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Leptospirosis

Pathology, Bacteriology and Serology

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1. Introduction

Pathogenic leptospires, which were formerly all included in the species *Leptospira interrogans*, are classified on the basis of agglutinating antigens into over 200 serovars contained within 23 serogroups. These cause several disease conditions in domestic animals, wildlife and humans. Infection may also be asymptomatic or subclinical.

On the basis of DNA studies, pathogenic leptospires have recently been reclassified into six species two of which are *L. interrogans* (including serovars *pomona* and *copenhageni*) and *L. borgpetersenii* (including serovar *hardjo*) (Yasuda *et al.*, 1987; Ramadass *et al.*, 1990).

Clinical leptospirosis of domestic animals in Australia is largely confined to cattle (serovars *hardjo* and *pomona*) and pigs (serovar *pomona*). There is serological evidence for the infection of sheep, particularly with serovar *hardjo*, and sporadic evidence that sheep occasionally experience disease (Gordon, 1980; McCaughan *et al.*, 1980; Cousins and Robertson, 1986; Cousins *et al.*, 1989). Infection of horses may also occur, although again most is subclinical (Slatter and Hawkins, 1982; Hogg, 1983). Infection of dogs with serovar *copenhageni* occasionally occurs in Australia (Watson *et al.*, 1976). Human leptospirosis is most commonly associated with contact with cattle in milking sheds, and with pigs at abattoirs.

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 has only limited value in identifying individual infected pigs (Jones *et al.*, 1987).

A valuable general reference for most aspects of leptospirosis in livestock and humans is Faine (1982).

Diagnosis of leptospirosis depends on serology (primarily involving the microscopic agglutination test) or on the detection of leptospiral organisms. Serology has an important role, but detecting the organism is preferable. Isolation of the bacteria from urine or tissues remains important, but culture of leptospires is slow, laborious and expensive. The alternatives of microscopic examination of urine, and histochemical staining, are of value. DNA hybridisation will certainly be important in the future.

Table 1. Formula for EMJH Basal Medium

		g/L
Disodium hydrogen phosphate	Na ₂ HPO ₄	1.0
Potassium dihydrogen phosphate	KH ₂ PO ₄	0.3
Sodium chloride	NaCl	1.0
Ammonium chloride	NH ₄ Cl	0.25
Thiamine		0.005
Glycerol		0.1

2. Bacterial Culture

2.1. Introduction

Culture serves two purposes: the isolation of leptospires from clinical material, and the maintenance of leptospiral strains for use in the microscopic agglutination test (see 3.2).

Leptospires grow in liquid medium. The medium of choice is the Johnson and Harris modification of the Ellinghausen and McCullough medium, known as EMJH (Johnson and Harris, 1967; Turner, 1970). This is supplemented by sodium acetate (CH₃CO₂Na) and sodium pyruvate (C₃H₃O₃Na) (see 2.2.). Semisolid medium is of value when isolating from field material, but laboratory-adapted strains grow well in liquid medium.

2.2. 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 commercially from Difco [medium base code 0794; enrichment broth code 0795, available from BioScientific Pty Ltd, PO Box 78, Gympie, NSW 2227, Tel. (008) 25 1437; Fax (02) 542 3037].

For many laboratories the use of the commercial medium is to be preferred. However it is cheaper to prepare the medium from basic components (analytical reagent grade, AR), according to the formulae given in Tables 1 and 2. Recommendations for preparing the medium using stock solutions are given in Johnson and Harris (1967) and Turner (1970).

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 solution. The iron sulfate must be predissolved in water before it is added to the other components, or precipitation occurs. The 10 times concentrate is prefiltered before sterile filtration, no part of it being

Table 2. Formula for EMJH Enrichment Broth

	g/L
Bovine albumin, fraction V	100
Calcium chloride	CaCl ₂ ·2H ₂ O 0.1
Magnesium chloride	MgCl ₂ ·6H ₂ O 0.1
Zinc sulfate	ZnSO ₄ ·7H ₂ O 0.04
Copper sulfate	CuSO ₄ ·5H ₂ O 0.003
Iron sulfate	FeSO ₄ ·7H ₂ O 0.5
Vitamin B12	0.002
Tween 80	12.5

autoclaved. It can then be stored for several months in the refrigerator and diluted 1/10 in sterile distilled water before use.

2.3. Isolation from Field Material

Leptospire are fastidious and slow growing. When isolating leptospire 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 30°C 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 x200 is suitable for screening the cultures, and x400 can be used for close examination of the leptospire.

It is important to use freshly collected material. Furthermore the likelihood of isolating leptospire from field material can be increased by any of the following: performing the culture at a series of dilutions; adding antimicrobial agents to the culture medium; and 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 serially dilute this 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 has been developed by Adler *et al.* (1986). This medium 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 leptospire from kidney tissue.

Semisolid medium, containing 0.1–0.3% agar (Bacto), can be of value for field isolation, particularly for serovar *hardjo*.

The use of a diuretic (such as Furosemide, 0.8 mg/kg body weight) greatly enhances the success of isolating leptospire from bovine, ovine or porcine urine.

2.4. Storage of Leptospiral Cultures

Leptospire 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 30°C, 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, by the method of Palit *et al.* (1986). The procedure 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 -70°C to -80°C.
- (e) Transfer to a liquid nitrogen tank for long-term storage.

Resurrection of the culture is achieved by thawing a vial at room temperature or in a water bath at 30°C, dispensing into fresh EMJH liquid medium, incubating at 30°C, and subsequently subculturing to dilute out the DMSO. Glycerol should not be used as a cryopreservative, as it is toxic for leptospire.

3. Serology

3.1. Introduction

The only widely accepted serological test for leptospirosis is the microscopic agglutination test (MAT). The MAT employs live leptospiral bacteria as antigens, and these must be routinely maintained by the diagnostic laboratory.

Agglutination of leptospire is serogroup-specific, and a different antigen must be employed for each serovar or serogroup of interest.

Complement fixation and haemagglutination tests have been applied for the detection of antileptospiral antibodies, but have not found favour as routine tests. The experimental use of enzyme immunoassays (also called ELISAs, or enzyme-linked immunosorbent assays) in leptospirosis diagnosis has become fairly widespread: in Australia they have been applied to the sera of cattle (Adler *et al.*, 1982), sheep (Cousins and Robertson, 1986) and pigs (Ballard *et al.*, 1984). Assays designed to specifically detect IgM antibody may give a better indication of current infection than the MAT does. However, enzyme immunoassays for leptospirosis diagnosis are not yet sufficiently well standardised for adoption as routine diagnostic tests.

3.2. Microscopic Agglutination Test

3.2.1. Maintenance of Stock Cultures for Antigen

Original cultures should be obtained from The WHO-FAO Leptospirosis Reference Laboratory, State Health Laboratories, George Street, Brisbane, Qld 4000; Tel. (07) 224 5545; Fax (07) 221 9737.

Cultures for routine use should be subcultured twice weekly (each three to four days). Inoculate about one drop of culture into 5 mL of EMJH liquid medium. Up to five drops can be added when the culture is unhealthy or unusually slow growing. Incubate at 30°C. Cultures are grown for two to three days until ready to use. The density of leptospire can be assessed in a bacterial counting chamber, and adjusted if necessary to about 2×10^8 /mL. An approximate assessment of culture density can be made visually by an experienced person. Long-term stock cultures may be stored in semisolid medium as described in 2.4.

3.2.2. Pretreatment of Sera

Sera in good condition do not require pretreatment. However, sera that are obviously contaminated or in poor condition can if desired be spun in a microcentrifuge, or filtered through a 0.45 µm filter, prior to testing. This can make the result of the MAT easier to read with such sera.

3.2.3. Titration

Sera are screened at final dilutions from 1/50 to 1/400.

- (a) Dilute test serum 1/25 in PBS, pH 7.2, and mix well.
- (b) Add 25 µL of diluted test serum to the first of four wells of a flat bottomed microtitre plate. Include on each plate, for each serovar tested on that plate, a positive standard serum and a negative serum, and a dilution series for saline instead of serum as a control for autoagglutination of the antigen.
- (c) Add 25 µL of PBS to all wells in the plate, including those containing serum.
- (d) Serially dilute each serum from the first well (final dilution 1/50) to the fourth well (1/400), by successive 25 µL transfers.
- (e) Add 25 µL antigen to each well, agitate by hand, cover the plate with a lid and incubate at 37°C for 60 min.
- (f) Remove plate from incubator and examine each well at a magnification of x40 using dark field illumination. Plates should be read within one hour of removal from the incubator.

Positive reactions usually appear as very dense, intense white clumps of leptospire. The grey background of motile leptospire generally clears to a very dark background when agglutination occurs. The formation of the agglutinated cells may vary with dilution, and occasionally a lacework agglutination appears at low dilutions.

Alternatively a loop from each well can be transferred to a microscope slide, and examined under dark field illumination. This is more time-consuming than examining the results in the microtitre plate, but can make the result easier to read.

Sera that are positive at a dilution of 100 can if desired be titred out across eight wells, from 1/50 through to 1/6400, or across 12 wells to 1/102 400.

The titre of each serum should be read as the reciprocal of the highest dilution showing detectable agglutination. The dilution recorded is expressed as the final dilution before addition of antigen. The results of control sera should be recorded for every plate used.

3.3. Interpretation of Serological Results

The interpretation of the results of the MAT is often not straightforward, and should be approached with considerable caution. The antibodies measured by the test are long-lasting, and so a low or moderate titre may indicate infection that has long passed. Conversely, the MAT is occasionally 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.

The following general guidelines may be used. A titre of 200 or greater indicates probable recent infection. 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.

Serovar *pomona* is more immunogenic than serovar *hardjo*, and animals infected with the former are much more likely to have high titres. Detection of recent infection by serovar *hardjo* in a herd of cattle is improved if a group of 10 or more animals is sampled on two occasions at least two weeks apart.

Leptospirosis serology is often not required for export purposes, as valuable animals can be treated with streptomycin to eliminate infection. Active infection in a herd is indicated when graphical representation of the distribution of titres in the herd indicates a bimodal distribution (Durfee and Allen, 1980).

Except in clear-cut cases, serological diagnosis is generally less satisfactory than detecting the leptospiral organism itself.

4. Histopathology

Tissues fixed in 10% buffered neutral formalin and processed in paraffin 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.

4.1. Kidney

Changes are virtually confined to the cortex. There are varying degrees of damage to the tubular epithelium cells resulting in the formation of tubular casts. However, the most prominent change is interstitial infiltration, principally by lymphoreticular cells but sometimes with significant numbers of neutrophils. Plasma cells are prominent in advanced cases. At times a notable lesion is the formation of syncytial masses resembling Langhans' giant cells by regenerating tubular epithelium.

4.2. Liver

Acute leptospirosis may cause lesions in this organ. There is disorganisation of the hepatic chords resulting from dissociation of hepatocytes. Centrilobular necrosis is present and there are additional foci of necrosis with infiltration by neutrophils 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.

5. Histological Stains for Leptospire

5.1. Introduction

A diagnosis of leptospirosis may be confirmed by demonstration of leptospire in tissues by nonspecific 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 leptospiuria. Pepsin digestion can be used to enhance the access of immunofluorescent or immunogold stains to leptospiral antigens. Application of immunohistochemical staining to aborted foetal tissues 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.

5.2. The Warthin–Starry Technique

5.2.1. Introduction

The technique described is based on the modification by Young (1969) of the original method of

Warthin and Starry (Warthin and Starry, 1920). Elliott (1988) has described a further modification in which sections are treated with iodine then decolourised with thiosulfate before treating with silver nitrate: this modification is stated to improve the reproducibility of the technique.

5.2.2. Solutions

5.2.2.1. 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.

5.2.2.2. Developer solution

The following three solutions should be all brought to 55°C 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

5.2.3. Staining Technique

- Take thin paraffin sections through xylene and alcohol to buffer.
- Impregnate with 1% silver nitrate in buffered water at 55–60°C for one hour (warm the solution first). Prepare developer solutions and place in oven or water bath. Place tap water in oven or water bath to heat.
- Place slides in fresh developer at 55°C for 1.5–3.5 min. The sections should become a golden brown colour.
- Drain sections briefly, rinse for two to three minutes in warm tap water (55–60°C) and then cool in cool buffer solution.
- Dehydrate, clear and mount.

Leptospire are seen as black in pale yellowish–brown tissues.

5.3. Immunochemical Staining

5.3.1. Introduction

The immunofluorescence method given is as described by Skilbeck (1986), and the immunogold silver staining method is as described by Skilbeck and Chappel (1987). The pepsin treatment outlined greatly enhances the subsequent staining of leptospire in each case.

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 x100 or x200, and viewed closely with x400 or x800.

5.3.2. Solutions

5.3.2.1. Solutions for immunofluorescent and immunogold staining

5.3.2.1.1. Washing solution. 0.05% Tween 20 in 0.85% saline.

5.3.2.1.2. 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.

5.3.2.2. Solutions for immunogold staining only

5.3.2.2.1. 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 21°C) before use.

5.3.2.2.2. 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.

5.3.2.2.3. 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.

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

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

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

5.3.3. Immunofluorescent Staining

- Cut paraffin sections of about 4 μ m, and dry in an oven at 56–60°C. Drying in an oven helps the section to adhere to the slide.
- Dewax by washing through three 10-min changes of Histolene or xylene, dehydrate by three changes of absolute alcohol, and rinse in tap water.
- Digest the sections in freshly-prepared 1% pepsin in 0.02 mol/L hydrochloric acid at 37–50°C 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.
- Wash with very slowly running tap water, then in washing solution. (Slides may be left overnight in washing solution if desired.)
- Quickly drain the slides, and dry around the tissue sections with absorbent paper.
- Cover the sections with 100 μ L 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.

- Incubate in a moist chamber at room temperature for 30 min to two hours.
- Wash three times for five to 10 min each in washing solution.
- Repeat step (e).
- Cover the sections with 100 μ L of an appropriate dilution of fluorescein isothiocyanate-conjugated sheep or goat antirabbit IgG (code RF, Silenus Laboratories Pty Ltd, PO Box 398, Hawthorn, Vic. 3122, Australia. Tel. (008) 33 7430; Fax (03) 818 6977).
- Repeat steps (g) and (h).
- Counterstain with 0.1% Evans blue (C.I.23860) for five minutes, rinse with washing solution, mount in buffered glycerol pH 8.6, and examine sections using an incident-light fluorescence microscope.

5.3.4. Immunogold Silver Staining

Steps (a–i) are as given in 5.3.3.

- Cover the sections with 60–100 μ L of an appropriate dilution (about 1/40) of gold-conjugated goat anti-rabbit IgG in antiserum diluent. [Product 'AuroProbe LM GAR' available from Amersham Australia Pty Ltd, Unit A, 7 Lyon Park Rd, North Ryde, NSW 2113, Australia. Tel. (008) 22 2216; Fax (02) 888 9305, Catalogue No. RPN450.]
- Repeat steps (g) and (h).
- Incubate slides in 2% glutaraldehyde solution at room temperature for 15 min.
- Rinse well in distilled water.
- Before going to the dark room, prepare solutions A and B (see 5.3.2), weigh the silver lactate for solution C, and prepare commercial photographic fixer and the stop bath solution.
- 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.
- Develop for about 100 s. The development time is of critical importance: 100 s is about right if the developer is at 21°C.
- Transfer slides to the stop bath solution for about 30 s, then to the fixer for 30–60 s.
- Rinse well in slowly running tap water.
- Counterstain in eosin (see 5.3.2) for 5–15 min.
- Rinse three times extremely quickly in absolute alcohol. (Alcohol is necessary for dehydration, but excessive alcohol washing removes the counterstain.) Rinse three times in Histolene or xylene to remove the

alcohol, then mount with DePex and a cover slip. [DePex is available from Crown Scientific Pty Ltd, 1 Florence St, Burwood, Vic. 3125. Tel. (008) 134 175, (03) 808 0366; Fax (03) 808 0610.]

- (u) View under a light microscope.

6. Examination of Urine

Leptospire are shed in the urine for most of the period during which an animal is infected, and these may be viewed under dark field illumination. However, bacterial numbers are sometimes too low for them to be found.

It is necessary to examine urine that is as fresh as possible, certainly within a few hours of obtaining the sample, so that leptospire are still living and motile. The urine sample should be kept at room temperature until it is examined. The bacteria can then be recognised by their spiral shape and characteristic rapid motion. Some workers believe that leptospire are more likely to be detected in the pellet obtained after centrifugation of urine at 3000 rpm for 20 min.

Treating urine with formalin kills the leptospire and is not recommended.

A magnification of x200 is suitable for scanning the sample under examination, and x400 for close inspection.

Laboratory safety procedures should be observed to prevent accidental human infection.

7. DNA Hybridisation

DNA hybridisation procedures for the detection of leptospire, using a whole genomic probe prepared from serovar *pomona*, have been applied to the detection of leptospire in porcine urine and in porcine kidney extracts (Millar *et al.*, 1987; McCormick *et al.*, 1989). The published techniques have some diagnostic value, but lack sensitivity, and appear to cross react widely with other leptospiral serovars.

Without doubt, as a result of the recent application of polymerase chain reaction technology (Van Eys *et al.*, 1989), DNA hybridisation procedures which are both highly sensitive and specific for individual serovars of interest will become available in the next few years. These are likely to greatly reduce our dependence on culture, on microscopic examination of urine, and on histology and histochemical techniques.

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9. References

- Adler, B., Cousins, D.V., Faine, S., and Robertson, G.M. (1982). The bovine IgM and IgG response to *Leptospira interrogans* serovar hardjo as measured by enzyme immunoassay. *Veterinary Microbiology* 7, 577-85.
- Adler, B., Faine, S., Christopher, W.L., and Chappel, R.J. (1986). Development of an improved selective medium for isolation of leptospire from clinical material. *Veterinary Microbiology* 12, 377-81.
- Ballard, S.A., Adler, B., Millar, B.D., Chappel, R.J., Jones, R.T., and Faine, S. (1984). The immunoglobulin response of swine following experimental infection with *Leptospira interrogans* serovar *pomona*. *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene, Abt. Orig. A* 256, 510-17.
- Cousins, D.V., Ellis, T.M., Parkinson, J., and McGlashan, C.H. (1989). Evidence for sheep as a maintenance host for *Leptospira interrogans* serovar *hardjo*. *Veterinary Record*, 124, 123-4.
- Cousins, D.V., and Robertson, G.M. (1986). Use of enzyme immunoassay in a serological survey of leptospirosis in sheep. *Australian Veterinary Journal* 63, 36-9.
- Durfee, P.T., and Allen, J.D. (1980). Serological titres of dairy cows over a 63-week period following natural infection with *Leptospira interrogans* serovar *hardjo*. *Australian Veterinary Journal* 56, 574-9.
- Elliott, K.G. (1988). A simple technique to enhance for demonstration of *Leptospira* in Young's Warthin-Starry technique. *Stain Technology* 63, 122-4.
- Faine, S. (1982). Guidelines for the control of leptospirosis. *WHO Offset Publication*, 67.
- Gordon, L.M. (1980). Isolation of *Leptospira interrogans* serovar *hardjo* from sheep. *Australian Veterinary Journal* 56, 348-9.
- Hogg, G.G. (1983). Equine leptospirosis. *Proceedings of the Postgraduate Committee in Veterinary Science, University of Sydney* 65, 221-41.
- Johnson, R.C., and Harris, V.G. (1967). Differentiation of pathogenic and saprophytic leptospire. I. Growth at low temperatures. *Journal of Bacteriology* 94, 27-31.
- Jones, R.T., Millar, B.D., Chappel, R.J., and Adler, B. (1987). Macroscopic kidney lesions in slaughtered pigs are an inadequate indicator of current leptospiral infection. *Australian Veterinary Journal* 64, 258-9.
- McCaughan, C.J., Gordon, L.M., Rahaley, R.S., Slee, K.J., and Presidente, P.J.A. (1980). Evidence for infection of sheep in Victoria with leptospire of the Hebdomadis serogroup. *Australian Veterinary Journal* 56, 201-2.
- McCormick, B.M., Millar, B.D., Monckton, R.P., Jones, R.T., Chappel, R.J., and Adler, B. (1989). Detection of leptospire in pig kidney using DNA hybridisation. *Research in Veterinary Science* 47, 134-5.
- Millar, B.D., Chappel, R.J., and Adler, B. (1987). Detection of leptospire in biological fluids using DNA hybridisation. *Veterinary Microbiology* 15, 71-8.
- Palit, A., Haylock, L.M., and Cox, J.C. (1986). Storage of pathogenic leptospire in liquid nitrogen. *Journal of Applied Bacteriology* 61, 407-11.
- Ramadass, P., Marshall, R.B., and Jarvis, B.D.W. (1990). Species differentiation of *Leptospira interrogans* serovar *hardjo* strain *Hardjobovis* from strain *Hardjoprajitno* by DNA slot blot hybridization. *Research in Veterinary Science* 49, 194-7.
- Skilbeck, N.W. (1986). Immunofluorescent staining of leptospire in pepsin-treated histologic sections. *Stain Technology* 61, 273-8.
- Skilbeck, N.W., and Chappel, R.J. (1987). Immunogold silver staining for visualization of leptospire in histologic sections. *Journal of Clinical Microbiology* 25, 85-6.

- Slatter, D.H., and Hawkins, C.D. (1982). Prevalence of leptospiral titres in normal horses. *Australian Veterinary Journal* 59, 84-6.1
- Turner, L.H. (1970). Leptospirosis III. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 64, 623-46.
- Van Eys, G.J.J.M., Gravekamp, C., Gerritsen, M.J., Quint, W., Cornelissen, M.T.E., Ter Schegget, J., and Terpstra, W.J. (1989). Detection of leptospire in urine by polymerase chain reaction. *Journal of Clinical Microbiology* 27, 2258-62.
- Warthin, A.S., and Starry, A.C. (1920). A more rapid and improved method of demonstrating spirochetes in tissues. *American Journal of Syphilis* 4, 97-103.
- Watson, A.D.J., Wannan, J.S., Porges, W.L., and Testoni, F.J. (1976). Leptospiral agglutinins in dogs in Sydney. *Australian Veterinary Journal* 52, 425-6.
- Yasuda, P.H., Steigerwalt, A.G., Sulzer, K.R., Kaufmann, A.F., Rogers, F., and Brenner, D.J. (1987). Deoxyribonucleic acid relatedness between serogroups and serovars in the family Leptospiraceae with proposals for seven new *Leptospira* species. *International Journal of Systematic Bacteriology* 37, 407-15.
- Young, B.J. (1969). A reliable method for demonstrating spirochaetes in tissue sections. *Journal of Medical Laboratory Technology* 26, 248-52.