CHAPTER 2.1.9.

LEPTOSPIROSIS

SUMMARY

Leptospirosis is a transmissible disease of animals and humans caused by infection with any of the pathogenic members of the genus Leptospira. Laboratory diagnosis of leptospirosis can be complex and involves tests which fall into two groups. One group of tests is designed to detect anti-leptospiral antibodies and the other group of tests is designed to detect leptospires, 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 leptospires in:

a) 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 leptospires 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 leptospires 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 leptospires 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.

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 plus 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 and the screening of experimental animals for use in challenge studies. 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 and diagnostic biologicals: 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 have been tested, commercial vaccines are, with few exceptions, 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

Leptospirosis is a transmissible disease of animals and humans caused by infection with the spirochete Leptospira. All the pathogenic leptospires were formerly classified as members of the species Leptospira interrogans; however the genus has recently been reorganised and pathogenic leptospires are now identified in 17 named species and four genomospecies of Leptospira (14, 61, 71, 105). There are more than 200 distinct leptospiral serovars recognised and these are arranged in 23 serogroups (52, 98).

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 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. 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 to life and serve as reservoirs of the infection for other animals and humans.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The demonstration of leptospires 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 leptospires 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 leptospires 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 leptospires at the time of testing. Collection of urine following treatment of the animals with a diuretic enhances the chances of detecting the organism (63). In important cases involving individual animals (e.g. clearing an infected stallion to return to breeding), negative tests on three consecutive weekly urine samples has been considered to be good evidence that an animal is not shedding leptospires in the urine.

The demonstration of leptospires 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.

In experienced hands, the isolation of leptospires is the most sensitive method 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 semisolid (0.1–0.2% agar) medium containing BSA and either Tween 80 (e.g. Tween 80/BSA medium or EMJH) (44) or containing BSA and a combination of Tween 80 and Tween 40 (30). Contamination may be controlled by the addition of a variety of selective agents, e.g. 5-fluorouracil (48), nalidixic acid (49), fosfomycin (64), and a mixture of rifamycin, polymyxin, neomycin, 5-fluorouracil, bacitracin, and actidione (1). However, use of selective agents may reduce the chances of isolation when there are only small numbers of viable leptospires, and some strains of leptospires will not grow in selective media containing multiple antibiotics. Addition of 0.4–1% 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 (30). 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.

Leptospires may also be demonstrated by a variety of immunochromic staining techniques, e.g. immunofluorescence (in pathological material that is unsuitable for culture or where a rapid diagnosis is required. As the success of these techniques is dependant 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. Leptospires do not stain satisfactorily with aniline dyes, and silver-staining techniques lack sensitivity and specificity, although they are a useful adjunct for histopathological diagnosis (7).

Polymerase chain reaction (PCR)-based assays are now used in some diagnostic and many reference laboratories for the detection of leptospires in tissues and body fluids of animals. A variety of primer sets for the conduct of PCR assays have been described (3, 6, 13, 37, 39, 44, 50, 51, 54, 59, 60, 66, 84, 93, 97, 103) with some primers only specific for the genus *Leptospira* and others designed to identify only pathogenic species. These assays do not identify the infecting serovar, although some primer sets may permit further identification to the species or serovar 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. Therefore, the individual laboratory is generally responsible for the validation of the particular assay they use for the tissue, fluid, and species being tested. PCR assays can be quite sensitive, but lack of specificity (i.e. false-positive results) can be a problem. 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 diagnosis of leptospirosis requires careful attention to laboratory design and workflow to prevent contamination of reagents, and use of appropriate control samples (29, 57). In addition, sample processing for PCR is critical and must be suited to the tissue, fluid, and species being tested. A procedure for the preparation of urine samples for PCR using magnetic beads coated with anti-leptospiral antibody shows promise in enhancing the detection of pathogenic leptospires in urine (88).

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 (46, 47); PCR-assay-based amplification of 23S rDNA (102); and G+C content of DNA (46).

New leptospiral species have been identified based on DNA–DNA hybridisation analysis (14, 71, 105). In most cases, the type strain for each serovar was used in these analyses; for a few serovars, clinical isolates have also been tested to determine the new species designations. Different isolates belonging to a single serovar usually belong to the same species, but this is not always the case. Species identification of field isolates is still cumbersome but can be done by sequence analysis of the 16S rDNA, by genetic analysis of the 16S or 23S ribosomal RNA genes (17, 53, 61, 68, 70, 100, 101), by multilocus sequence typing (4, 77), by sequencing the DNA gyrase subunit B encoding gene (82), or by PCR using species-specific *ompL1* primer sets (73).

Strains belonging to *Leptospira* can be differentiated to the serogroup level by cross-agglutination reactions (28). Further 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: factor analysis (28); monoclonal antibodies (MAbs) (89, 90); restriction endonuclease analysis (43, 56, 91, 92); and various other molecular strategies (17, 21,
A wide variety of serological tests, which show varying degrees of serogroup and serovar specificity, have been needed to detect the organism in urine or the genital tract of chronic carriers. 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.

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, which 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).

a) 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 definitively identify 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 (36, 62).

The strains selected should be grown in liquid leptospiral culture medium (e.g., EMJH, Tween 80 BSA, or other suitable medium) at 29 ± 1°C and the culture should be at least 4 days old, but no more than 8 days. Live cultures with densities of approximately $2 \times 10^8$ leptospires 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. 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). 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. The microtitration plates are incubated at 29 ± 1°C for 2–4 hours. 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). Many laboratories perform a screening test at a final serum dilution of 1/100 and then retest sera with titres of ≥100 to determine an endpoint using doubling dilutions of sera at a starting titre of 1/100 through to 1/12,800 or higher.

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 (45). 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 cloned culture with a density of approximately $2 \times 10^8$ leptospires/ml. 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 ≥1/12,800, the rabbit is anaesthetised and
b) 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. Information regarding the surface antigens of Leptospira has been reviewed (25). In general, ELISAs are quite sensitive, but lack the serovar specificity of the MAT. An ELISA that measures canine IgG and IgM against various leptospiral serovars has been developed and evaluated in Europe (40, 41). Anti-leptospiral IgM is detectable in this assay as early as 1 week after infection, before agglutinating antibodies are present. IgG antibodies are detectable in infected dogs beginning 2 weeks after infection and persist for long periods of time. Therefore, dogs with acute leptospirosis have high IgM titres and relatively low IgG titres; dogs that are vaccinated or have had previous leptospiral infections have high IgG titres but low IgM titres. Similar assays to detect anti-leptospiral bovine, porcine, and ovine antibodies have also been developed (2, 19, 24, 58, 74, 85–87, 94, 104). The major identified role of ELISA in livestock species is the use of an IgM ELISA for identification of recent infections (24) and for screening herds in regions where vaccination for leptospirosis is not practiced. A total-Ig ELISA is useful in identification of fully susceptible animals suitable for experimental challenge work (34). 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. 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. New ELISAs have been developed based on detection of antibodies against surface proteins or lipoproteins of Leptospira (12, 27, 55, 65, 67) but these tests are not yet widely available.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

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°C. Alternatively, the serum can be stored in 2 ml volumes at −15 to −20°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.

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 may be a low survival rate of leptospires 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.

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 (32) and in the identification of renal or genital carriers (30). 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% (30). 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. (20) 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. (42) 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.
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. While experimental vaccines based on cellular extracts have been tested (9), commercial vaccines are, with few exceptions, whole-cell products. 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.

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.

**1. Seed management**

**a) Characteristics of the seed**

Proper selection of vaccine production strains is of utmost importance. Immunity induced by vaccination is largely serovar specific (18). 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.

**b) Methods of culture**

Each component strain to be included in the final vaccine should be grown separately in liquid medium; preferably in a protein-free (8, 80) or low-protein medium (8).

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.

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 (28) to ensure purity and homology. MAbs may also be used for this purpose.

**c) Validation as a vaccine**

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 (11). A draft monograph for the efficacy testing of serovar Hardjo vaccines has been prepared and specifies the use of more natural routes of challenge (35).

**2. Method of manufacture**

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 ± 1°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.

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 (47), incubating at 29 ± 1°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.

4. Batch control

a) Sterility

Selected samples of the completed vaccine should be tested for the absence of viable bacteria and fungi (16, 22, 23, 95). Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

b) Safety

Samples of completed product should be tested for safety. Methods for this have been described elsewhere (15, 22, 96). 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.

c) 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 between 10 and 10,000 LD₅₀ (50% 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 8/10 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.
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 (76). These assays are being standardised using reference vaccines and correlation with existing hamster or antibody-based potency assays and target–host efficacy data.

d) Duration of immunity
Duration of immunity should be determined in the animal species for which the vaccine is intended using natural routes of challenge (11). 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.

e) 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.

5. Tests on the final project
a) Safety
See Section C.4.b.

b) Potency
See Section C.4.c.

REFERENCES


35. EUROPEAN PHARMACOPOEIA DRAFT MONOGRAPH; Bovine Leptospirosis vaccine (inactivated); PA/PH/Exp. 15V/T (01) 28.


Chapter 2.1.9. - Leptospirosis


95. UNITED STATES DEPARTMENT OF AGRICULTURE STANDARD REQUIREMENTS § 113.26.

96. UNITED STATES DEPARTMENT OF AGRICULTURE STANDARD REQUIREMENTS § 113.38.


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**NB:** There are OIE Reference Laboratories for Leptospirosis (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).