Leptospirosis

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Summary
Leptospirosis is caused by the members of the genus *Leptospira*. *Leptospira* are motile, helical spirochaetes that metabolise long-chain fatty acids as their sole carbon source. The genus *Leptospira* contains 17 species and are divided serologically into serovars of which there have been over 200 described.[1]

Leptospirosis is one of the world's most widespread zoonotic diseases. Native, feral and domestic animals, may serve as reservoirs, with rats and other rodents recognised as the most important sources.[1] Human infections result from contact with contaminated soil, vegetation or water, or with the body fluids of infected animals.[2]

There have been 24 *Leptospira* serovars identified in Australia. The average annual incidence of leptospirosis in humans in Australia is 1.0 per 100,000 of population over the last 10 years.[3] The incidence of leptospirosis in Australian animals, domestic or otherwise, is unknown.

The annual incidence of human leptospirosis in New Zealand is approximately 2.4 per 100,000 per year.[4] The incidence of leptospirosis in the animal population in New Zealand is unknown.

The diagnosis of leptospirosis is based on serology but nucleic acid-based methods, such as the polymerase chain reaction (PCR), are used in some diagnostic and research laboratories. The selection of diagnostic method is still influenced by the role of a laboratory and the degree of automation desirable to meet diagnostic demand and budget.[5]

The microscopic agglutination test (MAT) remains the reference serological method with isolation being the definitive standard for evidence of infection or carrier status. The MAT is used to identify the most probable infecting serovar or serogroup. The MAT relies on the use of live or formalised cultures as the source of antigen representative of local serovars. The MAT is generally limited to reference laboratories due to the inherent risks of handling cultures of the live organisms and the high cost of commercial media. The validity of results of the MAT is strongly linked to the content of serovars in the panel. Serum is the recommended sample type.

Rapid serological tests, such as the enzyme immuno assays (EIA) have been developed both as 'in house' and commercial tests. EIA can detect different classes of antibody, can be automated for handling large numbers of sera but may require confirmation of results by the MAT.

The PCR or nucleic acid-based assays have been used increasingly over recent years to detect a large number of microorganisms.[5,6] The sensitivity of PCR or nucleic acid-based assays obviates the need for isolation and culture. The PCR can be applied to a range of specimens from blood to urine.

Isolation of leptospires is not the method of choice for fast diagnosis because specialised media and resources are required to maintain the cultures for six to thirteen weeks.

Part 1 - Diagnostic Overviews

Aetiology

The genus *Leptospira* belongs to a group of distinctive bacteria called spirochaetes. They are a helical gram-negative aerobic bacteria 6-12 µm long and 0.1 µm in diameter. They are highly motile, spinning on their long axis.[2]

The species of *Leptospira* as determined by DNA-DNA hybridisation can be further divided into pathogenic, non-pathogenic and opportunistic/possibly pathogenic groups. Pathogenic *Leptospira* species include *L interrogans*, *L kirschneri*, *L santarosai*, *L weilii*, *L alexanderi*, *L borgpetersenii*, *L* genomospecies 1 and *L noguchii*. Non-pathogenic *Leptospira* include *L biflexa*, *L meyeri*, *L wolbachii*, *L* genomospecies 3, *L* genomospecies 4, *L* genomospecies 5. Opportunistic/intermediate *Leptospira* include *L broomi*, *L fainei* and *L inadai*.[7]

Clinical Signs

Leptospirosis affects a range of domestic animals including cattle, pigs, sheep, horses, goats and deer. Classically the serovar linkages are cattle with serovar Hardjo and pigs with serovars Pomona and Tarrasovi. There is serological evidence for the infection of sheep, particularly with serovar Hardjo, and sporadic evidence that sheep occasionally experience disease.[8,9,10] Infection of horses may also occur, although again most is subclinical.[11,12] Infection of dogs with serovar Copenhageni occasionally occurs in Australia.[13]

The clinical signs of infection can range from the inapparent to severe and be influenced by factors such as species, inoculation dose, immune status and age of the animal. Some of the clinical signs associated with acute disease include high fever, jaundice, haemoglobinuria, pulmonary congestion and death. The clinical signs most associated with chronic infections tend to be infertility and reproductive failure. Agalactia can be associated with clinical signs of the disease, particularly in dairy cattle.[2] Leptospirosis sometimes leads to lesions of the kidney, both visible and microscopic. In pigs at slaughter, visible kidney lesions ('white spotting') are often used as an indication that a group of pigs carries leptospirosis infection. However, white spotting may be of limited value in identifying individual pigs infected with *Leptospira*.[14]

Epidemiology

Leptospirosis, a zoonotic bacterial disease of worldwide importance, affects humans and domestic and wild animals. The bacteria are spread through the urine of infected animals due to a chronic infection of the renal tubules. The host may be incidental or develop a maintenance role, depending on the serovar type and animal species, and be responsible for transfer of the agent among similar species or other animals. The transmission success is governed by many factors such environment, climate and population density.[2]

Rodents are the most common carrier of *Leptospira*. Most mammals are considered as carriers of the bacteria but the most commonly encountered domestic animal carriers are pigs and cattle.

Transmission between animals and to humans depends on the excretion of leptospires from carrier animals. Transmission occurs either through direct transmission of the infection via contamination of mucous membranes, contact with *Leptospira*-contaminated water or soil, or venereal transmission through presence of the organism in reproductive tracts.[1,2] Leptospires shed in urine may remain infective in water and soil for more then two months under favorable conditions. Leptospires die rapidly in a dry environment and do not tolerate a pH below 6.8 but can survive in alkaline conditions up to 7.7-7.9. Most antibiotics are effective against leptospires.[1,2]

Human to human transmission has been recorded rarely and is not a recognised path of transmission for the disease.[2] In the case of where *Leptospira* isolates have been recovered from humans only (and where they have undertaken no overseas travel at the time) this supports the case for a local animal source of the particular infecting serovar. Serovars can have a range of animal species hosts, for example, serovar Canicola can be found in a range of animal species and is not limited to canines as a host or carrier.

Occurrence and Distribution

In Australia, 24 serovars have been isolated from domestic, native and feral animals. These belong to the following *Leptospira* species – *interrogans, borgpetersenii, santarosai, weilii* and *meyeri*. Cattle have been the source of isolates for serovars Australis, Celledoni, Grippotyphosa, Pomona, Zanoni, Hardjo and Topaz. Serovars Pomona and Hardjo have been recovered from sheep, serovars Hurstbridge, Pomona and Tarassovi from pigs, serovars Icterohaemorrhagiae, Copenhageni and Australis from dogs and serovar Pomona from horses. Most serovars however have been recorded as recovered from native or feral animal species. Serological investigations have shown flying foxes as possible carriers of leptospirosis.[15] Serological survey has shown cats in Australia to have antibodies against a range of serovars but without isolation studies an interpretation cannot be finalised, the same applying for serovars Bratislava in pigs and Tarassovi in cattle in Australia.[16, 17,18,19]

The incidence of leptospirosis and the extent of carriers in the Australian domestic animal population are unknown; vaccination programmes have helped to reduce the incidence of the known endemic serovars but recently discovered serovars such as Topaz and Arborea may have the potential to infect domestic animals and cause leptospirosis.

In New Zealand the main serovars isolated from animals belong to the *Leptospira* species *borgpetersenii* and *interrogans*. Serovars from these species have been reported in rats, mice, hedgehogs, pigs, dogs, possums, deer and cattle in that country. Serovars reported as isolated are Hardjo, Ballum, Tarassovi, Balacanica, Pomona and Copenhageni.[20] Serovars Australis and Canicola have been isolated from humans in a small number of cases. These serovars have not been reported in animals.

Leptospirosis is widespread throughout the pig populations in New Zealand.[21] Pigs are maintenance hosts for Tarassovi and Pomona, while cattle are maintenance hosts for Hardjo. Serovars Copenhageni and Ballum have been isolated from herds of healthy calves, which showed no clinical signs attributable to leptospirosis.[22] Sheep have been shown to maintain serovar Pomona with significant economic losses on affected properties. Farmed deer are maintenance hosts for serovars Hardjo, Pomona and Copenhageni. Few data are available on the clinical presentation and epidemiology of the disease in these deer populations but it is considered to be aligned to that of cattle in New Zealand.[15]

A study reported in 2005 found serological evidence to suggest leptospirosis was present in colonies of fur seals (*Arctocephalus forsteri*) on the Otago Peninsula of New Zealand; the potential for impact on other mammalian species was suggested.[23]

Pathology

Tissues fixed in 10% buffered neutral formalin and processed in paraffin blocks are suitable for staining both by haematoxylin-and-eosin and by immunohistochemical techniques. The most consistent microscopic lesions occur in the kidney and liver. Changes in other organs are referable to endothelial damage to capillaries and are variable in occurrence.

Diagnostic Tests (General)

The selection of diagnostic method is influenced by the role of a facility. The microscopic agglutination test (MAT) is the preferred serological method for diagnosis with isolation of the organism providing opportunity to definitively identify the infecting serovar.[5] The use of nucleic acid detection methods, such as the polymerase chain reaction (PCR), are becoming more widespread in diagnostic and research laboratories.

Attempted isolation of leptospires is infrequently used in laboratories but should not be ignored. The successful isolation from an animal may indicate a current infection or carrier status, but a negative result does not exclude an infection with the agent. Cultures must be examined weekly. It may be difficult to see growth in fluid media until the concentration of leptospires has become very high, when opalescence is discernible when the tube is gently agitated. Urine is the preferred sample but aseptic technique should used to avoid contaminating the culture medium.

The use of darkfield microscopy can be applied for examining body fluids and cultures. *Leptospira* are too thin to be seen by conventional light phase microscopy and need to be visualised by darkfield microscopy. Experienced staff are necessary to ensure accurate assessment of specimens. The method is considered to lack sensitivity and specificity and is related to the quality and type of type of specimen and the presence of fibrin and other protein threads in blood samples, which can be mistaken as leptospires.[1]

For serology the MAT is the preferred test. Other tests such as the EIA can detect different classes of antibody but may be subject to false positive reactions and will require confirmation of these results by the MAT.[2] The diagnostic performance of the MAT is strongly linked to the content of serovars in the panel used for each locality. Test sensitivity depends on the standardisation of culture densities to meet the required $2x10^8$ leptospires per mL with accurate reading to the 50% endpoint. Positive and negative predictive values can be subjective in animals but enhanced by the use of acute and convalescent specimens.

Nucleic acid-based assays have been used increasingly over recent years to detect a large number of microorganisms.[5] The sensitivity of PCR often precludes the need for isolation, thus making it ideal for the rapid detection of organisms involved in active infections. Moreover, by real-time PCR, it is possible to quantify the amount of template and therefore the number of target organisms. The drawback with PCR is the inability to identify the infecting serovar. The PCR can be applied to a range of specimens from tissues, blood to urine. Test sensitivity depends on the ability of the primers to detect pathogenic species of the genus circulating in the population plus the type of PCR adopted by the laboratory.

Positive and negative predictive values are very high if the designed primers are able to detect pathogenic species of *Leptospira*. All PCR-based tests must undergo appropriate evaluation and laboratories should follow the guidelines developed by SCAHLS for the use of PCR technology.

Part 2 - Test Methods

1. Microscopic Agglutination Test (MAT)

This method is used for the detection of serovar-specific *Leptospira* antibodies in serum. The following method applies to the use of flat-bottom microtitre plates. In selecting the plate optical clarity is important to facilitate reading of agglutination. Do not use round bottom plates.

Total microscopic magnification for reading the MAT should be 80-120 otherwise determinations of endpoint readings may become inaccurate.

The test may be carried out using either suspensions of living cultures, or cultures killed by the addition of neutralised formalin.

Screening of sera

(a) Sera and positive controls are diluted 1/25 in Phosphate Buffered Saline (PBS) pH 7.4 as follows:

PBS	1200 μL
Serum	50 μL

A negative control consisting of 25 μL of diluent (PBS pH 7.4) per well must also be run for each serovar. This checks for diluent sterility and as an auto agglutination check.

(b) Dispense 25 μL of the diluted serum to each well (in the horizontal row) of the microtitre plates in its appropriate batch position.

Add 25 μL of diluted positive control (relevant hyperimmune serum) into the first row as follows:

MAT Control 1:	wells 1 to 5
MAT Control 2:	wells 6 to 10
MAT Control 3:	wells 11 to 15
MAT Control 4:	wells 16 to 22

(c) Using a single channel multistepper pipette, add 25 μL of a well-mixed serovar culture to the appropriate vertical rows of the microtitre plate.

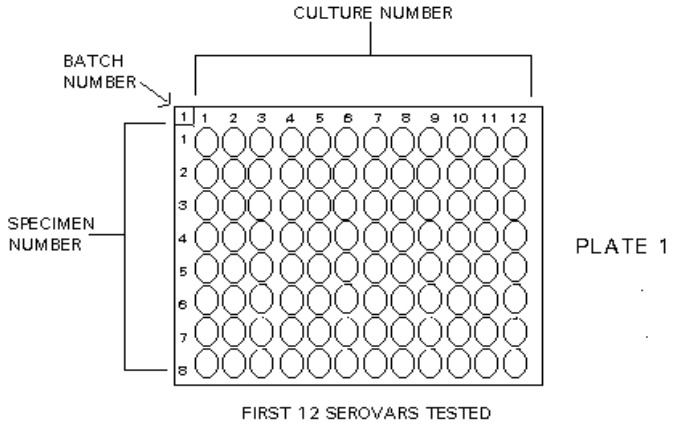
NB. It is important that only one drop be dispensed in each of steps (a) and (b).

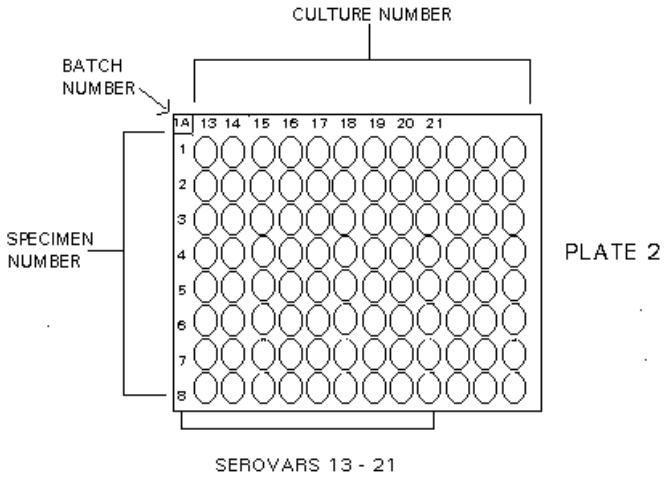
(d) Batch plates are shaken to mix, stacked no more than 6 high and each stack covered with a plastic plate cover.

(e) Plates are then incubated at 30°C for 90 minutes. Do not leave plates in incubator longer as bacterial overgrowth will make them difficult or impossible to read.

(f) Plates are then viewed under dark-field microscopy for agglutination. Serovars showing agglutination are marked on the worksheet with a cross.

Microtitre Plate Configuration





Quantitative test

Prepare serum dilutions in a flat-bottom microtitre plate by the following method. This gives a final serum dilution of 1:50 to 1:6400 once the antigen has been added.

(a) Add 25 µL PBS to wells 2 to 8 with a multistepper pipette.

(b) Dilute serum and positive controls 1:25 with PBS in plastic tubes as follows:

PBS	1200 µL
Serum	50 µL

(c) Run positive controls corresponding to those serovars that were found positive for sera in the screening test.

(d) For both samples and positive controls, add 25 μL of diluted serum 1:25 to wells 1 and 2 with multistepper pipette.

(e) Prepare doubling dilutions with multichannel pipetter from well 2 to 8.

(f) Add 25 μL of antigen to wells 1 to 8.

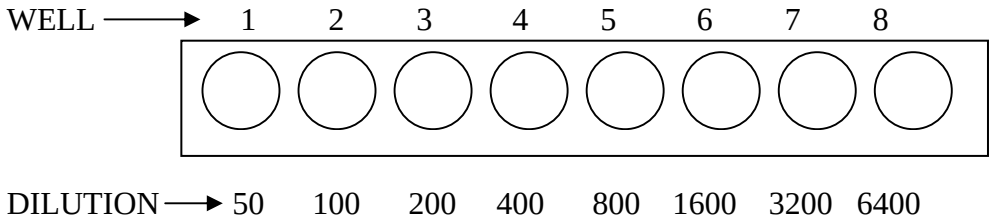
(g) Shake manually to mix.

(h) Incubate at 30°C for 90 minutes.

(i) View plates under dark-field microscopy for agglutination.

(j) Record titre as the highest dilution showing 50% agglutination.

Final dilutions in each well are as follows:



Result definition

The clumps of living *Leptospira* differ in appearance to the clumps of killed organisms. Living *Leptospira* will agglutinate into highly refractive spheroids of various sizes, some of which may be joined to produce elongated masses of confluent spheroids. The degree of agglutination ranges from 100%, where no free *Leptospira* can be seen between the clumps, through lesser degrees - as the serum becomes more dilute - to nil, as the presence of antibody becomes less. As fewer *Leptospira* are agglutinated, more can be seen free (that is, un-agglutinated) in the reaction wells. The degree of agglutination can be assessed only in terms of the proportion of free leptospires. The accepted end point of an MAT is the final dilution of serum at which 50% or more of the leptospires remain agglutinated.

Interpretation of test results

The interpretation of serology results is often not straightforward, and should be viewed with caution. The antibodies measured by tests such as the MAT are long lasting, and so a low or moderate titre may indicate infection that has long passed. Conversely, the MAT may be negative in infected animals. Antibodies induced by vaccination are generally short lived (weeks) and low. However, vaccinated animals sometimes become infected, and may show reduced serological responses to infection compared with unvaccinated animals.

It is often desirable to take two serum samples, two weeks apart, for comparison. A four-fold increase in titre between such paired sera indicates active infection, and a four-fold decrease in titre between paired sera indicates recent infection.

1.1 Serology

While the MAT is the test of choice for serological determination of an infection and the likely infecting serotype, EIA have a role in diagnosis of leptospirosis. Assays designed to specifically detect IgM antibody may give a better indication of current infection than the MAT does. However,

EIA for leptospirosis diagnosis is not yet sufficiently well standardised for adoption as routine diagnostic tests. Complement fixation and haemagglutination tests have been applied for the detection of antileptospiral antibodies, but have not been adopted as routine tests.

The interpretation of results is influenced by the type of specimen, clinical state of the animal and background information such as vaccination history of the animal or population of animals under assessment. The consideration of serology in combination with these other tests will help define the interpretation.

1.2 Culture Maintenance

Successful culture maintenance is dependent on the quality of media and integrity of seed cultures.

For many laboratories the use of the commercial medium is recommended (see Part 3). However it may be cheaper to prepare the medium from basic components (analytical reagent grade, AR), according to the formulae given in Tables 1 and 2. (See Part 3) Recommendations for preparing the medium using stock solutions are given in Johnson and Harris[24] and Turner.[25]

The pH values of both the medium base and the enrichment broth are adjusted to 7.4.

The following supplements are also recommended: sodium pyruvate and sodium acetate, each 0.1 g/L.

An alternative practical approach is to prepare the entire medium (medium base plus enrichment broth plus supplements) as a combined 10 times concentrated (10X) solution. The iron sulphate (Table 2) must be dissolved in water before it is added to the other components, or precipitation occurs. The 10X solution is filtered by sterile filtration, no part of it being autoclaved. It can then be stored at 2-5^{o}C for up to 3 months and diluted 1/10 in sterile distilled water before use.

Cultures for routine use in MAT serology should be used 3-6 days after subculture. For subculture inoculate about 0.5 mL of a 7-day-old culture into 3-5 mL of EMJH liquid medium and incubate in air. Up to five drops can be added when the culture is unhealthy or unusually slow growing. Healthy cultures can only be determined by inspection using darkfield microscopy to check for contamination. After 7 days of incubation, the culture medium should be turbid due to the density of the organisms. If contamination is confirmed the culture should be discarded. The density of leptospires can be assessed in a bacterial counting chamber, and adjusted if necessary to about 2×10^{8} leptospires per mL. An experienced person can make an approximate assessment of culture density visually.

2. PCR-Based Methods

All PCR-based tests must undergo appropriate evaluation and laboratories should follow the guidelines developed by SCAHLS for the use of PCR technology. In particular the design and layout of the PCR testing facility must be such as to minimise contamination.

The use of PCR has evolved steadily over recent years with a shift away from gel-based methods to real-time methods, which incorporate specific probes and primers and can be very sensitive, detecting down to 2-3 organisms per sample. Using real-time PCR, it is possible to quantify the amount of template and therefore the number of target organisms. Such quantification can be achieved using a number of approaches, for example employing Taqman chemistry. Smythe et al have published an example of design of a primer and probe set used for detection of Leptospira.[26]

3. Histopathology

Liver and kidney fixed in 10% buffered neutral formalin and processed in paraffin are suitable for staining both by haematoxylin-and-eosin and by immunohistochemical techniques.

Lesions in the kidney, which are confined to the cortex, include interstitial infiltration, principally by lymphoreticular cells but sometimes with significant numbers of neutrophils, and varying degrees of damage to the tubular epithelium cells resulting in the formation of tubular casts. Plasma cells are prominent in advanced cases. Syncytial masses, formed by regenerating tubular epithelium and resembling Langhans' giant cells, may be seen[27, 28].

In acute leptospirosis, dissociation of hepatocytes in the liver disorganises hepatic chords. Centrilobular necrosis is seen and additional foci of necrosis with infiltration by neutrophils are scattered through the parenchyma. The Kupffer cells contain varying amounts of haemosiderin. The livers of piglets, aborted as a result of infection with serovar Pomona, may show a characteristic focal necrosis with little or no associated inflammatory reaction.

3.1 Histological Stains for Leptospires

A diagnosis of leptospirosis may be confirmed by demonstration of leptospires in tissues by non-specific silver staining (the Warthin-Starry technique) or by the more sensitive immunohistochemical techniques. Of the latter, immunofluorescent staining and immunogold silver staining are the most useful. Immunogold silver staining, unlike immunofluorescent staining, has the advantage that it produces a permanent image that does not fade with time. Furthermore a fluorescence microscope is not required.

Organisms can be demonstrated in the renal tubules of animals with kidney infection and leptospiruria. Pepsin digestion can be used to improve the access of immunofluorescent or immunogold stains to leptospiral antigens. Application of immunohistochemical staining to aborted foetal tissue is possible, but may be hindered by autolytic changes.

Irrespective of the technique used, a positive control section should be included in the preparation of each batch of histological sections.

The Warthin-Starry Technique [29,30, 31]

Solutions

Buffer solution

Combine 1.5 mL of 0.2 mol/L sodium acetate with 18.5 mL of 0.2 mol/L glacial acetic acid (11.8 mL glacial acetic acid made to 1 L with distilled water), and make to 50 mL with distilled water. The pH should be 3.6; if necessary adjust up with 0.2 mol/L sodium acetate or down with 0.2 mol/L acetic acid.

Developer solution

The following three solutions should be all brought to 55ºC and mixed in the order given just before use:

Silver nitrate, $AgNO_3$, 2%, in buffer	7.5 mL
Gelatin, 5%, in buffer	37.5 mL
Hydroquinone (quinol), 3%, in buffer	2.5 mL

Staining Technique
(a) Take thin paraffin sections through xylene and alcohol to buffer.

(b) Impregnate with 1% silver nitrate in buffered water at 55–60ºC for one hour (warm the solution first).

(c) Prepare developer solutions and place in oven or water bath. Place tap water in oven or water bath to heat.

(d) Place slides in fresh developer at 55ºC for 1.5–3.5 min. The sections should become a golden brown colour.

(e) Drain sections briefly, rinse for 2–3 min in warm tap water (55–60ºC) and then cool in cool buffer solution.

(f) Dehydrate, clear and mount.

Leptospires are seen as black in pale yellowish-brown tissues.

Immunochemical Staining

Introduction

The optimum incubation periods, within the range indicated, may be found to vary from one laboratory to another depending on the material under study. They should, if possible, be determined experimentally using known positive and negative material.

Positive and negative controls should be run with each batch of unknown samples, with either of the following techniques. Stained slides can be scanned at a magnification of ×100 or ×200, and viewed closely with ×400 or ×800.

Solutions

Solutions for immunofluorescent and immunogold staining
Washing solution. 0.05% Tween 20 in 0.85% saline.

Antiserum diluent. PBS pH 7.2, containing 0.05% v/v Tween 20, 1 g/L bovine albumin, 0.1% v/v Triton X-100, and 0.1 g/L thiomersal.

Solutions for immunogold staining only
Developer solution A. 0.5 mol/L hydroquinone.
Prepare on the day of use by adding 1.65 g to 30 mL of water. Heat to dissolve and allow to return to room temperature (about 21ºC) before use.

Developer solution B. Citrate buffer, pH 4.0.
Prepare stock solution by adding 100 mL of 0.5 mol/L citric acid ($C_6H_8O_7$) to 75 mL of 0.5 mol/L trisodium citrate ($C_6H_5Na_3O_7$). On the day of use, dilute 20 mL of stock solution with 120 mL of water.

Developer solution C. 0.037 mol/L silver lactate ($C_3H_5AgO_3$, 0.22 g in 30 mL water).
Do not add water to silver lactate until under safe light. Store silver lactate in the dark by enclosing the bottle, and the weighed-out material, in aluminium foil.

Stop bath solution. 2% v/v glacial acetic acid.

Fixer solution. Commercial fixer is used. Ilford rapid fixer CP4143 is suitable.

Eosin counterstain.
Prepare stock solution of 1% eosin Y in 80% alcohol. Dilute stock for use, 1:4 in 80% alcohol, and add 500 μL glacial acetic acid per 100 mL stain.

Immunofluorescent Staining[32]

(a) Cut paraffin sections of about 4 μm, and dry in an oven at 56–60ºC. Drying in an oven helps the section to adhere to the slide.

(b) Dewax by washing through three 10-min changes of an appropriate clearing agent, dehydrate by three changes of absolute alcohol, and rinse in tap water.

(c) Digest the sections in freshly prepared 1% pepsin in 0.02 mol/L hydrochloric acid at 37–50ºC for one to four hours. The optimal temperature and incubation time depend on the type of tissue being treated, the degree of any autolysis, and the source of the pepsin used.

(d) Wash with very slowly running tap water, then in washing solution. (Slides may be left overnight in washing solution if desired.)

(e) Quickly drain the slides, and dry around the tissue sections with absorbent paper.

(f) Cover the sections with 100 μL of an appropriate dilution, in antiserum diluent, of rabbit antiserum against leptospiral cells. Rabbit antisera with an MAT titre of 2000 or more can be diluted about 1/200 or more. Antiserum against one leptospiral serovar can be expected to cross-react with other serovars.

(g) Incubate in a moist chamber at room temperature for 30 min to two hours.

(h) Wash each three times for 5–10 min in washing solution.

(i) Repeat step (e).

(j) Cover the sections with 100 μL of an appropriate dilution of fluorescein isothiocyanate-conjugated sheep or goat antirabbit IgG.

(k) Repeat steps (g) and (h).

(l) Counterstain with 0.1% Evans blue (C.I.23860) for 5 min, rinse with washing solution, mount in buffered glycerol pH 8.6, and examine sections using an incident-light fluorescence microscope.

Immunogold Silver Staining[33]
Steps (a-i) above

(j) Cover the sections with 60–100 μL of an appropriate dilution (about 1/40) of gold-conjugated goat anti-rabbit IgG in antiserum diluent.

(k) Repeat steps (g) and (h).

(l) Incubate slides in 2% glutaraldehyde solution at room temperature for 15 min.

(m) Rinse well in distilled water.

(n) Before going to the dark room, prepare solutions A and B, weigh the silver lactate for solution C, and prepare commercial photographic fixer and the stop bath solution.

(o) In the dark room, quickly dissolve the silver lactate in the required volume of water to give solution C, mix solutions A (30 mL) and B (140 mL), add solution C (30 mL) to this mixture to prepare the developer (200 mL), and immediately place slides in the developer.

(p) Develop for about 100 s. The development time is of critical importance: 100 s is about right if the developer is at 21ºC.

(q) Transfer slides to the stop bath solution for about 30 s, then to the fixer for 30–60 s.

(r) Rinse well in slowly running tap water.

(s) Counterstain in eosin for 5–15 min.

(t) Rinse three times extremely quickly in absolute alcohol. (Alcohol is necessary for dehydration, but excessive alcohol washing removes the counterstain.) Rinse three times in xylene or similar agent to remove the alcohol, and then mount with a suitable medium and a cover slip.

(u) View under a light microscope

4. Isolation from Field Material

Leptospires are fastidious and slow growing. When isolating leptospires from field material (usually urine or kidney), contamination with faster-growing microorganisms often presents a problem.

A culture established from field material should be maintained at 30ºC for at least two months before being considered as negative. Drops of culture medium should be thoroughly examined under a dark field microscope at least once every two weeks during this period.

Cultures can be occasionally topped up to their original volume with fresh medium if evaporation occurs. A magnification of ×200 is suitable for screening the cultures, and ×400 can be used for close examination of the leptospires.

It is important to use freshly collected material. Furthermore, the likelihood of isolating leptospires from field material can be increased by:
- performing the culture at a series of dilutions;
- adding antimicrobial agents to the culture medium; or
- using semisolid medium.

Dilution of the inoculum serves both to reduce the likelihood of contamination and to reduce any growth inhibition caused by lipids or other components of the inoculum. A suitable procedure for kidney is to prepare in a Stomacher™ a 10–20% tissue suspension in sterile phosphate buffered saline (PBS) or in EMJH base, and to dilute this serially into culture medium at final concentrations of 1, 0.1 and 0.01%. Urine can be added at the same final concentrations.

The addition of 0.1 g/L 5-fluorouracil and 0.1 g/L actidione is recommended. However, these will not prevent all contamination.

A selective medium, consisting of six antimicrobial agents added to EMJH medium[34], is effective against many potential contaminants, and has been used to isolate serovars Pomona and Hardjo from urine and from blood. However, it has not yet been demonstrated to be suitable for isolating leptospires from kidney tissue.

Semisolid medium, containing 0.1–0.3% agar, can be of value for field isolation, particularly for serovar Hardjo.

The use of a diuretic greatly increases the success of isolating leptospires from bovine, ovine or porcine urine.

5. Storage of Leptospiral Cultures

Leptospires adapt progressively to culture, and can be maintained with regular subculturing every few days into fresh liquid medium. During this process they lose virulence and become unsuitable for infection into the animal host.

If maintaining virulence is not important, isolates can be grown to reasonable density in semisolid EMJH medium at 30ºC, and subsequently stored in the dark at room temperature. These cultures can survive up to three years, but are best subcultured at intervals of six months.

Leptospiral cultures may be stored long term in liquid nitrogen, with the maintenance of virulence. The procedure[35] is as follows:

(a) Grow culture well into log phase (10^9 organisms/mL is suitable) in liquid EMJH medium.

(b) Add dimethyl sulfoxide (DMSO) to the culture to a final concentration of 2.5%. The DMSO should be sterilised by filtration before use.

(c) Dispense into plastic vials suitable for liquid nitrogen storage.

(d) Wrap vials in cotton wool and freeze slowly overnight in a freezer of -70ºC to -80ºC.

(e) Transfer to a liquid nitrogen tank for long-term storage.

Cultures are revived by thawing a vial at room temperature or in a water bath at 30ºC, dispensing into fresh EMJH liquid medium, incubating at 30ºC, and subsequently subculturing to dilute out the DMSO. Glycerol should not be used as a cryopreservative, as it is toxic for leptospires.

6. Risk

Leptospira according to the *Australia/New Zealand Standard – Safety in Laboratories. Part 3: Microbiological aspects and containment facilities* are Risk Group level 2 and should be handled as specified in the standard.

Part 3. Reagents and Kits

1. Kits and Reagents in Common Use in Australia

The supply of Leptospira cultures and antisera can be obtained from the:

WHO/FAO/OIE Collaborating Centre for Reference & Research on Leptospirosis
Western Pacific Region
Queensland Health Forensic and Scientific Services
39 Kessels Road, COOPERS PLAINS QLD 4108
Phone: (07) 3274 9064
Fax: (07) 3274 9175

EMJH Culture Medium

EMJH medium is generally prepared as a medium base, which can be autoclaved, and an enrichment broth, which is sterilised by filtration. This arrangement makes it unnecessary to filter the entire medium. The base and the enrichment broth are combined in the ratio 9:1. EMJH medium is available from:

Becton Dickinson (BD Diagnostics) Pty Ltd
Business Development, Biopharm & Industry, Asia Pacific
Phone: +61 7 3347 4700
Fax: +61 7 3347 4747
Street Address: 69 Brandl Street, Eight Mile Plains, QLD 4113
Postal Address: PO box 4021, Eight Mile Plains, QLD 4113
or
BioScientific Pty. Ltd
PO Box 78
Gymea NSW 2227 Australia
International: +61 (0)2 9521 2177
Australia: 1300 BIOSCI (246724)
New Zealand: 0800 444 157
Facsimile: +61 (0)2 9542 3100

Table 1. Formula for EMJH Basal Medium

		g/L
Disodium hydrogen phosphate	Na_2HPO_4	1.0
Potassium dihydrogen phosphate	KH_2PO_4	0.3
Sodium chloride	NaCl	1.0
Ammonium chloride	NH_4Cl	0.25
Thiamine		0.005
Glycerol		0.1

Table 2. Formula for EMJH Enrichment Broth

		g/L
Bovine albumin, fraction V		100
Calcium chloride	$CaCl_2.2H_2O$	0.1
Magnesium chloride	$MgCl_2.6H_2O$	0.1
Zinc sulphate	$ZnSO_4.7H_2O$	0.04
Copper sulphate	$CuSO_4.5H_2O$	0.003
Iron sulphate	$FeSO_4.7H_2O$	0.5
Vitamin B12		0.002
Tween 80		12.5

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