

CLINICAL LABORATORY DIAGNOSIS OF LEPTOSPIROSIS

1. Choice, Timing And Interpretation Of Specific Diagnostic Tests

There are separate aims and needs for diagnosis of acute, chronic or carrier infection, and for epidemiological or epizootological screening. In acute infections of individual humans or animals an answer to the question: "Is the disease leptospirosis?" is needed as quickly as possible to indicate or confirm management, treatment and prognosis, to eliminate alternative diagnoses which may confuse strategies for management and treatment, and to indicate the directions for urgent epidemiological control and preventive measures. Additional information about the type of leptospire will be important later, rather than immediately, to help with prognosis and to identify sources. In chronic and carrier infections the questions are: "Is there evidence of continuing leptospiral infection in a person or animal? Are they infectious for others, by spreading leptospores in the environment through their urine?" Both speed and knowledge of the type of leptospire are important to identify a potential animal source of an acute outbreak, and to indicate public health risks in handling or transporting animals. Finally, in screening populations of animals or people for epidemiological surveillance, speed is usually less important than knowledge of the prevalence of leptospirosis, and the types of leptospores and their likely origins. In endemic areas, an understanding of the underlying prevalence and persistence of anti-leptospiral antibodies and of leptospirosis in the local populations of humans or animals is necessary for interpretation of serological tests in individuals.

Occupational history in humans is also important, because of the strong occupational incidence of leptospirosis.

2. Bacteriological diagnosis of acute leptospirosis in humans and animals

The principles, and in general, the practices of specific diagnosis in the acute stages are similar enough in human and animal leptospirosis to be treated together. Leptospiremia occurs simultaneously with the onset of the first symptoms and fever, so that efforts for early diagnosis are directed towards the detection of leptospores or their DNA or antigens in blood, CSF, urine and tissues. The techniques available are direct examination for leptospores, culture, hybridization with nucleic acid probes or amplification of *Leptospira*-specific target DNA using polymerase chain reaction (PCR). It is harder to find leptospores in specimens of blood or tissues, or to culture them from these specimens, after 5-7 days; there is little chance of detecting them other than in the "privileged sites", (where they are not exposed to antibodies and phagocytes), in the brain, anterior chamber of the eye and the renal tubules. In severe infections accompanied by a massive leptospiremia the chances of recovering viable leptospores in later stages are increased a little, provided the patient survives. There are reports of isolation of unidentified leptospores from blood cultures taken from older patients, in whom relative immunosuppression was postulated, as long as 90 days after initial infection.

2.1. Direct examination of blood, body fluids and tissues

During the first week to 10 days, especially days 3-7, leptospores may be seen on direct microscopic examination of blood, peritoneal or pleural exudates and urine. The advantage of direct observations is speed, leading to a definitive early diagnosis. The disadvantages are the technical difficulties of obtaining suitable specimens, the short time in which positive results are likely, and the subjective interpretation in reading results.

Specimens should be taken aseptically and sent to a laboratory without delay; they must not be frozen. Oxalate, citrate or heparin anticoagulant may be used for blood or pleural fluid. Volumes of approximately 1-10 mL may be taken; the larger volumes can be concentrated by centrifugation to improve sensitivity. The uncoagulated blood is centrifuged to remove cells, and leptospores sought in the plasma. Cerebrospinal fluid and urine taken as midstream, catheter or bladder-tap specimens may be obtained as indicated clinically. Tissues may be removed aseptically for surgical or experimental reasons, in animals. Otherwise, autopsy specimens, tissues from carcasses or abortion products will usually be contaminated, sometimes heavily.

Dark field examination is not recommended; even experts can occasionally confuse threads of fibrin and protein in wet preparations with leptospores. It is helpful to use a thin preparation with a higher magnification dry objective (x40) and a thin coverslip, to overcome diffraction of light in the darkfield by cells and fragments. Sensitivity is sacrificed by using the higher magnification. Observations of CSF and urine may be more reliable if the specimens are free of cellular elements. Results of direct examination must be supported by other

observations. Leptospire can be visualized by light microscopy in fluids or tissues during this period by immunostaining, using primary anti-leptospiral monovalent or polyvalent antisera, conjugated to a secondary antibody labelled with a fluorochrome (e.g. fluorescein isothiocyanate), gold enhanced with silver, or peroxidase. Avidin-biotinylated horseradish peroxidase complex alone binds to and stains leptospire. Radioimmunoassay has been used to detect antigen in urine. Use of immunostains presupposes a knowledge of the likely infecting serovar, whose antigens must react with the immunoconjugate, or requires use of a polyvalent antiserum. On the other hand, reactions of immunostains can usually presumptively identify the leptospire at least to serogroup level. In addition, leptospiral antigen in forms which no longer look like intact leptospire can be identified in tissues, phagocytes and in fluids, even in partially autolyzed tissues, especially if the specimens have been refrigerated during transport to the laboratory. The older silver stains are effective but non-specific for serovar, and some of them are prone to stain tissue fibrils nonspecifically; they cannot identify degenerated leptospire and antigen.

2.2. Culture of leptospire from clinical specimens

Cultures taken in appropriate conditions and media can be positive frequently, during the first few days, especially during fever. Incubation at 28-30° C is preferred because leptospire do not survive long at 37° C in culture media.

2.2.1. Blood cultures.

Volumes of 0.1-0.2 mL (1-5 drops) of blood, taken with usual blood culture aseptic precautions, are inoculated into each of 4-5 bottles containing approximately 5 mL - 10 mL of medium. EMJH (tween-albumin) medium supports growth of nutritionally demanding fresh isolates better, but serum-containing media (Korthof, Stuart, Fletcher) may be used. Too much blood can be inhibitory, especially after the fourth day when antibodies, and possibly antibiotics, are present; hence the need for adequate dilution. Addition of 0.5-1.0% sodium polyanethane sulphate, (Liquoid®), prolongs leptospiral survival in culture media. adding 0.1-0.15% agar (to make it semi-solid), and 0.4-1% rabbit serum, and or lactalbumin hydrolysate to the media. blood culture media for leptospire are not available, blood mixed with sterile anticoagulant (heparin, oxalate, or citrate) or clotted blood may be sent to a laboratory where it will be inoculated into media; leptospire can be grown from the clot or the serum. Since leptospirosis is frequently not among the diagnoses considered in febrile patients for whom blood cultures are requested, survival of leptospire in conventional bacterial blood culture medium is important but relatively unexplored; some strains tested survived in aerobic, unflushed commercial media with compatible peptones in their formulations. Rapid detection of growth from relatively small inocula was recorded in Stuart's medium supplemented with Middlebrook TB(12A) medium containing unspecified ¹⁴C-labeled fatty acids, designed to grow mycobacteria, especially of tuberculosis, in a BACTEC apparatus. Stuart's medium alone, supplemented with L-[¹⁴C].asparagine, was also effective.

Selective media, containing sodium sulfathiazole (50 µg/mL), neomycin sulfate (5 µg/mL) and cycloheximide (actidione) (0.5 µg/mL) may be used to reduce risks of contamination. Subcultures should be made within 48 hr to minimize the inhibitory effect of the selective agents on leptospire.

Media should be inspected initially for growth or contamination after 1, 3 and 5 days. The blood pigments often turn light brown, and the supernatant medium remains clear or very faintly turbid preceding visible growth, which may sometimes be seen as small grey and translucent balls, representing colonies, in the fibrin clot. Birefringent turbidity indicating established growth can occur after from 1-2 days incubation with heavy inocula, to 30 days or longer with slowly adapting small inocula. Negative cultures should not be discarded before final careful examination, including darkfield microscopy, at 30 days. Some keep cultures up to 90 days, or even 6 months. Apparent growth is checked by darkfield examination, and several replicate subcultures should be made as soon as growth appears, using relatively large inocula into prewarmed media. Isolates are often lost at this critical subculture stage. Contamination is often indicated by a purple, magenta discoloration of the blood in the medium, and turbidity.

2.2.2. CSF and urine

In the first 5-10 days, CSF cultures may be positive; volumes of 0.5 mL of CSF are inoculated into 5 mL of semisolid medium.

Urine specimens must be taken aseptically, by clean-catch or midstream collection procedures, catheter or bladder tap. In animals, furosemide may be used to encourage micturition. Urine should be alkaline when voided or made alkaline soon afterwards because leptospire die rapidly in acid urine. In humans, voided urine can be made alkaline by the usual clinical practices of administration of sodium bicarbonate or potassium citrate. The urine of carnivores or omnivores can be made alkaline by making drinking water alkaline.

In the acute febrile stages, the urine may not contain many leptospire. It is nevertheless worth culturing, in the same way as for blood cultures. The risk of contamination is very high in voided specimens, especially in those from immobile or unconscious severely ill patients. Uncontaminated specimens can be obtained by suprapubic aspiration of urine from the bladder. Contaminated specimens may negate the possibility of a result. Selective media (see above) should be used to reduce contamination. Although leptospiruria may continue beyond the febrile stage, during convalescence, significant leptospiruria due to renal tubular colonization is not seen until 14-28 days after infection, or about 1-3 weeks after onset of symptoms. In humans leptospiruria at this stage is infrequent, usually associated with an alkaline diet. It is transient, lasting days or weeks. In animals, this period may be prolonged and signifies the carrier stage, dealt with below.

2.2.3. Tissues

Tissues are seldom cultured in the first few days. Usually only specimens obtained at autopsies are available, but death is rare at this stage. Aborted or stillborn fetuses, fetal tissue, placentas and abortion products affected by acute leptospirosis are more usual specimens.

Consistent criteria are required for the definition of presumptive and confirmed cases using serological diagnosis; otherwise epidemiological patterns can not be validly compared. Samples of up to 1 g of tissue taken aseptically are either macerated or ground in a little sterile culture medium. The supernatant is inoculated in small volumes of 0.1-1 mL at a dilution of approximately 1:100 into medium, or serially diluted, to reduce the inhibitory effects of anaerobic conditions created by autolysis of the tissues. Blind subcultures after one day's incubation can improve chances for isolation. EMJH medium is recommended, but for more difficult leptospire, tween 80/40/lactalbumin hydrolysate medium, or EMJH medium, each with 0.15% agar and 100-200 µg/mL of 5-fluorouracil and 0.4%-1.0% rabbit serum, may be used; they allowed growth on primary isolation of serovar bratislava, an extremely fastidious and slow-growing leptospire.

3. Molecular approaches of the biological diagnosis of leptospirosis.

The first approach, direct probing on DNA extracted or purified from biological specimens, was limited by a relatively high threshold of detection of DNA and uncertainty about the specificities of the probes. PCR-based strategies for detecting specific leptospiral DNA were more useful; they require selection of specific primers to allow amplification of all strains that are classified as pathogenic or potentially pathogenic, including *L. inadai* and *L. fainei*. The two main targets have been a unique set of primers derived from leptospiral ribosomal 16S RNA gene (*rrs* gene) demonstrated as universal for the species *Leptospira* or a combination of two sets of primers derived from genomic libraries of serovars icterohaemorrhagiae and bim that cover seven species. Apart from minor differences in the methodologies, new data from testing human biological samples showed that leptospiral DNA can be detected early in the course of the disease (from day 2) with an average persistence of *Leptospira* in serum estimated at 12 days with a maximum around 40 days. In urine, leptospire were estimated to persist up to one year in some patients, indicating that leptospire may continue to be shed in urines longer than assumed using bacteriological methods. Ophthalmologic complications of leptospirosis can be diagnosed directly and positively by PCR on aqueous humor, supporting evidence of the direct involvement of leptospire in the genesis of ocular lesions. Similarly, PCR improved the reliability of diagnosis of leptospiral "aseptic" meningitis. PCR diagnosis also improves the accuracy of epidemiological evaluation of leptospirosis, allowing a better evaluation of the real incidence of the disease, especially in tropics. The same methods are available for diagnosis of veterinary leptospirosis.

Overall, PCR based strategies have improved the diagnosis of leptospirosis through their advantages of speed, sensitivity and specificity. They have also advanced knowledge of the natural history of the human disease, overcoming some of the limitations of bacteriological or serological investigations. The main limitations of PCR-based methods as routine diagnostic procedures remain the need of special equipment, the relative high cost of the reagents and the absence of automated and standardized procedures allowing the testing of large sets of samples, particularly in tropical countries where the disease is endemic.

4. Inoculation of laboratory animals

Virulent leptospires will cause an infection in suitable laboratory animals, which can be used for primary isolation of leptospires from clinical or environmental specimens. Hamsters, gerbils and guineapigs are used most often, although they are susceptible to a relatively small number of known serovars. The animals should be checked before use to make sure they are not carriers of leptospires.

Specimens of blood, CSF, urine or tissue suspensions from humans or animals for diagnosis are inoculated intraperitoneally, and the animals watched for signs of weight loss, fever, and clinical illness. (See Chapter 13). Leptospires will be found in the peritoneal fluid after the third day, sometimes transiently, in infected animals. Peritoneal fluid or more commonly blood may be cultured in conventional blood culture media to isolate the circulating leptospires. The typical appearances of animals infected with leptospirosis are described in Chapter 13. Leptospires may be seen and or isolated from blood or organs at autopsy. Surviving animals may become carriers in 14-28 days after inoculation. The investigation of their carrier state is described below. Animals should be used with discretion, only when necessary, in minimal numbers. All experiments must conform to accepted international protocols of humane treatment of laboratory animals. Precautions must be taken to protect staff handling and caring for the animals from inadvertent infection with leptospires. (Chapter 19).

5. Serological diagnosis in acute leptospirosis.

The need for non-bacteriological methods for diagnosis increases with time after onset, as the likelihood of detecting leptospires decreases; the diagnosis is frequently not considered during the early stages when leptospires can be detected. In addition, culture is slow and often uncertain. Traditionally, serology has been used to provide evidence of current or recent leptospirosis. IgM agglutinating antibodies usually appear within 3-10 days; fetuses, stillborn or neonatal victims have IgM antibodies in cord blood and in the placenta.

The main methods used are the microscopic agglutination test (MAT), and enzyme-linked immunoassay (EIA, ELISA), with a variety of antigens. Much more rarely, in very few places, micro-capsule agglutination, immune hemagglutination or hemolysis of antigen-sensitized erythrocytes, and complement fixation are used. The specimens are samples of serum obtained from blood, taken aseptically and sent to the laboratory in sterile containers, without anticoagulant. At autopsy, blood clot, or serum which has separated, may be taken. Alternatively a square or circle of sterile filter paper which will absorb a standard volume of 0.1 mL of blood can be used to soak up capillary blood from a finger or ear prick. The specimen is dried, labeled for identification and sent to a laboratory, where the serum dried on the paper is eluted and used for titration. At all times, standard biosafety precautions for handling blood and body fluids should be taken.

In general, blood for serology should be taken as soon as possible in the illness. A second specimen should be taken 5-7 days later, and repeated at similar intervals if necessary. The test may be negative in the early stages, but the second specimen may be positive or show a rise in titer compared with the first. A third or later successive specimen usually shows a rise in titer to a peak in 2-3 weeks. Sometimes antibodies do not appear until 3-4 weeks after infection; delays of months have been recorded. There are views that early antibiotic treatment can eliminate leptospires so quickly that antibodies cannot be detected later; in such cases diagnosis depends on successful culture from blood or other specimens. In the latter study, it is probable that infections with both serovars pomona and hardjo were epidemic, but only pomona was recognized, by serology, at the time. Some successfully treated patients who did not respond serologically to pomona antigens had titers to serovar medanensis, which cross-reacts a little with hardjo; response to hardjo was not tested.

Antibody levels drop over weeks or months, but may persist for 2-10 years at least in humans, and for comparable periods at low levels in animals, where they may persist throughout the life of the animal. In endemic areas, where many people or animals may have had leptospirosis, the interpretation of a single low titer may be impossible; a rising titre in successive specimens is then mandatory for serological diagnosis. Where there are antibodies to several serovars detected in early sera, the highest titer may not be against the infecting serovar. The highest titer in later sera tends to be more serovar-specific. MAT involves the reaction of any class or classes of immunoglobulin; the class cannot be determined from the routine MAT test. IgM antibodies produced early in infection can be detected with specific anti-IgM ELISA. The early IgM antibodies react broadly against a variety of serovars more than do the later IgG. However, some people produce little or no IgG. Thus it may not be possible to recognize the infecting serovar from the results of serological tests.

In some laboratories serum for MAT is serially diluted from an initial dilution of 1 in 100, to avoid low-level cross reactions. This procedure too insensitive to detect lower titer antibodies at a very early stage of infection, when they have most clinical significance. MAT tests for diagnosis should start at a titer of 1 in 50 or less. The maximum titer varies, depending largely on the serovar. Titers to 20-30,000 may follow infections with Icterohaemorrhagiae serogroup leptospire while serovar hardjo infections produce much lower levels, typically 1600-3000, in people or animals, corresponding to the relatively lower levels seen after immunization with hardjo. Low level early responses are thus especially significant with hardjo infections, in which maximum titers of 100 have been recorded in patients whose diagnosis of leptospirosis was proved by blood culture.

The MAT is specific for the infecting serovar or closely antigenically related serovars. Thus the likely infecting serovar must be known, or a battery of serovars used to ensure that there is a good chance of detecting antileptospiral antibodies. Most laboratories use at least 6, up to 15 serovars, representative of the locally common serogroups. Some reference laboratories use representative serovars of all serogroups. In the early diagnosis of clinical leptospirosis in humans it is vital to know whether the patient has leptospirosis rather than what sort of leptospirosis. The MAT has the disadvantage that it is tedious and wasteful to test against a large battery of serovars to ensure that the infecting serovar is included. Less serovar-specific agglutination tests used for diagnosis and epidemiological screening include agglutination of *L. biflexa* serovar *patoc* strain Patoc I, which was found empirically to be agglutinated by sera from patients and animals convalescent from infection with many serovars. In the author's laboratory, where infections are almost exclusively hardjo, pomona and tarassovi forms of leptospirosis, there was no significant difference between the proportion of "positive" reactions with the Patoc antigen in leptospirosis and non-leptospirosis patients. Other less tedious and less specific tests are a macroscopic agglutination reaction, as a slide test using pools of formalin-fixed antigens or heated cultures of strain Patoc I; immune hemagglutination and complement fixation; and microcapsule agglutination. They are all less sensitive than MAT, and are thus less useful for early clinical diagnosis. ELISA tests using broadly reactive antigens derived from boiled leptospire have not been used routinely because of poor correlation with MAT. ELISA using sonicated leptospire or LPS correlate better in human or animal leptospirosis and are more sensitive. IgM ELISA is more promising for detection of early leptospirosis but further evaluations are needed. Immunodot ELISA was comparable with Patoc agglutination for sensitivity, while a similar "line blot" immunoassay was comparable with Patoc agglutination and more sensitive than MAT starting at 1 in 100 dilution. A further adaptation of Elisa-IgM consists in a dipstick assay using a classical heat-extract of *L. biflexa* as antigen. Its main appeal is its ease of use, compatible with field conditions encountered where there are few medical resources. This type of test is essentially useful only for screening as it is limited by the same constraints that apply to conventional ELISA methods. In epidemiological studies the data provided by this type of method need to be validated using MAT in parallel as a reference method.

A general recommended standard is that, in an endemic area, a serological diagnosis of leptospirosis is **confirmed** in human patients or animals with a compatible clinical illness, where the microscopic agglutination titre (MAT) in sera taken 5-10 days apart rises from less than the starting dilution of the test (usually 1:50, or 1:100), to more than 100, or 4-fold or more, or is initially more than 400. In a non-endemic area, a single titre of 50 or more, with a clinically compatible illness, indicates likely leptospirosis; the titre will usually rise 4-fold or more in a second specimen a few days later. However, a single titre of 400 or more can lead to only **presumptive** diagnosis, indicating recent contact with leptospire which are not necessarily the cause of the current illness. The titre to some serovars is consistently higher than to others. Consistent criteria are required for the definition of presumptive and confirmed cases using serological diagnosis. These two categories need to be separated, otherwise diagnosis is subjective and epidemiological patterns can not be validly compared.

6. Bacteriological diagnosis in chronic leptospirosis, including carrier-excretor stages.

Leptospire will be found after the first 8-10 days only in the brain, anterior chamber of the eye in cases of uveitis, genital tract in animals, and the kidneys in humans or animals. The specimens and methods of examination are the same as for the diagnosis of acute infections. Molecular methods (probes or PCR-based methods) have given a new insight on the capacity of leptospire to persist in the infected host. They detected leptospiral DNA in a variety of biological samples, such as autopsy or biopsy specimens, urines, aqueous humor or CSF. Like for others spirochetes infecting humans (*Borrelia* or *Treponema*), the possible persistence of leptospire in protected sites is documented more and more frequently, as evidenced in the anterior chamber of the eye or in the urinary tract. Clearly, this new knowledge will influence therapeutic strategies.

The most important needs are to discover whether farm animals or wildlife are carriers of leptospire. Usually, renal carriage of leptospire is investigated, but carriage in the genital tract is important especially in

breeding stock. The usual specimens are urine and kidneys, and in genital infections, the internal genital organs (uterus, testes and adnexa). The main problems are that urinary excretion is sporadic and variable in amount, in many individuals. Concentrations of up to 10^8 leptospire/mL and down to the limits of detectability have been recorded in urine, sometimes from the same animal at different times. Excretion may continue for periods from several weeks, months or years, to the lifetime of the animal. An association between serum IgM ELISA antibody and renal excretion has been recorded in pigs. Urinary antibody does not preclude excretion of viable leptospire.

Specimens of urine obtained by usual methods, including stimulation of urination by furosemide, are examined and cultured as described above for acute infections. It is almost essential to use of selective media under field conditions.

The kidneys of chronic carrier animals vary in appearance from normal, (as frequently seen in naturally infected rodents), to scarred, pale shrunken kidneys characteristic of chronic nephritis, (as seen in some dogs). Most frequently, in cattle, pigs and sheep, the kidneys have grey spots beneath the capsule, with or without scarring and abnormal indentations. For cultivation of leptospire, the kidneys are removed, the surface seared and cut with a sterile knife or scalpel, and the cortical and subcortical areas sampled. Alternatively, the surface can be scarified and a plug of tissue removed, or a segment of the kidney removed to be ground and extracted as described above. In each case, cultures are set up as described above. Tissue sections may be prepared by removing blocks of suspect tissue, cutting them to not more than 5mm thick, and placing them in 10% formalin in saline, buffered to pH7.4 with 0.15M phosphate buffer. They may then be stained with immunostains or silver.

A comparison of immunoperoxidase staining of formalin fixed, paraffin embedded sections of pig kidneys with culture for finding leptospire in 72 pig kidneys showed 89% agreement. Staining detected 78% of infections confirmed by culture. In another study of 102 kidneys with white spots among 368 kidneys from abattoir-slaughtered pigs, 44 infected pigs could be identified by one or more of culture, immunogold staining or high MAT. Infection was found in 7.5% of macroscopically normal kidneys and 13.5% of those with spots. The relative sensitivities of the methods were 95% for MAT, 82% for IgM ELISA, 61% for culture, 55% for white spots, 52% for immunogold staining and 20% for silver staining.

Leptospire could be cultured from 85-95% of kidneys from experimentally infected cattle after storage in transport medium at 4°C for 0-8 days after slaughter.

7. Serological diagnosis in chronic infection or carrier stages

Serological testing differs little from testing in acute infection. Similar serum specimens are sent. However, in the late stages after titers have reached their peak, static high titers reflect recent infection.

The firm diagnosis of prolonged or chronic leptospirosis in humans is difficult. Symptoms of continuing lassitude, debility, neurological symptoms or depression may persist for periods of six months to two years after acute leptospirosis. There is a need to relate the condition to a firm initial diagnosis of leptospirosis, which is sometimes not available. There are usually no positive results from bacteriological specimens. Urine examination is fruitless. CSF is usually not available and diagnostic lumbar puncture cannot be justified; results are negative. Persistence of antibodies indicates nothing because immunoglobulins persist in many people for months or years and some remain IgM indefinitely. In view of the clinical and medicolegal importance of chronic leptospirosis in workers compensation claims for occupational illness, more work should be done to follow up patients prospectively and document accurately the syndrome of chronic or prolonged leptospirosis in humans.

ADDITIONAL INFORMATION:

WHO ILS GUIDELINES ON HUMAN LEPTOSPIROSIS

http://www.leptonet.net/html/who-ils_guidelines.asp