The prevalence of Leptospira interrogans infection in mature cattle in the United States

by

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INTRODUCTION

Leptospirosis is an infectious disease of domestic animals, humans, and wildlife with almost worldwide distribution (Torten, 1979). The annual loss due to leptospirosis in United States livestock has been estimated at $215 million (Stalheim, 1976). Losses occur due to abortions, stillbirths, infertility, acute and chronic illnesses, decreased milk production, and death. Cattle and swine are the species principally affected among the domestic animals in the United States. Horses, sheep, goats, and dogs are also susceptible. Infected domestic animals and wildlife are potential sources of infection for other animals and humans (Hanson, 1982).

The Leptospira interrogans serovars most frequently isolated from domestic animals in this country are canicola, grippotyphosa, hardjo, icterohaemorrhagiae, and pomona. On a worldwide basis, over 200 pathogenic serovars have been isolated and characterized, and all of them could potentially infect domestic animals or humans (Torten, 1979). The ability of leptospires to colonize the kidney and be shed in the urine for long periods is the most important factor in the dissemination of leptospirosis (Baldwin and Atkins, 1987). Shedding has been demonstrated for periods of months in cattle, swine, sheep, and horses and for years in dogs (Hanson, 1976*a*). Transmission of leptospirosis occurs directly by contact of urine from infected animals with mucus membranes or abraded skin (Michna, 1970), and indirectly through urinary contamination of food, water, or soil (Babudieri, 1958).

Historically, the diagnosis of leptospirosis has been difficult. Serological tests have been used since the early 1900s, but the interpretation of results has been complicated by cross-reactivity among Leptospira serovars, uncertainty as to what is a diagnostic titer, and failure of some infected animals to seroconvert. Improvements in media formulations and culture techniques have increased the probability of isolation of leptospires from clinical and post mortem specimens (Thiermann, 1984).

Serological surveys for prevalence of leptospirosis in cattle have been conducted in several states and the data have been tabulated to estimate prevalence in some regions (Stoenner and Glosser, 1975). Prevalence surveys based on isolation have been limited to states or small areas surrounding outbreaks (White et al., 1982; Nelson et al., 1973). A nationwide survey for prevalence of leptospirosis in cattle based on serological tests or isolation of leptospires has not been done.

The first objective of this study was to determine the effect of transportation time on isolation of leptospires from bovine kidneys (Manuscript 1).

The second objective was to estimate the prevalence of Leptospira interrogans infection in mature cattle in the United States (Manuscript 2).

The third objective was to determine the relationships between leptospira prevalence rates in cattle and regional, climatic, and seasonal factors (Manuscript 3).

EXPLANATION OF THESIS FORMAT

This thesis consists of an introduction, a literature review, three separate manuscripts, a general summary and discussion, and a list of references. The candidate, David Alan Miller, is the senior author and principal investigator for each of the manuscripts.

LITERATURE REVIEW

Taxonomy of the Genus Leptospira

Leptospira is the single genus in the Family Leptospiraceae of the Order Spirochetales. Leptospires are obligately aerobic, tightly coiled, motile, rod-shaped bacteria that are 0.1 um in diameter and 6 to over 12 um in length and characteristically have one or both ends hooked (Johnson and Faine, 1984). The cells consist of a helical protoplasmic cylinder contained by a cytoplasmic membrane. An external membrane envelopes the whole organism. A flagellum referred to as an axial filament is inserted subterminally at each end of the protoplasmic cylinder. The flagella extend toward the middle of the organism and do not usually overlap. Leptospires have a characteristic rotatory motility that may be accompanied by flexing, boring, or serpentine movements (Faine and Stallman, 1982). Leptospires stain poorly with the aniline dyes and the unstained organisms cannot be seen by brightfield microscopy, but can be visualized by darkfield or phase-contrast microscopy (Johnson and Faine, 1984). The organisms may be observed with silver and Giemsa stains (Faine and Stallman, 1982).

Leptospires can be cultivated in media containing serum, serum albumin, or long-chain fatty acids with 14 carbons or more as sources of carbon and energy. They utilize inorganic ammonium salts, but not amino acids, as sources of nitrogen (Faine and Stallman, 1982). Thiamine (vitamin B_1) and cyanacobalamine (vitamin B_{12}) are required for growth (Wooley and Van Eseltine, 1968). The optimum pH range is 7.2 to 7.6 and the optimum growth

temperature range is 28 to 30 C (Faine and Stallman, 1982). Purines, but not pyrimidines, are utilized. Therefore, leptospires are resistant to the inhibitory action of 5-fluorouracil to which other spirochetes are sensitive (Johnson and Rogers, 1964). Growth appears as faint turbidity in liquid media, bluish-gray subsurface discs in semisolid media, and diffuse to discrete surface or subsurface colonies in soft agar plate media (Faine and Stallman, 1982).

Two species are currently recognized. L. interrogans includes leptospires which are pathogenic to humans and animals. L. biflexa includes leptospires which are not pathogenic, but are commonly found in soil and water. The basic taxon of both species is the serovar which is determined by antigenic composition based on serological tests (Johnson and Faine, 1984) and cross agglutinin-absorption tests (Wolff, 1954). Another classification scheme based on DNA hybridization has been proposed (Yasuda et al., 1987). Comparison of fragments after digestion of genomes with restriction endonucleases has become a widely used method of identification of isolates (Marshall et al., 1981; Robinson et al., 1982; Hathaway et al., 1985; LeFebvre et al., 1985; Thiermann et al., 1986; Thiermann and Ellis, 1986; Gregoire et al., 1987; LeFebvre et al., 1987). Some serologically indistinguishable serovars have been shown to be genetically different (LeFebvre et al., 1987). Serovars within species cannot be differentiated on the basis of biochemical tests (Alexander, 1985).

L. interrogans and L. biflexa can be differentiated on the basis of pathogenicity, serological tests, growth at 13 C, resistance to

8-azoguanine, and conversion to spherical forms in 1 M NaCl (Johnson and Faine, 1984). A third species, L. illini (Hanson et al., 1974), differs from L. interrogans and L. biflexa in some phenotypic characteristics and in DNA annealing tests (Johnson and Faine, 1984). A new genus, "Leptonema", has been proposed for L. illini (Hovind-Hougen, 1979), but it is currently included in the Genus Leptospira as a *species insertae sedis* (Johnson and Faine, 1984). "L. parva" is somewhat different from other leptospires in morphology and DNA base composition, and has been proposed to be included in the genus Leptospira as a *species nova* (Hovind-Hougen et al., 1981).

The species L. interrogans currently contains 214 recognized serovars (C. R. Sulzer, Centers for Disease Control, Atlanta, GA, personal communication, 1988). New or previously unrecognized serovars are being characterized and reported frequently (Santa Rosa et al., 1972; Santa Rosa et al., 1975; Everard et al., 1980; Jones et al., 1982; Sulzer et al., 1984; Jones et al., 1984; Schmid et al., 1986; Coghlan and Kmety, 1987). Antigenically related serovars are classified in serogroups, and there are currently 23 recognized serogroups.

Leptospirosis: A True Zoonosis

A zoonosis is defined as a disease which is naturally transmitted between vertebrate animals and humans. Leptospirosis is an infection with one or more serovars of L. interrogans, and has been described as the zoonosis with the widest geographical distribution. Pathogenic serovars have

been isolated in 77 countries and clinical or serological evidence indicates that the disease is present in domestic animals and humans in almost every country in the world (Torten, 1979). Human infection is not essential for maintenance of leptospirosis in nature (Walton, 1985). Humans appear to be accidental hosts who become infected through occupational exposure, invasion of wildlife environments, or close contact with infected pets (Hanson, 1982). Infected humans may shed leptospires in the urine for periods of weeks or months; shedding for a period of 11 months was reported in one case of serovar australis infection (Michna, 1970). Although transmission of leptospirosis among humans has rarely been documented, one case of intrauterine transmission (Faine et al., 1984), one case of suspected venereal transmission (Michna, 1970), and one case of transmission from mother to infant through breast feeding (Songer and Thiermann, 1988) have been reported.

Leptospirosis was first described as a distinct clinical entity in humans in 1886 by H. A. Weil, a German physician, 30 years before the etiological agent was shown to be a spirochete (Inada et al., 1916). The term "Weil's disease" became a widely used common name for the malady described as "a febrile illness with jaundice in epidemic or endemic form", and is usually associated with serovar icterohaemorrhagiae infections. The recognition that serovar pomona infections were transmitted from swine to humans led to the term "swineherd's disease" in Europe (Alston and Broom, 1958). Over 20 other synonyms for leptospirosis based on clinical signs ("hemorrhagic jaundice", "pretibial fever"), locations of outbreaks ("Fort

Bragg fever", "swamp fever"), or occupations of patients ("cane cutter's disease", "fish handler's disease") have been used (Torten, 1979). Although Stimson (1907) described a spirochete observed in tissues of a patient suspected of having yellow fever, the first proven case of human leptospirosis in the United States was not reported until 1922 (Wadsworth et al.). Stiles and Sawyer (1942) showed a definite association between activities or occupations of individuals (swimmers, sewer workers, fish handlers, farmers, abattoir workers, and veterinarians) and the occurrence of leptospirosis. The disease has also been described as one of soldiers. Much of the epidemiological data that are available came from leptospira infections of military personnel in training exercises or combat zones in swampy tropical or subtropical areas (Noguchi, 1918; Jeghers et al., 1934; Gochenour et al., 1953; Wisseman et al., 1955; McCrumb et al., 1957; Calero et al., 1957; Berman et al., 1973; Torten, 1979). The prevalence of leptospirosis has been characteristically higher in males than in females (Stiles and Sawyer, 1942; Giestfield, 1975; Martone and Kaufmann, 1979; Swart et al., 1983; Everard et al., 1984).

The unsuspected animal carrier of leptospirosis has been described as a major hazard for humans (Walton, 1985). Contact with rats and dogs was recognized early in the recorded history of the disease as a source of human infection (Jeghers et al., 1934; Meyer et al., 1938). Robertson et al. (1981) reported an outbreak of leptospirosis in trout farm workers in which there was evidence of rat infestation around ponds and feed storage sheds. Shenberg et al. (1977*b*) described five cases of serovar hardjo infection in

dairy farmers in Israel and demonstrated leptospira titers in the dairy cows and in rats and hedgehogs trapped in the vicinity. Serovar hardjo infection has been described as endemic in the majority of New Zealand dairy herds (Ryan et al., 1982) and over 1,000 human cases have been reported there annually (Deas, 1981). Heath et al. (1965) analyzed 483 human cases that occurred in the United States between 1949 and 1961 and observed that the majority of patients with serovar icterohaemorrhagiae infections had been exposed to rats, those with serovar canicola to dogs, and those with serovar pomona to cattle and swine. Swart et al. (1983) diagnosed leptospirosis caused by serovars hardjo, pomona, and tarassovi in 208 of 2,516 patients in Australia suspected of having some zoonosis and observed that 101 of the patients were farmers, 44 were meat workers, and 11 were meat inspectors.

Of 138 reported cases of human leptospirosis in the United States between 1971 and 1973 for which there was a probable source identified, 55% were associated with dogs, 21% with cattle and swine, 14% with rodents, and 9% with wildlife (Giestfield, 1975). An outbreak involving 19 human cases in Florida was reported in 1978, in which the source was identified as dairy cattle shedding the serovar balcanica (Martone and Kaufmann, 1979). Many human outbreaks involving significant numbers of people have been associated with swimming in ponds or streams to which domestic animals had access (Schaeffer, 1951; Cockburn et al., 1954; Williams et al., 1956; Braun, 1961; Gillespie and Ryno, 1963; Nelson et al., 1973). The majority of these outbreaks were traced to infected cattle and swine, although there

was evidence of infection in dogs, horses, and mules in some cases.

The incubation period of human leptospirosis may range from 3 to 30 days, but is usually 10 to 12 days (Alexander, 1985). Symptoms may vary widely. Among the most frequently observed symptoms in 746 cases reported between 1971 and 1978 in the United States were fever, headache, myalgia, jaundice, and stiff neck. Less frequently observed were nausea and vomiting, chills, anuria, rashes, and conjunctivitis. Initial clinical impressions in these cases indicated that leptospirosis accounted for approximately 26% of the diagnoses. Other common diagnoses were meningitis, fever of unknown origin, hepatitis, encephalitis, viral syndrome, and pneumonia (Giestfield, 1975; Martone and Kaufmann, 1979). Other clinical manifestations of leptospirosis may be interstitial nephritis (Lai et al., 1982), acute renal failure (Winearls et al., 1984), pulmonary involvement (Pathy, 1960), anterior uveitis (David and Barkay, 1976; Heath et al., 1965), extensive rashes over the pretibial areas of the legs (Bowdoin, 1942; Daniels and Grennan, 1943; Gochenour et al., 1952), anemia, cardiac involvement which may be severe enough to cause dilatation or left ventricular failure, delirium, hallucinations, psychoses, and mania (Edwards and Domm, 1966). Although most recognized cases of human leptospirosis are those with acute manifestations, Nicolescu and Andreescu (1984) observed that human infections may become chronic.

Giestfield (1975) stated that leptospirosis is undoubtedly underdiagnosed in the United States because it is not usually suspected by clinicians and is difficult for laboratories to diagnose. Prevalence estimates

in many countries probably lack accuracy because leptospirosis is not a reportable disease (Szatalowicz et al., 1969; Szyfres, 1976).

Occurrence of L. interrogans Serovars in the United States

Thirty-one pathogenic serovars belonging to 13 serogroups of leptospires have been isolated from humans, animals, or environmental sources in the United States. Other leptospires have been isolated and identified as to serogroup, but not to the serovar level (Ward et al., 1956; Roth et al., 1962; Gorman et al., 1962; Roth, 1964; Redetzke and McCann, 1980). Serovar dakota of the Icterohaemorrhagiae group has been isolated only from water (Alexander et al., 1962).

Serovars of the Autumnalis (Gochenour et al., 1952), Canicola (Meyer et al., 1938), Icterohaemorrhagiae (Wadsworth et al., 1922), Mini (Goley et al., 1960), and Pomona groups (Hale and Cathey, 1958; Nelson et al., 1973) have been isolated from human infections. Serovar bratislava of the Australis group has been isolated only from swine, and has not been associated with clinical signs of leptospirosis in the United States (Ellis and Thiermann, 1986). Other serovars isolated from swine were canicola (Ward et al., 1956), grippotyphosa (Hanson et al., 1971*a*), icterohaemorrhagiae (Schnurrenberger et al., 1970) and copenhageni of the Icterohaemorrhagiae group (D. A. Miller, National Veterinary Services Laboratories, Ames, IA, unpublished data, 1986), and pomona (Gochenour et al., 1952) and kennewicki (Thiermann et al., 1985) of the Pomona group. Serovars canicola (Turner et al., 1958), grippotyphosa (Hanson et al.,

1964*a*), icterohaemorrhagiae (Schnurrenberger et al., 1970), copenhageni (Miller, 1985), szwajizak of the Mini group (Glosser et al., 1974), pomona (Baker and Little, 1948), kennewicki (Nelson et al., 1973), and hardjo (Roth and Galton, 1960) and balcanica (White et al., 1982) of the Sejroe group have been isolated from cattle. Serovars isolated from dogs include canicola (Meyer et al., 1938), grippotyphosa, ballum (Cole et al., 1982), icterohaemorrhagiae (Randall and Cooper, 1944), and pomona (Murphy et al., 1958). Serovars pomona (Bryans, 1955) and kennewicki (D. A. Miller, National Veterinary Services Laboratories, Ames, IA, unpublished data, 1987) have been isolated from horses, and pomona has been isolated from goats (Hirschberg et al., 1956).

Twenty-four of the 31 serovars have been isolated from wildlife. Serovars australis of the Australis group, autumnalis, mooris, and rachmati of the Autumnalis group, arboreae of the Ballum group, paidjan of the Bataviae group, orleans and louisiana of the Louisiana group, mozdok of the Pomona group, myocastoris and zanoni of the Pyrogenes group, sejroe of the Sejroe group, and atchafalaya, atlantae, bakeri, and tarassovi of the Tarassovi group have been isolated only from wildlife (Roth, 1964; Galton, 1966; Sulzer, 1975; Zygmont, 1981). Of the 50 States, sejroe has been isolated only in Hawaii (Minette, 1964).

Leptospirosis in Animals

Host species and serovars incriminated

Although the etiology was unknown, leptospirosis was first recognized as a clinical entity in animals in 1850. A leptospirosis epidemic began in dogs in Stuttgart, Germany, in 1898 and the resulting name "Stuttgart disease" has been widely used as a synonym for canine leptospirosis (Torten, 1979). After the discovery of Inada et al. (1916) that spirochetes were the etiological agents of Weil's disease, it was observed that Stuttgart disease and Weil's disease were caused by identical microorganisms. Within 3 years after the discovery of the etiological agent, leptospires were shown to cause a severe icterohemorrhagic disease in puppies (Torten, 1979). Peaks in the widespread European epidemic occurred from 1921 to 1925 and from 1946 to 1952, and attracted considerable attention to the clinical manifestations of leptospirosis (Hartman, 1984). Kirkwood and Horning first reported canine leptospirosis in the United States in 1923, and observed that the disease which they referred to as "sore mouth" was spread by the urine of infected rats. Many surveys done since the advent of serological tests have indicated a worldwide distribution of canine leptospirosis (Michna and Ellis, 1973). The first reported case in cattle occurred in Russia in 1935 (Michin and Azinov, 1937), and it was observed that the etiological agent resembled the spirochetes isolated from cases of Weil's disease in humans. Jungherr described the first recognized cases of leptospirosis in cattle in the United States in 1944, and concluded that cattle should be considered in addition to humans, dogs, and rodents in the

transmission of the disease. During the 1940s and 1950s, leptospirosis was established as a disease of major significance in veterinary medicine (Torten, 1979).

Leptospires have been isolated from 220 host species and have the potential to infect all mammals. Both intraspecies and interspecies dissemination of leptospirosis are possible. Some species appear to be more important than others as carriers of leptospirosis in certain areas (Torten, 1979). As examples, raccoons are carriers of serovar grippotyphosa and swine are reservoirs of serovar pomona in the United States (Roth, 1964; Martin et al., 1967; Hanson, 1984), hedgehogs appear to be reservoirs of serovar bratislava in England (Hathaway et al., 1983), and dogs are carriers of serovar canicola in many countries (Rosenberg, 1951; Michna and Ellis, 1973; Hartman, 1984). Rats appear to be almost universal carriers of leptospires of the Icterohaemorrhagiae group, and have been incriminated in many leptospirosis outbreaks on a worldwide basis (Jeghers et al., 1934; Meyer et al., 1938; Babudieri, 1958; Shenberg et al., 1977*b*; Robertson et al., 1981; Thiermann, 1981*a*).

The serovars which affect domestic animals vary widely from country to country. Generally, pomona, tarassovi, canicola, and icterohaemorrhagiae infections are common in swine (Michna, 1967), hardjo, pomona, and grippotyphosa in cattle, pomona in sheep, goats, and horses, and canicola and icterohaemorrhagiae in dogs. Although antibodies against several serovars have been detected in cats, clinical feline leptospirosis has rarely been reported (Michna, 1970). Experimentally infected birds are capable of

producing antibodies, but do not react clinically or become shedders (Torten et al., 1965).

In a particular region, an animal species may be infected by serovars maintained by that species, or by serovars maintained by other species. The prevalence of incidental interspecies infections is determined by opportunities for contact and transmission provided by management and environmental factors (Ellis, 1986). The concept of susceptibility of a particular host species to infection by a given serovar, or a definite host-serovar specificity, is no longer accepted as valid. Endemic leptospirosis may occur in cattle and swine herds, sheep flocks, and dog kennels and may be transmitted from one species to others and to humans (Michna, 1970). The host-serovar relationships in some areas have been shown to change, in that a particular leptospira serovar may replace one that had been recognized for a long period of time as most predominant in a given host species. The converse has also been observed in which the apparent host of a particular serovar may change over a period of time (Shenberg et al., 1977*a*; Torten, 1979).

Clinical manifestations and pathogenesis

Michna (1970) stated that leptospirosis may occur in animals in at least four clinical forms. Subclinical infections may occur in rodents, wildlife, cattle, and swine which appear to be healthy carriers. Acute or subacute forms may be manifested by fever, depression, anorexia, loss of milk production in lactating cattle and swine, icterus and hemoglobinuria in calves, dogs, and pigs, and neurological signs in dogs and cattle.

Reproductive disorders may appear in the form of sporadic or epidemic abortions which are more common in cattle and swine and less common in ewes and mares, or breeding difficulties and mastitis in cattle. Ocular disease occurs more frequently in humans and horses, but may affect swine in the form of iridocyclitis and cataracts.

Leptospires colonize the proximal convoluted tubules of the kidney and are shed in the urine (Marshall, 1976). Transmission of the disease occurs by contact of urine from infected animals with abraded skin or mucus membranes of the eyes, nose, mouth, or genitalia (Michna, 1970). Entrance through the intact skin is unlikely (Hanson, 1976*a*), but it has been suggested that leptospires can penetrate water-softened skin (Michna, 1970). Indirect transmission may occur through urinary contamination of food, water, or soil (Babudieri, 1958). Inhalation of aerosolized urine droplets has been suggested as a possible mode of transmission (Baker and Little, 1948). Evidence that venereal transmission may occur has been provided by the isolation of leptospires from the genital tracts of sows and boars (Ellis et al., 1985*a*) and from the semen of bulls (Sleight et al., 1964; Kiktenko et al., 1976). It is unlikely that transmission occurs through artificial insemination with leptospire-infected semen because of the dilution of semen, antibiotics in the extender, and the temperatures at which semen is stored (Sleight, 1965). Leptospires may be shed in the milk of infected cattle (Ellis and Michna, 1976; Higgins et al., 1980; Thiermann, 1982), and human outbreaks have resulted from the consumption of raw milk (Michna, 1970). Leptospires have been isolated from the mammary glands of

sows and hamsters, and evidence indicates that transmission of leptospirosis from sows to nursing pigs through milk probably occurs (Tripathy et al., 1981). Vectors are not usually considered important in the transmission of leptospirosis, although serovars grippotyphosa and canicola have been isolated from ticks, and it has been suggested that flies may act as mechanical vectors (Michna, 1970).

After entry into a susceptible host, leptospires are transported to the visceral tissues through the blood stream (Jungherr, 1944). The bacteremic stage is relatively short, usually lasting 4 to 5 days (Michna, 1970). Primary infection and multiplication occur in the liver. After an incubation period of 2 to 16 days, an elevation in temperature of 2 to 5 F may occur which persists for 6 to 48 hours. When the acute disease occurs, hemorrhages, anemia, icterus, hemoglobinuria, malaise, and agalactia may be observed. Meningitis and encephalitis occur less frequently (Hanson, 1976*a*). A noninflammatory mastitis may be seen in cattle (Hanson, 1984). Nephritis and hepatitis are common in dogs (Baldwin and Atkins, 1987). Signs associated with the acute stage result from multiplication of leptospires in various tissues and toxic reactions (Hanson, 1976*a*).

Infection of the kidneys results in an acute nephritis followed by a chronic infection which may persist for months or years. Leptospires appear in the urine within a few days after infection and increase in number during the first week. Following a period of weeks in which large numbers of leptospires are shed in the urine, shedding may become intermittent for an extended period. The period of shedding depends on the

serovar involved and host factors, particularly urine pH. Shedding of serovars pomona and tarassovi from swine has been demonstrated for up to one year, and pomona from cattle, horses, and sheep for 100 days, 3.5 months, and 9 months respectively (Babudieri, 1958). Thiermann (1982) demonstrated shedding in cattle experimentally infected with serovar hardjo for a minimum of 30 days and in one cow for at least 542 days. Dogs have shed serovar canicola for over 4 years (Hanson, 1976*a*) and rats have been shown to shed serovar icterohaemorrhagiae for years, probably throughout their lifetimes. Survival of leptospires is usually longer in the urine of herbivorous animals which has a neutral or slightly alkaline pH than in the urine of carnivores which is usually acidic. The acidic urine of rats and dogs is normally fatal to leptospires, but a predominantly vegetable diet can raise the pH of the urine to a point that will allow leptospires to survive (Babudieri, 1958).

Ocular lesions commonly develop in some hosts, and may vary from acute to chronic with mild to severe visual impairment (Hanson, 1976*a*). For many years, recurrent uveitis, also referred to as iridocyclitis, periodic ophthalmia, and moon-blindness, was believed to be the major clinical manifestation of leptospirosis in horses (Hanson et al., 1969).

Abortion is the most commonly recognized sequela of leptospirosis in cattle, swine, horses, sheep, and goats, and frequently occurs without premonitory signs (Hanson, 1976*a*; Kirkbride, 1984; Ellis, 1986). Leptospires are transported through the maternal blood to the placenta and then migrate to the fetus. The results may be an active infection which results

in fetal death and subsequent expulsion or a nonlethal infection which persists after birth. Infection during the first trimester does not usually interrupt fetal development. Abortions and stillbirths are most common in animals infected in the last trimester of pregnancy. Fetuses may be retained for 2 to 5 weeks prior to expulsion (Hanson, 1976*a*).

Leptospirosis in Cattle

Serovars incriminated

Cattle, as well as other domestic animals, could potentially be infected by any of the known pathogenic serovars (Torten, 1979). Generally, serovar hardjo, pomona, and grippotyphosa infections are common in cattle (Faine, 1982). Serovar pomona infections have been frequently demonstrated in the cattle of many countries since the 1940s (Baker and Little, 1948; Hoag and Bell, 1954*b*; Hadlow and Stoenner, 1955; Spradbrow and Seawright, 1963; Higgins and Cayouette, 1978; Carter et al., 1982; Herr et al., 1982). On a worldwide basis, serovar hardjo infection has emerged as one of the most frequently diagnosed leptospiroses in cattle (Carlos et al., 1979; Higgins et al., 1980; Aycardi et al., 1982; Bahaman et al., 1984; Thiermann, 1984; Ellis et al., 1985*b*; Schonberg et al., 1986; Bahaman et al., 1987; Skilbeck et al., 1988).

Bovine infections with serovar grippotyphosa have been reported less frequently (Hanson et al., 1964*a*; Diesch et al., 1967; Burdin, 1963; Michna, 1970). Other serovars associated with bovine leptospirosis are australis and peruviana of the Australis group (Kita et al., 1960; Galton,

1966), autumnalis of the Autumnalis group (Kita et al., 1960), ballum of the Ballum group (Sulzer, 1975), argentiniensis, bataviae, and paidjan of the Bataviae group (Galton, 1966; Sulzer, 1975), canicola and galtoni of the Canicola group (Turner et al., 1958; Sulzer, 1975), guaracuris, goiano, hebdomadis, and kremastos of the Hebdomadis group (Kita et al., 1960; Sulzer, 1975), copenhageni and icterohaemorrhagiae of the Icterohaemorrhagiae group (Schnurrenberger et al., 1970; Sulzer, 1975), szwajizak of the Mini group (Glosser et al., 1974), kennewicki and mozdok of the Pomona group (Nelson et al., 1973; Sulzer, 1975), an unidentified serovar of the Pyrogenes group (Sulzer, 1975), balcanica and sejroe of the Sejroe group (White et al., 1982; Galton, 1966), and tarassovi of the Tarassovi group (Galton, 1966).

In the United States, pomona was recognized as the most common serovar in cattle from the time of its identification as an etiological agent in bovine leptospirosis in 1948 (Baker and Little) until 1968 when serological test results began to indicate that serovar hardjo infection was of approximately equal prevalence (York et al., 1957; Hanson et al., 1964*b*; Morter et al., 1967; Morter et al., 1968). Isolations of serovars canicola (Turner et al., 1958), hardjo (Roth and Galton, 1960), grippotyphosa (Hanson et al., 1964*a*), icterohaemorrhagiae (Schnurrenberger et al., 1970), szwajizak (Glosser et al., 1974), and balcanica (White et al., 1982) from cattle provided evidence that the etiology of bovine leptospirosis in the United States was complex. By 1971, the serovars canicola, grippotyphosa, hardjo, icterohaemorrhagiae, and pomona were recognized as important in

cattle (Hanson et al., 1971*b*). By 1975, hardjo was recognized as the most prevalent serovar in United States cattle (Stoenner and Glosser, 1975).

Clinical manifestations

Investigators disagree as to the classification of clinical features of bovine leptospirosis, but most agree that the disease may vary from clinically inapparent to a severe icterohaemorrhagic syndrome. According to Freund (1947) bovine leptospirosis may occur in three clinical forms. The peracute form is seen mainly in calves and is characterized by a sudden onset, anorexia, high fever, hemoglobinuria, icterus, uremia, and possibly death in 3 to 5 days. Acute and subacute forms have a slower onset, last about 2 weeks, are characterized by nephritis, mastitis, and reduced milk production, are often followed by a slow recovery, and are usually seen in older cattle and lactating cows. In the chronic and recurrent form, abortions, retained placentas, and breeding difficulties may be seen or there may be no apparent clinical signs. Amatredjo and Campbell (1975) classified the clinical forms as acute, subacute, and chronic, observed that abortions may occur in the acute stage as well as in the chronic stage, and added stillbirths and weak calves to the list of chronic signs. Mazzonelli (1984) divided the clinical patterns into five categories: peracute, characterized by a sudden onset, icterus, hemoglobinuria, and death within a few hours; acute, seen more frequently in calves, and characterized by icterus, hemoglobinuria, leucopenia, neurological signs, abortions, a course of 2 to 10 days, and possibly death; subacute, occurring mainly in adult cattle, with signs similar to the acute form but less severe, lasting 2 to

7 days, with abortions less common than in the acute form; chronic, resulting from acute or subacute forms, persisting for months with recurring icterus and hemoglobinuria, weight loss, and reduced milk production, with a few deaths occurring 7 to 8 months after the onset; and occult disease, the most common form in endemic areas, characterized by mild or unrecognizable clinical signs, possibly some febrile episodes, frequently diagnosed only by serology, and having abortion as the only constant feature.

Clinical signs in cattle may vary with the serovars involved and with the degree of herd susceptibility. Distinct differences may exist between leptospiroses caused by serovar hardjo and those caused by serovars pomona and grippotyphosa (Hanson, 1984). Although morbidity in hardjo outbreaks may be high, mortality in adults is rare (Thiermann, 1980). Two clinical conditions have been reported in cattle infected with hardjo: an agalactia of sudden onset associated with bacteremia, and abortion which occurs as a sequela to a chronic infection. In dairy herds with endemic hardjo infection, new clinical cases may be seen only in replacement heifers (Ellis et al., 1985c). The severe signs of acute leptospirosis are often associated with pomona and grippotyphosa infections. Serovar pomona, which has swine as a reservoir, usually produces a more severe infection of short duration while hardjo, which has cattle as its natural reservoir, produces infections which are less severe but persist longer (Hanson, 1984). Mortality in pomona epidemics in some herds has reached 10% (Stoenner, 1976). The high prevalence of hardjo infections and the lower prevalence of pomona infections in some areas suggests that hardjo infection is endemic and

pomona infection is sporadic in cattle (Bahaman et al., 1984). Epidemics in cattle caused by grippotyphosa have been reported in Russia, Israel, and Hungary, and have been associated with high mortality in calves (Michna, 1970).

Leptospires usually produce their major effects on the kidneys, causing acute nephritis followed by chronic nephritis that may persist for months or years (Hanson, 1976*a*). Acute nephritis results from penetration of leptospires through interstitial spaces into the glomeruli and proximal tubules and is characterized by edema, hemorrhages, and cellular necrosis. The lumen of the proximal convoluted tubules is the primary site of multiplication of leptospires in the kidney. Extensive interstitial nephritis may result if the disease progresses (Hanson, 1976*b*). The kidneys may be grossly enlarged and uremia may develop in the terminal stages (Michna, 1970). Hemorrhagic nephritis resulting in hematuria and anemia has been observed in some outbreaks (Hanson, 1976*b*). When only scattered renal lesions develop, no clinical signs are apparent (Hanson, 1984). A host-parasite equilibrium may be established with little effect on the kidney or renal function (Michna, 1970). Chronic infections are characterized by interstitial nephritis with marked infiltration of lymphocytes, plasma cells, and fibrous connective tissue (Hanson, 1976*a*). Chronic changes appear to result from hypersensitivity reactions (Hanson, 1976*b*).

The immune response may be effective in controlling the acute disease or may contribute to the development of chronic lesions (Hanson, 1976*a*). IgM antibodies against serovars hardjo and pomona in cattle have been

detected within 3 to 8 days and peaked at 10 to 20 days post-inoculation (Fairbrother, 1985*a*). The initial antibody response leads to a reduction in the level of leptospiremia, localization of leptospires in the kidney tissue, and the subsequent leptospiruria (Hanson, 1976*a*; Michna, 1970). In relapses, leptospires have been detected in the blood simultaneously with significant levels of antibodies (Taylor et al., 1970). IgG antibodies appear later but persist for longer periods than IgM (Negi et al., 1971*a*). Fairbrother (1985*a*) found that IgG against serovars hardjo and pomona was the predominant class of antibodies detectable at 35 to 80 days post-inoculation, and the peak total antibody level occurred at 10 to 30 days post-inoculation. The total antibody level against serovar hardjo remained relatively high for 240 days post-inoculation, but that against serovar pomona declined to a low level after 30 days and remained there until at least 63 days post-inoculation. Antibodies have been demonstrated in naturally infected cattle for months after infection (Michna, 1970), and as long as 6 years in one herd infected with hardjo (Hanson et al., 1972). The immune response has been associated with adverse inflammatory and degenerative reactions in chronic infections and may contribute to the development of chronic nephritis and ocular lesions (Hanson, 1976*a*).

Abortions and stillbirths are the signs most frequently recognized in bovine leptospirosis (Hanson, 1976*b*). Stalheim (1976) observed that 20 to 40% of cattle infected in the last trimester of pregnancy may abort. Ellis et al. (1985*c*) diagnosed leptospira infection, which was almost entirely hardjo, in 57% of 505 calves in Ireland which included 472 aborted fetuses,

20 stillbirths, and 13 perinatal deaths. Higgins et al. (1981) studied 92 bovine abortions in Canada and associated leptospirosis with 28% of them. Although abortions may be preceded by icterus, hemoglobinuria, anemia, agalactia, or fever, they frequently occur without premonitory signs. Abortions are most common in the last trimester of pregnancy, but may occur at any stage of gestation. Placentitis occurs rarely, and the fetus may have icterus, interstitial nephritis, or a mild suppurative pneumonia. The fetus may be born alive and weak, soon after death, or may be retained up to 72 hours after death (Kirkbride, 1984). Periods between fetal death and expulsion in experimental infections have ranged from 16 to 30 days (Mazzonelli, 1984).

Leptospira infection of the bovine udder produces a characteristic clinical syndrome. The udder becomes flaccid and the milk may be thickened, yellow, and blood-tinged. Although leptospires can be isolated from the milk, the so-called "cold mastitis" is noninflammatory. The lesions are primarily degenerative with only minor infiltration of lymphocytes and plasma cells. An agalactia which may be severe follows the mastitis and may persist for 1 or 2 weeks (Hanson, 1984). The occurrence of mastitis in leptospirosis outbreaks varies considerably and prevalences of 5% (Ellis et al., 1985c), 10% (Durfee and Allen, 1980), 15% (Hathaway and Little, 1983) and 50% (Higgins et al., 1980) have been reported. Infection with hardjo may result in a sudden decrease in milk production with uniformly flaccid udders (Durfee and Allen, 1980). Unexplained low milk production and

agalactia without typical signs of leptospira mastitis may occur in some herds with hardjo infection (Higgins et al., 1980).

Abortions, stillbirths, decreased milk production, and death cause obvious losses in cattle, but losses may also result from less apparent or less frequent problems. Infertility has been associated with various serovars (Michna, 1970), but more commonly with hardjo and szwajizak. Retained placentas commonly occur following abortions and may contribute to infertility problems which result from chronic low grade infections. Decreased weight gain may be seen in calves (Hanson, 1984). Meningitis (Hoag and Bell, 1954*a*; Stoenner et al., 1963), ocular lesions (Hoag and Bell, 1954*b*; Hanson, 1976*a*), orchitis, lymphadenitis, pneumonia, enteritis, peritonitis, skin necrosis (Michna, 1970), necrosis of ear tips, photosensitization, stomatitis, and diarrhea (Mazzonelli, 1984) may occur.

Prevention and control

Methods employed in the prevention and control of bovine leptospirosis fall into five categories: serological testing, vaccination, use of antibiotics, simultaneous vaccination and use of antibiotics, and carrier and environment control.

Stoenner (1976) suggested that breeding stock replacements should be seronegative and originate from herds shown to be free of leptospirosis on the basis of a complete herd test. Efforts to certify herds as being free of leptospirosis have been proposed and are based on the methods used for brucellosis eradication with modifications to fit the unique problems of

leptospirosis (Stalheim, 1976). Annual serological testing may be a useful method of estimating progress in a long term control program in which vaccination is primarily used for prevention (Amatredjo and Campbell, 1975). Serological testing may be of limited value in hardjo infections. Ellis et al. (1981) demonstrated titers below the commonly used screening titer of 100 in 46% of cattle proven to be renal carriers.

Since the recognition in the early 1950s that leptospirosis caused economic loss due to abortion, vaccination of cattle has been widely practiced (Hanson, 1973). Bacterins should contain the specific serovars affecting individual herds, since there appears to be little or no cross-protection among serovars (Amatredjo and Campbell, 1975; Thiermann, 1980). In the United States, bacterins usually contain serovars canicola, grippotyphosa, hardjo, icterohaemorrhagiae, and pomona. Vaccination of entire herds at 6- or 12-month intervals has been suggested as the most economical preventive procedure (Hanson, 1976*b*). Morter (1972) observed that the duration of immunity after vaccination may be limited to 6 months. In endemic herds where new additions are common, vaccination at 6-month intervals is suggested (Hanson, 1981). Vaccination of calves under 4 months of age is generally avoided since maternal antibodies transferred to calves in colostrum may interfere with development of active immunity (Hanson, 1976*b*). Adult cattle should be vaccinated to provide the greatest degree of protection in the last two-thirds of gestation, and feedlot cattle should be vaccinated and held in isolation for 2 weeks before being mixed with other cattle (Stoenner, 1976). McDonald and Rudge (1957) showed

that vaccination of dams in late pregnancy prevented infection of calves during the first month of life. Vaccination usually induces immunity which is adequate to prevent losses due to death, abortions, stillbirths, weak calves, and reduced milk production, but does not prevent infection, bacteremia, and leptospiruria (Hanson et al., 1972). However, vaccination appears to prevent leptospiruria in some cattle and shorten its duration in others (Mackintosh et al., 1980). Hancock et al. (1984) showed that only 2.7% of heifers vaccinated at 9 to 10 months of age developed leptospiruria after natural challenge compared to 58.5% of unvaccinated controls. Killinger et al. (1976) showed that renal tissue damage was less severe in vaccinated cattle challenged with pomona than in unvaccinated controls.

Elimination of leptospires from the kidneys of carrier cattle with penicillins, streptomycin, and tetracyclines has been attempted for many years and reports indicate conflicting results (Michna, 1970). Cameron (1977) found 14 New Zealand isolates highly susceptible to benzyl penicillin *in vitro*. Amatredjo and Campbell (1975) stated that large doses of penicillin early in the course of the disease may be required to be effective. However, Watt et al. (1988) demonstrated significant differences in human patients receiving penicillin late in the course of leptospirosis when compared to patients receiving placebos. After establishment of bovine renal infection, dihydrostreptomycin (DSM) appears to be the drug of choice (Amatredjo and Campbell, 1975). Broughton and Flack (1986) found amoxycillin and erythromycin much more effective than tetracycline or oxytetracycline against an icterohaemorrhagiae isolate *in vitro*. Prescott and

Nicholson (1988) demonstrated the susceptibility of 18 Canadian hardjo isolates to peniclin G, ampicillin, tetracycline, erythromycin, and streptomycin *in vitro*. Alexander and Rule (1986) demonstrated leptospires in surviving hamsters infected with bataviae which were treated with doxycycline, chlortetracycline, cyclacillin, and piperacillin, but not in those treated with ampicillin, bacampicillin, mezlocillin, cefotaxime, or moxalactam. Initial treatment of mature cattle with tetracycline followed by DSM has been shown to be somewhat successful in epidemics caused by pomona (Stoenner, 1976). Stalheim (1969) concluded that bovine renal leptospirosis could be successfully treated with one dose of DSM. However, Ellis et al. (1985*d*) demonstrated hardjo infections in the kidneys or genital tracts of heifers after one or two doses of DSM. Since neither negative serological status nor DSM treatment insure against the purchase of carrier cattle, Ellis et al. (1985*d*) suggested that a combination of the two methods may be the best approach currently available.

South and Stoenner (1974) reported that simultaneous vaccination of all cattle in a herd and treatment of pregnant cows with one injection of DSM were effective in controlling leptospirosis when the disease was diagnosed early in an outbreak. The rationale for this approach was that during the period that the cattle were protected by the antibiotic, active immunity was being stimulated by the vaccine. Vaccination without concurrent antibiotic therapy may be of questionable value in an outbreak, since assembling and confining cattle for vaccination may increase exposure to the urine of infected animals (South and Stoenner, 1974; Stalheim, 1976).

Stoenner (1976) suggested that feeder cattle which originate from infected herds should be vaccinated, treated with DSM, and segregated for 2 weeks before being mixed with other cattle. Endemic infections with hardjo may be more difficult to contain than pomona infections, but semi-annual vaccination along with a single treatment of cows with DSM during the dry period may be effective (Hanson, 1984).

Stoenner (1976) stated that control of leptospirosis is possible with proper herd management and consideration for the complex epidemiology of leptospirosis in developing a control program. Once cattle become infected, the disease may remain on the premises for years, and the elimination of carriers, especially rodents, is important (Michna, 1970). Cattle appear to be exclusive shedders of serovar hardjo, and the maintenance of closed herds could be expected to prevent the entry of hardjo into a herd. However, cattle, swine, skunks, raccoons, opossums, and dogs may be carriers of pomona and the maintenance of closed herds would not be expected to prevent entry of that serovar (Roberts, 1958; Stoenner, 1976). Other livestock, such as swine, sheep, and goats, should be considered as potential carriers and tested for leptospira antibodies (Hanson, 1981). Sanitary measures, especially the removal of animal secretions and wastes and disinfection of premises in confinement operations, appear to be valuable in eradicating leptospirosis (Michna, 1970). Direct sunlight, desiccation, chemical disinfectants, detergents, soaps, and high salinity are rapidly detrimental to leptospires. The organisms are destroyed within 5 minutes at 50 C (Michna, 1970). Leptospires may survive in diluted bovine urine

for 35 days and up to 6 months in soil saturated with urine. Bovine milk appears to be leptospiracidal within 3 days, but leptospires may survive in diluted milk up to 60 days. Diluted bull semen without antibiotics has been shown to support leptospires up to 60 days at 2 C (Michna, 1970). Diesch (1971) observed that pomona survived in cattle manure for 61 days during the summer and 18 days under winter conditions. Since leptospires may survive in surface water such as ponds and streams, either limited access or fencing cattle away from these sources completely may be necessary (Hanson, 1981).

Laboratory Diagnosis of Leptospirosis

Serological tests

For many years, the only accurate methods of diagnosing leptospirosis other than by clinical signs were isolation of leptospires from blood in acute cases or from urine several weeks after an acute attack, and demonstration of seroconversion or increasing titer by serological methods. Since culture techniques were considered impractical or impossible in the field, serological tests were the only practical aid to diagnosis (Roberts, 1958).

The agglutination-lysis (AL) test described by Schuffner and Mochtar (1926) which utilized live cultures as antigens, became the basis for many test procedures used for the detection of leptospira antibodies. The "lysis balls" were considered to be remnants of leptospires which had first agglutinated and then lysed, but were later recognized as clumps of

leptospires which had agglutinated very strongly (Lawrence, 1955; Babudieri, 1961). Since 1953, the AL test has been modified, simplified, and adapted to general diagnostic use (Roberts, 1958), and during this period the procedure became known as the microscopic agglutination test (MAT) (Galton et al., 1965). Wolff's (1954) modification of the AL tube test into a procedure conducted in porcelain plates gave results which were difficult to reproduce in some laboratories (Sulzer and Jones, 1973*a*). The MAT was modified into a microtechnique utilizing smaller quantities of reagents in disposable microtitration plates by Galton et al. (1965) and Sulzer and Jones (1973*a*). Other modifications have been suggested to aid in the reading and interpretation of the degree of agglutination (Carter and Ryan, 1975). In addition to serum, the MAT has been used for detection of leptospira antibodies in milk (Van Der Hoeden, 1955; Carbrey and Packer, 1961), whey (Hussaini, 1976), urine (Stuart, 1956), and semen (Lyle et al., 1972).

The MAT technique described by Cole et al. (1973) and refined by Cole et al. (1979) is the reference serological test for leptospirosis in animals (Ellinghausen, 1979; Diesch, 1980). The test procedure consists of reacting standardized antigens grown in liquid media (Ellinghausen and McCullough, 1965; Johnson and Harris, 1967*a*) with serum diluted in phosphate buffered saline (PBS) in microtitration plates. Reactions are read under a darkfield microscope and endpoints are determined as the greatest serum dilutions in which 50% of leptospires are agglutinated. Sera are usually screened at 1:50 or 1:100 dilutions and are titrated against

reacting antigens in serial two-fold dilutions from 1:100 through 1:12,800 (Higgins and Cayouette, 1978; National Veterinary Services Laboratories, 1987). MAT titers $\geq$ 100 are generally considered significant. Leptospira infection may be diagnosed when a majority of seropositive animals in a herd have titers $\geq$ 1000, or when paired samples show a four-fold rise or fall or a conversion from negative to positive (Cole et al., 1979).

The MAT detects both IgM and IgG immunoglobulin fractions (Morris and Hussaini, 1974; Fairbrother, 1985*a*). Treatment of sera with 2-mercapto-ethanol has been used to estimate the contribution of IgM to the agglutination reaction (Ellinghausen, 1979; Fairbrother, 1985*a*). Paradoxical and prozone reactions may occur, making interpretation of results difficult (Galton et al., 1958; Malkin, 1984). Malkin (1984) found that heating sera to 56 C for 10 minutes eliminated prozone reactions and increased the number of serological positives in sheep and goat sera by 9%. This finding indicated that complement may interfere with the agglutination reaction to some extent. Although the MAT has been described as being relatively serovar specific (Morris and Hussaini, 1974; Fairbrother, 1985*a*), cross-reactivity may be seen among serovars especially in sera with high homologous titers. There is also evidence that Campylobacter (Stoenner, 1954), Shigella (Rothstein, 1957), and Treponema spp. (Miller et al., 1989) have antigens in common with Leptospira serovars and may cross-react in the MAT. Heat-inactivated and formalized antigens tend to lose homogeneity during storage and commonly result in nonspecific reactions (Kelen and Labzoffsky,

1960). Titers with killed antigens are usually lower than with live antigens (Babudieri, 1961).

Agglutinating antibodies may persist for months after infection and may produce high titers in animals that have recovered and are no longer shedding (Hodges and Ris, 1974). In a study of a large herd of cattle by Roberts (1958), agglutinins were found to persist for years or even for the lifetime of some animals, and no animal with a high titer ever became serologically negative thereafter. The interval between clinical infection and abortion in hardjo infections may be 6 to 12 weeks, and the titer is frequently falling at the time of abortion (Ellis and Michna, 1977). Vaccination history must be considered when MAT results are interpreted (Cole et al., 1979). Most animals develop lower MAT titers after vaccination than after infection (Tripathy et al., 1976). However, some cattle may develop significant post-vaccination titers which are not distinguishable from titers produced by infection (Morter, 1980; Diesch, 1980; Ris and Hamel, 1982; Stringfellow et al., 1983). The MAT appears to be more accurate in the detection of infections due to serovar pomona than to hardjo. Elder et al. (1985) were able to show a correlation between high pomona MAT titers and abortions in cattle, but found no significant relationship between levels of hardjo titers and the occurrence of abortions. Ellis et al. (1981) found that 46% of hardjo carrier cattle had MAT titers < 100, and approximately 20% of them had no detectable titers. Thiermann (1983*a*) isolated hardjo and grippotyphosa from the kidneys of seronegative cattle. Kingscote (1985*b*) found hardjo infection of the kidneys and cerebrospinal

fluid to be independent of the presence or level of MAT titers in cattle.

Several macroscopic agglutination tests which utilize formalized antigens have been described. Stoenner (1953) described a capillary tube test which compared favorably in sensitivity and specificity with the AL test. Macroscopic tests have been conducted on plates (Hoag et al., 1953; Stoenner, 1954; Lepherd, 1969; Sandhu and White, 1972), in tubes (Howarth, 1956; Muraschi, 1958), on slides (Starbuck and Ward, 1942; Galton et al., 1958; Rao and Murthy, 1983), and on cards (Bragger and Adler, 1976). Muraschi (1958) described a latex agglutination test which was evaluated by Kelen and Labzoffsky (1960) utilizing antigens which were formalized, boiled, ultrasonicated, or pyridine-treated. Pooled antigens have been commonly used in the macroscopic tests for screening purposes (Muraschi, 1958; Galton et al., 1958; Lepherd, 1969; Bragger and Adler, 1976; Rao and Murthy, 1983). Although most investigators found macroscopic tests to be comparable to the AL test or MAT, Starbuck and Ward (1942) noted that the slide test was less sensitive, and Babudieri (1961) stated that macroscopic titers are usually 5 to 10 times lower than MAT titers. Cross reactions have also been observed with macroscopic tests (Stoenner, 1954; Howarth, 1956; Galton et al., 1958; Sandhu and White, 1972). Solarzano (1967) attributed an increased incidence of canicola and icterohaemorrhagiae reactions in bovine serum to over-sensitivity of plate antigens. Sandhu and White (1972) concluded that the plate test was unreliable for detection of canicola antibodies because of false positive reactions. Stoenner (1972) noted that hardjo plate antigen is weakly antigenic, and peak

antibody titers are usually < 40. Ellis et al. (1982*a*) observed that 31% of cattle which aborted hardjo-infected fetuses were serologically negative on the plate test, and concluded that the plate test has no value in testing sera from cases of hardjo abortion.

Complement fixation (CF) tests for leptospira antibodies were developed by Pot and Dornickx (1936) and Boerner and Lukens (1941), which eliminated the need for maintenance of live antigens used in the AL test. Randall et al. (1949) described a CF test utilizing sonicated cells which improved the sensitivity and specificity of the test. York (1952) and Robertson and Boulanger (1963) found the CF test useful for diagnosis of leptospirosis in cattle. The CF test may be more useful than agglutination tests for detection of recent infections. CF antibodies have been detected primarily during the period of maximum urinary shedding of pomona in pigs (Hodges, 1973). A closer association with the duration of leptospiruria in calves was found with CF antibodies than with agglutinating antibodies (Hodges and Ris, 1974). Screening bovine sera for a variety of serovars in a single CF test with polyvalent antigen has been suggested (Hodges, 1974). A semi-automated CF procedure for testing large numbers of serum samples has been described (Hodges and Weddell, 1977) which compared favorably with the MAT in the detection of infected cattle (Hodges and Carter, 1979). Like the agglutination tests, cross-reactivity among serovars has been observed with the CF test (Hodges, 1973; Hodges and Ris, 1974).

In their evaluation of an indirect immunofluorescence (IF) test, Sandhu and White (1972) observed good correlation with the MAT and

suggested the IF as a screening test for pomona antibodies in bovine serum. The indirect hemagglutination (IHA) test described by Cox (1955) detects primarily IgM and may be useful in the diagnosis of recent infections (Morris et al., 1977). The hemolytic (HL) test described by Cox (1955) appears to be useful in diagnosing human infections, but lacks the sensitivity necessary for detection of antibodies in animal sera. The hemagglutination (HA) test (Negi et al., 1971*a*) also appears to detect IgM and may be more sensitive than the MAT (Negi et al., 1971*b*). The passive microcapsule agglutination (MCA) test described by Arimitsu et al. (1982) also appears to be more sensitive than the MAT, but is less specific.

Tests employed for the detection of infection may not be sufficient for the detection of protective antibodies produced post-vaccination (Tripathy et al., 1971). The passive hemagglutination (PHA) test described by Sulzer and Jones (1973*b*) did not detect significant differences between vaccinated gilts and controls (Dawe et al., 1976). An *in vitro* growth inhibition test, also referred to as a neutralization test, described by Tripathy et al. (1971) appeared to be more sensitive than other serological tests in gilts (Dawe et al., 1976). The hamster passive protection test (Huhn et al., 1970) and the leptospiracidal activity (LA) test (Johnson and Muschel, 1966; Johnson and Harris, 1967*b*; Bey and Johnson, 1978*a*) have been found effective in evaluating the protection afforded by bacterins (Huhn et al., 1975; Bey and Johnson, 1978*b*). The LA test appeared to be more sensitive for detecting antibodies against canicola and icterohaemorrhagiae than the MAT, but little difference in sensitivities was observed for

pomona and grippotyphosa antibodies. Both the hamster passive protection test and the LA test appear to be more sensitive than the MAT for detection of antibodies against hardjo (Bey and Johnson, 1978*b*).

Several enzyme-linked immunosorbent assay (ELISA) procedures for detection of leptospira antibodies have been described. Antigens used in ELISA have been whole cells (Cursons and Pyke, 1981; Fairbrother, 1984; Takase and Yanagawa, 1988), sonicated cells (Adler et al., 1980; Adler et al., 1981; Waltman and Dawe, 1983; Fairbrother, 1984; Banfi et al., 1984; Cousins et al., 1985; Milner et al., 1985), formalized and sonicated cells (Fairbrother, 1984), ethanol extracts (Fairbrother, 1984; Pappas et al., 1985), heat extracts (Terpstra et al., 1985), alkali extracts (Fairbrother, 1984), desoxycholate extracts (Kirkbride and Halley, 1982), phenol extracts (Thiermann and Garrett, 1983; Thiermann, 1983*b*), outer envelope proteins (Hartman et al., 1984*a*), and sodium dodecyl sulfate (SDS) extracts (Biancifiori and Cardaras, 1983; Hartman et al., 1984*a*). Reactions have been read visually (Kirkbride and Halley, 1982; Hartman et al., 1984*b*; Pappas et al., 1985) and photometrically (Thiermann and Garrett, 1983; Biancifiori and Cardaras, 1983; Milner et al., 1985). Antigen quality appears to be a critical factor in the ELISA (Banfi et al., 1984; Terpstra et al., 1985).

Although the ELISA appears to measure a different antigen-antibody system (Adler et al., 1981; Adler et al., 1982; Biancifiori and Cardaras, 1983; Ballard et al., 1984; Milner et al., 1985), it has frequently been compared to the MAT. The ELISA has the advantages of the use of killed

antigens, objective reading of results, measurement of both IgM and IgG responses (Cousins et al., 1985), and automated reading and analysis of results with a computer (Milner et al., 1985). The IgM-specific ELISA appears to be more useful than the MAT for detection of recent infections (Adler et al., 1982; Ballard et al., 1984; Cousins et al., 1985; Terpstra et al., 1985). Adler et al. (1982) suggested that a positive IgM-ELISA indicated infection within the previous month in cattle. The ELISA has been reported to be more sensitive than the MAT (Adler et al., 1980; Thiermann and Garrett, 1983; Biancifiori and Cardaras, 1983), and may detect an IgM response earlier than the MAT (Hartman et al., 1984*b*; Mailloux et al., 1984; Milner et al., 1985). However, some infected animals have been found positive with the MAT but negative with the ELISA (Adler et al., 1981; Thiermann and Garrett, 1983). Cousins et al. (1985) observed that ELISA results did not correlate any better than MAT results with leptospiruria in cattle. Urinary antibodies have been detected in swine with the MAT when the ELISA was negative (Ballard et al., 1984). False positive reactions may also occur with the ELISA (Adler et al., 1981; Waltman and Dawe, 1983; Hartman et al., 1984*b*; Pappas et al., 1985). Cross-reactivity among Leptospira serovars (Adler et al., 1980; Cursons and Pyke, 1981; Kirkbride and Halley, 1982; Thiermann and Garrett, 1983; Thiermann, 1983*b*; Terpstra et al., 1985) and with other genera (Pappas et al., 1985) have been observed. The degree of cross-reactivity appears to depend upon the method of antigen preparation (Hartman et al., 1984*a*). Difficulties in distinguishing between post-infection and post-vaccination immune responses

have also been experienced with the ELISA (Thiermann and Garrett, 1983; Hartman et al., 1984*a*; Thiermann et al., 1984).

Schonberg et al. (1980) evaluated a sensitivity test for the diagnosis of leptospirosis by injection of leptospirin intradermally into humans, cattle, goats, pigs, horses, and dogs. The leptospirin contained antigens of serovars canicola, grippotyphosa, icterohaemorrhagiae, pomona, and tarassovi. Although the test results correlated well with MAT results in humans, a few false positive reactions and many false negative reactions were observed in animals.

Isolation

Isolation of leptospires has been described as essential for an unequivocal diagnosis of leptospirosis (Nervig and Garrett, 1979; Everard et al., 1980; Thiermann et al., 1984). The recovery and identification of isolates is necessary for diagnosis, epidemiological studies, selection of bacterins for prophylaxis, and selection of antigens for serological tests (Everard et al., 1980; Thiermann et al., 1984; Kingscote, 1985*a*; Ellis and Thiermann, 1986). Isolation has not been commonly used as a diagnostic method due to the difficulties in media preparation, the low percentage of isolates recovered from suspected cases of leptospirosis, the cost of culturing large numbers of specimens, and the delay between collection of field specimens and receipt in laboratories (Kirkbride and Halley, 1982; Thiermann et al., 1984; Palmer et al., 1984; DeLange et al., 1987). In recent years, the likelihood of isolating leptospires from clinical or post mortem specimens has been increased by the development of better quality

media (Ellinghausen and McCullough, 1965; Johnson and Harris, 1967*a*; Palmer et al., 1984; Ellis et al., 1985*d*), the commercial availability of media concentrates (Thiermann et al., 1984), and improvements in culture techniques (Orr and Little, 1979; Thiermann et al., 1984; Palmer et al., 1984; Kingscote, 1985*a*; Ellis et al., 1985*d*).

Leptospires can be cultivated in liquid media (Wolff, 1954), semisolid media containing 0.2 to 0.5% agar (Alston and Broom, 1958; Turner, 1970), and soft agar plate media often referred to as solid media containing approximately 1.0% agar (Cox and Larson, 1957; Turner, 1970). Gellan gum has been successfully used as a replacement for agar (Rule and Alexander, 1986). Since the growth of leptospires in semisolid media is more rapid, reaches a greater density, and is sustained longer than in liquid media, the semisolid media are preferred for isolation. Although the exact mechanism by which agar increases the multiplication of leptospires is unknown, it has been suggested that agar may absorb leptospira metabolites or inhibitory substances in the inoculum (Turner, 1970). The motility of leptospires has been shown to be more rapid and more efficient in viscous environments than in water (Berg and Turner, 1979). Solid media are frequently used for isolation of leptospires from contaminated specimens and for recovering leptospires from contaminated cultures (Roth et al., 1961; Turner, 1970). Thiermann (1981*b*) found solid media superior to semisolid media for isolation of leptospires of the Hebdomadis group from milk, but noted that more isolates from urine were obtained with the semisolid form. Solid media have not been routinely used because the solid form tends to be less

reliable and the greater volume of solid media used in plates is more expensive than semisolid media in tubes (Turner, 1970).

Serum was used as an enrichment factor in the semisolid media of Vervoort, Chang, Korthof, Fletcher, Noguchi, Stuart, and Cox (Cox and Larson, 1957; Roth et al., 1961; Turner, 1970). Sera from horses, cattle, bovine fetuses, sheep, swine, and guinea pigs have been used, but use of rabbit serum has been most successful (Turner, 1970). Rabbit serum may have undesirable properties such as the presence of specific or nonspecific antibodies (Turner, 1970) and inconsistent physical and nutritional qualities (Johnson and Sieter, 1976). Formation of a film on the surface of media, formation of particulate matter from lipids (Turner, 1970), denaturation and subsequent precipitation of globulins upon aeration (Johnson and Sieter, 1976), and difficulties in sterilization (Bey and Johnson, 1978*c*) have been observed with media containing serum.

Ellinghausen-McCullough (EM) medium containing bovine serum albumin (BSA) as a substitute for rabbit serum and polysorbate 80 as a source of fatty acids (Ellinghausen and McCullough, 1965) and modifications of this medium referred to as EMJH (Johnson and Harris, 1967*a*; Johnson et al., 1973) have been widely used for isolation of leptospires (Johnson and Sieter, 1976; Schotts, 1976; Everard et al., 1980; Thiermann et al., 1984). The shelf life of the polysorbate 80-bovine albumin (P80-BA) media has been shown to be at least 36 months, and may be as long as 5 years at 25 C (Ellinghausen, 1975). The antigenicity, immunogenicity, and virulence of leptospires cultivated in P80-BA media do not differ from those grown in

media containing serum (Johnson and Sieter, 1976). Leptospires have been isolated in P80-BA media which could not be isolated in media containing serum (Turner, 1970; Johnson and Sieter, 1976; Orr and Little, 1979; Herr et al., 1982). Conversely, leptospires have been isolated in P80-BA media with rabbit serum that could not be isolated in the conventional P80-BA media (Tripathy et al., 1980; Ellis et al., 1982*b*; TeBrugge and Louw, 1985). Ellis et al. (1982*b*) achieved a significantly higher number of hardjo isolates from cattle with freshly prepared EMJH medium as compared to Stuart's, Korthof's, Fletcher's, or commercially prepared EMJH media. The T80/40/LH medium described by Ellis et al. (1985*d*) contains both polysorbate 80 and polysorbate 40, lactalbumin hydrolysate, superoxide dismutase, and rabbit serum in addition to the basic ingredients in P80-BA media. Leptospires have been isolated in T80/40/LH medium which could not be isolated in EMJH medium (Ellis and Thiermann, 1986).

Several serum-free, chemically defined, synthetic media have been described (Vogel and Hutner, 1961; Shenberg, 1967; Van Eseltine et al., 1967; Bey and Johnson, 1978*c*). Shenberg (1967) was successful in serially subculturing 52 strains belonging to 12 serogroups in a protein-free medium, but noted some difficulty in maintaining growth of ballum, javanica, and tarassovi strains. Bey and Johnson (1978*c*) evaluated a protein-free medium with serial subcultures of five serovars and observed that difficulties in growing grippotyphosa could be overcome by addition of 0.1% BSA to the medium. Schonberg (1983) evaluated protein-free media containing ingredients used in both the media of Shenberg (1967) and Bey and Johnson

(1978*c*), and observed that the growth of grippotyphosa and tarassovi was not satisfactory. Mazzonelli et al. (1984) also observed difficulties in growing grippotyphosa and tarassovi in the medium of Bey and Johnson (1978*c*) and in modifications of it.

Turner (1970) observed that different strains of the same serovar and different serovars have different nutritional requirements. Since there is no standard medium which can provide optimal requirements for all strains, the use of at least two different types of media will increase the likelihood of isolating leptospires from clinical or post mortem specimens (Ellis et al., 1982*b*; Alexander, 1985; Kingscote, 1985*a*; TeBrugge and Louw, 1985; Ellis and Thiermann, 1986).

Contamination with other microorganisms is likely to reduce the chances of isolating leptospires by direct culture due to overgrowth of contaminants or production of substances lethal to leptospires (Turner, 1970), and is a major problem when attempts are made to culture urine or aborted bovine fetal tissues (Adler et al., 1986). In some cases, contaminants seem to have little adverse effect while growing along with leptospires (Turner, 1970), but interfere with the subsequent identification of isolates by serological or restriction enzyme methods (Babudieri, 1971; Marshall et al., 1981). Sulfadiazine, sulfaguanidine, sulfanilamide, dihydrostreptomycin, chloramphenicol, sulfathiazole, neomycin sulfate, actidione (Cousineau and McKiel, 1961), 5-fluorouracil (5-FU) (Johnson and Rogers, 1964), furazolidone (Myers, 1975), naladixic acid (Ellis et al., 1985*d*), fosfomycin (Oie et al., 1986), bacitracin, polymyxin B, and

rifampicin (Adler et al., 1986) have been used either singly or in various combinations in efforts to develope selective media. Although the growth of some strains of leptospires has been slowed or inhibited by 5-FU (Ris, 1974; Alexander, 1985), it has been commonly used in media for primary isolation of leptospires (Turner, 1970; Schotts, 1976; Johnson and Sieter, 1976; Ris and Hamel, 1978; Thiermann, 1984). Ingredients in Fletcher's medium have been found to annul the bacteriostatic action of 5-FU (Ris, 1974; Kingscote, 1985*a*). Some specimens may have a level of contamination beyond the ability of 5-FU to control (Thiermann et al., 1984; Kingscote, 1985*a*). Ris (1974) found that more contaminants survive in media containing 5-FU when incubated at 20 C than at 30 C. The combination of 5-FU and naladixic acid has been successfully used in T80/40/LH medium (Ellis et al., 1985*d*; Ellis and Thiermann, 1986). No inhibitor has been found which would prevent the overgrowth of Pseudomonas spp. while allowing growth of leptospires (Adler et al., 1986; Oie et al., 1986).

The isolation of leptospires from animal tissues and fluids requires a medium in which growth from a small inoculum can be effectively initiated (Johnson and Sieter, 1976). Fastidious serovars such as hardjo have been found to be extremely demanding of high quality media (Ellinghausen, 1975). Of the ingredients in the P80-BA media, the quality of BSA has been found to be most critical (Ellinghausen, 1975; Thiermann and Ellinghausen, 1981). Thiermann and Ellinghausen (1981) evaluated five lots of BSA from commercial sources and found that only two lots supported growth of a recently isolated hardjo strain, although all five lots supported the growth of

laboratory adapted strains. Ellinghausen (1982) found that the effect of BSA quality was not apparent when $\geq 2 \times 10^6$ leptospires/ml were used as inocula, but became a limiting factor when lower numbers of leptospires were used. Ris and Hamel (1978) tested media with serial ten-fold dilutions of pomona and found that growth occurred in all cultures inoculated with $\geq$ 10 leptospires and in two of three cultures inoculated with 1 leptospire. Thiermann and Ellinghausen (1981) suggested that acceptable quality media should be capable of growing a culture from 100 cells of a recently isolated field strain of leptospires within 60 days.

Leptospires have been isolated from kidney (Orr and Little, 1979), liver, spleen, brain (Turner, 1970), lung (Hathaway and Little, 1983), eye (Ellis et al., 1982*b*), renal lymph node (DeLange et al., 1987), urethra, seminal vesicles, bulbo-urethral gland, prostate, testes, placenta (Ellis et al., 1985*a*), oviduct, uterus, vagina (Ellis et al., 1985*b*), blood (Turner, 1970), cerebrospinal fluid (CSF) (Kingscote, 1985*a*), milk (Higgins et al., 1980), urine (Ris and Hamel, 1978), aqueous humor (Thiermann, 1984), vitreous humor (Ryan et al., 1977), amniotic tissue, placentomes, fetal pleural fluid, peritoneal fluid, stomach contents, liver, kidney, and blood (Smith et al., 1970). From live animals, specimens for isolation are limited to blood, urine, milk, and CSF. In the leptospiremic phase, blood and vascular organs such as liver, spleen, and kidney, and CSF are specimens of choice (Turner, 1970). Since leptospires are often present in the blood only during the first 7 to 10 days after the onset of infection, and the acute stage of the disease is frequently inapparent in animals, blood

cultures are not usually successful (Turner, 1970; Schotts, 1976; Alexander, 1985). Culturing CSF may have value since leptospires have been isolated from CSF in chronic infections (Kingscote, 1985*b*). During the leptospiruric phase, kidney tissue and urine appear to be the most productive specimens (Turner, 1970). Leptospires have been isolated from bovine urine collected after an intravenous injection of furosemide that could not be isolated from urine collected by manual stimulation (Nervig and Garrett, 1979). A decrease in the osmolarity of urine leading to increased survival of leptospires (Nervig and Garrett, 1979) or an effect on the kidney characterized by flushing of leptospires from the tubules (Schnurrenberger et al., 1970) have been suggested as reasons for success in culturing urine from cattle treated with diuretics.

Various culture techniques have been successfully used to isolate leptospires. Fluids have been inoculated directly into multiple tubes of media or serial dilutions of the fluid made in buffered saline or 1% BSA diluent have been used as inocula (Schotts, 1976; Sulzer and Jones, 1980; Kingscote, 1985*b*). Urine has been cultured directly (Sulzer and Jones, 1980) or the sediment obtained after centrifugation has been cultured (Kingscote, 1985*a*). Small sections of tissue (Ris and Hamel, 1978), tissue scrapings (Schotts, 1976), or larger blocks of tissue (Thiermann, 1984) have been used to prepare inocula. In most cases, tissues have been ground or homogenized in some physiological diluent and serial ten-fold dilutions of the suspension inoculated into multiple tubes of media (Schotts, 1976; Orr and Little, 1979; Sulzer and Jones, 1980; Ellis et al., 1981).

Buffered saline (Sulzer and Jones, 1980), liquid culture media (Orr and Little, 1979), and 1% BSA solution (Ellis et al., 1981) have been used as diluents. One percent BSA solution has also been used as a transport medium for shipment of both fluid and tissue specimens from field locations to laboratories (Flint and Liardet, 1980; Ellinghausen and Painter, 1976) and appears to have a protective effect on leptospires (Ellinghausen, 1973).

Dilution of specimens prior to culturing has been used to dilute substances which may be toxic to leptospires. Stalheim (1965) showed that crude lipids, phospholipids, triglycerides, and fatty acids in homogenized renal tissue or whole milk rapidly lysed the serovar pomona. The destruction of leptospires by urine and products of tissue autolysis was demonstrated by Fairbrother (1985*b*) who observed that the rate of destruction proceeded more rapidly during storage at 20 C than at 4 C. Turner (1970) suggested that inocula should be made in small volumes or diluted in order to avoid the inhibitory effects of antibodies present and the anaerobic culture conditions created by autolyzing tissues. Flint and Liardet (1980) proposed the dilution of urine in a medium containing 5-FU to decrease the toxic effect of urine on leptospires and to control contaminant bacterial growth. The control of contaminants with extensive dilution and use of 5-FU was also suggested by Sulzer and Jones (1980), Thiermann (1984), and DeLange et al. (1987).

Cultures should be incubated at 28 to 30 C in a dark area for 5 to 6 weeks or more (Sulzer and Jones, 1980). Leptospires have been detected

within 6 to 14 days of incubation (Alexander, 1985), but periods of 19 to 21 days (Higgins et al., 1980), 28 to 131 days (Te Brugge and Louw, 1985), and 1 to 26 weeks (Ellis et al., 1985*d*) have been reported. Cultures have usually been examined by darkfield microscopy at weekly or biweekly intervals (Ellis et al., 1985*d*; Ellis and Thiermann, 1986) for the presence of leptospires.

Inoculation of clinical or post mortem specimens into laboratory animals has been used for indirect isolation of leptospires. Although guinea pigs, chicks, and gerbils have been sucessfully used, the weanling hamster appears to be the animal of choice (Sulzer and Jones, 1980). Following intraperitoneal inoculation, blood withdrawn from the heart at 3- to 4-day intervals from day 4 through day 20 may be inoculated into semisolid media (Alexander, 1985). Alternatively, the urine, kidney, liver, or brain may be cultured when the animal shows a rise in temperature or at 21 to 28 days post-inoculation (Sulzer and Jones, 1980). Leptospires have been recovered from contaminated cultures by culturing heart blood drawn from hamsters 10 to 15 minutes after intraperitoneal inoculation (Sulzer and Jones, 1980; Alexander, 1985). Ris and Hamel (1978) examined urine specimens from infected cattle with darkfield microscopy, hamster inoculation, and culture methods and concluded that hamster inoculation was the least sensitive method for demonstration of leptospires.

Fluorescent antibody tests

Detection of leptospires in animal tissues and fluids with fluorescent antibody (FA) conjugates has been attempted for many years with

investigators reporting varying degrees of success. Sheldon (1953) demonstrated the serovar icterohaemorrhagiae in human muscle and concluded that the muscle lesions observed in Weil's disease were caused by leptospires. Moulton and Howarth (1957) demonstrated canicola in experimentally infected hamster kidneys and showed that fluorescence could be blocked by treatment of the tissue with unlabeled leptospira antiserum prior to staining with an FA conjugate. White and Ristic (1959) demonstrated pomona with an FA test in the urine of experimentally infected guinea pigs and cattle. Leptospires were observed by immunofluorescence in the urine of 14 of 17 (82%) naturally infected dogs from which canicola and icterohaemorrhagiae were subsequently isolated (White et al., 1961). Boulanger and Robertson (1961) found leptospires in 2 of 19 (11%) swine kidneys from which pomona was isolated. Ellis et al. (1985*b*) demonstrated hardjo infection by FA in 10 of 12 (83%) calves from experimentally infected heifers. Miller et al. (1989) demonstrated leptospires with an FA test in 9 of 21 (43%) naturally infected bovine kidneys from which hardjo, grippotyphosa, and pomona were isolated. Both the direct (Kirkbride and Halley, 1982; Ellis et al., 1982*b*) and the indirect (Cook et al., 1972) FA tests have been successfully used to demonstrate leptospires.

Various methods of antiserum and conjugate preparation have been described. Single serovar antisera (Maestrone, 1963*a*; Smith et al., 1966; Kirkbride and Halley, 1982), antisera against multiple serovars (Boulanger and Robertson, 1961), pooled single serovar antisera (Miller et al., 1989), and monoclonal antibodies (Stevens et al., 1985; Ainsworth et al., 1985)

have been labeled with fluorescein isothiocyanate. Whole globulin preparations (Hodges and Ekdahl, 1973) and IgG fractions (Hookey et al., 1987) have been used to prepare conjugates. To eliminate nonspecific fluorescence, absorption of conjugates with various proteins (Maestrone, 1963*a*; Coffin and Maestrone, 1962; Kirkbride and Halley, 1982), and counterstaining with nonspecific rhodamine conjugates (Smith et al., 1966), eriochrome black (Smith et al., 1967), Evan's blue, Congo red (Cook et al., 1972), and flazo-orange (Miller et al., 1989) have been used.

Fluids and tissue homogenates (Smith et al., 1966), tissue scrapings (Cook et al., 1971), sediments obtained by centrifugation (Boulanger and Robertson, 1961), frozen tissue sections (Sheldon, 1953), tissue impressions (Kirkbride and Halley, 1982), and formalin-fixed specimens (Cook et al., 1972) have been used in FA tests. Maestrone (1963*b*) suggested that formalinized specimens were suitable for the FA test if they were treated with ammonium hydroxide or sodium bisulphite. The use of an incident-light rather than a transmitted-light fluorescent microscope has been suggested as a critical factor in leptospira FA tests (Kirkbride and Halley, 1982; Ellis et al., 1982*b*).

Smith (1973) suggested that the detection of leptospires with the FA test depends on the presence of a relatively large number of leptospires in specimens. Although the FA test does not appear as sensitive as culture techniques (Boulanger and Robertson, 1961; Miller et al., 1989), advantages of the FA test include screening large numbers of specimens in a relatively

short time (Hookey et al., 1987) and detection of non-viable leptospires in autolyzing tissues and contaminated specimens (Smith et al., 1967).

Other diagnostic methods

Leptospires may be detected by darkfield microscopic examination of fluids and tissue homogenates. The routine darkfield examination of blood is not recommended due to the low numbers present (Faine, 1982) and the possibility of mistaking cellular fibrils and fibrin strands for leptospires (Schotts, 1976; Smith et al., 1979; Faine, 1982). Schotts (1976) suggested that the technique is most useful for examination of specimens which contain high concentrations of leptospires, such as peritoneal fluid or tissue homogenates from animals used for indirect isolation. Podgwaite et al. (1955) observed leptospires in peritoneal and thoracic fluids from aborted bovine fetuses infected with pomona. Hoare and Claxton (1972) observed leptospires in centrifuged urine samples from 10 of 14 (71%) cattle which had recently shown clinical signs of leptospirosis. Ris and Hamel (1978) examined 46 urine samples from infected cattle and detected leptospires in 28 (61%) by darkfield examination and 35 (76%) by culture methods. Cordes et al. (1982) and Herr et al. (1982) also reported higher percentages of bovine urine samples positive with culture methods than with darkfield examinations. However, Kingscote (1985*b*) reported a higher rate of detection of leptospires in bovine kidney tissue homogenates and cerebrospinal fluid with darkfield examination than with culture methods. Schotts (1976) stated that the failure to detect leptospires by darkfield

examination does not rule out their presence and a diagnosis based on darkfield examination alone should not be considered confirmatory.

Several staining techniques have been used to demonstrate leptospires in tissue specimens. The more commonly used techniques involve silver impregnation or deposition on tissues and may be valuable in situations where cultural or serological procedures are not possible or have proven ineffective (Schotts, 1976). Bridges (1958) successfully used a modified Warthin-Starry silver stain to demonstrate leptospires in fetal membranes and aborted bovine fetuses. Shive et al. (1969) demonstrated leptospires in kidney and liver tissues with a Warthin-Starry stain in three fatal cases of icterohaemorrhagiae infection in Barbary Apes. Smith et al. (1970) demonstrated leptospires in the spleen, liver, kidney, and placentomes of infected ovine fetuses with a Warthin-Starry technique. Ellis and Michna (1977) demonstrated leptospires in the kidneys and cotyledons of infected cattle with Faine's (1965) silver staining technique. Slee and Skilbeck (1983) demonstrated leptospires with a Levaditi silver stain in the liver, kidney, intestine, and heart of bovine fetuses experimentally infected with hardjo. Elliott (1988) observed that silver stains of leptospires in paraffin sections often lacked specificity, it was not often possible to find well stained leptospires, and some serovars were more difficult to demonstrate than others. Congo red and Giemsa stains have also been used to demonstrate leptospires (Babudieri, 1961). Herr et al. (1982) concluded that darkfield examination of wet smears was a more sensitive method for detection of leptospires in urine than Giemsa-stained smears.

However, the detection rates reported by Ris and Hamel (1978) were equal for unstained and Giemsa-stained preparations of urine. Schotts (1976) observed that staining has the same limitations as darkfield microscopy in that relatively high numbers of leptospires are required.

Several techniques utilizing the hybridization of labeled DNA and genomic DNA of leptospires have been described. Millar et al. (1987) observed that a pomona DNA probe would react with pomona, hardjo, and tarassovi, but not with other bacterial genera. LeFebvre (1987) described a hardjo probe which reacted with the hardjo-bovis strains, but not with other serovars known to be present in the United States. The evaluation of a hardjo DNA probe by Van Eys et al. (1988) indicated that the *in situ* method would allow visualization of leptospira morphology in addition to specific hybridization. The authors suggested that the sensitivity and specificity of hybridization with recombinant probes would allow detection and classification of the infecting serovar in one assay. Probes have been labeled with biotin (Van Eys et al., 1988) and radioisotopes (Millar et al., 1987; LeFebvre, 1987).

PART I THE EFFECT OF TRANSPORTATION TIME ON ISOLATION OF *Leptospira interrogans* FROM BOVINE KIDNEYS

THE EFFECT OF TRANSPORTATION TIME ON ISOLATION OF *Leptospira interrogans* FROM BOVINE KIDNEYS

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SUMMARY

The probability of isolation of leptospires from infected tissues submitted to diagnostic laboratories was examined. Variable factors influencing isolation rates such as sampling techniques and sample preparation were considered. Leptospires were isolated from 95%, 90%, and 90% of experimentally and naturally infected bovine kidney tissues held in transport medium at 4 C for 3, 6, and 8 days respectively prior to inoculation of culture media.

INTRODUCTION

Leptospira infection is usually diagnosed in the laboratory by microscopic agglutination test (MAT) results from serum samples (Cole et al., 1973). The limitations of the MAT have been described (Stoenner, 1975; Stringfellow et al., 1983; Thiermann, 1984). Demonstration of leptospires by fluorescent antibody test (FAT) or isolation provides definitive evidence of infection (Ellis et al., 1981; Ellis et al., 1982). In addition, isolation of the organism provides the antigen needed for identification of the infecting serovar. Improvements in media formulations (Ellinghausen and McCullough, 1965; Johnson and Harris, 1967; Ellis et al., 1985) and isolation techniques (Ellis et al., 1981; Thiermann and Handsaker, 1985) have increased the recovery rate of leptospires in the laboratory.

Ellinghausen (1973) proposed the use of a diluent composed of 1% bovine serum albumin (BSA) solution which is frequently used with inhibitors such as 5-fluorouracil (5-FU) as a transport medium for submission of samples to laboratories for leptospira isolation. A generally accepted principle is that specimens must be cultured as soon as possible after collection in order to isolate Leptospira interrogans serovars (Thiermann et al., 1984; Fairbrother, 1985). However, Michna (1959) reported recovery of serovar canicola from porcine kidney tissue at 12 days post mortem. Ho and Blackmore (1979) isolated serovar pomona from 7 of 9 naturally infected swine kidneys held at 0 C for 14 days and from 8 of 10 kidneys held at -15 C for 30 days. Ellis et al. (1982) have shown that leptospires can be isolated from a significant number of aborted bovine fetuses if proper

culture techniques are used, even though some fetuses may have undergone advanced autolysis. The rate of destruction of leptospires by urine and products of tissue autolysis has been shown to be more rapid during storage at 20 C than at 4 C (Fairbrother, 1985). Kirkbride (1984) stated that leptospires are more likely to remain viable if specimens are held at 4 C rather than being frozen.

The purpose of the present study was to determine the effect of transportation time on the recovery rate of leptospires from infected bovine kidney tissues.

MATERIALS AND METHODS

Kidneys from eight cattle experimentally infected with bovine serovar hardjo isolates and 400 bovine kidneys with gross lesions suggestive of leptospira infection (Amatredjo et al., 1976) obtained from a slaughter plant were collected for leptospira isolation. Of the experimentally infected cattle, one heifer was inoculated intravenously with a single dose of 10^6 leptospires and seven heifers were inoculated by conjunctival installation of 10^8 leptospires on four occasions (Days 1, 2, 3, and 21) (C. A. Bolin, National Animal Disease Center, Ames, IA, personal communication, 1988). Shedding of leptospires from the heifers was confirmed by culture or FAT of urine samples taken at irregular intervals between inoculation and euthanasia of the cattle at 2 to 4 months post-inoculation. Slaughter plant specimens were collected from mature cows and bulls which were recently culled from cow-calf operations because of age, reproductive failure, or other physical problems. Lesions observed in the slaughter plant specimens were interstitial nephritis, petechial hemorrhages, fibrosis, and infarctions.

Four specimens were taken from each kidney with 10 ml single-use syringes which had been modified by cutting the barrels off at a 45-degree angle. The syringes were inserted into the kidneys to a depth of 2 to 3 cm and were rotated to cut the tissue loose. The core samples which contained both cortical and medullary tissue were separated from the remainder of the kidney by prying them out with the tip of the modified syringe. Size of specimens ranged from 2.5 to 3.5 g. One specimen from each kidney was

cultured within 24 hours after collection. Three specimens were placed in polypropylene bottles containing 18 ml of 1% BSA solution with 200 ug/ml of 5-FU and held at 4 C for periods of 3, 6, and 8 days respectively before being cultured.

Specimens were cultured by the method described by Ellis et al. (1981) with some modifications. Specimens were placed in WhirlPaks[a] with 30 ml of 1% BSA solution and were homogenized in a Stomacher 400.[b] Three serial ten-fold dilutions of homogenates were made in 1% BSA solution and from each dilution 0.5 ml aliquots were inoculated into 9 ml quantities of polysorbate-80 bovine albumin (P80-BA) semisolid medium containing 200 ug/ml of 5-FU. Cultures were incubated at 29 C for 16 weeks and were examined by visual observation and darkfield microscopy on a weekly basis for 4 weeks and twice monthly thereafter. Isolates were subcultured in P80-BA medium immediately after darkfield confirmation of leptospira growth. Bacterial and fungal contaminants were removed from positive cultures by dilution (1:20) in P80-BA liquid medium and filtration through 0.45 or 0.22 um filters.[c] Isolates were identified to the serovar level with agglutinin-absorption tests as previously described (Kmety et al., 1970).

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[a]Nasco, Ft. Atkinson, WI.

[b]Tekmar, Inc., Cincinnati, OH.

[c]Falcon Filters; Becton, Dickinson, and Co., Cockeysville, MD.

RESULTS

Leptospires were isolated from all of 8 kidneys from experimentally infected cattle and from 12 (3%) of 400 kidneys obtained from the slaughter plant. All of 32 samples taken from experimentally infected cattle and 40 (83%) of 48 samples from naturally infected cattle were culturally positive. Leptospires were isolated from 17 (85%) of 20 positive tissues cultured on day 1, 19 (95%) on day 3, 18 (90%) on day 6, and 18 (90%) on day 8.

Leptospires were isolated from all specimens from experimentally infected cattle inoculated on days 1, 3, 6, and 8 (Table 1). Of the 12 positive slaughter plant specimens, leptospires were found in 9 (75%) of the cultures inoculated on day 1, 11 (92%) on day 3, 10 (83%) on day 6, and 10 (83%) on day 8 (Table 2). Eight (67%) of the 12 specimens were positive in cultures inoculated on all four days. Leptospires were found in first dilutions of all 20 positive cultures, in second dilutions of 11 cultures, and in third dilutions of 9 cultures, and were recovered from 4 contaminated cultures. Of the 12 isolates from slaughter plant specimens, 6 (50%) were identical to the kennewicki strain of the serovar pomona, 5 (42%) were identified as serovar hardjo, and 1 (8%) as serovar grippotyphosa (Table 2).

Table 1. Culture results from kidney tissues of cattle experimentally infected with serovar hardjo

Sample No.	Days After Collection 1	3	6	8
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
8	+	+	+	+

+ Leptospira isolated

Table 2. Culture results from bovine kidneys collected at slaughter

Sample No.	Serovar Isolated	Days After Collection 1	3	6	8
S- 1	hardjo	+	+	+	+
S- 2	kennewicki	+	+	+	+
S- 3	kennewicki	+	+	+	+
S- 4	grippotyphosa	+	+	+	+
S- 5	kennewicki	n	+	n	n
S- 6	hardjo	+	+	+	+
S- 7	hardjo	+	+	+	+
S- 8	kennewicki	+	+	+	+
S- 9	hardjo	n	+	n	n
S-10	kennewicki	+	+	+	+
S-11	kennewicki	n	+	+	+
S-12	hardjo	+	n	+	+

+ Leptospira isolated, n Negative

DISCUSSION

Delays of 3, 6, and 8 days between collection of samples and inoculation of media were used to simulate various lengths of time needed for transportation of specimens to a diagnostic laboratory. Isolation from 95% of positive tissues on day 3 and 90% on days 6 and 8 indicates that leptospires can be recovered from a relatively high percentage of infected kidney tissues held in transport medium under refrigeration. Most specimens are received in diagnostic laboratories within 3 days after collection. A summary of data from 277 submissions sent to the National Veterinary Services Laboratories for leptospira isolation from nine states during fiscal year 1987 indicated a range of 1 to 7 days (mean 2.2) between collection and receipt of specimens (D. A. Miller, National Veterinary Services Laboratories, Ames, IA, unpublished data, 1987). The results of this study indicate that the probability of isolation from infected tissues received within this range of time is relatively high.

Although specimens cannot be shipped under a controlled temperature of 4 C as was used in this study, they can be protected from undergoing severe autolytic changes by shipping them with ice packs in well-insulated containers. Protection of specimens from heat, the protective effect for leptospires of 1% BSA, and selective inhibitors which prevent overgrowth of other bacteria can provide the diagnostician with good quality specimens.

A difference was observed between the percentages of culturally positive samples from experimentally infected cattle (100%) and naturally

infected cattle (83%). There appear to be two possible explanations for this difference. First, the experimentally infected cattle were inoculated with serovar hardjo strains which had been passaged twice in P80-BA medium since they were originally isolated, and might have become less fastidious than other field strains due to medium adaptation. Secondly, there was probably a difference in stages of infection between the two groups. The experimentally infected cattle were euthanized at 2 to 4 months post-inoculation, and were actively infected as evidenced by frequent demonstrations of leptospires in the urine by isolation or FAT. Amatredjo et al. (1976) observed that rates of identification of leptospires with culture or silver stain techniques were higher in acute clinical cases than in subclinical cases, whether experimental or natural. Limited histories of the naturally infected cattle indicated that they were culled from cow-calf operations because of age, reproductive failure, or other physical problems. The lesions observed in these culled animals were suggestive of chronic infections in which the number of leptospires is low and leptospires are not distributed uniformly throughout the kidney (Amatredjo et al., 1976). Urinary shedding in the chronically infected animal is often intermittent, and urine cultures are frequently negative. Sampling multiple sites of the kidney in this type of animal could be expected to increase the probability of isolation (Amatredjo et al., 1976; Michna et al., 1974).

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PART II A SURVEY TO ESTIMATE THE PREVALENCE OF *Leptospira interrogans* INFECTION IN MATURE CATTLE IN THE UNITED STATES

A SURVEY TO ESTIMATE THE PREVALENCE OF *Leptospira interrogans* INFECTION IN MATURE CATTLE IN THE UNITED STATES

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SUMMARY

A total of 5,142 kidney tissue samples and 5,111 serum samples from mature cattle in 49 States and Puerto Rico were collected at slaughter. The range in ages of the cattle sampled was 1 to 16 years (mean 6.6). Leptospires were isolated from 88 (1.7%) of the kidney tissues, and 2,493 (49%) of the sera were positive against one or more of 12 leptospira antigens. Forty-one (0.8%) of the kidney tissues were positive in a leptospira fluorescent antibody test (FAT). Seventy-three (83%) of the isolates were identified as serovar hardjo, 11 (12.5%) as serovar pomona, and 4 (4.5%) as serovar grippotyphosa with agglutinin-absorption tests. With restriction endonuclease analysis (REA) methods, all isolates differed from reference serovars, but were identical to strains previously isolated from cattle or swine in the United States. Of the serovar hardjo isolates, 62 (85%) were identical to REA type hardjo-bovis A and 11 (15%) were identical to REA type hardjo-bovis B. Serovar pomona isolates were identical to the kennewicki type A (64%) or type B (36%) strains, and serovar grippotyphosa isolates were identical to the RM 52 strain. Isolation rates were significantly higher for beef cattle than for dairy cattle and higher for bulls than for cows. The combined culture and FAT results indicated that 2% of mature cattle sampled were renal carriers of leptospires.

INTRODUCTION

Since the 1950s, there have been many case reports describing abortions, weak and stillborn calves, infertility, hemolytic anemia, and mastitis associated with leptospira infection (Turner et al., 1958; Dacres and Kiesel, 1958; Sulzer et al., 1964; Diesch et al., 1967; Smith et al., 1972; Glosser et al., 1974; Hanson, 1984). However, the data published on prevalence and distribution of leptospira infection in cattle have been limited to cultural studies in small geographical areas and serological surveys.

For 20 years after the original isolation of Leptospira interrogans serovar pomona in 1948, pomona was generally accepted as the predominant serovar in cattle (Hanson and Brodie, 1967). Evidence supporting the etiologic role of other serovars began to accumulate in the 1950s and 1960s. Turner et al. (1958) documented the infection of a newborn calf in Alabama with serovar canicola. Roth and Galton (1960) described the isolation of serovar hardjo from cattle in Louisiana. Sulzer et al. (1964) and Hanson et al. (1965) reported isolations of serovar hardjo from cattle following abortions in Nebraska and Illinois, respectively. Hanson et al. (1964) isolated serovar grippotyphosa from the urine of a cow 7 days after abortion in Illinois. Diesch et al. (1967) reported isolations of serovars grippotyphosa and hardjo from aborting cattle in Iowa. Schnurrenberger et al. (1970) described isolations of serovars hardjo from beef cattle and icterohaemorrhagiae from dairy cattle in Illinois. Glosser et al. (1974) isolated serovar swzajizak from dairy cattle in Oregon with metritis,

anestrus, mastitis, agalactia, and stillbirths. The kennewicki strain of serovar pomona was isolated from cattle in Washington subsequent to a human outbreak of leptospirosis (Nelson et al., 1973). Serovars hardjo, balcanica, and pomona were isolated in a survey of slaughter cattle in Florida in which the overall leptospira isolation rate was 27% (White et al., 1982).

In a study of microbial causes of abortion in Michigan, leptospira antibodies were found in sera from 6 of 50 fetuses and leptospires were detected by darkfield examination in 3 of 98 aborted bovine fetuses (Moojen et al., 1983). Maestrone (1963) demonstrated leptospires with a fluorescent antibody test (FAT) in 5 of 21 bovine urine specimens and 7 of 10 aborted fetuses from suspected cases of leptospirosis. Leptospires were detected by FAT in 9 of 57 aborted fetuses in Massachusetts (Smith et al., 1967) and in 11 of 1,309 aborted calves in South Dakota (Kirkbride and Halley, 1982).

Serological surveys in Wisconsin (Ruedy et al., 1964; Lyle, 1967), Georgia (Cole et al., 1972), Florida (Rubin, 1977), Iowa (Nervig et al., 1980), and Arizona (Songer et al., 1983) have clearly shown that antibodies against serovar hardjo are widely distributed in the United States cattle population. A summary of serological test results from 18 states indicated a leptospira infection rate of 16% in cattle (Hanson, 1974). Stoenner's (1975) analysis of microscopic agglutination test results from 13 diagnostic laboratories indicated the prevalence of infections with serovars pomona and hardjo was greater in the southeastern and western states than

in the midwest, and the nationwide prevalence of serovar hardjo infections was greater than that of serovar pomona.

The purpose of the present study was the collection and analysis of statistically valid data from cultural, serological, and fluorescent antibody tests to estimate the prevalence of L. interrogans infection in mature cattle in the United States.

MATERIALS AND METHODS

The bovine population sampled in this survey consisted of mature cows and bulls recently culled from dairy and beef herds. A list of 1,455 Federally inspected packing plants which were processing mature cattle and the number of cattle slaughtered annually by each plant were obtained from the United States Department of Agriculture Food Safety and Inspection Service (FSIS). Data for plants in operation and the number of cattle slaughtered were updated quarterly during the survey. Plants to be sampled each week were randomly selected. The annual slaughter rates of the plants ranged from one to 270,000. For each 60,000 cattle slaughtered annually, one sample per week was requested. A total of 110 samples per week was requested from selected slaughter plants during the period February 23, 1986, through February 20, 1987. Instructions and materials for collection and submission of samples were mailed to the plants 2 weeks prior to the dates of sample collection. FSIS Inspectors-in-Charge (IICs) were asked to select a cow or bull at random on designated dates without regard for the presence or absence of disease signs for collection of a kidney tissue sample and a blood sample. Core samples of kidney tissue (2.5 to 3.5 g) were collected with modified 10 ml disposable syringes (Miller et al., 1989*b*) and 13 ml serum separation tubes were used for collection of blood samples. Kidney samples were placed in polypropylene bottles containing 18 ml of a transport medium composed of 1% BSA solution (Ellinghausen, 1973) with 200 ug/ml of 5-fluorouracil (5-FU) (Johnson and Rogers, 1964). IICs furnished available information as to sex, type of animal (dairy or beef),

backtag and eartag numbers, state of origin, and estimated age for each animal sampled. Samples were shipped to the National Veterinary Services Laboratories (NVSL), Ames, Iowa, in styrofoam containers with ice packs by priority mail as soon as possible after collection.

Samples received from Monday through Friday of each week were processed immediately after receipt. Samples that arrived on weekends or holidays were held at 4 C and were processed on the next workday. Kidney tissues were cultured by the method described by Ellis et al. (1981) with some modifications. Tissues were removed from transport medium and homogenized (1:10) with a Stomacher 400[a] in fresh transport medium. From the homogenate, three serial ten-fold dilutions were made in 1% BSA, and 0.5 ml of each dilution was inoculated into polysorbate 80-bovine albumin (P80-BA) semisolid medium (Ellinghausen and McCullough, 1965) with 200 ug/ml of 5-FU and T80/40/LH semisolid medium with 200 ug/ml of 5-FU and 20 ug/ml of nalidixic acid (Ellis et al., 1985*a*). Each lot of medium was pretested for growth of leptospires by inoculation with serial ten-fold dilutions of field isolates of serovars hardjo and copenhageni recently passaged in hamsters (Ellinghausen, 1975). Lots of media were used only if growth of serovar hardjo could be achieved from an inoculum of 10 to 100 cells within 4 weeks. Procedures for incubation and examination of cultures and recovery of leptospires from contaminated cultures have been previously described (Miller et al., 1989*b*).

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[a]Tekmar Inc., Cincinnati, OH.

Rabbit antisera against isolates were produced by the method described by Sulzer and Jones (1980) except that P80-BA medium was used rather than Fletcher's medium. Agglutinin-absorption tests were used to compare isolates with reference serovars for identification as previously described (Kmety et al., 1970). Isolates were also identified with the restriction endonuclease analysis (REA) method described by LeFebvre et al. (1985) as modified by Ellis (W. A. Ellis, Veterinary Research Laboratories, Belfast, Northern Ireland, personal communication, 1988). Genomic DNA of serovar hardjo and grippotyphosa isolates was treated with restriction enzyme Hha I, and that of serovar pomona with a combination of Hha I and Bgl II.[b] In addition to the reference serovars hardjo Hardjoprajitno, pomona Pomona, and grippotyphosa Andaman, isolates were compared to hardjo-bovis type A and B strains of serovar hardjo, the kennewicki type A and B strains of serovar pomona, and the RM 52 strain of serovar grippotyphosa which were previously isolated from cattle or swine in the United States (Thiermann et al., 1984; Thiermann et al., 1985; Thiermann et al., 1986; C. A. Bolin, National Animal Disease Center, Ames, IA, personal communication, 1987).

Sera were separated from blood samples and were screened at a 1:100 dilution with the microscopic agglutination test (MAT) (National Veterinary Services Laboratories, 1987) against 12 antigens representative of the L. interrogans serogroups known to occur in the United States (Table 2).

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[b]Bethesda Research Laboratories, Rockville, MD.

Positive sera were titrated against reacting antigens in serial two-fold dilutions from 1:100 to 1:12,800.

Two ml aliquots of homogenates were stored at -70 C and were examined with a direct fluorescent antibody test (FAT) as previously described (Miller et al., 1989*a*) after completion of culture work. Samples were considered positive only if fluorescent cells with typical leptospira morphology were observed.

Serological and cultural prevalence rates among sexes, types, and ages of cattle were compared in chi-square tests of independence. Geometric mean serum titers (GMTs) were calculated for cattle from which tissue samples were culturally positive and negative. GMTs were tested for a significant difference with Student's t test.

RESULTS

A total of 5,142 kidney tissue samples and 5,111 serum samples were received from 49 States and Puerto Rico. No samples were received from Alaska. Less than 50 samples were received from each of 16 States and Puerto Rico. Eighty-two samples were collected from dairy bulls, 2,543 from dairy cows, 356 from beef bulls, and 2,161 from beef cows (Table 1). The age range of the cattle was 1 to 16 years (mean 6.6). A total of 4,754 (92%) of the samples were collected from cattle between 4 and 10 years of age. Time between collection of samples and receipt in the laboratory ranged from 1 to 14 days (mean 4.6).

A total of 2,493 (49%) of the sera were positive in the MAT against one or more serovars at dilutions $\geq$ 1:100. Positives against more than one serovar were found in 1,609 (65%) of the positive sera. A total of 29% of the sera were positive against serovar hardjo, 23% against serovar pomona, 20% against serovar copenhageni, 14% against serovar wolffi, 11% against serovar canicola, and 6% against serovar grippotyphosa. Lower positive rates were observed against other serovars.

The percentage of positive sera was significantly higher for beef cattle (53%) than for dairy cattle (44%) ($p < .001$), and higher for cows (49%) than for bulls (43%) ($p < .05$). The highest rate of seropositives was seen in beef cows (55%), and the lowest rate in dairy bulls (33%). MAT results by serovar and type of cattle are presented in Tables 2 and 3.

Leptospires were isolated from 88 (1.7%) of the kidney tissues. Positive cultures were detected by darkfield examination from 10 to 46 days post-inoculation (mean 18.5). Isolations were made from 1 sample in T80/40/LH medium only, from 12 samples in P80-BA medium only, and from 75 samples in both media. Leptospires were found in first dilutions of 88 cultures, second dilutions of 65 cultures, and third dilutions of 39 cultures. Leptospires were recovered from 28 contaminated cultures.

A total of 73 (83%) of the isolates were identified as serovar hardjo, 11 (12.5%) as serovar pomona, and 4 (4.5%) as serovar grippotyphosa with agglutinin-absorption tests. All of the isolates had REA patterns which differed from those of reference serovars (Figures 1 and 2). Sixty-two (85%) of the serovar hardjo isolates had REA patterns identical to hardjo-bovis type A, and 11 (15%) were identical to hardjo-bovis type B. All serovar pomona isolates differed from the reference strain in both the agglutinin-absorption test and REA. Seven (64%) were identical to kennewicki type A, and 4 (36%) were identical to kennewicki type B. All serovar grippotyphosa isolates had REA patterns identical to the RM 52 strain.

Isolation rates were significantly higher for beef cattle samples than for dairy cattle samples ($p < .001$), and higher for bull samples than for cow samples ($p < .001$). The highest isolation rate was obtained from beef bull samples and the lowest rate from dairy cow samples (Table 3). Leptospires were isolated from cattle 2 to 12 years of age (mean 6.3) (Table 4).

Table 1. Survey samples received by state and type of cattle

State	Dairy		Beef		Total
	Bulls	Cows	Bulls	Cows	
AL	0	12	6	51	69
AZ	0	6	0	10	16
AR	1	17	12	52	82
CA	7	134	19	55	215
CO	2	32	15	73	122
CT	0	14	0	0	14
DE	0	1	0	0	1
FL	1	37	4	28	70
GA	1	26	6	42	75
HI	0	2	4	1	7
ID	2	57	5	56	120
IL	4	44	2	23	73
IN	2	41	4	10	57
IA	4	106	8	76	194
KS	1	44	20	106	171
KY	3	61	13	50	127
LA	0	26	4	38	68
ME	0	13	2	2	17
MD	0	15	0	2	17
MA	0	17	1	0	18
MI	4	101	0	3	108
MN	10	282	10	32	334
MS	0	25	9	59	93
MO	2	70	16	103	191
MT	0	5	12	81	98

Table 1. (Continued)

State	Dairy		Beef		Total
	Bulls	Cows	Bulls	Cows	
NE	1	39	18	168	226
NV	0	0	3	4	7
NH	0	1	0	0	1
NJ	1	8	0	1	10
NM	0	26	3	34	63
NY	4	180	2	7	193
NC	1	26	5	26	58
ND	3	39	4	54	100
OH	0	68	3	13	84
OK	0	33	24	128	185
OR	0	25	7	34	66
PA	5	176	7	17	205
RI	0	1	0	0	1
SC	0	4	2	10	16
SD	1	64	19	121	205
TN	3	58	6	52	119
TX	6	78	60	428	572
UT	0	21	4	14	39
VT	0	20	1	0	21
VA	1	39	4	18	62
WA	1	62	4	33	100
WV	0	12	0	2	14
WI	6	364	0	10	380
WY	1	6	2	31	40
PR	4	5	6	3	18
Total	82	2543	356	2161	5142

Table 2. MAT results by serovar and type of cattle

Serovar[a]	Beef		Dairy		No. Positive[b]	% Positive[c]
	Cow	Bull	Cow	Bull		
AS	17	2	11	0	30	0.6
AT	68	11	51	2	132	2.6
BM	90	14	81	4	189	3.7
BT	19	5	20	0	44	0.9
CA	224	17	313	3	557	10.9
GR	133	13	147	5	298	5.8
HA	703	100	680	18	1501	29.4
IC	453	38	519	4	1014	19.8
PO	566	57	536	10	1169	22.9
PY	97	12	116	2	227	4.4
TA	53	7	59	3	122	2.4
WO	382	61	281	13	737	14.4

[a]AS australis, AT autumnalis, BM ballum, BT bataviae, CA canicola, GR grippotyphosa, HA hardjo, IC copenhageni, PO pomona, PY pyrogenes, TA tarassovi, WO wolffi.

[b]1609 (64.5%) of positive sera were positive against more than one serovar; positive $\geq$ 100 titer.

[c]Percentage positive of total number of samples.

Table 3. Summary of serological and cultural results by type of cattle

	Beef	Dairy	Total
Cow	55[a](2.0)[b]	45 (0.9)	49 (1.4)
Bull	45 (5.3)	33 (3.7)	43 (5.0)
Total	53 (2.5)	45 (1.0)	49 (1.7)

[a]Percentage of positive sera; positive $\geq$ 100 titer against 1 or more of 12 antigens.

[b]Percentage of isolates in parentheses = no. isolates divided by no. samples.

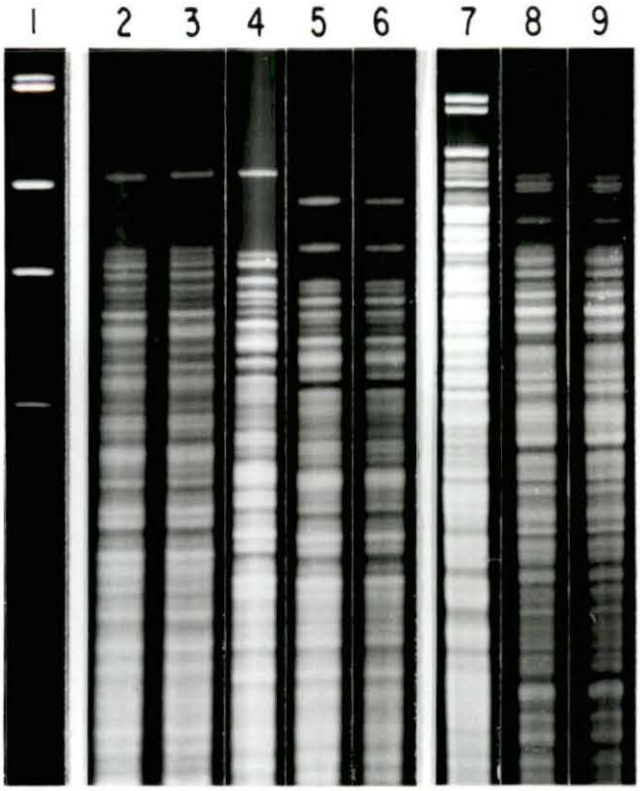

Figure 1. Electrophoretic patterns of a Hind III digest of lambda DNA (1); Hha I and Bgl II double digests of kennewicki type A reference (2), kennewicki type A isolate (3), pomona Pomona reference (4), kennewicki type B isolate (5), and kennewicki type B reference (6); and Hha I digests of grippotyphosa Andaman reference (7), grippotyphosa isolate (8), and grippotyphosa RM 52 reference (9)

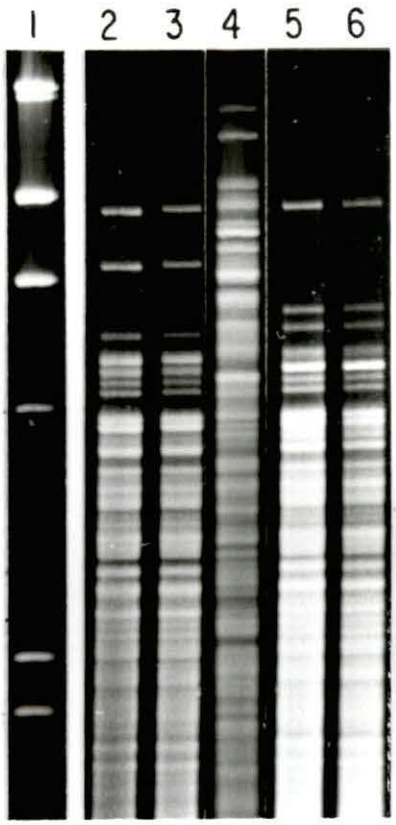

Figure 2. Electrophoretic patterns of a Hind III digest of lambda DNA (1) and Hha I digests of hardjo-bovis type A reference (2), hardjo-bovis type A isolate (3), hardjo Hardjoprajitno reference (4), hardjo-bovis type B isolate (5), and hardjo-bovis type B reference (6)

Differences in isolation rates by ages of cattle were not significant. Of 62 isolates from beef cattle, 52 (84%) were serovar hardjo, 6 (10%) were serovar pomona, and 4 (6%) were serovar grippotyphosa. Of 26 isolates from dairy cattle, 21 (81%) were serovar hardjo and 5 (19%) were serovar pomona (Table 5).

Forty-one (0.8%) of the kidney samples were positive in the FAT. The number of leptospires observed ranged from less than one to 100 per microscopic field at 250X magnification. More leptospires were observed in samples from which serovars grippotyphosa and pomona were isolated (usually 20 to 50 per field) compared to the low numbers observed in samples from which serovar hardjo was isolated (1 to 5 per field).

With the exception of one sample from which serovar pomona was isolated, the sera from cattle which were culturally positive were also serologically positive. MAT titers ranged from 100 to 12,800 against hardjo, 1,600 to 12,800 against pomona, and 400 to 1,600 against grippotyphosa in cattle from which the respective serovars were isolated. A significant difference in GMTs for cattle from which companion tissue samples were culturally positive (879) and negative (119) was observed ($p < .001$). Of 85 samples which were both serologically and culturally positive, 72 (85%) of the sera exhibited the highest titer against the serovar which was isolated, 11 (13%) reacted to the same titer against both the serovar isolated and another serovar, and 2 (2%) reacted more strongly against another serovar than against the serovar isolated. Of 71 samples from which serovar hardjo was isolated and companion serum samples were available, 5 (7%) of

Table 4. Number of isolates and isolation rates by age of cattle

Age (years)	2	3	4	5	6	7	8	9	10	11	12
No. Samples	77	188	411	1124	973	694	712	220	620	42	48
No. Isolates	3	8	8	20	12	9	15	2	7	1	3
Isolation Rate (%)	3.9	4.2	1.9	1.8	1.2	1.3	2.1	0.9	1.1	2.4	6.3

Table 5. Isolates by serovar and type of cattle

Serovar	Beef		Dairy		Serovar Total
	Cows	Bulls	Cows	Bulls	
hardjo-bovis type A	27	15	18	2	62
hardjo-bovis type B	8	2	1	-	11
(*hardjo* total)	(35)	(17)	(19)	(2)	(73)
kennewicki type A	3	1	3	-	7
kennewicki type B	2	-	1	1	4
(*pomona* total)	(5)	(1)	(4)	(1)	(11)
grippotyphosa	3	1	-	-	4
Total Isolates	43	19	23	3	88

the sera were positive against serovar hardjo only, 47 (66%) were positive against serovars hardjo and wolffi, and 19 (27%) had MAT titers ranging from 100 to 6,400 against serovars ballum, bataviae, canicola, copenhageni, pomona, pyrogenes, tarassovi, and/or wolffi in addition to hardjo. A total of 52 (73%) of the sera had titers against only serovar hardjo and the closely related serovar wolffi. Of 11 samples from which serovar pomona was isolated, 1 (9%) serum was negative, 1 (9%) was positive against serovars pomona and autumnalis, and 9 (82%) were positive against multiple serovars, predominantly the serovars autumnalis and copenhageni, in addition to hardjo. Of four samples from which serovar grippotyphosa was isolated, one serum was positive against serovar grippotyphosa only and three sera had multiple titers against serovars grippotyphosa, hardjo, pomona, and/or wolffi.

FAT positives were observed in 31 (35%) of 88 culturally positive samples and 10 samples which were culturally negative. Of 73 tissues from which serovar hardjo was isolated, 11 from which serovar pomona was isolated, and 4 from which serovar grippotyphosa was isolated, 20 (27%), 8 (73%), and 3 (75%) were also FAT positive, respectively. Of the 40 FAT positive samples with companion sera, 5 (12%) of the sera were negative in the MAT and 35 (88%) were positive against multiple serovars, predominantly canicola, grippotyphosa, hardjo, and pomona.

DISCUSSION

Compared to the total United States cattle population, the collection of samples in proportion to the annual slaughter rates of individual plants led to a disproportionate number of samples being collected from dairy cattle in 1986 due to the sale of large numbers of dairy cattle through the USDA Dairy Termination Program in that year.

The seropositive rate (49%) observed in this study approximated that observed in 1986 in a summary of results from diagnostic samples from 25 states (Miller, 1986). One possible explanation for the fact that beef cattle had a higher seropositive rate and a higher isolation rate than dairy cattle is that beef cattle are often free ranging and are frequently exposed to stagnant water, rodents, and other wildlife carriers. Dairy cattle are more often kept in dry lot areas and may not have as much exposure to these sources of infection. Since dairy cattle are commonly artificially inseminated and beef cattle are commonly bred naturally, venereal transmission (Ellis et al., 1985*a*) could have been a contributing factor to the difference observed. Seroprevalences of serovars hardjo and pomona were also observed to be higher in beef cattle than in dairy cattle in Ontario (Prescott et al., 1988), but the opposite relationship has been demonstrated in serovar hardjo abortions in Ireland by cultural and immunofluoresence methods (Ellis et al., 1985*b*) and in serovar hardjo and pomona abortions in Australia by serological methods (Elder et al., 1985).

Antibodies against serovar hardjo were most commonly detected, followed by serovars pomona and copenhageni. This confirmed Stoenner's observation in 1975 that the nationwide seroprevalence of serovar hardjo was greater than that of serovar pomona. Since serovars hardjo and wolffi are closely related, the reaction of sera with wolffi antigen was probably cross-reactivity with serovar hardjo antibodies. For many years, titers against serovar sejroe were observed in bovine serum, but it was later shown that this reactivity could be absorbed with serovar hardjo antigen (Alexander and Evans, 1962). Some seropositives may have been due to residual antibodies from vaccination, although this variable could not be related to serological results since the vaccination status of cattle was unknown. Morter (1980) observed titers > 1,000 at 6 months post-inoculation in some cattle vaccinated with two doses of a pentavalent leptospira bacterin. Ris and Hamel (1982) reported geometric mean titers ranging from 1,300 to 10,000 at 2 to 3 months post-inoculation in calves vaccinated with a hardjo-pomona bacterin. Stringfellow et al. (1983) observed titers up to 3,200 in cattle vaccinated with a pentavalent bacterin, and noted that discrimination between post-infection and post-vaccination titers in individual animals might be difficult during a 20-week period following vaccination. Hodges and Day (1987) found that MAT titers ranging from 100 to 3,000 persisted for 7 to 23 weeks post-vaccination in heifers inoculated with a hardjo-pomona bacterin. Other seropositives in this study may have been due to residual antibodies in animals that were no longer renal carriers (Orr and Little, 1979; Hodges and Ris, 1974) or antibodies in animals with too few leptospires to be detected with culture or FAT methods (Miller

et al., 1989*a*). The GMTs of 879 for culturally positive cattle and 119 for culturally negative cattle could be interpreted as evidence that most cattle with titers of approximately 800 are infected and those with titers of approximately 100 are not. However, leptospires have been isolated from seronegative cattle (Ellis et al., 1981), and isolation attempts failed in some cattle with high titers in the present study.

The overall isolation rate was much lower than the seropositive rate and the isolation rate was higher for bulls than for cows. White et al. (1982) reported an isolation rate of 27% for Florida cattle at slaughter and also found the isolation rate below the seropositive rate (71%) and the isolation rate for steers (38%) greater than that for bulls (25%) and cows (12.5%). In a survey of Iowa cattle at slaughter, Thiermann (1983) reported an isolation rate of 6% for cows. Both of the previous studies were conducted in one slaughter plant over a limited period of time (3 months and 2 months, respectively) with a limited number of samples (306 and 204, respectively). In the present study, mature cattle were sampled in an effort to measure the rate of infection in animals used for reproduction. Other studies in Ireland (Ellis et al., 1981), England (Orr and Little, 1979), Quebec (Gregoire et al., 1987), and Australia (Skilbeck et al., 1988) have generally indicated that steers in confinement have higher rates of leptospira infection than brood cattle on pasture. Apparently, transmission occurs more rapidly and more extensively through these concentrated groups of cattle. Possible reasons for this could be increased chances for contact of urine with mucus membranes of the eyes, nose, and mouth and

retention of infective urine in pools on feedlot floors for prolonged periods. No relationship was apparent between ages of cattle and culturally positive status. The mean age of culturally positive cattle (6.3 years) was approximately the same as the mean age of all cattle sampled (6.6 years). Ellis et al. (1981) found a much higher prevalence of infection in younger cattle in Ireland and concluded that younger animals played an important role in the epidemiology of leptospira infection.

Serovar hardjo was most frequently isolated (83% of the isolates), followed by serovar pomona (12%). These findings agreed with the order but not the magnitude of seropositive rates: 29% and 23%, respectively. The serovar grippotyphosa ranked third among isolates and fifth in seropositive rate, excluding wolffi. The third- and fourth-ranked serovars in seropositivity, copenhageni and canicola, were not isolated in this study, although they have been previously isolated from cattle in the United States (Turner et al., 1958; Miller, 1985). All isolates differed in the REA from the reference serovars which were isolated in other countries. This finding may have importance in future epidemiological studies, identification of isolates, and selection of antigens for serological tests and bacterins (Thiermann, 1984).

Leptospires were detected in 41 samples with the FAT, including 5 samples which were serologically negative and 10 samples which were culturally negative. Although the FAT detection rate of culturally positive samples was low (35%), leptospires were found in 10 samples which would not have otherwise been detected. Leptospires were detected with the combination of

culture and FAT methods in 98 (2%) of the kidney tissues. If the rate of renal infection was constant throughout the estimated 1986 mature cattle population of 47 million (Anonymous, 1986), it could be estimated that approximately one million mature cattle were renal carriers of leptospires in the United States during this period.

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PART III RELATIONSHIPS BETWEEN *Leptospira interrogans* PREVALENCE RATES IN CATTLE AND REGIONAL, CLIMATIC, AND SEASONAL FACTORS

RELATIONSHIPS BETWEEN *Leptospira interrogans* PREVALENCE RATES IN CATTLE AND REGIONAL, CLIMATIC, AND SEASONAL FACTORS

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SUMMARY

The distribution of Leptospira interrogans serovars and prevalence rates based on serological and cultural results from mature cattle were compared by state and region of the United States. Relationships between isolation rates and time of collection, mean temperature, and mean precipitation were examined. Isolation rates and seropositive rates were significantly higher for Southeastern, South Central, and Pacific Coastal Regions. The hardjo-bovis type A, kennewicki, and grippotyphosa isolates appeared to be randomly distributed. The hardjo-bovis type B isolates came from a southern area of the country from Georgia to New Mexico. This report is believed to contain the first recorded isolation of serovar hardjo from Hawaii. Although no significant relationship was demonstrated between isolation rates and month or season of the year, seroprevalence rates for summer, fall, and winter were significantly higher than for spring. Regional isolation rates were related more to mean temperature than to mean precipitation levels.

INTRODUCTION

Environmental factors have been frequently shown to influence the occurrence of leptospirosis (Torten, 1979). In addition to direct contact, transmission of leptospirosis may occur through exposure to infective materials in environments which permit survival of leptospires (Ellis, 1986). Generally, the survival of pathogenic leptospires outside the host requires a warm, moist environment with a pH that is near neutral (Michna, 1970; Ellis, 1986). Direct sunlight, dessication, and high salinity are rapidly detrimental to leptospires (Michna, 1970). Survival of serovar pomona has been demonstrated for 42 days in soil under simulated winter conditions (Hellstrom and Marshall, 1978), 183 days in wet soil, and 94 days in river water (Okazaki and Ringen, 1957). Other studies have demonstrated significant variation in length of survival of various serovars depending on the pH of soil or water (Smith and Self, 1955; Smith and Turner, 1961). Leptospires can survive longer in areas with high moisture and abundant vegetation than in areas with low moisture and little shade to protect them from direct sunlight (Karaseva et al., 1973). Areas of high rainfall and high levels of subsurface water and areas near the equator have become endemic zones (Torten, 1979). Kingscote (1970) observed that leptospirosis was more common in areas with clay soil, and correlated the prevalence of leptospirosis with the type of underlying bedrock in different areas of Canada. The purpose of the present study was to examine the relationships between leptospira serological and cultural prevalence rates

in cattle and regional, climatic, and seasonal factors in the United States.

MATERIALS AND METHODS

The methods of sample collection, cultural and serological techniques, and identification of isolates used in a survey to estimate the prevalence of Leptospira interrogans infection in mature cattle in the United States have been described (Miller et al., 1989). For purposes of this study, all sera which exhibited a microscopic agglutination test (MAT) titer $\geq$ 100 against one or more of 12 L. interrogans serovars were considered positive.

Data from the previous survey were used to determine the state of origin and the month in which samples were collected. Isolation rates were calculated by dividing the number of isolates by the total number of samples per month, season of the year, and state of origin. For purposes of this study, the seasons were considered to be March through May (Spring), June through August (Summer), September through November (Fall), and December through February (Winter). Regional prevalence rates were determined by the sums of serological and cultural results from individual states.

Cultural and serological prevalence rates by month, season, and region were compared in chi-square tests of independence. Correlation coefficients (r) were calculated to examine the relationships between regional isolation rates and 55-year averages of mean temperature (Anonymous, 1986*a*) and mean precipitation (Anonymous, 1986*b*) (Table 1). Student's t tests were used to determine the significance of correlation coefficient values.

Table 1. Mean temperature and precipitation by region[a,b]

Region	Mean Temperature (degrees F)	Mean Precipitation (inches)
Northeastern	46.93	41.27
North Central	45.02	22.79
Southeastern	62.23	51.05
South Central	61.84	33.97
Northern Plains	48.89	36.04
Rocky Mountain	48.60	13.45
Pacific Coastal	53.50	26.96

[a]Anonymous, 1986*a*.

[b]Anonymous, 1986*b*.

RESULTS

Significantly higher percentages of positive samples were seen in sera from Southeastern, South Central, and Pacific Coastal Regions than from Northeastern, North Central, Northern Plains, and Rocky Mountain Regions of the United States ($p < .001$) (Figure 1). Seropositive rates for summer, fall, and winter were significantly higher than for spring ($p < .001$) (Table 3).

Leptospires were isolated from samples from 27 (55%) of the 49 states from which samples were received. The 11 hardjo-bovis type B isolates came from a southern area of the United States including Georgia, Mississippi, Louisiana, Texas, Oklahoma, Kansas, and New Mexico. The more common hardjo-bovis type A isolates (62) did not appear to be distributed according to any particular pattern, but originated from 24 states including Hawaii. The kennewicki types A and B and serovar grippotyphosa isolates also appeared to be randomly distributed (Figure 2). Among states from which 50 or more samples were received, isolation rates above the mean of this group (2.3%) were obtained from Oklahoma, Washington, Missouri, Tennessee, Virginia, Kentucky, Oregon, Alabama, Louisiana, Texas, and Georgia. A summary of the number of isolates and isolation rates by state of origin is presented in Table 2. Isolation rates were significantly higher for the South Central, Southeastern, and Pacific Coastal Regions than for other regions of the United States ($p < .001$) (Figure 3).

The isolation rate was highest from samples collected in October (2.7%) and lowest from samples collected in May (0.7%) (Figure 4). Isolation rates were higher from samples collected in summer and fall than in winter and spring (Table 3). Forty-four (50%) of the isolates were obtained from samples collected between July and October. Differences in isolation rates by month and season were not significant.

There appeared to be a linear relationship between isolation rates and mean regional temperatures ($r = .83$; $p < .05$), but a similar relationship was not apparent between isolation rates and mean regional precipitation levels ($r = .34$).

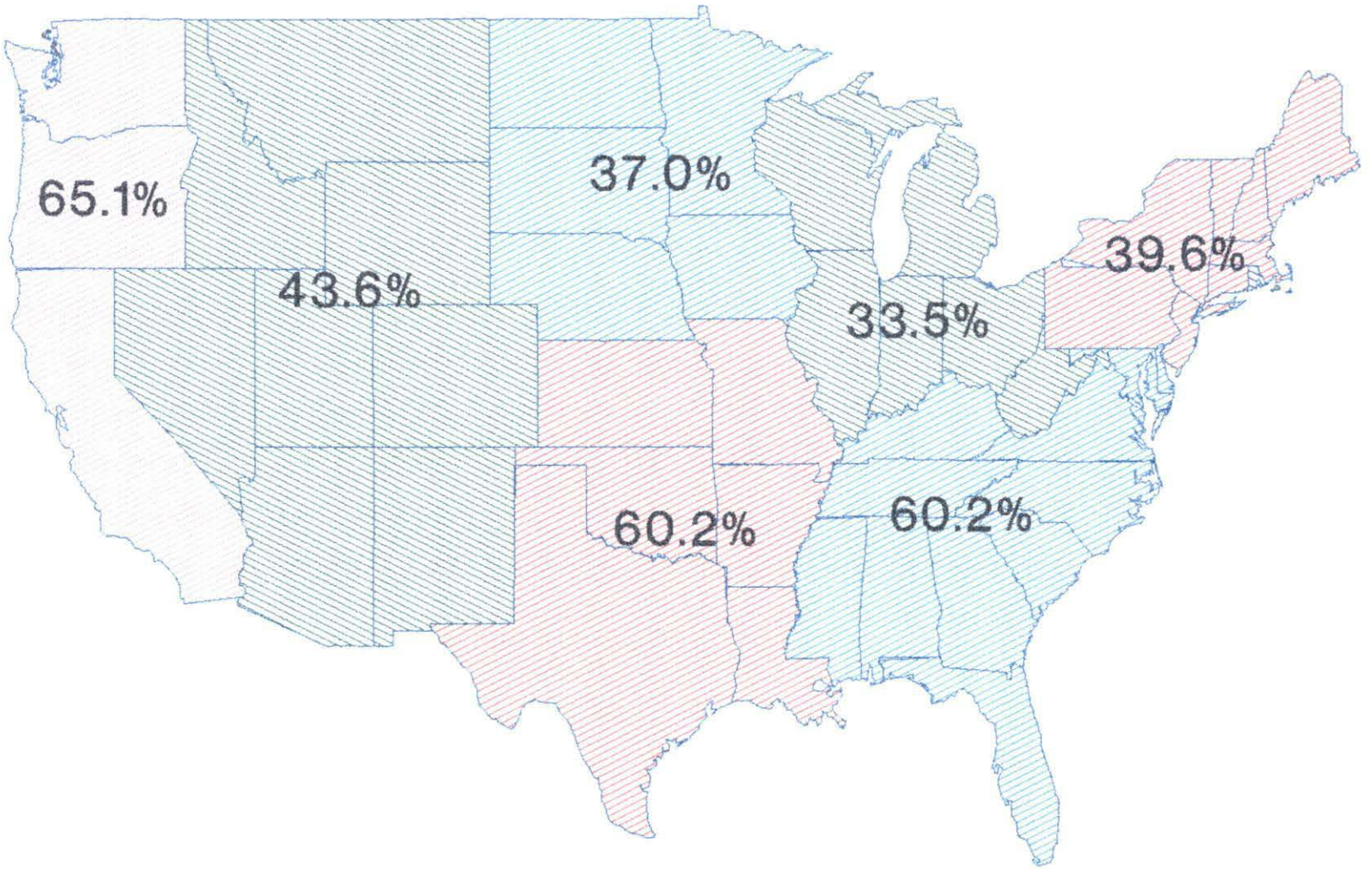

Figure 1. Leptospira seroprevalence by region

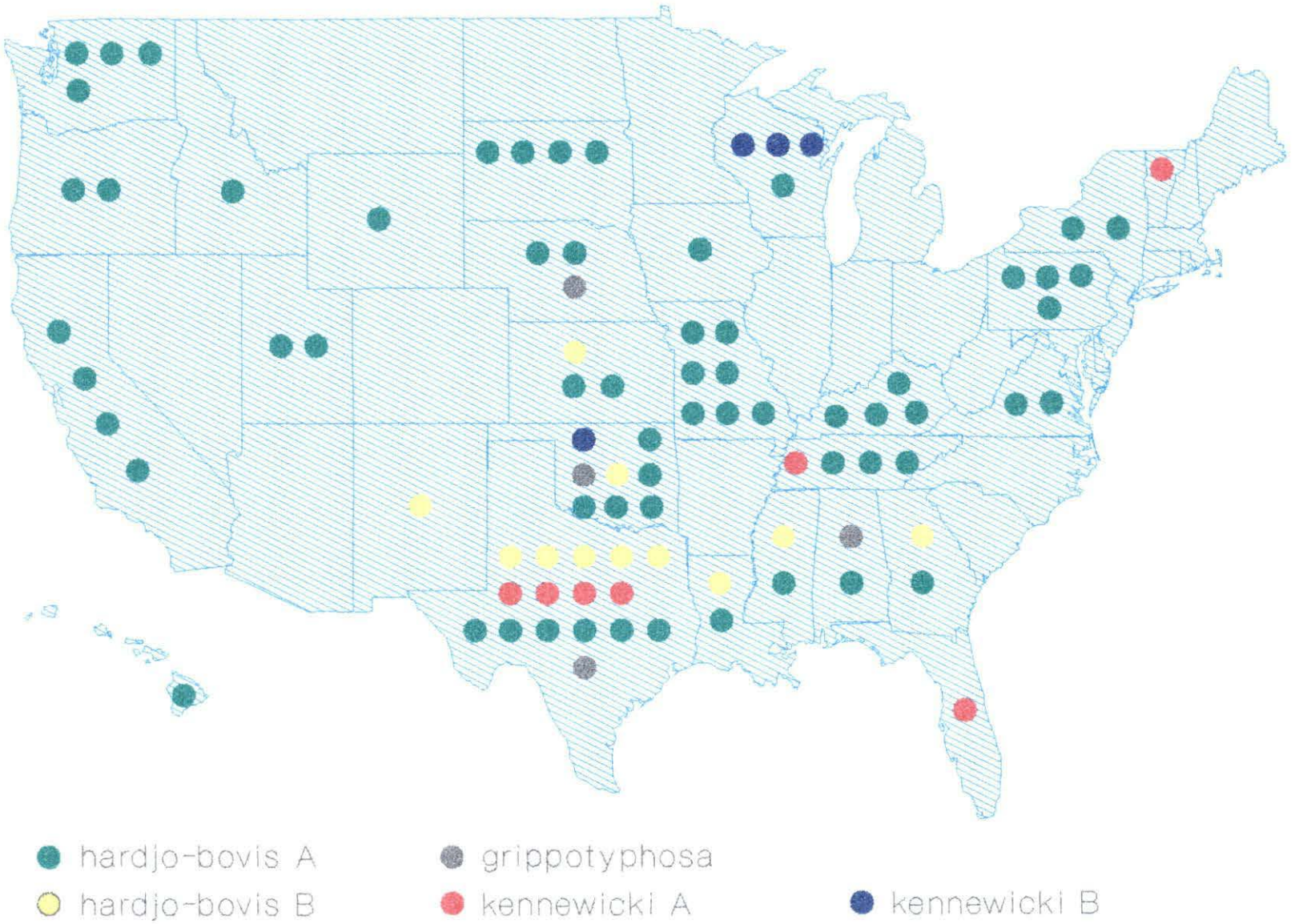

Figure 2. Distribution of isolates by serovar and state

Table 2. Number of isolates and isolation rates by state of origin

State	No. Samples	No. Isolates	Isolation Rate (%)
AL	69	2	2.9
CA	215	4	1.9
FL	70	1	1.4
GA	75	2	2.7
HI[a]	7	1	---[b]
ID	120	1	0.8
IA	194	1	0.5
KS	171	3	1.8
KY	127	4	3.1
LA	68	2	2.9
MS	93	1	1.1
MO	191	7	3.7
NE	226	4	1.8
NM	63	1	1.6
NY	193	2	1.0
OK	185	8	4.3
OR	66	2	3.0
PA	205	4	2.0
SD	205	4	2.0
TN	119	4	3.4
TX	572	16	2.8
UT[a]	39	2	---[b]
VT[a]	21	1	---[b]
VA	62	2	3.2
WA	100	4	4.0
WI	380	4	1.1
WY[a]	40	1	---[b]
Totals		88	1.7

[a]Less than 50 samples received from these states.

[b]Insufficient data for accurate estimates.

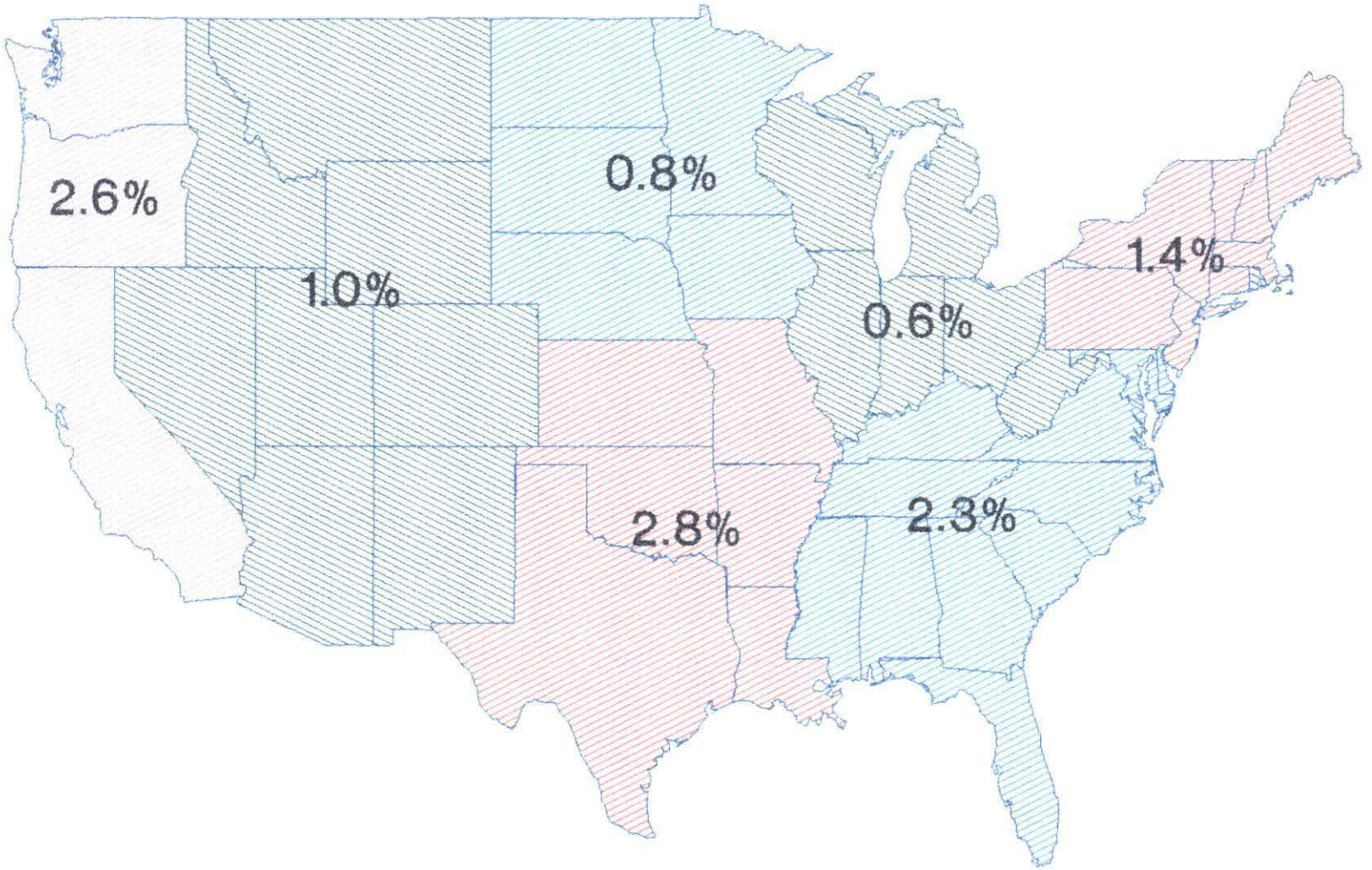

Figure 3. Isolation rates by region

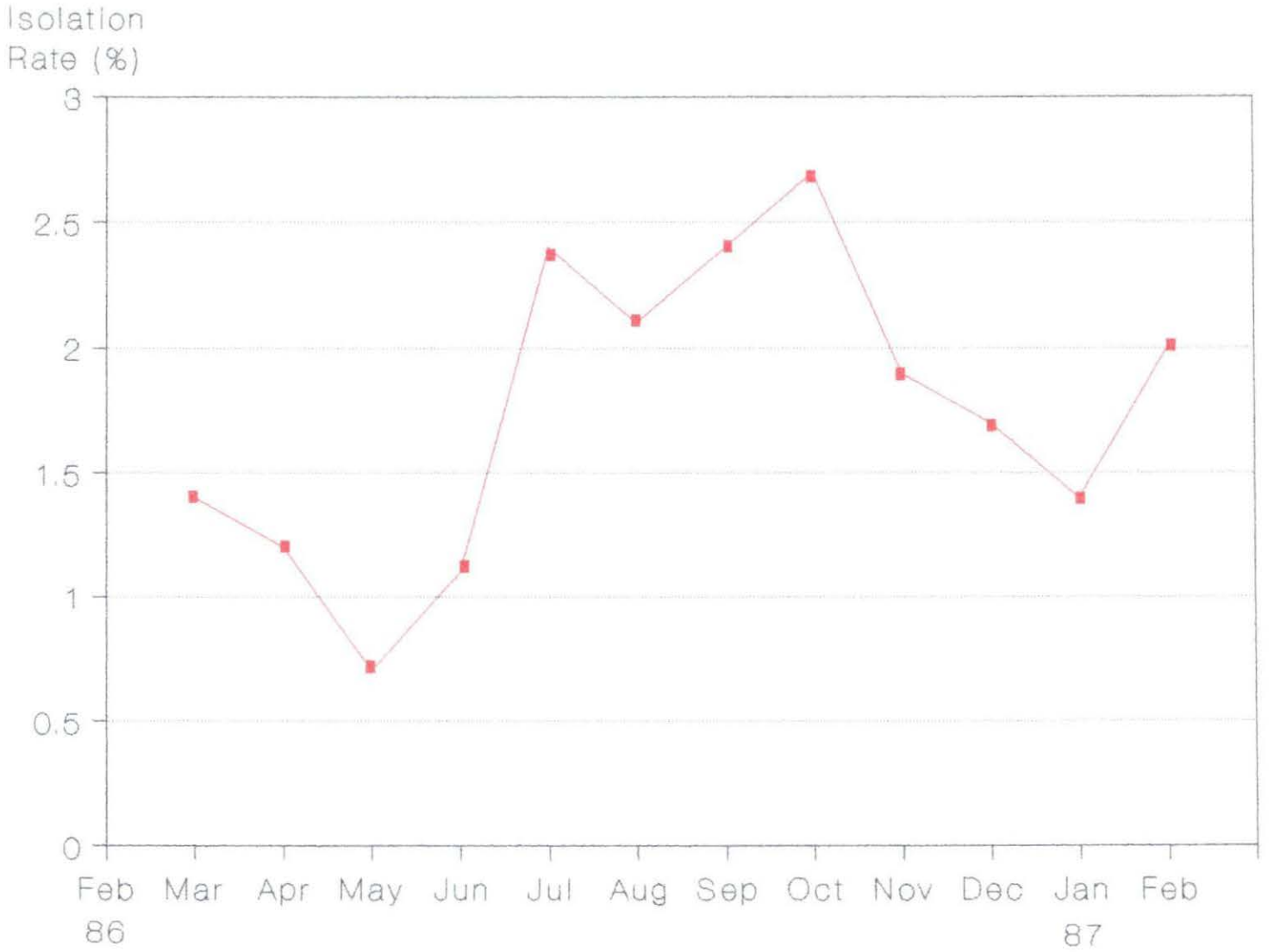

Figure 4. Isolation rates by month

Table 3. Isolation rates and seropositive rates by season of the year

Season	Spring	Summer	Fall	Winter
Isolation Rate (%)	1.1	1.9	2.3	1.5
Seropositive Rate (%)	41.4	51.7	49.3	51.5

DISCUSSION

The high seropositive rate in the Southeastern Region of the country observed in this study has been previously recognized (Cole et al., 1972; Rubin, 1977), and was equaled or exceeded only by the South Central and Pacific Coastal Regions.

Findings in the present study were in agreement with those of Thiermann et al. (1986), who observed that hardjo-bovis type A was the most common serovar hardjo isolate in North America. The type A isolates seemed to be randomly distributed throughout the country, including one isolate from Hawaii. Although the closely related serovar sejroe has been isolated in Hawaii (Minette, 1964), this is believed to be the first reported isolation of serovar hardjo from that state. All of the hardjo-bovis type B isolates came from states in a southern area of the country from Georgia to New Mexico. Thiermann et al. (1986) also reported evidence that type B occurred in the southern part of the United States in their description of serovar hardjo isolates from Florida. In the present study, no pattern of distribution was apparent for serovar kennewicki or grippotyphosa isolates.

Although 50% of the isolates were recovered from samples collected between July and October and isolation rates for the summer-fall period were higher than for the winter-spring period, differences were not statistically significant. However, the significantly higher seropositive rates of summer, fall, and winter compared to spring indicated some seasonal influence on prevalence rates. It might be expected that more cattle would

become infected in warmer seasons of the year when conditions are favorable for survival of leptospires in surface water and animal waste (Michna, 1970; Ellis, 1986). Once cattle become infected, they may retain their renal carrier status for months or years (Thiermann, 1982) and leptospira infection may be detected by sampling chronic carriers during any season of the year.

Isolation rates appeared to be related more to regional temperature than to precipitation levels. The Southeastern, South Central, and Pacific Coastal Regions of the United States are recognized as areas of higher mean temperature (Anonymous, 1986*a*) and isolation rates for these regions were significantly higher than for other regions. No significant relationship was apparent between isolation rates and mean regional precipitation levels. Torten (1979) suggested that high levels of precipitation were not required for the occurrence of leptospirosis in his observation that oases in arid lands and deserts may become well-defined endemic zones by introduction of carrier animals.

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GENERAL SUMMARY AND DISCUSSION

The isolation of leptospires from 90 to 95% of bovine kidney tissues held under refrigeration in transport medium provided a basis for the methods of sample collection and submission for the prevalence survey. The difference between isolation rates from naturally and experimentally infected cattle was believed to be due to a lack of uniform distribution of leptospires in chronically infected kidneys. Since the sampling methods resulted in isolation of leptospires from 83% of naturally infected kidney tissues, it was expected that the same percentage would be detected in kidney tissues collected at random by the same methods in the prevalence survey. Based on this hypothesis, the 1.7% isolation rate would represent 83% of the total number of infected cattle, which would be 2%. This is the infection rate actually detected by the combination of cultural and FAT results.

Samples were collected from Federally inspected slaughter plants for two reasons: (1) most cattle are slaughtered in Federally inspected plants, and (2) the FSIS agency provided a network of communication with all plants under its authority and had the necessary data for plants in operation and number of cattle slaughtered. Less than 7% of mature cattle slaughtered in 1986 were slaughtered in state-inspected plants (Federal-State Relations Staff, FSIS, Washington, DC, personal communication, 1988) and these plants had no effective communication system in the form of a central organization. The state of origin of each animal sampled in the prevalence survey was taken from backtag and eartag numbers or the IIC's records, and in very

few cases were there doubts as to the state of origin of a particular animal. In those cases, the location of the slaughter plant was used as the state of origin.

The high rate of seropositives detected in the survey and the predominance of antibodies against serovar hardjo have been previously recognized. Although the vaccination history of cattle was unknown, many of the seropositives were believed to be due to residual antibodies from vaccination since companion tissue samples were neither culture- nor FAT-positive. Cross-reactivity among serovars was apparent in the reaction of some sera with as many as nine antigens. Serovars hardjo, pomona, and grippotyphosa have been previously recognized as bovine pathogens. The overall infection rate of 2%, the identification of 83% of the isolates as serovar hardjo, the regional distribution of isolates, and the isolation of serovar hardjo from Hawaii have not been previously reported. No relationship between infection rates and ages of cattle was apparent. The recognition that all isolates differed from the reference serovars may provide valuable information for epidemiological studies and selection of antigens for serological tests and bacterin production. Identification of isolates with the REA method not only saved 50% in time required, but allowed the identification of subtypes within recognized serovars.

Historically, leptospirosis has occurred more frequently in humans and animals in areas of high rainfall and temperature. The results of this study are in partial agreement. Mean temperature appeared to be useful in predicting which regions of the country would have higher infection rates.

Although higher infection rates were detected in some regions with higher mean rainfall levels, mean precipitation alone was not an accurate predictor of infection rates. Although the seroprevalence rates for summer, fall, and winter were significantly higher than for spring, no significant differences were detected in isolation rates among samples collected in different months or seasons of the year.

Future studies should be directed toward the estimation of prevalence and identification of serovars in swine, horses, and wildlife, the chain of transmission between wildlife carriers and domestic animals, and a more specific serological test for diagnostic and surveillance purposes.

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