 10% of all patients with leptospirosis have the icteric form of the disease (273). The jaundice occurring in leptospirosis is not associated with hepatocellular necrosis, and liver function returns to normal after recovery (476). Serum bilirubin levels may be high, and many weeks may be required for normalization (177). There are moderate rises in transaminase levels, and minor elevation of the alkaline phosphatase level usually occurs.

The complications of severe leptospirosis emphasize the multisystemic nature of the disease. Leptospirosis is a common cause of acute renal failure (ARF), which occurs in 16 to 40% of cases (2, 177, 473, 631). A distinction may be made between patients with prerenal azotemia (non-ARF) and those with ARF. Patients with prerenal azotaemia may respond to rehydration, and decisions regarding dialysis can be delayed for up to 72 h (417). In patients with ARF, oliguria (odds ratio [OR], 9.98) was a significant predictor of death (142).

Serum amylase levels are often raised significantly in association with ARF (18, 175, 422), but clinical symptoms of pancreatitis are not a common finding (174, 401, 439). Necrotizing pancreatitis has been detected at autopsy (175, 544). Thrombocytopenia (platelet count of $<100 \times 10^9$/liter) occurs in ≥50% of cases and is a significant predictor for the development of ARF (176). However, thrombocytopenia in leptospirosis is transient and does not result from disseminated intravascular coagulation (179, 419).

The occurrence of pulmonary symptoms in cases of leptospirosis was first noted by Silverstein (525). Subsequent reports have shown that pulmonary involvement may be the major manifestation of leptospirosis in some clusters of cases (294, 510, 614, 664) and in some sporadic cases (63, 461). The severity of respiratory disease is unrelated to the presence of jaundice (283, 294). Patients may present with a spectrum of symptoms, ranging from cough, dyspnea, and hemoptysis (which may be mild or severe) to adult respiratory distress syndrome (15, 22, 59, 89, 110, 151, 165, 200, 399, 426, 472, 527, 664, 666). Intra-alveolar hemorrhage was detected in the majority of patients, even in the absence of overt pulmonary symptoms (171). Pulmonary hemorrhage may be severe enough to cause death (294, 581, 659, 664).

The incidence of respiratory involvement varies. In a Chinese series of anicteric cases, more than half had respiratory symptoms, while 67% had radiographic changes (614); in a similar Korean series, 67% of patients had respiratory symptoms and 64% had radiographic abnormalities (294), whereas

in a series of jaundiced patients in Brazil, only 17% had clinical evidence of pulmonary involvement, but 33% had radiographic abnormalities (415). In a large Chinese series, moist rales were noted in 17% of cases (115). Rales are more common in icteric than in nonicteric leptospirosis (18). Concurrent hemoptysis and pulmonary infiltrates on chest radiographs were noted in 12% of 69 nonfatal cases in the Seychelles (659). Cigarette smoking was reported as a risk factor for the development of pulmonary symptoms (375).

Radiography generally reveals diffuse small opacities which may be widely disseminated or which may coalesce into larger areas of consolidation, with increasing severity of symptoms (342, 415, 525, 614, 659, 664). Pleural effusions may occur (342, 560). The patchy infiltrates which are commonly seen reflect areas of intra-alveolar and interstitial hemorrhage (294, 419, 472, 614, 664). Both alveolar infiltrates (OR 7.3) and dyspnea (OR 11.7) are poor prognostic indicators in severe leptospirosis (172). Similarly, in icteric leptospirosis in Brazil, respiratory insufficiency (OR 4.6) was associated with death (332).

Cardiac involvement in leptospirosis is common but may be underestimated. Fatal myocarditis was first described in 1935 (400). Clinical evidence of myocardial involvement, including abnormal T waves, was detected in 10% of 80 severe icteric cases in Louisiana (536), while similar electrocardiographic (ECG) abnormalities were detected in over 40% of patients in China, India, Sri Lanka, and the Philippines (353, 467, 471, 618), including both icteric and nonicteric cases. However, in a prospective study in Malaysia, identical ECG changes were found in patients with either leptospirosis or malaria (445), and it was concluded that such ECG changes were nonspecific. Other ECG abnormalities have been reported less frequently (470). The presence of myocarditis was strongly associated with the severity of pulmonary symptoms in anicteric Chinese patients (353). A mortality rate of 54% was reported in severe leptospirosis cases with myocarditis (341). Repolarization abnormalities on ECG were considered a poor prognostic indicator (OR 5.9) in severe leptospirosis cases (172), as were arrhythmias (OR 2.83) in a Brazilian series (332).

Ocular Involvement

Ocular manifestations of severe leptospirosis were noted in early reports (622, 624). Conjunctival suffusion is seen in the majority of patients in some series (377). Conjunctival suffusion in the presence of scleral icterus is said to be pathognomonic of Weil's disease (596). Anterior uveitis, either unilateral or bilateral, occurs after recovery from the acute illness in a minority of cases (53). Uveitis may present weeks, months, or occasionally years after the acute stage. Chronic visual disturbance, persisting 20 years or more after the acute illness, has been reported (521).

The incidence of ocular complications is variable, but this probably reflects the long time scale over which they may occur. In the United States the incidence was estimated at 3% (273), while in Romania an incidence of 2% was estimated between 1979 and 1985 (28). However, in abattoir workers with evidence of recent leptospirosis, the latter authors reported an incidence of 40% (28).

In most cases uveitis is presumed to be an immune phenomenon, but leptospires have been isolated from human and equine eyes (16, 209), and more recently, leptospiral DNA has been demonstrated in aqueous humor by PCR (114, 209, 389). Late-onset uveitis may result from an autoimmune reaction to subsequent exposure (211).

Recently, a large cluster of cases of uveitis was reported from Madurai in southern India following an outbreak of leptospirosis which occurred after heavy flooding (114, 477, 478). The majority of affected patients were males, with a mean age of 35 years (477). Eyes were involved bilaterally in 38 patients (52%), and panuveitis was present in 96% of eyes. Other significant ocular findings included anterior chamber cells, vitreous opacities, and vasculitis in the absence of visual deficit (114).

Other Complications

Acute infection in pregnancy has been reported to cause abortion (116) and fetal death (122, 214), but not invariably so. In one of the cases reported by Chung et al. (116), leptospires were isolated from amniotic fluid, placenta, and cord blood; the infant was mildly ill and was discharged at 2 weeks of age. In another case, a neonate developed jaundice and died 2 days after birth (356). Leptospires were demonstrated in the liver and kidneys by silver staining, but serological evidence of leptospiral infection in the mother was only obtained 2 weeks after delivery. Leptospires have been isolated from human breast milk (116), and in one case serovar hardjo was probably transmitted from an infected mother to her infant by breastfeeding (70).

Rare complications include cerebrovascular accidents (224, 346), rhabdomyolysis (133, 374, 537), thrombotic thrombocytopenic purpura (336), acute acalculous cholecystitis (44, 401, 600), erythema nodosum (157), aortic stenosis (91), Kawasaki syndrome (291, 636), reactive arthritis (633), epididymitis (285), nerve palsy (516, 578), male hypogonadism (437), and Guillain-Barré syndrome (403). Cerebral arteritis, resembling Moyamoya disease, has been reported in a series of patients from China (650).

Chronic or Latent Infection

Anecdotal reports suggest that leptospirosis may induce chronic symptoms analogous to those produced by other spirochetal infections, such as Lyme disease. However, there is very little objective evidence to support or disprove this hypothesis. The possibility of chronic human infection was suggested, without evidence of infection other than serology (420). A single case of late-onset meningitis following icteric leptospirosis has been described (406), in which leptospires were isolated from both cerebrospinal fluid (CSF) and urine. This patient exhibited a negligible antibody response to the infecting strain, suggesting the presence of an immune defect.

Of the sequelae of acute leptospirosis described above, uveitis is a potentially chronic condition and is a recognized chronic sequel of leptospirosis in humans and horses. Equine recurrent uveitis appears to be an autoimmune disease (358, 443), and Faine (211) suggested that late-onset uveitis in humans may result from an autoimmune reaction to subsequent exposure. Immune involvement in retinal pathology has been demonstrated in horses with spontaneous uveitis (318). Leptospires have been isolated from the human eye (16), and more

recently, leptospiral DNA has been amplified from aqueous humor (114, 367, 389) of patients with uveitis. In these cases, uveitis has occurred relatively soon after the acute illness.

One follow-up study of 11 patients with a mean time of 22 years (range, 6 to 34 years) after recovery from acute leptospirosis has been reported (521). Four patients complained of persistent headaches since their acute illness. Two patients complained of visual disturbances; both had evidence of past bilateral anterior uveitis. No biochemical or hematologic abnormalities were detected to suggest continuing liver or renal impairment. No studies to date have attempted to confirm the persistence of leptospires in the tissues of patients who have subsequently died of other causes.

Pathology

Leptospirosis is characterized by the development of vasculitis, endothelial damage, and inflammatory infiltrates composed of moncytic cells, plasma cells, histiocytes, and neutrophils. On gross examination, petechial hemorrhages are common and may be extensive (35), and organs are often discolored due to the degree of icterus (459). The histopathology is most marked in the liver, kidneys, heart, and lungs (665), but other organs may also be affected according to the severity of the individual infection. The overall structure of the liver is not significantly disrupted, but there may be intrahepatic cholestasis (35, 169). Hypertrophy and hyperplasia of Kupffer cells is evident (148), and erythrophagocytosis has been reported (35, 169). In the kidneys, interstitial nephritis is the major finding, accompanied by an intense cellular infiltration composed of neutrophils and moncytes (447). Leptospires can be seen within the renal tubules (35, 447, 665). By electron microscopy, the tubular cell brush borders are denuded, the tubular basement membrane is thickened, and tubular cells exhibit mitochondrial depletion (147). In addition, minor changes are seen in the glomeruli, suggesting an anatomical basis for proteinuria in leptospirosis (147).

Pathological findings in the heart include interstitial myocarditis with infiltration of predominantly lymphocytes and plasma cells, petechial hemorrhages (particularly in the epicardium), mononuclear infiltration in the epicardium, pericardial effusions, and coronary arteritis (34, 146, 149, 202, 341, 472). In the lungs, pulmonary congestion and hemorrhage are common (35, 664), and infiltration of alveolar spaces by monocytes and neutrophils occurs (472). Hyaline membrane formation may occur (472, 666). Leptospires may be seen within endothelial cells in interalveolar septa, and attached to capillary endothelial cells (419).

In skeletal muscles, particularly of the leg, focal necrosis of isolated muscle fibers occurs, with infiltration of histiocytes, neutrophils, and plasma cells (169, 589). This evidence of myositis correlates with the intense myalgia reported by some patients (325). In brain, perivascular cuffing is observed (35, 665).

Treatment

Treatment of leptospirosis differs depending on the severity and duration of symptoms at the time of presentation. Patients with mild, flu-like symptoms require only symptomatic treatment but should cautioned to seek further medical help if they develop jaundice. Patients who present with more severe anicteric leptospirosis will require hospital admission and close observation. If the headache is particularly severe, a lumbar puncture usually produces a dramatic improvement.

The management of icteric leptospirosis requires admission of the patient to the intensive care unit initially. Patients with prerenal azotemia can be rehydrated initially while their renal function is observed, but patients in acute renal failure require dialysis as a matter of urgency. This is accomplished satisfactorily by peritoneal dialysis (250, 408, 556). Cardiac monitoring is also desirable during the first few days after admission (172).

Specific antibiotic treatment was reported soon after penicillin became available, with mixed results (42). Oxytetracycline was also used (497). Early experience was summarized by Alston and Broom in their monograph (24). Few well-designed and well-controlled studies of antibiotic treatment have been reported (252). A major difficulty in assessing the efficacy of antibiotic treatment results from the late presentation of many patients with severe disease, after the leptospires have localized in the tissues.

Doxycycline (100 mg twice a day for 7 days) was shown to reduce the duration and severity of illness in anicteric leptospirosis by an average of 2 days (382). Patients with severe disease were excluded from this study. Two randomized studies of penicillin produced conflicting results. One study included 42 patients with severe leptospirosis, of whom 19 were jaundiced (619); no patient required dialysis and there were no deaths. Intravenous penicillin was given at a dosage of 6 MU/day for 7 days and found to halve the duration of fever. A second study included 79 patients with icteric leptospirosis, of whom 4 died (178). Patients in the treatment group received intravenous penicillin at a dose of 8 MU/day for 5 days. No difference was observed between treatment and control groups in outcome or duration of the illness. There have been no controlled trials of penicillin versus doxycycline for treatment of leptospirosis.

A consistent finding of these studies has been the prevention of leptospiruria or a significant reduction in its duration (178, 382, 619). This finding alone is sufficient justification for antibiotic use, but any antibiotic treatment should be started as early as possible and should not replace other therapeutic measures. Jarisch-Herxheimer reactions have been reported after penicillin administration (200, 227, 598, 615). However, the apparently low risk should not preclude the use of penicillin (620).

Doxycycline (200 mg orally, once weekly) has been shown to be effective for short-term prophylaxis in high-risk environments (245, 511, 551). Similar findings have been reported in rhesus monkeys challenged experimentally (199). In a recent controlled trial, doxycycline significantly reduced the incidence of clinical disease but not serological evidence of infection (511). Anecdotal evidence suggests that doxycycline but not penicillin may be used successfully after exposure in laboratory accidents (239). An evidence-based review of antibiotic prophylaxis has been published (251).

Immunization

Immunity to leptospirosis is largely humoral (7) and is relatively serovar specific. Thus, immunization protects against

disease caused by the homologous serovar or antigenically similar serovars only. Vaccines must therefore contain serovars representative of those present in the population to be immunized. Immunization has been widely used for many years as a means of inducing immunity in animals and humans, with limited success. Early vaccines were composed of suspensions of killed leptospires cultured in serum-containing medium, and side effects were common. Modern vaccines prepared using protein-free medium are generally without such adverse effects (64, 113). In developed countries, pigs and cattle are widely immunized, as are domestic dogs, but in most developing countries, vaccines which contain the locally relevant serovars are not available. Most vaccines require booster doses at yearly intervals.

Most bovine and porcine vaccines contain serovars hardjo and pomona; in North America, commercial vaccines also contain serovars canicola, grippotyphosa, and icterohaemorrhagiae. Protection against hardjo infection has been suboptimal, but one vaccine has recently been shown to offer good protection (C. A. Bolin, D. P. Alt, and R. L. Zuerner, Abstr. 2nd Int. Leptospirosis Soc. Meet., 1999. abstr. 18) and induces a cell-mediated immune response.

Canine vaccines generally contain serovars canicola and icterohaemorrhagiae. Vaccines protect against disease and renal shedding under experimental conditions (82), but transmission of serovar icterohaemorrhagiae from immunized dogs to humans has been reported (221). Moreover, immunized dogs may be infected with serovars other than those contained in commercial vaccines (83, 123, 206, 261, 464). A vaccine has been released recently which includes serovars grippotyphosa and pomona in addition to the traditional vaccine strains, in response to the increasing incidence of canine infection with these serovars.

Human vaccines have not been applied widely in Western countries. Immunization with polyvalent vaccines has been practiced in the Far East, where large numbers of cases occur in ricefield workers, such as in China (111) and Japan. In France, a monovalent vaccine containing only serovar icterohaemorrhagiae is licensed for human use. A vaccine containing serovars canicola, icterohaemorrhagiae, and pomona has been developed recently in Cuba (376).

PATHOGENESIS

The mechanisms by which leptospires cause disease are not well understood. A number of putative virulence factors have been suggested, but with few exceptions their role in pathogenesis remains unclear. These are reviewed briefly below, with an emphasis on recent developments.

Toxin Production

The production of toxins by pathogenic leptospires in vivo was inferred by Areán (35, 36). Endotoxic activity has been reported in several serovars (159, 300, 379, 421). Leptospiral LPS preparations exhibit activity in biological assays for endotoxin, but at much lower potencies (159, 300, 379).

Serovar pomona is notable for the production of hemolytic disease in cattle, while serovar ballum produces similar symptoms in hamsters. Hemolysins from several serovars have been characterized. The hemolysins of serovars ballum, hardjo, pomona, and tarassovi are sphingomyelinases (62, 154). Virulent strains exhibit chemotaxis towards hemoglobin (663). Plasma has been shown to prevent hemolysis (576). Phospholipase C activity has been reported in serovar canicola (655). A hemolysin from serovar lai is not associated with sphingomyelinase or phospholipase activity and is thought to be a pore-forming protein (343).

Strains of serovars pomona and copenhageni elaborate a protein cytotoxin (119, 394, 651), and cytotoxic activity has been detected in the plasma of infected animals (331). In vivo, this toxin elicited a typical histopathologic effect, with infiltration of macrophages and polymorphonuclear cells (651). A glycolipoprotein fraction with cytotoxic activity was recovered from serovar copenhageni (602). A similar fraction from serovar canicola inhibits Na^+,K^+ ATPase (662). Inhibitory activity was associated with unsaturated fatty acids, particularly palmitic and oleic acids (90). However, equal activity was demonstrated in *L. biflexa* serovar patoc (90), implying that other virulence factors might be of greater significance.

Attachment

Leptospires have been shown to attach to epithelial cells. Virulent leptospires adhere to renal epithelial cells in vitro, and adhesion is enhanced by subagglutinating concentrations of homologous antibody (48). Leptospires are phagocytosed by macrophages (118, 448) in the presence of specific antibody (49, 604). Inhibition of macrophage activity increased sensitivity to infection (301). Virulent leptospires become associated with neutrophils, but are not killed (117, 613). Phagocytosis occurs only in the presence of serum and complement (385), suggesting that the outer envelope of leptospires possesses an antiphagocytic component. Leptospiral LPS stimulated adherence of neutrophils to endothelial cells (166, 298) and platelets, causing aggregation and suggesting a role in the development of thrombocytopenia (298).

Immune Mechanisms

The second stage of acute leptospirosis is also referred to as the immune phase, in which the disappearance of the organism from the bloodstream coincides with the appearance of antibodies. The clinical severity of the disease often appears to be out of proportion to the histopathological findings. Immune-mediated disease has been proposed as one factor influencing the severity of the symptoms.

The production of immune complexes leading to inflammation in the central nervous system has been postulated (578). Levels of circulating immune complexes were correlated with severity of symptoms (233), and in patients who survived, circulating immune complex levels fell concurrently with clinical improvement. However, in experimental infections in guinea pigs, leptospiral antigen localized in the kidney interstitium, while immunoglobulin G (IgG) and C3 were deposited in the glomeruli and in the walls of small blood vessels (656).

The pathogenesis of equine recurrent uveitis appears to involve the production of antibodies against a leptospiral antigen which cross-react with ocular tissues (358, 443). Retinal damage in horses with uveitis is related to the presence of B lymphocytes in the retina (318). Antiplatelet antibodies have been demonstrated in human leptospirosis (144, 339). In lep-

tospirosis and septicemia, such antibodies are directed against cryptantigens exposed on damaged platelets and do not play a causal role in the development of thrombocytopenia (592). Other autoantibodies have been detected in acute illness, including IgG anticardiolipin antibodies (495) and antineutrophil cytoplasmic antibodies (127). However, the significance of antineutrophil cytoplasmic antibodies in the pathogenesis of vascular injury in leptospirosis has been questioned (1).

Virulent leptospires induce apoptosis in vivo and in vitro (388, 391). In mice, apoptosis of lymphocytes is elicited by LPS via induction of tumor necrosis factor alpha (TNF-α) (299). Elevated levels of inflammatory cytokines such as TNF-α have been reported in patients with leptospirosis (203).

Surface Proteins

The outer membrane of leptospires contains LPS and several lipoproteins (outer membrane proteins [OMPs]) (254). The LPS is highly immunogenic and is responsible for serovar specificity (107, 152). An inverse relationship between expression of transmembrane OMPs and virulence was demonstrated in serovar grippotyphosa (259). Outer membrane lipoprotein LipL36 is downregulated in vivo (56) and is not recognized by the humoral immune response to host-adapted leptospirosis in hamsters (257). Other OMPs are also downregulated in vivo (418). Outer membrane components may be important in the pathogenesis of interstitial nephritis (56, 256). A fibronectin-binding protein produced only by virulent strains was described recently (390).

Immunity

Immunity to leptospirosis is largely humoral in nature (7). Passive immunity can be conferred by antibodies alone (6, 316, 505). A serovar-specific antigen (F4) extracted from LPS (215) lacked endotoxic activity and induced protective immunity in rabbits, guinea pigs, and mice (216). A similar antigen (TM), which inhibited agglutination by homologous antisera (3), was shown to be distinct from F4 (10) but had a common epitope (12). Sodium dodecyl sulfate extracts of whole cells induced production of protective antibody, which was also agglutinating and complement fixing (326). Immunity is strongly restricted to the homologous serovar or closely related serovars. Serovar specificity is conferred by the LPS antigens (317, 392, 605). Broadly reactive genus-specific antigens have also been described (13, 411, 431, 538).

Several of the leptospiral OMPs are highly conserved (256, 515), and the potential for subunit vaccines which can generate broadly cross-protective immunity has been suggested by recent studies using OmpL1 and LipL41 (258), which induced synergistic protection.

Cell-mediated immune responses to leptospirosis have been reported (480). However, suppression of the cell-mediated immune response has been reported (652), with reduction in the number of $CD4^+$ lymphocytes and in their responsiveness to some mitogens. Anecdotal evidence for lack of a significant cell-mediated component in the immune response to leptospirosis was provided by the clinical course of cases occurring in patients with AIDS (143, 416).

LABORATORY DIAGNOSIS

General Clinical Laboratory Findings

In anicteric disease, the erythrocyte sedimentation rate is elevated, and white cell counts range from below normal to moderately elevated (180). Liver function tests show a slight elevation in aminotransferases, bilirubin, and alkaline phosphatase in the absence of jaundice. Urinalysis shows proteinuria, pyuria, and often microscopic hematuria. Hyaline and granular casts may also be present during the first week of illness (180).

Lumbar puncture will usually reveal a normal or slightly elevated CSF pressure (57) and may serve to reduce the intensity of headache. CSF examination may initially show a predominance of polymorphs or lymphocytes, but later examination almost invariably shows that lymphocytes predominate (57, 96). CSF protein may be normal or elevated, while CSF glucose is usually normal. In patients with severe jaundice, xanthochromia may occur (96, 180, 634). CSF abnormalities are common in the second week of illness, and CSF pleocytosis can persist for weeks (180).

In severe leptospirosis, a peripheral leukocytosis occurs with a shift to the left, whereas in dengue, atypical lymphocytes are commonly observed. Thrombocytopenia is common and may be marked (176). Renal function impairment is indicated by raised plasma creatinine levels. The degree of azotemia varies with severity of illness (24). In icteric leptospirosis, liver function tests generally show a significant rise in bilirubin, with lesser increases in transaminases and marginal increases in alkaline phosphatase levels (177). The increase in bilirubin is generally out of proportion to the other liver function test values (179). Similar findings were reported for serum creatinine phophokinase levels (313). Serum amylase may also be elevated, particularly in patients with ARF.

The nonspecific nature of these changes can only suggest a diagnosis of leptospirosis. For confirmation of the diagnosis, specific microbiological tests are necessary.

Microscopic Demonstration

Leptospires may be visualized in clinical material by dark-field microscopy or by immunofluorescence or light microscopy after appropriate staining. Dark-field microscopic examination of body fluids such as blood, urine, CSF, and dialysate fluid has been used but is both insensitive and lacking specificity. Approximately 10^4 leptospires/ml are necessary for one cell per field to be visible by dark-field microscopy (587). A quantitative buffy coat method was recently shown to have a sensitivity of approximately 10^3 leptospires/ml (335). A method which involved repeated microscopic examination of double-centrifuged anticoagulated blood demonstrated leptospires in 32% of patients whose leptospirosis was confirmed by animal inoculation (634). Microscopy of blood is of value only during the first few days of the acute illness, while leptospiremia occurs. In volunteers infected with serovar grippotyphosa, leptospires were detected as early as 4 days prior to the development of symptoms (24). None of the positive samples reported by Wolff (634) were taken more than 6 days after onset of symptoms. Most authorities agree that there are too few leptospires in CSF for detection by dark-field microscopy (24,

634). Direct dark-field microscopy of blood is also subject to misinterpretation of fibrin or protein threads, which may show Brownian motion (213, 587, 634).

Staining methods have been applied to increase the sensitivity of direct microscopic examination. These have included immunofluorescence staining of bovine urine (72, 284), water, and soil (275) and immunoperoxidase staining of blood and urine (562). A variety of histopathological stains have been applied to the detection of leptospires in tissues. Leptospires were first visualized by silver staining (542), and the Warthin-Starry stain is widely used for histologic examination. Immunofluorescence microscopy is used extensively to demonstrate leptospires in veterinary specimens (195). More recently, immunohistochemical methods have been applied (256, 589, 664, 665).

TABLE 8. Genus-specific serological tests for diagnosis of leptospirosis

Method	Reference(s)
Complement fixation test	463
Sensitized erythrocyte lysis	105
Macroscopic slide agglutination	234
Immunfluorescence	33, 580
Patoc slide agglutination test	76, 362, 381, 557
Indirect hemagglutination	295, 431, 499, 546, 547
Counterimmunoelectrophoresis	410, 567
ELISA	11, 363, 565, 566
Microcapsule agglutination	38, 39, 514
Dot-ELISA	438, 617, 350a
IgM dipstick	253, 533, 350a
Latex agglutination	534

Antigen Detection

Detection of leptospiral antigens in clinical material would offer greater specificity than dark-field microscopy while having the potential for greater sensitivity. An evaluation of several methods concluded that radioimmunoassay (RIA) could detect 10^4 to 10^5 leptospires/ml and an enzyme-linked immunosorbent assay (ELISA) method could detect 10^5 leptospires/ml, but countercurrent immunoelectrophoresis and staphylococcal coagglutination were much less sensitive (4). RIA was more sensitive than dark-field microscopy but less sensitive than culture when applied to porcine urine (109). A double-sandwich ELISA could detect 10^4 leptospires of serovar hardjo but was less sensitive for other serovars (103). A chemiluminescent immunoassay (612) was applied to human blood and urine (433) but was no more sensitive than earlier ELISA. More recently, immunomagnetic antigen capture was combined with fluoroimmunoassay to detect as few as 10^2 leptospires/ml in urine of cattle infected with serovar hardjo (654). Inhibitory substances have been reported in urine (4, 109, 654), indicating the need for treatment of urine prior to testing.

Isolation of Leptospires

Leptospiremia occurs during the first stage of the disease, beginning before the onset of symptoms, and has usually finished by the end of the first week of the acute illness (384). Therefore, blood cultures should be taken as soon as possible after the patient's presentation. One or two drops of blood are inoculated into 10 ml of semisolid medium containing 5-fluorouracil at the patient's bedside. For the greatest recovery rate, multiple cultures should be performed, but this is rarely possible. Inoculation of media with dilutions of blood samples may increase recovery (548). Rapid detection of leptospires by radiometric methods has been described (366). Leptospires survive in conventional blood culture media for a number of days (434). Rarely, leptospires have been isolated from blood weeks after the onset of symptoms (303).

Other samples that may be cultured during the first week of illness include CSF and dialysate. Urine can be cultured from the beginning of the second week of symptomatic illness. The duration of urinary excretion varies but may last for several weeks (46). Survival of leptospires in voided human urine is limited, so urine should be processed immediately (587) by centrifugation, followed by resuspending the sediment in phosphate-buffered saline (to neutralize the pH) and inoculating into semisolid medium containing 5-fluorouracil.

Cultures are incubated at 28 to 30°C and examined weekly by dark-field microscopy for up to 13 weeks before being discarded. Contaminated cultures may be passed through a 0.2-μm or 0.45-μm filter before subculture into fresh medium (487).

Identification of leptospiral isolates. Isolated leptospires are identified either by serological methods or, more recently, by molecular techniques. Traditional methods relied on cross-agglutinin absorption (162). The number of laboratories which can perform these identification methods is very small. The use of panels of monoclonal antibodies (327, 333, 334, 520, 563, 564) allows laboratories which can perform the microscopic agglutination test to identify isolates with relative rapidity. Molecular methods have become more widely used (279, 451) and are discussed below.

Susceptibility testing. Leptospires are susceptible to β-lactams, macrolides, tetracyclines, fluoroquinolones, and streptomycin (21, 213). MBCs are several orders of magnitude higher than MICs (423, 554). Problems in the determination of susceptibility include the long incubation time required (183), the use of media containing serum (423, 648), and the difficulty in quantifying growth accurately. These constraints have limited the development of rapid, standardized methods for susceptibility testing.

Serological Diagnosis

Most cases of leptospirosis are diagnosed by serology. Antibodies are detectable in the blood approximately 5 to 7 days after the onset of symptoms. Serological methods can be divided into two groups: those which are genus specific (Table 8) and those which are serogroup specific. The use of agglutination tests was described soon after the first isolation of the organism (373, 506). At that time few serovars were recognized, and there was little attempt to standardize the methodology between laboratories. Many other methodologies have since been applied to serological diagnosis, but the definitive serological investigation in leptospirosis remains the microscopic agglutination test (MAT).

Microscopic agglutination test. The reference method for serological diagnosis of leptospirosis is the MAT, in which patient sera are reacted with live antigen suspensions of lep-

tospiral serovars. After incubation, the serum-antigen mixtures are examined microscopically for agglutination, and the titers are determined. Formerly, the method was known as the agglutination-lysis test because of the formation of lysis balls (506) or lysis globules (596) of cellular debris in the presence of high-titered antiserum. However, these are tightly agglutinated clumps of leptospires containing live cells and not debris (586).

Several modifications of earlier methods (124, 235, 549, 634) led to an MAT method which can be performed and read in microtiter trays. Protocols for performing the MAT have been described in detail (17, 210, 322, 548). The MAT is a complex test to control, perform, and interpret (586). Live cultures of all serovars required for use as antigens must be maintained. This applies equally whether the test is performed with live or formalin-killed antigens. The repeated weekly subculture of large numbers of strains presents hazards for laboratory workers, and laboratory-acquired infections have been reported (16, 460). Other drawbacks include the continuous risk of cross-contamination of the antigen cultures, necessitating periodic verification of each serovar. MAT titers are affected by the culture medium in which the antigens are grown (409).

The range of antigens used should include serovars representative of all serogroups (210, 586) and all locally common serovars (579). Antibody titers to local isolates are often higher than titers to laboratory stock strains of serovars within the same serogroup. It is usual to include one of the serovars of the nonpathogenic species *L. biflexa* (276, 557). Such a wide range of antigens is used in order to detect infections with uncommon or previously undetected serovars (320). Contrary to a widely held belief, the MAT is a serogroup-specific assay. In many reports which purport to show serovar specificity, a limited range of serogroups were tested, each represented by only a single serovar. Moreover, few studies have attempted to correlate the presumptive serogroup determined by MAT with the results of culture. However, the ability of convalescent-phase MAT titers to predict even the infecting serogroup may be as low as 40% (P. N. Levett, Abstr. 2nd Int. Leptospirosis Soc. Meet. 1999, abstr. 29).

The MAT is read by dark-field microscopy. The end point is the highest dilution of serum at which 50% agglutination occurs. Because of the difficulty in detecting when 50% of the leptospires are agglutinated, the end point is determined by the presence of approximately 50% free, unagglutinated leptospires compared to the control suspension (210). Considerable effort is required to reduce the subjective effect of observer variation, even within laboratories.

Interpretation of the MAT is complicated by the high degree of cross-reaction that occurs between different serogroups, especially in acute-phase samples. This is to some extent predictable, and patients often have similar titers to all serovars of an individual serogroup. Of note, "paradoxical" reactions (Fig. 3), in which the highest titers are detected to a serogroup unrelated to the infecting one, are also common (24, 577). The broad cross-reactivity in the acute phase, followed by relative serogroup specificity in convalescent-phase samples, results from the detection in the MAT of both IgM and IgG antibodies (6, 41, 112, 404, 431, 491, 578) and the presence of several common antigens among leptospires (6, 108, 355).

Paired sera are required to confirm a diagnosis with certainty. A fourfold or greater rise in titer between paired sera confirms the diagnosis regardless of the interval between samples. The interval between the first and second samples greatly depends on the delay between onset of symptoms and presentation of the patient. If symptoms of overt leptospirosis are present, an interval of 3 to 5 days may be adequate to detect rising titers. However, if the patient presents earlier in the course of the disease or if the date of onset is not known precisely, then an interval of 10 to 14 days between samples is more appropriate. Less often, seroconversion does not occur with such rapidity, and a longer interval between samples (or repeated sampling) is necessary. MAT serology is insensitive, particularly in early acute-phase specimens (33, 77, 140). Moreover, patients with fulminant leptospirosis may die before seroconversion occurs (84, 140, 484).

Acute infection is suggested by a single elevated titer detected in association with an acute febrile illness. The magnitude of such a titer is dependent on the background level of exposure in the population and hence the seroprevalence. Thus, in the current CDC case definition, a titer of $\geq$200 is used to define a probable case with a clinically compatible illness (97). Although this may be appropriate for use in a population in which exposure to leptospirosis is uncommon, a higher cut-off titer is necessary for defining probable cases of leptospirosis in most tropical countries. In areas where leptospirosis is endemic, a single titer of $\geq$800 in symptomatic patients is generally indicative of leptospirosis (212), but titers as high as $\geq$1,600 have been recommended (17).

Titers following acute infection may be extremely high ($\geq$25,600) and may take months or even years to fall to low levels (24, 67, 359, 493). Often, it is not possible to distinguish a predominant serogroup until months after infection, as cross-reacting titers decline at different rates (359). If possible, it is important to examine several sera taken at intervals after the acute disease in order to determine the presumptive infecting serogroup. Rarely, seroconversion may be delayed for many weeks after recovery, and longer serological follow-up will be necessary to confirm the diagnosis.

Some patients have serological evidence of previous infection with a different leptospiral serogroup. In these cases, serological diagnosis is complicated further by the "anamnestic response," in which the first rise in antibody titer is usually directed against the infecting serovar from the previous exposure. Only later does it become possible to identify the serovar or serogroup responsible for the current infection, as the titer of specific antibody rises. Paradoxical reactions also occur in patients who have such infections, and interpretation of serology is further complicated.

Formalized antigens have been used in the MAT to overcome some of the difficulties associated with the use of live antigens. Titers obtained with these antigens are somewhat lower, and more cross-reactions are detected (210, 243, 368, 435, 548, 634). Agglutination of formalin-treated antigens is qualitatively different from that seen with live antigens (17); however, for laboratories without the staff or expertise to maintain live antigens, formalin-treated and lyophilized antigens may represent a good alternative.

The MAT is also the most appropriate test to employ in epidemiological serosurveys, since it can be applied to sera from any animal species and the range of antigens used can be

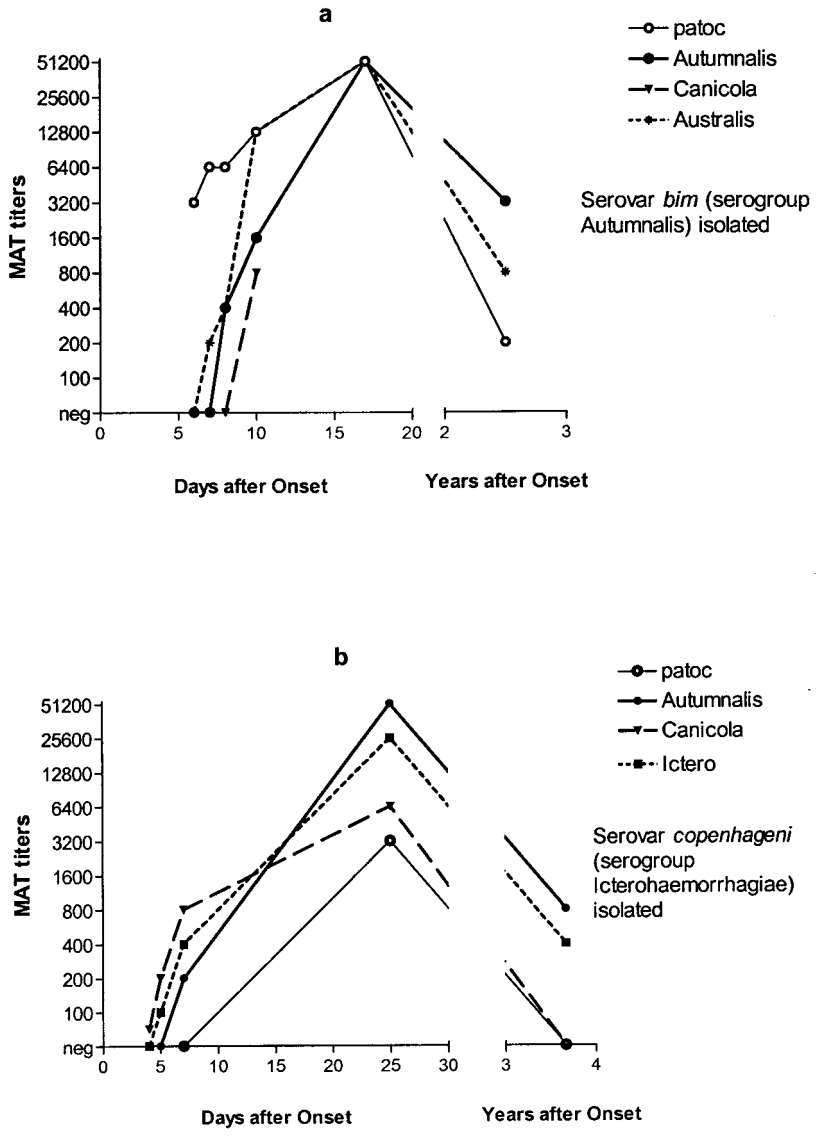

FIG. 3. Paradoxical immune response to acute infection with serovar bim, in which the presumptive serogroup (Autumnalis) was identified during follow-up (a), and copenhageni, in which serogroup Icterohaemorrhagiae was never identified as the predominant serogroup (b).

expanded or decreased as required. It is usual to use a titer of ≥100 as evidence of past exposure (210). However, conclusions about infecting serovars cannot be drawn without isolates; at best, the MAT data can give a general impression about which serogroups are present within a population.

Other serological tests. Because of the complexity of the MAT, rapid screening tests for leptospiral antibodies in acute infection have been developed (Table 8). Complement fixation (CF) was widely used (24, 586, 595, 634), but methods were not standardized. CF was applied to veterinary diagnosis, but species-specific differences were noted (488). CF tests have generally been replaced by ELISA methods (11, 365, 440, 565, 566). IgM antibodies become detectable during the first week of illness (11, 112, 173, 351, 617), allowing the diagnosis to be confirmed and treatment initiated while it is likely to be most effective. IgM detection has repeatedly been shown to be more sensitive than MAT when the first specimen is taken early in the acute phase of the illness (140, 484, 632).

IgM antibodies have been detected by ELISA in CSF from patients with icteric leptospirosis (94). In patients with meningitis without a proven etiology, IgM was detected in the CSF in 15% (522). IgM has been detected in saliva (524), and a dot-ELISA using polyester fiber was developed to facilitate collection of saliva directly onto the support material (523).

ELISA methods have been applied in a number of modifications. An IgM-specific dot-ELISA was developed in which polyvalent leptospiral antigen was dotted onto nitrocellulose filter disks in microtiter tray wells, allowing the use of smaller volumes of reagents. Further modifications of this approach have been used to detect IgG and IgA in addition to IgM (524) and have employed an immunodominant antigen (485) and a polyester fabric-resin support in place of nitrocellulose (523).

A commercial IgM dot-ELISA dipstick has been shown to be as sensitive as a microtiter plate IgM-ELISA (350a). Another dipstick assay (253) has been extensively evaluated in several populations (512, 533, 661). A dot immunoblot assay using colloidal gold conjugate allowed completion of the assay within 30 min (455).

In contrast to the applications of ELISA for diagnosis of human infection, in which broadly reactive assays are generally desirable and few serovar-specific assays have been developed (395), veterinary applications have been directed towards detection of serovar-specific antibodies, particularly for detection of infection in food animals. ELISA methods have described for detection of serovar pomona (134, 573) and hardjo (5, 58, 573, 653) infection in cattle and hardjo in sheep (9). Several assays are available commercially for serodiagnosis of bovine hardjo infection and have been evaluated (642). IgM detection by ELISA has also been applied to canine diagnosis (264, 265, 623).

A macroscopic slide agglutination test was described in which 12 serovars were combined into four pools for the rapid screening of sera from humans and animals (234). Despite the use of an expanded antigen range, false-negative results were reported for sera from populations in areas of endemic leptospirosis (635). Several modifications of this test have used a single serovar antigen, usually serovar patoc (76, 364, 369, 621). Some studies have reported that the patoc slide test is insensitive (369, 546, 616), but a commercial slide agglutination assay was recently found to be as sensitive and specific as an IgM-ELISA while remaining reactive for a shorter time after recovery than either the IgM-ELISA or the MAT (77).

A number of methods using sensitized red blood cells have been described. The extraction of an erythrocyte-sensitizing substance led to the development of both a hemolytic assay requiring complement (135, 136) and a hemagglutination assay (383, 547), and a number of modifications of the latter have been described (295, 499). These assays detect both IgM and IgG antibodies (351, 431). The indirect hemagglutination assay (IHA) developed at CDC (547) was shown to have a sensitivity of 92% and specificity of 95% compared with the MAT (546). This assay is available commercially and for many years as the only U.S. Food and Drug Administration-approved product for serological diagnosis of leptospirosis. Recent estimates of the sensitivity of the IHA in populations in which leptospirosis is endemic have varied. In one study, IHA detected all patients with leptospirosis but was positive in only 44% of first acute-phase samples taken a mean of 5 days after onset of symptoms (351). Other studies have reported lower overall sensitivities, partly due to differences in case ascertainment and study design (181, 661).

A microcapsule agglutination test using a synthetic polymer in place of red blood cells (39, 514) has been evaluated extensively in Japan and China (40, 139). In an international multicenter evaluation, the microcapsule agglutination test was more sensitive than either the MAT or an IgM-ELISA in early-acute-phase samples (38), but failed to detect infections caused by some serovars (38, 513). An advantage of this direct agglutination method is that it can be applied without modification to sera from other animal species (37).

Other techniques applied to the detection of leptospiral antibodies include immunofluorescence (33, 580), RIA (323), counterimmunoelectrophoresis (410, 567, 657), and thin-layer immunoassay (50). These methods have not been widely used.

Molecular Diagnosis

Leptospiral DNA has been detected in clinical material by dot-blotting (393, 569) and in situ hybridization (568). A recombinant probe specific for pathogenic serovars was prepared from serovar lai (143). Probes specific for serovar hardjobovis were developed (344, 594, 673) and applied to the detection of leptospires in bovine urine (72). However, the sensitivity of ^{32}P-labeled probes was approximately 10^3 leptospires (393, 569, 673), much lower than the sensitivity of PCR, and probes have not been used extensively for diagnosis since PCR became available.

Several primer pairs for PCR detection of leptospires have been described, some based on specific gene targets (483), most frequently 16S or 23S rRNA genes (287, 386, 407, 607, 637, 639, 667) and repetitive elements (428, 502, 643, 670, 671), while others have been constructed from genomic libraries (247, 248, 324, 594). However, few have been shown to amplify leptospiral DNA from either human (247, 386) or veterinary (378, 559, 594, 606, 643, 670) clinical material, and of these, only two methods have been subject to extensive clinical evaluation (84, 387). Both methods were found to be more sensitive than culture, but differences in analysis of the data render direct comparisons between the two approaches impossible.

In one analysis, culture and PCR were positive in 48 and 62% of confirmed cases of leptospirosis, respectively, but serology was positive in 97% (84). However, PCR was positive for two patients who died before seroconversion and was also positive for 18% of seronegative first acute-phase samples.

Both these approaches have limitations. The primers described by Merien et al. (386) amplify a 331-bp fragment of the *rrs* (16S rRNA) gene of both pathogenic and nonpathogenic leptospires, which in the unlikely event of contamination of specimens with nonpathogenic leptospires might produce a false-positive result, whereas the G1 and G2 primers described by Gravekamp et al. (247) do not amplify *L. kirschneri* serovars, necessitating the use of two primer pairs for detection of all pathogenic serovars (248).

Despite these potential shortcomings, these two primer pairs have been the most widely used for clinical studies. Leptospiral DNA has been amplified from serum (84, 247, 387), urine (46, 84, 387), aqueous humor (389), CSF (387, 492, 601), and a number of tissues obtained at autopsy (unpublished data).

The detection of leptospiral DNA in bovine urine has also been investigated. Primers which amplified several serovars of serogroup Sejroe were described (593), and a method specific for serovar hardjo genotype hardjobovis was developed (643). An assay based on the IS*1533* insertion sequence (670) facilitated both detection and identification of serovars directly from urine. Another assay was developed and applied to both bovine and porcine urine samples (607). To overcome the problem of inhibitors present in bovine urine, a magnetic immunocapature PCR assay for serovar hardjo was developed (559).

A recent study evaluated five PCR methods, culture, and immunofluorescence for detection of serovar hardjo in bovine urine samples (606). Primers derived from rRNA gene se-

quences were the least specific, and none of the methods was 100% sensitive. A combination of two detection methods chosen from PCR, immunofluorescence, and culture was the most sensitive.

A limitation of PCR-based diagnosis of leptospirosis is the inability of most PCR assays to identify the infecting serovar. While this is not significant for individual patient management, the identity of the serovar has significant epidemiological and public health value. Strategies designed to overcome this obstacle have included restriction endonuclease digestion of PCR products (85, 502), direct sequencing of amplicons (424), and single-strand conformation analysis (SSCP) (380, 647). Leptospiral genomospecies but not individual serovars can be differentiated following PCR by electrophoresis in nondenaturing polyacrylamide gels, followed by silver staining (424), without the additional step of purification and denaturing.

PCR has been used to distinguish pathogenic from nonpathogenic serovars (407, 444, 639). Recently, a fluorescent-probe 5′ exonuclease PCR assay was described for the rapid detection of pathogenic leptospires (637).

Molecular Typing

Because of the difficulties associated with serological identification of leptospiral isolates, there has been great interest in molecular methods for identification and subtyping (279, 561). Methods employed have included digestion of chromosomal DNA by restriction endonucleases (REA), restriction fragment length polymorphism (RFLP), ribotyping, PFGE, and a number of PCR-based approaches.

REA has been studied extensively (270, 288, 370, 372, 490, 555, 563, 575). Distinct genotypes within serovar hardjo were demonstrated (490). Bovine isolates from North America have all been found to be of genotype hardjobovis, of which subtypes A, B, and C could be recognized (574). In Northern Ireland, both genotypes hardjobovis and hardjoprajitno were found among bovine isolates (371). Antigenic differences were also reported among hardjobovis isolates (345). Moreover, serovar balcanica isolates in North America were indistinguishable from genotype hardjobovis isolates by REA (574). Further analysis of RFLP in genotype hardjobovis isolates by REA, Southern blotting, and PFGE has shown the existence of multiple genetic clones resulting from genomic rearrangement (675). These clones were usually localized within geographical locations and thus are of epidemiological significance (675).

Similar subserovar differences were detected within serovar pomona, isolates from North America being identified as subtype kennewicki (563, 575), while European isolates were of serovar pomona (270, 563) or mozdok (269, 270). More recently, differences between subtype kennewicki isolates were correlated with host animal source (71). Differences between serovars copenhageni and icterohaemorrhagiae were demonstrated by some workers (372), but not all (288, 555). However, all isolates of these two serovars are indistinguishable by PFGE (281).

Ribotyping has demonstrated reasonably good correlation with the phylogenetic classification of leptospira into 11 genomospecies. Using *Eco*RI for digestion and 16S and 23S rRNA from *Escherichia coli* as the probe, a large database was constructed (451, 452). Many serovars gave unique profiles, while other serovars could not be distinguished from each other by ribotyping, particularly those that were known previously to be closely related, such as icterohaemorrhagiae and copenhageni (288, 555). Ribotypes of serovars within genomospecies could be grouped together by the possession of common fragments. This database is available at the Institut Pasteur website (http://www.pasteur.fr/recherche/Leptospira/Ribotyping.html). Ribotyping has been shown to discriminate accurately between the serovar hardjo genotypes hardjobovis and hardoprajitno (453).

An alternative approach to ribotyping used three restriction enzymes and a PCR-derived 16S rDNA probe. Use of only 16S rDNA gave fewer bands, but this was counterbalanced to some extent by the use of multiple restriction enzymes, giving three different patterns for each serovar (286). Relatively few serovars were examined but all gave distinct ribotypes with the exception of serovars icterohaemorrhagiae and copenhageni. A range of other probes have been used to generate RFLPs (429, 593, 672, 673). A probe based on the repetitive sequence element from serovar hardjo genotype hardjobovis was also used to detect leptospires in bovine urine (673).

PFGE has proven useful to characterize leptospiral serovars (279). In contrast to its application in strain typing of other organisms, PFGE has shown that the genomes of leptospiral serovars are remarkably conserved, both over time and across wide geographical distributions (279, 281). Importantly, recent clinical isolates gave the same banding patterns as reference strains of the same serovar which have been maintained for many years by repeated subculture (279). Using the enzyme *Not*I, most but not all serovars gave unique PFGE patterns. *L. interrogans* serovars bratislava, lora, jalna, and muenchen gave identical patterns when digested with *Not*I but were differentiated when digested with *Sgr*AI (282). Other serovars which were difficult to differentiate included *L. borgpetersenii* serovars arborea and castellonis. The *L. interrogans* serovars copenhageni and icterohaemorrhagiae were indistinguishable by PFGE, confirming their close relationship. PFGE analysis has become the de facto standard for molecular characterization of leptospiral isolates, and other molecular typing methods will in future have to be validated against this method.

A limiting factor in all methods which analyze chromosomal DNA is the requirement for large quantities of purified DNA. As a result, several methods based on the analysis of PCR-amplified sections of leptospiral DNA have been employed. Sequence variation within the 285-bp fragment amplified by the G1 and G2 primers (248) led to different electrophoretic mobilities which were detected by polyacrylamide gel electrophoresis and silver staining (424). This approach allowed serovars of *L. interrogans* sensu stricto to be differentiated from *L. noguchii* serovars.

Sequence variation is also exploited in SSCP. Using this method, serovars prevalent in China were shown to have different mobilities corresponding to *L. interrogans* and *L. borgpetersenii* (647). The Chinese isolates were studied using a sequence amplified from the 16S rRNA gene, the highly conserved nature of which may account for the inability to distinguish serovars from one another. In contrast, SSCP analysis of the G1-G2 amplicon allows serovar identification within each genomospecies studied (380). A restriction on the use of the latter sequence is the inability of the G1 and G2 primers to amplify *L. kirschneri* (248). An alternative application of these

primers is their use under low-stringency conditions, generating a mixture of specific and nonspecific products (150). Under these conditions, the G1 and G2 primers amplify all species, including *L. biflexa*. Polymorphisms were detected which allowed discrimination of serovars with the exception of closely related serovars, including copenhageni and icterohaemorrhagiae (85, 150).

The presence of multiple copy insertion sequences has been exploited for serovar identification (481, 502, 670, 671). Methods based on IS*1533* have limited application because of the absence of this insertion sequence in *L. interrogans* (sensu stricto) and *L. noguchii* (481, 670). By amplifying the sequences between adjacent copies of IS*1500*, numerous genetic subgroups within serovar pomona type kennewickii were distinguished (671).

RFLP analysis of PCR-amplified 16S and 23S rRNA genes allowed the grouping of 48 serovars into 16 mapped restriction site polymorphism profiles (469). Using this approach, the genomospecies of *Leptospira* could be identified, and the genotypes hardjobovis and hardjoprajitno of serovar hardjo were clearly distinguished (453). The method was simplified to yield only five profiles by using a single restriction enzyme (638). One of the potential advantages of this RFLP approach is the ability to amplify leptospiral DNA from clinical material and to identify the infecting serovar or genomospecies rapidly in the absence of an isolate. Other workers have used primers that amplify only a restricted range of serovars (85, 502), limiting the utility of the approach unless several primer sets are used (85).

DNA fingerprinting using arbitrary primers (625, 629) has been studied extensively (85, 128, 129, 237, 453, 469), using different primers and conditions. Direct comparison between the results of these studies is therefore impossible, but it is clear that reproducibility is difficult to achieve without absolute standardization of experimental procedure. Profiles are affected markedly by the primer used, the quantity and quality of the DNA template (128, 380, 599), and the electrophoresis conditions (129). The greatest value of arbitrary primer techniques lies in their ability to differentiate between isolates when the range of potential serovars is limited, allowing rapid identification of freshly isolated strains (85, 128, 237). Arbitrary-primed PCR was used to derive species-specific probes for identification of *L. interrogans* (sensu stricto), *L. borgpetersenii*, and *L. kirschneri* by dot blotting (347). A cluster of 43 *L. interrogans* sensu strico isolates from a number of Brazilian outbreaks were shown to have identical arbitrary-primed PCR fingerprints (449) despite the inclusion of isolates of serovars copenhageni and canicola.

CONCLUSION

The etiology and epidemiology of leptospirosis have been understood for many years, and this knowledge has led to the development of effective preventive strategies. In developed countries, leptospirosis continues to be a disease of considerable economic significance in animal husbandry, but the major burden of human disease remains in tropical and subtropical developing countries. Several recent outbreaks of leptospirosis have drawn attention to the potential effects of climate change and human activity on the incidence of the disease and the broad spectrum of clinical manifestations. The development of several promising approaches to rapid diagnosis has been based largely on the recognition that early initiation of antibiotic therapy is important in acute disease, but also on the need for simpler assays which can be used more widely. However, many of these diagnostic advances will be unavailable to those populations for which they would be most useful. At a more fundamental level, understanding of the mechanisms of pathogenesis remains incomplete, but recent advances in the molecular biology of leptospires offer the prospect of more rapid progress in the future.

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