

LEPTOSPIROSIS

SUMMARY

Definition of the disease: *Leptospirosis is a transmissible disease of animals and humans caused by infection with any of the pathogenic members of the genus Leptospira.*

Description of disease: *Acute leptospirosis should be suspected in the following cases: sudden onset of agalactia (in adult milking cattle and sheep); icterus and haemoglobinuria, especially in young animals; meningitis; and acute renal failure or jaundice in dogs. Chronic leptospirosis should be considered in the following cases: abortion, stillbirth, birth of weak offspring (may be premature); infertility; chronic renal failure or chronic active hepatitis in dogs; and cases of periodic ophthalmia in horses.*

Laboratory diagnosis of leptospirosis can be complex and involves tests that fall into two groups. One group of tests is designed to detect anti-leptospiral antibodies, and the other group is designed to detect leptospire, leptospiral antigens, or leptospiral nucleic acid in animal tissues or body fluids. The particular testing regimen selected depends on the purpose of testing (e.g. herd surveys or individual animal testing) and on the tests or expertise available in the area.

Identification of the agent: *The isolation or demonstration of leptospire in:*

- a) *several of the internal organs (such as liver, lung, brain, and kidney) and body fluids (blood, milk, cerebrospinal, thoracic and peritoneal fluids) of clinically infected animals gives a definitive diagnosis of acute clinical disease or, in the case of a fetus, chronic infection of its mother;*
- b) *the kidney, urine, or genital tract of animals without clinical signs is diagnostic only of a chronic carrier state.*

Isolation of leptospire from clinical material and identification of isolates is time-consuming and is a task for specialised reference laboratories. Isolation followed by typing from renal carriers is important and very useful in epidemiological studies to determine which serovars are present within a particular group of animals, an animal species, or a geographical region.

The demonstration of leptospire by immunochemical tests (immunofluorescence and immunohistochemistry) is more suited to most laboratory situations. However, the efficacy of these tests is dependent on the number of organisms present within the tissue, and these tests lack the sensitivity of culture. Unless specially prepared reagents are used, immunochemical tests do not identify the infecting serovar and results must be interpreted in conjunction with serological results. Reagents for immunofluorescence are best prepared with high IgG titre anti-leptospire sera, which are not available commercially. Rabbit leptospiral-typing serum or monoclonal antibodies can be used for immunohistochemistry and are available from leptospiral reference laboratories.

Genetic material of leptospire can be demonstrated in tissues or body fluids using a variety of assays based on the polymerase chain reaction (PCR), either in real-time or traditional formats. PCR assays are sensitive, but quality control procedures and sample processing for PCR are critical and must be adjusted to the tissue, fluid and species being tested. Like immunochemical tests, PCR assays do not identify the infecting serovar, although some will identify the infecting species.

Serological tests: *Serological testing is the most widely used means for diagnosing leptospirosis, and the microscopic agglutination test (MAT) is the standard serological test. Antigens selected for*

use in the MAT should include representative strains of the serogroups known to exist in the particular region as well as those known to be maintained elsewhere by the host species under test.

The MAT is used to test individual animals and herds. As an individual animal test, the MAT is very useful for diagnosing acute infection: a four-fold rise in antibody titres in paired acute and convalescent serum samples is diagnostic. To obtain useful information from a herd of animals, at least ten animals, or 10% of the herd, whichever is greater, should be tested and the vaccination history of the animals documented.

The MAT has limitations in the diagnosis of chronic infection in individual animals and in the diagnosis of endemic infections in herds. Infected animals may abort or be renal/genital carriers with MAT titres below the widely accepted minimum significant titre of 1/100 (final dilution).

Enzyme-linked immunosorbent assays (ELISAs) can also be useful for detection of antibodies against leptospires. Numerous assays have been developed and are primarily used for the detection of recent infections, the screening of experimental animals for use in challenge studies, and, in cattle, health schemes to assess levels of infection of serovar Hardjo – either as tests on individual animal blood or milk or as bulk milk tank tests. Animals that have been vaccinated against the serovar of interest may be positive in some ELISAs, thus complicating interpretation of the results.

Requirements for vaccines: Vaccines for veterinary use are most often suspensions of one or more serovars of *Leptospira* spp. inactivated in such a manner that immunogenic activity is retained. While a range of experimental vaccines based on cellular extracts has been tested, commercial vaccines are whole cell products. The leptospires are grown in suitable culture media, which often contain serum or serum proteins. If used, serum or serum proteins should be removed from the final products. Vaccines may contain suitable adjuvants.

A. INTRODUCTION

Definition of the disease: Leptospirosis is a transmissible disease of animals and humans caused by infection with the spirochete *Leptospira*.

Causal pathogen: All the pathogenic leptospires were formerly classified as members of the species *Leptospira interrogans*, however the genus has recently been reorganised. The genus *Leptospira* consists of 20 species and includes nine pathogenic, five intermediate and six saprophytic species (Picardeau, 2013). The majority of pathogenic serovars are found in the three species with a global distribution – *L. interrogans*, *L. borgpetersenii*, and *L. kirschneri*. The other pathogenic species are: *L. alexanderi*, *L. alstonii*, *L. kmetyi*, *L. noguchi*, *L. santarosai* and *L. weilii*. Speciation is based on DNA homology as determined by DNA–DNA hybridisation but because of technical difficulties, PCR-based methods are commonly used instead (Ahmed *et al.*, 2012a; 2012b). There are more than 300 distinct leptospiral serovars recognised and these are arranged in 25 serogroups (Picardeau, 2013).

In theory, any parasitic *Leptospira* may infect any animal species. Fortunately, only a small number of serovars will be endemic in any particular region or country. Furthermore, leptospirosis is a disease that shows a natural nidality, and each serovar tends to be maintained in specific maintenance hosts. Therefore, in any region, a domestic animal species will be infected by serovars maintained by a species or by serovars maintained by other animal species present in the area. The relative importance of these incidental infections is determined by the opportunity that prevailing social, management, and environmental factors provide for contact and transmission of leptospires from other species. An example of a host-maintained infection is serovar Hardjo infection in cattle. Limited host ranges allows for the development of control/eradication schemes.

Description of the disease: The use, interpretation, and value of laboratory diagnostic procedures for leptospirosis vary with the clinical history of the animal or herd, the duration of infection, and the infecting serovar. Acute leptospirosis should be suspected in the following cases: sudden onset of agalactia (in adult milking cattle and sheep); icterus and haemoglobinuria, especially in young animals; meningitis; and acute renal failure (in dogs). Chronic leptospirosis should be considered in the following cases: abortion, stillbirth, birth of weak offspring (may be premature); infertility; chronic renal failure (in dogs); and cases of periodic ophthalmia in horses. Two major chronic microbiological sequelae of leptospiral infection present particular diagnostic problems: the localisation and persistence of leptospires in the kidney and in the male and female genital tract. Chronically infected animals may remain carriers for years or life and serve as reservoirs of the infection for other animals and humans.

Zoonotic risk and biosafety requirements: *Leptospira* spp. are classed in Risk Group 2 for human infection and should be handled with appropriate measures as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*. Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4.

Differential diagnosis: 1) Diseases where acute milk drop may occur, such as acute viral infections and sudden absence of drinking water; 2) diseases with hepato-renal failure; and 3) diseases characterised by reproductive wastage – abortion, reduced litter size, stillbirth and infertility e.g. brucellosis, *Neospora*, Q fever and bovine viral diarrhoea infection in cattle, chlamydiosis and toxoplasmosis in sheep, Q fever in goats, etc.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of leptospirosis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification¹						
Isolation and identification	–	+++	–	+++	–	–
PCR	–	++	–	++	–	–
Detection of immune response						
MAT	–	+++	–	++	+++	–
ELISA	+++	–	+++	+++	++	+++

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.

Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

PCR = polymerase chain reaction; MAT = microscopic agglutination test; ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

The demonstration of leptospire in blood and milk of animals showing clinical signs suggestive of acute leptospirosis is considered to be diagnostic. However, isolation from blood is not often successful because bacteraemia is transient and not always accompanied by clinical signs. Dogs are often treated with antibiotics before samples are collected for testing for *Leptospira*, which further decreases the likelihood of identifying the agent in blood. The demonstration of generalised leptospiral infection in a range of organs taken at necropsy is also considered to be diagnostic. However, if the animal lives long enough or has been treated with antibiotics, it may be difficult to detect intact organisms systemically; immunohistochemistry can be particularly helpful in identifying residual leptospiral antigen in these cases. Demonstration of leptospire in the genital tract, kidneys, or urine only must be interpreted with full consideration of the clinical signs, and serological results as these findings may merely indicate that the animal was a carrier.

Failure to demonstrate leptospire in the urine of an animal does not eliminate the possibility that the animal is a chronic renal carrier, it merely indicates that the animal was not excreting detectable numbers of leptospire at the time of testing. Collection of urine following treatment of the animals with a diuretic enhances the chances of detecting the organism (Nervig & Garrett, 1979). In important cases involving individual animals (e.g. clearing an infected stallion to return to breeding), negative tests on three consecutive weekly urine samples have been considered to be good evidence that an animal is not shedding leptospire in the urine.

¹ A combination of agent identification methods applied on the same clinical sample is recommended.

The demonstration of leptospire in body fluids or internal organs (usually kidney, liver, lung, brain, or adrenal gland) of aborted or stillborn fetuses is considered to be diagnostic of chronic leptospirosis of the mother, and is evidence of active infection of the fetus.

1.1. Isolation of *Leptospira*

In experienced hands, the isolation of leptospire is one of the most specific methods of demonstrating their presence, provided that antibiotic residues are absent, that tissue autolysis is not advanced, that tissues are processed for culture rapidly after collection, and – in the case of urine – at a suitable pH. If tissues or fluids cannot be transported promptly to the laboratory for leptospiral culture, the sample should be kept at 2–5°C to prevent overgrowth with other bacteria and autolysis of tissue samples. Liquid culture medium or 1% bovine serum albumin (BSA) solution containing 5-fluorouracil at 100–200 µg/ml should be used as transport medium for the submission of samples.

Culture should be carried out in a liquid or semisolid (0.1–0.2% agar) medium containing BSA and either Tween 80 (e.g. EMJH²) (Johnson & Harris, 1967) or a combination of Tween 80 and Tween 40 (Ellis, 1986). Contamination may be controlled by the addition of a variety of selective agents, e.g. 5-fluorouracil (Johnson & Rogers, 1964), nalidixic acid (Johnson & Seiter, 1977), fosfomycin (Oie *et al.*, 1986), and a mixture of rifamycin, polymyxin, neomycin, 5-fluorouracil, bacitracin, and actidione (Adler *et al.*, 1986). However, use of selective agents may reduce the chances of isolation when there are only small numbers of viable leptospire, and some strains of leptospire will not grow in selective media containing multiple antibiotics. Addition of 0.4–5% rabbit serum to semisolid culture medium enhances the chances of isolating fastidious leptospiral serovars.

Cultures should be incubated at 29 ± 1°C for at least 16 weeks, and preferably for 26 weeks (Ellis, 1986). The time required for detection of a positive culture varies with the leptospiral serovar and the numbers of organisms present in the sample. Less fastidious serovars (e.g. Pomona and Grippotyphosa) may result in positive cultures as soon as 7–10 days after inoculation; other serovars (e.g. Hardjo and Bratislava) may take much longer. Cultures should be examined by dark-field microscopy every 1–2 weeks. It is important to use a 100 watt light source and a good quality dark-field microscope.

1.2. Immunochemical staining techniques

Leptospire may also be demonstrated by a variety of immunochemical staining techniques, e.g. immunofluorescence (Ellis *et al.*, 1982a), and various immunohistochemical techniques (Barnett *et al.*, 1999; Scanziani, 1991; Wild *et al.*, 2002). These are useful in diagnosing infection in pathological material that is unsuitable for culture or where a rapid diagnosis is required. As the success of these techniques is dependent on the number of organisms present, they are less suitable for diagnosing the chronic carrier state, where the numbers of organisms may be very low or localised. Leptospire do not stain satisfactorily with aniline dyes, and silver-staining techniques lack sensitivity and specificity, although they are a useful adjunct for histopathological diagnosis (Baskerville, 1986).

1.3. Nucleic acid detection methods

Polymerase chain reaction (PCR)-based assays are increasingly used for the detection of leptospire in tissues and body fluids of animals because of their perceived sensitivity and capacity to give an early diagnosis. Real-time PCR is faster than regular PCR and less sensitive to contamination (Picardeau, 2013). Assays fall into two categories based on the detection of genes that are universally present in bacteria, for example, *gryB*, *rrs* (16S rRNA gene) and *secY*, or the detection of genes restricted to pathogenic *Leptospira*, for example, *lipL21*, *lipL32*, *lipL41*, *ligA* and *ligB* (Thaipadunpanit *et al.*, 2011). These assays do not identify the infecting serovar, although some primer sets may permit further identification to the species or strain level if the PCR amplicons are sequenced. This further analysis is not a routine diagnostic method. Many of the PCR primer sets have been designed and evaluated for use in human rather than animal specimens and general agreement about the PCR primers to be used for testing of animal samples is lacking, although those based on the *lipL32* gene are the most commonly reported. Validation remains one of the outstanding issues surrounding the use of PCR in the diagnosis of animal leptospirosis (see Table 1), with the individual laboratory being responsible for the validation of the particular assay they use for the tissue, fluid, and species being tested. To date, only three real-time PCR have been presented with a solid validation (Ahmed *et al.*, 2009; 2012b; Slack *et al.*, 2007; Thaipadunpanit *et al.*, 2011). The presence of amplification inhibitors in clinical samples can cause false-negative results, particularly in animal specimens that may be compromised by contamination with faeces or autolysis. Quality control of PCR assays used for leptospirosis

2 EMJH: Ellinghausen-McCullough-Johnson-Harris

diagnosis requires careful attention to laboratory design and workflow to prevent contamination of reagents, and appropriate control samples should be used (Dragon *et al.*, 1993; McCreedy & Callaway, 1993). In addition, sample processing for PCR is critical and must be suited to the tissue, fluid, and species being tested.

1.4. Identification of leptospiral isolates

The identification of leptospiral isolates is a task for specialised reference laboratories. For complete identification, a combination of procedures is used to determine: 1) if the isolate is a pathogen or a saprophyte; 2) the species of *Leptospira* to which the isolate belongs; and 3) the serogroup and serovar of the isolate. A pure leptospiral culture may be identified as belonging to a pathogenic or saprophytic species by a variety of tests: the ability to infect animals; the relative resistance to 8-azaguanine; lipase activity; salt and temperature tolerance (Johnson & Faine, 1984; Johnson & Harris, 1967); and G+C content of DNA (Johnson & Faine, 1984).

Speciation is based on DNA–DNA hybridisation analysis (Brenner *et al.*, 1999) but increasingly other more rapid molecular techniques are used (Ahmed *et al.*, 2012a) of which the multilocus sequence typing (MLST) is the most robust (Ahmed *et al.*, 2006). Different isolates belonging to a single serovar usually belong to the same species, but this is not always the case.

Strains belonging to *Leptospira* can be differentiated to the serogroup level (a concept that no longer has any taxonomic validity but which is a useful preliminary step in identification and antigen selection for vaccines and serological tests) by cross-agglutination reactions (Dikken & Kmety, 1978). Subsequent differentiation to the serovar level was traditionally by cross-agglutination absorption, although for most isolates this is now being done using less time-consuming methods such as monoclonal antibodies (MAbs) (Terpstra *et al.*, 1985; 1987). A variety of molecular methods can give results that may be concordant with serotyping. These methods are not valid for establishing new serovars, however they provide useful guidance on identification and can provide useful molecular epidemiological information at the sub-serovar level. PCR-based approaches include multi-locus variable number of tandem repeats (Slack *et al.*, 2006; Zuerner & Alt, 2009), amplification of insertion elements (Zuerner & Bolin, 1995; Zuerner *et al.*, 1997), amplified fragment length polymorphisms (AFLP) and fluorescent-labelled AFLP (Vijaychari *et al.*, 2004) and arbitrarily primed PCR (Perolat *et al.*, 1994). BRENDA has proved very useful in epidemiological investigation of leptospires of food producing animals (Ellis *et al.*, 1991; Thiermann *et al.*, 1986).

2. Serological tests

Serological testing is the laboratory procedure most frequently used to confirm the clinical diagnosis, to determine herd prevalence, and to conduct epidemiological studies. Leptospiral antibodies appear within a few days of onset of illness and persist for weeks or months and, in some cases, years. Unfortunately, antibody titres may fall to undetectable levels while animals remain chronically infected. To overcome this problem, sensitive methods are needed to detect the organism in urine or the genital tract of chronic carriers.

A wide variety of serological tests that show varying degrees of serogroup and serovar specificity, have been described. Two tests have a role in veterinary diagnosis: the microscopic agglutination test (MAT) and the enzyme-linked immunosorbent assay (ELISA).

2.1. Microscopic agglutination test

The MAT using live antigens is the most widely used serological test. It is the reference test against which all other serological tests are evaluated and is used for import/export testing. For optimum sensitivity, it should use antigens representative of all the serogroups known to exist in the region in which the animals are found and, preferably, strains representing all the known serogroups. The presence of a serogroup is usually indicated by frequent reaction in serological screening but can only be definitively identified by isolation of a serovar from clinically affected animals. The sensitivity of the test can be improved by using local isolates rather than reference strains, but reference strains assist in the interpretation of results between laboratories.

The specificity of the MAT is good; antibodies against other bacteria usually do not cross-react with *Leptospira* to a significant extent. However, there is significant serological cross-reactivity between serovars and serogroups of *Leptospira* and an animal infected with one serovar is likely to have antibodies against the infecting serovar that cross-react with other serovars (usually at a lower level) in the MAT. Therefore, serology cannot be used to identify definitively the infecting serovar in an individual infection or outbreak – this requires isolation of the agent. However, in areas where the serovars of *Leptospira* present have been well described by isolation studies, serological examination

of the infected animal(s) may suggest, but not definitively identify, the infecting serovar. In addition, animals that have been vaccinated against leptospirosis may have antibodies against the serovars present in the vaccine used. Therefore, it is particularly important to consider the vaccination history of the animals under test. The two methods for carrying out the test have been described in detail (Faine *et al.*, 2000; USDA, 1987).

2.1.1. Test procedure

- i) The strains selected should be grown in liquid leptospiral culture medium (e.g. EMJH or other suitable medium) at $29 \pm 1^\circ\text{C}$ and the culture should be at least 4 days old, but no more than 8 days. Live cultures with densities of approximately 2×10^8 leptospores per ml are to be used as the antigens. The culture density can be determined by counting the cells directly using a bacterial counting chamber and dark-field microscopy. Alternatively, cell counts can be estimated by measuring transmittance in a spectrophotometer with a 400 nm filter or by nephelometry. If indirect methods are used, direct bacterial cell counts should be correlated with the readings on the specific instrument being used.
- ii) The number of antigens to be used is determined and a screening test may be performed with a 1/50 serum dilution (or a different starting dilution based on the purpose of the test).
- iii) A volume of each antigen, equal to the diluted serum volume, is added to each well, making the final serum dilution 1/100 in the screening test.
- iv) The microtitration plates are incubated at $30 \pm 1^\circ\text{C}$ for 1.5–4 hours.
- v) The plates are examined by dark-field microscopy.

The endpoint is defined as that dilution of serum that shows 50% agglutination, leaving 50% free cells compared with a control culture diluted 1/2 in phosphate buffered saline. The result of the test may be reported as the endpoint dilution of serum (e.g. 1/100 or 1/400) or as a titre that is the reciprocal of the endpoint serum dilution (e.g. 100 or 400).

2.1.2. Quality control of antigens used in the MAT

Identity of antigens is a crucial factor in conducting the MAT. Antigens should be evaluated for identity using hyperimmune rabbit sera, MABs, or a molecular method that confirms passages over time, preferably each time the test is run, but at least twice a year. Hyperimmune rabbit serum for this purpose can be obtained from a reference laboratory or prepared using a protocol such as that given by the Subcommittee on the Taxonomy of *Leptospira* (International Committee on Systematic Bacteriology, 1984). Briefly, healthy rabbits weighing 3–4 kg that lack detectable anti-leptospiral antibodies are selected. Each rabbit is given an intravenous injection in a marginal vein of the ear with a well-growing live or formalin-treated culture with a density of approximately 2×10^8 leptospores/ml. It may be necessary to include a washing step as BSA can cause shock. The culture should be grown in Tween 80 BSA medium or another appropriate medium. Five injections of 1 ml, 2 ml, 4 ml, 6 ml, and 6 ml each are given at 7-day intervals. One week following the final injection, the homologous antibody titre is determined by MAT. If the titre is $\geq 1/12,800$, the rabbit is anaesthetised and bled by cardiac puncture 7 days later (i.e. 14 days after the final injection). If the titre is $< 1/12,800$, a further injection of 6 ml of culture can be given; 7 days after this injection the homologous titre is again determined. Unless the titre is $\geq 1/12,800$ the procedure should be repeated with another rabbit. Two rabbits are used to prepare each antiserum. If the titres are satisfactory in both rabbits, the sera may be pooled. To preserve potency, it is preferable to freeze-dry the antiserum in 2-ml volumes and store it at $2-5^\circ\text{C}$. Alternatively, the serum can be stored in 1-ml volumes at -15 to -70°C . All animal inoculations should be approved and conducted according to the relevant standards for animal care and use. Other immunisation protocols may be considered based on the intended use of the antiserum and the need to reduce the number of rabbits used.

Purity of antigens used in the MAT should be checked regularly by culture on blood agar and in thioglycolate broth. Stock cultures of antigens may be stored at -70 to -80°C or in liquid nitrogen. There will be a low survival rate of leptospores after lyophilisation. Repeated passage of antigens in liquid medium results in a loss of antigenicity. In this case, a new liquid culture should be derived from the stock culture.

An annual international MAT proficiency scheme is available through the International Leptospirosis Society³.

3 Available at: <http://www.med.monash.edu.au/microbiology/staff/adler/ils.html#Otherinfo>

2.1.3. Interpretation and limitations of the MAT

A titre of 1/100 is taken as a positive titre for the purposes of international trade, but given the high specificity of the MAT lower titres can be taken as evidence of previous exposure to *Leptospira*.

As an individual animal test, the MAT is very useful in diagnosing acute infection; the demonstration of a four-fold change in antibody titres in paired acute and convalescent serum samples is diagnostic. In addition, a diagnosis of leptospirosis is likely, based on the finding of very high titres in an animal with a consistent clinical picture. The test has limitations in diagnosis of chronic infection in individual animals, both in the diagnosis of abortion (Ellis *et al.*, 1982b) and in the identification of renal or genital carriers (Ellis, 1986). This is particularly true with the host-adapted leptospiral infections, e.g. serovar Hardjo infection in cattle: when a titre of 1/100 or greater is taken as significant, the sensitivity of the test is only 41%, and even when the minimum significant titre is reduced to 1/10, the sensitivity of the test is only 67% (Ellis, 1986). The demonstration of antibodies in fetal blood is diagnostic, but the titres are often very low, i.e. 1/10, requiring a modified testing procedure for most laboratories.

As leptospirosis is a herd problem, the MAT has much greater use as a herd test. To obtain useful information, Cole *et al.* (1980) suggested that samples be taken from at least ten animals, or 10% of the herd, whichever is the greater. In a study of Hardjo infection in cattle, Hathaway *et al.* (1986) found that a 10-cow sample usually indicated the presence or absence of infection in a herd. Increasing the sample size markedly improved epidemiological information, investigations of clinical disease, and public health tracebacks.

In making a serological diagnosis of leptospirosis, the infecting serovar and the clinical condition involved must be fully considered. In the case of serovar Pomona-induced abortion in cattle, a high titre is commonly found at the time of abortion because the clinical incident occurs relatively soon after infection. Abortion in cattle due to serovar Hardjo is a chronic event; in this case, the serological response at the time of abortion is more variable, with some animals seronegative and others showing high titres. Cattle may experience a drop in milk production during the acute phase of Hardjo infection and this clinical sign is associated with high titres. Vaccination history must also be considered in the interpretation of MAT results as widespread vaccination contributes significantly to the number of seropositive animals and may mask the presence of chronic infections in the herd – particularly with serovar Hardjo.

2.2. Enzyme-linked immunosorbent assays

ELISAs for detection of anti-leptospiral antibodies have been developed using a number of different antigen preparations, assay protocols and assay platforms, including plate tests and dipstick tests. The antigen preparations have mainly been either whole cell preparations or outer membrane protein (OMP) preparations, with recent emphasis on developing tests using recombinant OMPs. The antigen used dictates the specificity of the ELISA. Recombinant OMP-based ELISAs are broadly reactive to antibodies to all pathogenic leptospires and so are of no value in epidemiological investigations. In contrast, lipopolysaccharide antigen-based ELISAs are serogroup specific and have value in epidemiological investigations and control schemes. IgM ELISAs have been shown to be useful in the diagnosis of acute infection (Cousins *et al.*, 1985; Hartman *et al.*, 1984; 1986). A total-Ig ELISA is useful in the identification of fully susceptible animals suitable for experimental challenge work (Ellis & Zygraich, 1986). ELISAs have also been developed for use in milk from individual cows or in bulk tank milk for the detection of serovar Hardjo antibodies. These tests have been helpful in identifying Hardjo-infected herds and in serovar Hardjo control/eradication programmes (Pritchard, 2001). However, herds that are vaccinated against serovar Hardjo will also be positive in these various ELISAs, decreasing their usefulness in regions where vaccination is a routine practice. OMP based tests are not yet widely available. While they may have a role in the diagnosis of incidental infections they are unlikely to have a role in control programmes for host maintained infections, such as serovar Hardjo, where naturally infected cattle produce weak or no response to outer membrane proteins, but where the major serological response is to outer envelope lipopolysaccharide antigens (Ellis *et al.*, 2000).

Problems with validation are a major constraint in assessing most ELISAs. Almost all have been validated against the MAT (using MAT titres of 1/100 or greater), which is an imperfect test, having a sensitivity of less than 50% in some chronic infections. Human investigators have attempted to overcome this problem by the use of Bayesian latent class models and random-effects meta-analysis studies (Limmathurotsakul *et al.*, 2012; Signorini *et al.*, 2013), but the best validation possible is using sequential sera from culture positive cases (Goris *et al.*, 2012). A small number of ELISAs for animals have been validated using sequential serum samples from experimental animals but not beyond 6 months post-challenge, while one commercial Hardjo ELISA has been validated against single serum samples from culture positive cattle.

C. REQUIREMENTS FOR VACCINES

1. Background

Leptospiral vaccines for veterinary use are suspensions of one or more strains of pathogenic *Leptospira* inactivated in such a manner that immunogenic activity is retained. Commercial vaccines are whole-cell products and are available globally for cattle, pigs and dogs. The leptospires are grown in suitable culture media that may contain serum or serum proteins. If used, serum or serum proteins should be removed from the final product. Vaccines may contain suitable adjuvants.

Vaccines are used in animals to protect both the animals and in-contact humans. They are a key tool in control or eradication programmes. Vaccines will not eliminate infection from an already infected host and therefore should be given prior to exposure. Commercial vaccines vary in their efficacy. A number of monovalent products used in cattle have been shown to produce clinical and microbiological protection for up to year, in contrast, a number of multivalent products have been shown to stimulate poor immunity. Vaccination programmes must be tailored to the target population and the efficacy of the product to be used. Ideally cattle should be vaccinated prior to possible exposure, and thereafter annually, with vaccination timed to precede major risk periods. A successful vaccination programme requires epidemiological studies to assess the incidence of different *Leptospira* serovars in a given population (Adler & de la Pena Moctezuma, 2010).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirement of vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

Proper selection of vaccine production strains is of utmost importance. Immunity induced by vaccination is largely serovar specific (Chen, 1986). A vaccine should be formulated for use in a particular animal species in a particular geographical region. It should contain only those serovars – and preferably those genotypes – that cause problems in the animal species, or that are transmitted by the animal species to other species in the region. Strains selected for use as master seed culture should be cloned on solid medium to ensure the absence of saprophytic *Leptospira* contaminants and uniformity of the culture.

Suitable strains should be further selected by their ability to grow to high yields under batch culture conditions.

Each component strain to be included in the final vaccine should be grown separately in liquid medium; preferably in a protein-free (Bey & Johnson, 1978; Shenberg, 1967) or low-protein medium (Bey & Johnson, 1978).

The volume of each master seed culture should be amplified by growth for 2–10 days at 29°C ±1°C in a series of subcultures until a volume sufficient for use as a production seed culture is achieved. Cultures should be aerated and agitated as required.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Each subculture of the master seed culture should be checked for purity and for satisfactory growth. Purity can be checked by inoculating a loopful of culture into blood agar plates or into thioglycolate broth for incubation at 35–37°C for 2–5 days, and by examining a Gram-stained smear of culture sediment. Growth can be checked by dark-field microscopy. Each production seed culture should also be checked against its' homologous rabbit antiserum (Dikken & Kmety, 1978) to ensure purity and homology. MAbs may also be used for this purpose.

2.1.3. Validation as a vaccine strain

There is a large volume of literature describing the efficacy of leptospiral vaccines. In most cases, vaccines provide significant protection against disease produced by homologous challenge under field conditions.

Vaccines are less efficacious at preventing infection in animals and a percentage of vaccinated animals will become infected with the relevant serovar and may shed the organism in their urine despite a lack of clinical signs of disease.

Efficacy trials and vaccine validation must be conducted in the target species for the vaccine. The vaccine should be administered as recommended on the label, and immunity should be tested by challenge with virulent field strains of each serovar by natural routes of infection, i.e. by conjunctival and/or vaginal challenge. Validation studies have often been conducted with challenge of immunity by intravenous or intramuscular injections of leptospires. Vaccines validated in this way have not always been shown to be protective against field challenge, which occurs by exposure of mucous membranes of the eye, mouth, and genital tract to leptospires. Most notably, commercial leptospiral vaccines containing serovar Hardjo have not always protected cattle from conjunctival or field challenge with serovar Hardjo. A draft monograph for the efficacy testing of serovar Hardjo vaccines has been prepared and specifies the use of more natural routes of challenge (European Pharmacopoeia monograph).

2.2. Methods of manufacture

2.2.1. Procedure

Manufacture is carried out by batch culture in appropriately sized fermentor vessels. These should be equipped with ports for the sterile addition of seed culture, air, and additional medium. They should also have sampling ports so that the purity and growth of the production culture can be monitored.

Ideally, low-protein or protein-free media are used for production. However, some strains require the presence of animal protein to achieve suitable yields; this is usually supplied as BSA. All media components that are not degraded by heat should be heat sterilised. This reduces the risk of contamination by water-borne saprophytic leptospires that are not removed by filter sterilisation.

After addition of the seed culture, the growth of the production culture is monitored at frequent intervals for the start of log-phase growth. Once this is observed, the vessel is then agitated and aerated. The final yield can often be improved by the addition of more Tween 80 to the culture when log-growth is first observed to be slowing down. Adequate growth may require up to 10 days of incubation at $29 \pm 1^\circ\text{C}$.

Inactivation is usually by the addition of formalin, but phenol, merthiolate, and heat inactivation have also been used.

After the appropriate inactivation period, the culture may be concentrated and extraneous protein material may be removed by ultrafiltration. Suitable volumes of the various strains to be included in the final vaccine can then be blended, and adjuvant and preservative added, if appropriate.

2.2.2. Requirements for ingredients

All products of biological origin, in particular BSA, must originate from a country with negligible risk of transmissible spongiform encephalopathies (see chapter 1.1.8).

2.2.3. In-process control

During production, daily or twice daily subsamples should be taken and monitored for growth of leptospires and absence of contaminants. Growth is monitored either by counting leptospires in a counting chamber under dark-field microscopy or by a nephelometer. The absence of contamination can be monitored by the microscopic examination of Gram-stained preparations of centrifuged culture.

Immediately prior to inactivation, a sample should be taken for checking against its homologous antibody in a MAT. The inactivated culture must be checked for freedom from viable leptospires. This is done by inoculating aliquots of inactivated culture into an appropriate growth medium, such as the medium of Johnson & Harris (1967), incubating at $29 \pm 1^\circ\text{C}$ for at least 4 weeks, and examining weekly by dark-field microscopy for the presence of viable leptospires.

After blending, the levels of free inactivating agents, minerals present in adjuvants (such as aluminum), and preservative (such as thiomersal) must be within prescribed limits.

2.2.4. Final product batch control

i) Sterility

Selected samples of the completed vaccine should be tested for the absence of viable bacteria and fungi (British Pharmacopoeia [Veterinary], 1985b; European Pharmacopoeia, 2002a; 2002b; 9CFR 113.26). Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Identity

Identity checks should be carried out on the product before inactivation, usually by checking with appropriate antisera,

iii) Safety

Samples of completed product should be tested for safety. Methods for this have been described elsewhere (British Pharmacopoeia [Veterinary], 1985a; European Pharmacopoeia, 2002a; 9CFR 113.38). The test should be carried out for each route of inoculation indicated on the label and in two healthy animals of each category (e.g. pregnant animals, young stock) for which the vaccine is intended. The animals must be susceptible to the serovars used in the vaccine and their sera must be free from agglutinating antibodies to those serovars. Each animal is given an injection of the vaccine by the recommended route with twice the recommended dose, as stated on the label. The animals are observed for 14 days and should show no adverse local or systemic effects attributable to the vaccine.

iv) Batch potency

Samples of completed vaccine should be tested for potency in hamsters or guinea-pigs. Potency is usually measured by the vaccine's ability to prevent the death of the animal when challenged with a lethal dose. With some serovars that are not hamster or guinea-pig lethal, such as serovar Hardjo, potency is measured against prevention of renal infection when the animals are challenged with between 10 and 10,000 ID₅₀ (50% infectious dose) or by induction of a suitable antibody titre in rabbits.

An example protocol is to inject 1/40 dog dose of the vaccine into each of ten healthy hamsters no more than 3 months old. After 15–30 days, each vaccinated hamster, and each of ten unvaccinated hamsters of the same age, is injected intraperitoneally with a suitable quantity of a virulent culture of leptospires of the serovar used to make the vaccine (or a suspension of liver or kidney tissue collected from an experimentally infected animal). In the case of bivalent vaccines, each serovar is tested separately. For the vaccine to pass the test, at least 80% of the controls should die showing typical signs of *Leptospira* infection and at least 80% of the vaccinated animals should remain in good health for 14 days after the death of the controls. Other protocols may apply to cattle and pig vaccines, which contain as many as five or six components. The European Pharmacopoeia uses five vaccinated and five control animals.

In-vitro potency tests for leptospiral vaccines are being developed based on quantifying the protective antigen in the vaccine using MAbs in a capture ELISA (Ruby *et al.*, 1992) and are currently coming into use (Klaasen *et al.*, 2013).

2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C.2.1 and C.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

In-process controls are part of the manufacturing process.

2.3.2. Safety requirements

Tests use single dose and repeat doses (taking into account the maximum number of doses for primary vaccination and, if appropriate, the first revaccination/booster vaccination) containing the maximum permitted payload and according to the case, the maximum number of vaccine strains.

i) General safety in the target animal

One example is to use no fewer than ten healthy target animals that do not have antibodies against *Leptospira*. Administer to each animal a double dose of the vaccine by the method recommended on the label. Observe the animals each day for 14 days. If adverse reactions attributable to the biological product occur during the observation period, the vaccine is unsatisfactory. If adverse reactions occur not attributable to the biological product, the test shall be declared inconclusive and has to be repeated (European Pharmacopoeia, 2002a; 9CFR; United States Department of Agriculture Standard Requirements § 9. CFR, 113).

ii) Safety in pregnant animals

If the vaccine is intended for use in pregnant animals, use no fewer than ten healthy animals at the stage of pregnancy that accords with the recommended schedule or at different stages of pregnancy. Administer to each animal a double dose of the vaccine by the method recommended on the label. Observe the animals at least until 1 day after whelping. The vaccine complies with the test if the animals do not show abnormal local or systemic reactions, signs of disease or die for reasons attributable to the biological product and if no adverse effects on the pregnancy or the offspring are noted.

iii) Precautions (hazards)

Vaccine should be identified as harmless or pathogenic for vaccinators. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection (including for adjuvants, oil-emulsion vaccine, preservatives, etc.) with warnings included on the product label or leaflet so that the vaccinator is aware of any danger.

2.3.3. Efficacy requirements

To register a commercial vaccine, a batch or batches produced according to the standard method and containing the minimum amount of antigen or potency value shall prove its efficacy (protection); each future commercial batch shall be tested before release to ensure that it has the same potency value demonstrated by the batch(es) used for the efficacy test(s).

A minimum of ten vaccinates and ten controls (in the case of cattle) and eight vaccinates and eight controls (in the case of dogs) should be challenged with each serovar included in the vaccine. Animals should be vaccinated according to the proposed field use. Challenge should take place by a natural route. Animals should be of an age and reproductive status appropriate to any subsequent claims. Animals should be slaughtered (if they have not previously died) 28–35 days post-challenge and appropriate tissues cultured. Daily clinical examinations should be carried out. Blood should be cultured on days 4–7 post-challenge and on any day pyrexia is detected. Urines should be examined for the presence of *Leptospira* 14, 21 and 28 days post-challenge. Kidney and urine should be cultured at slaughter. If protection of the genital track is to be included in a protection claim, uterus and oviduct should also be cultured at slaughter. In the event of an animal dying, urine, kidney and liver should be cultured. For a claim of efficacy, 80% of vaccinates should be protected and at least 80% of controls infected.

2.3.4. Duration of immunity

Duration of immunity should be determined in the animal species for which the vaccine is intended using natural routes of challenge. Duration of immunity should not be estimated based on the duration of MAT titres in vaccinated animals as protection against clinical disease may be present with very low titres. Vaccinal immunity should persist for at least 6 months or longer depending on the label claim.

2.3.5. Stability

When stored under the prescribed conditions, the vaccines may be expected to retain their potency for 1–2 years. Stability should be assessed by determining potency after storage at 2–5°C, room temperature, and 35–37°C.

REFERENCES

- ADLER B. & DE LA PEÑA MOCTEZUMA A. (2010). *Leptospira* and leptospirosis. *Vet. Microbiol.*, **140**, 287–296.
- ADLER B., FAINE S., CHRISTOPHER W.L. & CHAPPEL R.J. (1986). Development of an improved selective medium for isolation of leptospires from clinical material. *Vet. Microbiol.*, **12**, 377–381.
- AHMED A., ENGELBERTS M.F.M., BOER K.R., AHMED N. & HARTSKEERL R.A. (2009). Development and validation of a real-time PCR for detection of pathogenic leptospira species in clinical materials. *PLoS One*, **4**, e7093.
- AHMED A., GROBUSCH M.P., KLATSER P. & HARTSKEERL R.A. (2012a). Molecular approaches in the detection and characterization of *Leptospira*. *J. Bacteriol. Parasitol.*, **3**, 2 <http://dx.doi.org/10.4172/2155-9597.1000133>.
- AHMED A., KLAASEN H.L.B.M., VAN DER VEEN M., VAN DER LINDEN H., GORIS M.G.A. & HARTSKEERL R.A. (2012b). Evaluation of real-time PCR and culturing for the detection of leptospires in canine samples. *Adv. Microbiol.*, **2**, 162–170.
- AHMED N., MANJULATA DEVI S., VALVERDE M. DE LOS A., VIJAYACHARI P., MACHANG’U R.S., ELLIS W.A. & HARTSKEERL R.A. (2006). Multilocus sequence typing method for identification and genotypic classification of pathogenic *Leptospira* species. *Ann. Clin. Microbiol. Antimicrob.*, **5**, 28 (doi:10.1186/1476-0711-5-28).
- BARNETT J.K., BARNETT D., BOLIN C.A., SUMMERS T.A., WAGAR E., CHEVILLE N.F., HARTSKEERL R. & HAAKE D. (1999). Expression and distribution of leptospiral outer membrane components during renal infection of hamsters. *Infect. Immun.*, **67**, 853–861.
- BASKERVILLE A. (1986). Histopathological aspects of diagnosis of leptospirosis. *Curr. Top. Vet. Med. Anim. Sci.*, **36**, 33–43.
- BEY R.F. & JOHNSON R.C. (1978). Protein-free and low-protein media for the cultivation of *Leptospira*. *Infect. Immun.*, **19**, 562–569.
- BRENNER D.J., KAUFMANN A.F., SULZER K.R., STEIGERWALT A.G., ROGERS F.C. & WEYANT R.S. (1999). Further determination of DNA relatedness between serogroups and serovars in the family *Leptospiraceae* with a proposal for *Leptospira alexanderi* sp. nov. and four new *Leptospira* genomospecies. *Int. J. Syst. Bacteriol.*, **49**, 839–858.
- BRITISH PHARMACOPOEIA (VETERINARY) (1985a). *Leptospira* veterinary vaccines. 117.
- BRITISH PHARMACOPOEIA (VETERINARY) (1985b). Test for sterility of veterinary immunological products. Appendix xviA, (vet). 2, A127–A128.
- CHEN T.Z. (1986). Development and present status of leptospiral vaccine and technology of production of the vaccine in China. *Ann. Immunol. Hung.*, **26**, 125–151.
- CODE OF FEDERAL REGULATIONS (OF THE UNITED STATES OF AMERICA) (9CFR) United States Department of Agriculture Standard Requirements, 9 CFR 113.26.
- CODE OF FEDERAL REGULATIONS (OF THE UNITED STATES OF AMERICA) (9CFR) United States Department of Agriculture Standard Requirements, 9 CFR 113.38.
- COLE J.R., ELLINGHAUSEN H.C. & RUBIN H.L. (1980). Laboratory diagnosis of leptospirosis of domestic animals. *Proc. U.S. Anim. Health Assoc.*, **83**, 189–199.
- COUSINS D.V., ROBERTSON G.M. & HUSTAS L. (1985). The use of the enzyme-linked immunosorbent assay (ELISA) to detect the IgM and IgG antibody response to *Leptospira interrogans* serovars hardjo, pomona and tarassovi in cattle. *Vet. Microbiol.*, **10**, 439–450.
- DIKKEN H. & KMETY E. (1978). Serological typing methods of leptospires. *Methods Microbiol.*, **11**, 259–307.
- DRAGON E.A., SPADORO J.P. & MADEJ R. (1993). Quality control of polymerase chain reaction. In: Diagnostic Molecular Microbiology. Principals and Applications, Persing D.H., Smith T.F., Tenover F.C. & White T.J., eds. American Society for Microbiology, Washington DC, USA, 160–168.
- ELLIS W.A. (1986). The diagnosis of leptospirosis in farm animals. In: The Present State of Leptospirosis Diagnosis and Control, Ellis W.A. & Little T.W.A., eds. Martinus Nijhoff, Dordrecht, The Netherlands, 13–31.
- ELLIS W.A., MONTGOMERY J.M. & THIERMANN A.B. (1991). Restriction endonuclease analysis as a taxonomic tool in the study of pig isolates belonging to the Australis serogroup of *Leptospira interrogans*. *J. Clin. Microbiol.*, **29**, 957–961.
- ELLIS W.A., O'BRIEN J.J., NEILL S.D., FERGUSON H.W. & HANNA J. (1982a). Bovine leptospirosis: microbiological and serological findings in aborted fetuses. *Vet. Rec.*, **110**, 147–150.

- ELLIS W.A., O'BRIEN J.J., NEILL S.D. & HANNA J. (1982b). Bovine leptospirosis: Serological findings in aborting cows. *Vet. Rec.*, **110**, 178–180.
- ELLIS W.A., YAN K.T., MCDOWELL S.W.J., MACKIE D.P., POLLOCK J.M. & TAYLOR M.J. (2000). Immunity to Bovine Leptospirosis. *Proceedings of 21st World Buiatrics Congress, Punta del Este, Uruguay*, 10601–10611.
- ELLIS W.A. & ZYGRAICH N. (1986). Experimental studies with a *Leptospira interrogans* serovar hardjo vaccine. *Proceedings of the XIVth World Congress on Diseases of Cattle, Dublin, Eire*, 971–974.
- EUROPEAN PHARMACOPOEIA (2002a). Monograph 01/2002:0447: *Leptospira* vaccine for veterinary use. European Directorate for the Quality of Medicines and HealthCare (EDQM), Council of Europe, Strasbourg, France, p. 2270.
- EUROPEAN PHARMACOPOEIA (2002b). Chapter 2. Methods of analysis. European Directorate for the Quality of Medicines and HealthCare (EDQM), Council of Europe, Strasbourg, France, 13–231.
- EUROPEAN PHARMACOPOEIA MONOGRAPH: Bovine Leptospirosis vaccine (inactivated); PA/PH/Exp. 15V/T (01) 28.
- FAINE S., ADLER B., BOLIN C. & PEROLAT P. (2000). *Leptospira* and Leptospirosis, Second Edition. Medisci Press, Melbourne, Australia.
- GORIS M., LEEFLANG M., BOER K., GOEIJENBIER M., VAN GORP E., WAGENAAR J. & HARTSKEERL R.A. (2012). Establishment of valid laboratory case definition for human Leptospirosis. *J. Bacteriol. Parasitol.*, **3**, e1000132
- HARTMAN E.G., VAN DEN INGH T.S.G.A.M. & ROTHUIZEN J. (1986). Clinical, pathological and serological features of spontaneous canine leptospirosis. An evaluation of the IgM- and IgG-specific ELISA. *Vet. Immunol. Immunopathol.*, **13**, 261–271.
- HARTMAN E.G., VAN HOUTEN M., FRIK J.F. & VAN DER DONK J.A. (1984). Humoral immune response of dogs after vaccination against leptospirosis measured by an IgM- and IgG-specific ELISA. *Vet. Immunol. Immunopathol.*, **7**, 245–254.
- HATHAWAY S.C., LITTLE T.W.A. & PRITCHARD D.G. (1986). Problems associated with the diagnosis of *Leptospira interrogans* serovar hardjo infection in bovine populations. *Vet. Rec.*, **119**, 84–86.
- INTERNATIONAL COMMITTEE ON SYSTEMATIC BACTERIOLOGY (1984). Subcommittee on the taxonomy of *Leptospira*. *Int. J. Syst. Bacteriol.*, **34**, 258–259.
- JOHNSON R.C. & FAINE S. (1984). *Leptospiraceae*. In: *Bergey's Manual of Systematic Bacteriology*, Vol. 1. Williams & Wilkins, Baltimore, USA, 62–67.
- JOHNSON R.C. & HARRIS V.G. (1967). Differentiation of pathogenic and saprophytic leptospires. I. Growth at low temperatures. *J. Bacteriol.*, **94**, 27–31.
- JOHNSON R.C. & ROGERS P. (1964). 5-Fluorouracil as a selective agent for growth of Leptospirae. *J. Bacteriol.*, **87**, 422–426.
- JOHNSON R.C. & SEITER C.W. (1977). *The Leptospira and their cultivation – a monograph*. Reheis Chemical (a division of Armour Pharmaceutical), Phoenix, Arizona, USA.
- KLAASEN H.L.B.M., VAN DER VEE M., MOLKENBOER M.J.C.H. & SUTTON D. (2013). A novel tetravalent *Leptospira* bacterin protects against infection and shedding following challenge in dogs. *Vet. Rec.*, **172**, 181–181.
- LIMMATHUROTSAKUL D., TURNER E.L., WUTHIEKANUN V., THAIPADUNGPANIT J., SUPUTTAMONGKOL Y., CHIERAKUL W., SMYTHE L D., DAY N.P.J., COOPER B. & PEACOCK S.J. (2012). Fool's gold: why imperfect reference tests are undermining the evaluation of novel diagnostics: a re-evaluation of 5 diagnostic tests for Leptospirosis. *Clin. Infect. Dis.*, **55**, 322–331.
- MCCREEDY B.J. & CALLAWAYTH H. (1993). Laboratory design and work flow. In: *Diagnostic Molecular Microbiology. Principals and Applications*, Persing D.H., Smith T.F., Tenover F.C. & White T.J., eds. American Society for Microbiology, Washington D.C., USA, 149–159.
- NERVIG R.M. & GARRETT L.A. (1979). Use of furosemide to obtain bovine urine samples for leptospiral isolation. *Am. J. Vet. Res.*, **40**, 1197–1200.
- OIE S., KOSHIRO A., KONISHI H., & YOSHSII Z. (1986) *In vitro* evaluation of combined use of fosfomycin and 5-fluorouracil for selective isolation of *Leptospira* species. *J. Clin. Microbiol.*, **23**, 1084–1087.
- PEROLAT P., MERIEN F., ELLIS W.A. & BARANTON G. (1994). Characterization of *Leptospira* isolates from serovar hardjo by ribotyping, arbitrarily primed PCR, and mapped restriction polymorphisms. *J. Clin Microbiol.*, **32**, 1949–1957.
- PICARDEAU M. (2013). Diagnosis and epidemiology of leptospirosis. *Medecine et Maladies Infectieuses*, **43**, 1–9.

- PRITCHARD G. (2001). Milk antibody testing in cattle. *In Practice*, **23**, 542–548.
- RUBY K.W., CARDELLA M.A. & KNUDTSON W.U. (1992). Assay for measuring relative potency of leptospiral bacterins containing serovar pomona. *Biologicals*, **20**, 259–266.
- SCANZIANI E. (1991). Comparison between specific immunoperoxidase staining and bacteriological culture in the diagnosis of renal leptospirosis of pigs. *Res. Vet. Sci.*, **50**, 229–232.
- SHENBERG E. (1967). Growth of pathogenic *Leptospira* in chemically defined media. *J. Bacteriol.*, **93**, 1598–1606.
- SIGNORINI M.L., LOTTESBERGER J., TARABLA H.D. & VANASCO N.B. (2013) Enzyme-linked immunosorbent assay to diagnose human leptospirosis: a meta-analysis of the published literature. *Epidemiol. Infection.*, **141**, 22–32.
- SLACK A., SYMONDS M., DOHNT M.F. & SMYTHE L.D. (2006). An improved multiple-locus variable number of tandem repeats analysis for *Leptospira interrogans* serovar Australis: a comparison with fluorescent amplified fragment length polymorphism analysis and its use to redefine the molecular epidemiology of this serovar in Queensland, Australia. *J. Med. Microbiol.*, **55**, 1549–1557.
- SLACK A., SYMONDS M., DOHNT M., HARRIS C., BROOKES D. & SMYTHE L. (2007). Evaluation of a modified Taqman assay detecting pathogenic *Leptospira* spp. against culture and *Leptospira*-specific IgM enzyme-linked immunosorbent assay in a clinical environment. *Diagn. Microbiol. Infect. Dis.*, **57**, 361–366.
- TERPSTRA W.J., KORVER H., SCHOONE G.J., VAN LEEUWEN J., SCHÖNEMANN C.E., DE JONGE-AGLIBUT S. & KOLK A.H.J. (1987). Comparative classification of *Leptospira* serovars of the Pomona group by monoclonal antibodies and restriction-endonuclease analysis. *Zentralbl. Bakteriol. Mikrobiol. Hyg. [A]*, **266**, 412–421.
- TERPSTRA W.J., KORVER H., VAN LEEUWEN J., KLATSER P.L. & KOLK A.H.J. (1985). The classification of sejroe group serovars of *Leptospira interrogans* with monoclonal antibodies. *Zentralbl. Bakteriol. Mikrobiol. Hyg. [A]*, **259**, 498–506.
- THAIPADUNPANIT J., CHIERAKUL W., WUTHIEKANUN V., LIMMATHUROTSAKUL D., AMORNCHAI P., BOONSLIP S., SMYTHE L.D., LIMPAIBOON R., HOFFMASTER A.R., DAY N.P. J. & PEACOCK S.J. (2011). Diagnostic accuracy of real-time PCR assays targeting 16S rRNA and lipI32 genes for human Leptospirosis in Thailand: a case-control study. *Plos One*, **6**, e16236.
- THIERMANN A.B., HANDSAKER A.L., FOLEY J.W., WHITE F.H. & KINGSCOTE B.F. (1986). Reclassification of North American leptospiral isolates belonging to serogroups Mini and Sejroe by restriction endonuclease analysis. *Am. J. Vet. Res.*, **47**, 61–66.
- UNITED STATES DEPARTMENT OF AGRICULTURE: NATIONAL VETERINARY SERVICES LABORATORIES (1987). Microtitre technique for detection of *Leptospira* antibodies. *Proc. U.S. Anim. Health Assoc.*, **91**, 65–73.
- VIJAYACHARI P., HARTSKEERL R.A., SHARMA S., NATARAJASEENIVASAN K., ROY S., TERPSTRA W.J. & SEHGAL S.C. (2004). A unique strain of *Leptospira* isolated from a patient with pulmonary haemorrhages in Andaman Islands: a proposal of serovar Portblairi of serogroup Sehgal. *Epidemiol. Infect.*, **132**, 663–673.
- WILD C.J., GREENLEE J.J., BOLIN C.A., BARNETT J.K., HAAKE D.A. & CHEVILLE N.F. (2002). An improved immunohistochemical diagnostic technique for canine leptospirosis using antileptospiral antibodies on renal tissue. *J. Vet. Diagn. Invest.*, **14**, 20–24.
- ZUERNER R.L. & ALT D.P. (2009). Variable nucleotide tandem-repeat analysis revealing a unique group of *Leptospira interrogans* serovar pomona isolates associated with California sea lions. *J. Clin. Microbiol.*, **47**, 1202–1205.
- ZUERNER R.L., ALT D. & BOLIN C.A. (1995). IS1533-based PCR assay for identification of *Leptospira interrogans* sensu lato serovars. *J. Clin. Microbiol.*, **33**, 3284–3289.
- ZUERNER R.L. & BOLIN C. A. (1997). Differentiation of *Leptospira interrogans* isolates by IS1500 hybridization and PCR assays. *J. Clin. Microbiol.*, **35**, 2612–2617.

*
* *

NB: There are OIE Reference Laboratories for Leptospirosis
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list:
<http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/>).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for leptospirosis

NB: FIRST ADOPTED IN 1990; MOST RECENT UPDATES ADOPTED IN 2014.