Chapter 10

LISTERIOSIS

J.C. LOW

10.1 SUMMARY

Listeric infections, caused by micro-organisms of the genus *Listeria*, occur world-wide and in a variety of animals including man. The organism is widespread in the environment, in foodstuffs and many animals shed small numbers in their faeces. Two species, *Listeria monocytogenes* and *Listeria ivanovii* are pathogenic. The former causing encephalitis, abortion and septicaemia in a wide range of animal species, the latter is responsible for abortion and perinatal death in sheep, goats and occasionally cattle.

Direct diagnosis of listeric abortion is by isolation of the organism from abortive material on non-selective or selective media. Histological examination may be necessary for conclusive diagnosis since the organism may occasionally be isolated from normal placentas resulting from faecal contamination. Although serological tests are available for the detection of antibodies against *Listeria*, these are not used in the diagnosis of abortive listeriosis as antibodies are frequently found in the sera from healthy animals with no histories of listeric infections and many of the assays suffer from a lack of specificity.

10.2 INTRODUCTION

The bacterium now known as *Listeria monocytogenes* was first isolated from an epidemic disease of rabbits and guinea-pigs in a laboratory animal breeding unit in 1926 and causes infectious disease in both animals and man. Infection is truly widespread, having been recorded in more than forty mammalian species as well as in fowls, fish, crustaceans, flies and ticks and in countries in six continents. Listeriosis is of major veterinary importance in cattle, sheep and goats with encephalitis and uterine infections being most frequently identified.

The genus *Listeria*, formerly thought to contain only one species, *Listeria monocytogenes*, has now been expanded and contains : *L. monocytogenes* sensu stricto, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. murrayi*. Clinical infections are caused primarily by *L. monocytogenes* though *L. ivanovii* is also pathogenic, being particularly associated with abortion in ruminants. The remaining species are regarded as non-pathogenic. *Listeria monocytogenes* can be divided into 16 serovars [1] on the basis of somatic and flagella antigens with serovars 4b, 1/2a and 1/2b most commonly isolated from disease problems. Virulent strains produce a haemolysin, listeriolysin O, which is a major virulence factor and enables intracellular multiplication within macrophages, monocytes and other cells.

Listeria monocytogenes is a small Gram positive, non-sporing rod, 1-2µm in length and 0.5µm wide, with bacteria often lying parallel to each other in palisades, and thread like forms present in some cultures. The optimum temperature range is 30 to 37°C but growth occurs between 3 and 45°C and *Listeria* are among the few pathogenic bacteria that grow readily at 4°C. The organism multiplies in aerobic or microaerophilic conditions at pH values as high as 9.6. Growth is absent or scant in complete anaerobic conditions and multiplication is inhibited by pH values lower than 4.5. The bacterial colonies are small, smooth, slightly flattened and milky white by reflected light. When illuminated by obliquely transmitted light the colonies exhibit a characteristic blue/green colour. Organisms grown at 37°C show little or no motility but a characteristic tumbling motility can be demonstrated by incubation of cultures at room temperature with motile organisms commonly possessing many peritrichious flagellae.

Clinical disease in animals occurs mainly in the northern and southern latitudes and is much less commonly recognised in tropical and subtropical climates. Encephalitis, abortion and septicaemia are the most prevalent manifestations in sheep and goats. Though it is rare for an outbreak of encephalitis and abortion to occur simultaneously these may follow each other in a single flock or herd and septicaemia may occur alongside encephalitis or abortion. Occasionally mastitis, spinal myelitis or keratoconjunctivitis result from listeric infections.

Listeric abortion caused by *L. monocytogenes* occurs in ruminants and many other species of domestic animal. *Listeria ivanovii* is also recorded as a cause of abortion in sheep, goats and cattle but occurs less frequently than *L. monocytogenes*. Cases of listeric abortion in sheep and goats are usually sporadic although in exceptional circumstances up to 50 percent of a flock have been affected. Occasional massive outbreaks of septicaemia involving pregnant ewes have been described with clinically affected animals being pyrexic showing profuse diarrhoea and subsequently aborting. Experimentally, oral infection does not consistently produce abortion but the gravid uterus is highly susceptible to infection and abortion is readily produced by the intravenous injection of pregnant ewes with *L.monocytogenes* or *L. ivanovii*. In the pregnant animal invasion of the placenta and foetus occurs within 24 hours of the onset of bacteraemia and, following oedema and necrosis of the placenta, abortion arises 5-10 days post-infection. In sheep and goats abortion is seen from the 12th week of pregnancy onwards and there is a blood-stained vaginal discharge for several days. Infection in late pregnancy results in stillbirths or the delivery of young which develop a fatal septicaemia. Aborted foetuses are rarely mummified but may be autolysed. In most cases ewes or nanny goats make uneventful spontaneous recoveries and other forms of infection are unlikely to occur. On some farms listeric abortion may recur each year.

The natural habitat for all *Listeria* species including *L. monocytogenes*, is the environment and the organism is widespread having been isolated from surface soils, decaying vegetation, pasture herbage, silage, sewage sludge, animal slurry, factory effluent and surface and river waters. The organism has been shown to persist for up to 7 months on dry straw, up to months in damp soil and for more than 2 years in dry soil and faeces. In most animal species including ruminants *L.monocytogenes* can occasionally be isolated from the faeces, nasal secretions and milk of healthy animals. Factors that are believed to predispose the host to clinical disease include :

- factors that cause a lowering of the host animal's resistance

1. the stress of late pregnancy and parturition;

2. poor nutritional state;

3. sudden changes of weather to very cold and wet;

- or factors that increase the infection pressure of the organism, such as massive multiplication of *L. monocytogenes* in feed or the environment.

Although the organism is ubiquitous, listeric infections have been particularly associated with silage feeding. Much of this association is attributable to the high bacterial numbers which may be present in poorly preserved silage (>10^7 *L. monocytogenes* colony forming units (cfu) per kilogram). Abortive disease in sheep and goats can occur within 6-13 days of introduction to silage. It is unclear to what extent infection of other animals occurs following abortion but in the majority of instances abortions are sporadic and major outbreaks are associated with heavily contaminated foodstuffs.

Practically all common antibiotics, except cephalosporins, are active against *L.monocytogenes* in vitro. However, in vivo, a low efficiency may be expected and is probably in part due to the organism's intracellular location. Experimentally, ampicillin, amoxicillin are most active. Tetracycline and chloramphenicol though widely used are reportedly not the therapeutic agents of choice. Though antimicrobial resistance in clinical isolates is rare, resistance to chloramphenicol, erythromycin, streptomycin and tetracycline has been recorded.

Most attempts to produce a satisfactory vaccine have been unsuccessful, but a live attenuated vaccine has been in use in Norway for several years and is reported to reduce the annual incidence of listeric encephalitis from 4% to 1.5% in field trials. A commercial killed vaccine is available for the control of the disease in some other countries. However, the results of field trials for each of these vaccines are equivocal and no experimental model is available to test their efficacy. Since listeriosis is a disease of complex aetiology, further investigation into mechanisms of immunity and their role in the pathogenesis of disease are necessary before effective vaccines can be developed.

Although there is potential for zoonotic transmission of *Listeria* it would appear that the majority of human exposure to the organism, and risk for disease, results from contamination of foods during processing and from the particular ability of *Listeria* to grow at refrigerator temperatures. There is therefore no reason for statutory control measures to be taken to protect consumers following an outbreak of listeric abortion, though stock handlers should adopt routine hygiene precautions.

10.3 SAMPLES

Listeriosis can only be confirmed by isolation and identification of the specific aetiological agent. Samples of choice are the aborted placenta, foetus and vaginal excretions. Histopathological examination of aborted tissue samples is also advised for positive diagnosis.

10.3.1 Isolation of *Listeria*

Samples of placenta, the liver and spleen of the aborted foetus should be collected aseptically and placed in a suitable sterile container.

Stomach contents should be removed from the foetus as soon as possible after abortion using a sterile needle and either a sterile syringe or a sterile, evacuated glass tube (e.g. Vacutainer).

Vaginal swabs must be collected

within 48 hours after abortion. Immediately after collection the swabs should be placed in their protective sheaf and transported to the laboratory.

On arrival in the laboratory the samples should be cultured immediately or stored at 4°C.

10.3.2 Histopathology

Small representative samples of the placenta and the liver and spleen from the aborted foetus, of no more than 0.5cm thickness, should be collected as quickly as possible after abortion or death and placed in a glass sample jar containing a suitable fixative such as formol saline (0.85g NaCl dissolved in 10ml 40% formaldehyde and 90ml of water), with a ratio of fixative to tissue of at least 10:1 (v/v). The tissues should be fixed for at least two days before further processing.

10.4 RISKS TO HUMAN HEALTH

Despite the apparently low invasiveness of *L. monocytogenes* all suspect material should be handled with caution. Aborted foetuses and necropsy of septicaemic animals present the greatest hazard and such materials should only be cultured in properly equipped laboratories under the control of a skilled microbiologist. Personnel have rarely developed fatal meningitis, septicaemia and papular exanthema on the arms after handling aborted material. However, low-grade infection in man may be more common than suspected and, in pregnant women may lead to death of the foetus and for this reason pregnant women should not work with known cultures of *Listeria*.

10.5 DIRECT DIAGNOSIS

10.5.1 Gross pathology of placenta and aborted foetuses

Placental lesions are pin-point, yellowish, necrotic foci involving the tips of the cotyledonary villi with a focal to diffuse intercotyledonary placentitis covered in a red/brown exudate.

The foetus often shows few lesions because of advanced autolysis but miliary necrotic foci with pin-point greyish, white nodules are occasionally visible throughout the liver and spleen. There may also be small abomasal erosions and yellow-orange meconium.

10.5.2 Isolation of *Listeria*

Principle

Listeria usually grows on most non-selective culture media especially when blood is added. A wide range of selective media have also been developed providing increased sensitivity and specificity of isolation mainly in response to the requirement to detect low numbers of *Listeria* in human foodstuffs. Of these media LPM agar, Oxford agar, Modified Oxford agar and PALCAM agar have particular application to veterinary samples and are commercially available (Oxoid, Unipath Ltd., UK). 5% sheep or horse blood agar is still a suitable media for culture of *Listeria* from samples collected from aborted ewes and nanny goats in which *Listeria* numbers are high and contamination with other bacteria is minimal.

Materials and reagents

- 5% sheep or horse blood agar plates.

Procedure

1. Inoculate 0.1ml of tissue or foetal stomach contents onto the surface of the isolation medium. Smear vaginal swab

samples directly onto the surface.

2. Incubate at 37°C for up to 48 hours.

3. Examine by a plate microscope for typical colonies of *Listeria* species after 24 and 48 hours incubation.

Reading and interpretation of results

Following overnight culturing on blood containing solid media at 37°C, the smooth form of *Listeria* give rise to colonies 1-2mm in diameter which are round, translucent and slightly raised, have an entire margin and a watery consistency, and are easily emulisfiable.

Listeria monocytogenes, *L .ivanovii* and *L. seeligeri* are the only haemolytic species and colonies of these species grown on blood agar are surrounded by variable zones of haemolysis. Colonies of *L. monocytogenes* are surrounded by a narrow zone of b-haemolysis, those of *L. ivanovii* are surrounded by a wide indistinct zone of haemolysis and an inner clear haemolytic zone. *Listeria seeligeri* cultures produce a faint narrow zone of haemolysis.

Note

• The selective media often contain indicators to aid the identification of *Listeria species*. However, it is recommended that multiple suspect colonies are subcultured on blood agar plates to allow an examination for haemolysis and prior to further identification.

• Isolation from tissue held at 4°C for several weeks and recultured at intervals, "cold enrichment", has been historically used for the isolation of *Listeria*. The technique is particularly appropriate where bacterial numbers are low but has largely been superseded by modern isolation methods. It is unlikely to be relevant to the diagnosis of listeric abortion.

Preparation of reagents

- Blood agar

The method of preparation of blood agar is described in the chapter on Campylobacteriosis.

10.5.3 Identification of *Listeria* species

Colonies that appear to be *Listeria* species on blood agar plates can be confirmed as such using three simple tests :

1. Gram stain.
2. Motility at room temperature.
3. Catalase reaction.

Listeria monocytogenes and *L. ivanovii* can be distinguished from other *Listeria* species on the basis of the CAMP test and other simple biochemical reactions (Table 10.1)[2]. Recently a rapid ten test strip identification system for *Listeria* species differentiation has been developed [3] and is commercially available (Api *Listeria*, BioMérieux, France).

Table 10.1 : Identification of Listeria species

Character	*Listeria monocytogenes*	*Listeria ivanovii*	*Listeria seeligeri*
β-Haemolysis on blood agar	+	++	+
CAMP test with *Staphylococcus aureus*	+	-	-
CAMP test with *Rhodococcus equi*	+	+	-
Acid produced from:			
L-rhamnose	+	-	-
D-xylose	-	+	+
a- methyl D-mannoside	+	-	±

++Strongly positive reaction ; + Positive reaction ; - Negative reaction ; ± Variable.

10.5.3.1 Gram stain

The Gram stain should be carried out by the method described in the chapter on Campylobacteriosis using colonies of *Listeria* removed from 24 or 48 hour cultures.

Reading and interpretation of results

Gram-positive organisms appear blue-black, Gram-negative organisms appear red.

Listeria cells are short Gram-positive rods and have a distinctive appearance and disposition often occurring as regimented pairs or rows adjacent to each other.

Note

• *Listeria* species are relatively easy to over-decolourise in comparison to other Gram positive bacteria. The cellular morphology of isolates direct from selective media are often irregular or bizarre.

10.5.3.2 Motility test

Materials and reagents

- Bijoux bottles containing sterile broth (brain heart infusion or Nutrient broth n°. 2).
- Paraffin wax or "Blue-tac".

Procedure

1. Remove colonies from a 24 hour culture and inoculate into two bottles of broth.
2. Incubate for 4-6 hours, one bottle at room temperature and the other at 37°C.
3. Place single drops of the resultant broths on the underside of coverslips which are raised above a microscope slide on bridges of paraffin wax or "blue-tac" (hanging drop technique).
4. Examine cultures microscopically.

Reading and interpretation of results

A tumbling, rotating motility in cultures incubated at room temperature but not in cultures incubated at 37°C is characteristic of *Listeria* species. If motility is not seen at room temperature re-examine both cultures at 18 hours before discarding as negative.

Note

• Non-motile strains have been identified on rare occasions.

Preparation of reagents

- Brain Heart Infusion Broth

Add brain heart infusion (Oxoid-Unipath or Difco) according to manufacturer's directions to distilled water. Dispense 2.5ml volumes in sterile bijoux and autoclave at 15 psi for 15 minutes. Store at 4°C.

- Nutrient Broth N° 2

Add nutrient broth base n°2 (Oxoid-Unipath or Difco) according to manufacturer's directions to distilled water. Dispense 2.5ml volumes in sterile bijoux and autoclave at 15 psi for 15 minutes. Store at 4°C.

10.5.3.3 Catalase test

The catalase test should be carried out by the method described in the chapter on Campylobacteriosis using a single drop from a 4-6 hour culture of *Listeria*.

Reading and interpretation of results

Bubbling indicates a positive catalase test. *Listeria* species are all catalase positive.

False positive catalase reactions may occur if a colony is taken from a medium containing blood.

The test may be safely performed using inoculated medium described in 10.5.3.2.

10.5.3.4 CAMP test (Christie-Atkins-Munch-Peterson)

Principle

The CAMP test is used to differentiate between the three haemolytic species of *Listeria* on the basis of an enhancement of haemolysis that occurs when *L. monocytogenes* is cultured on blood agar plates alongside *Staphylococcus aureus* (NCTC 1803) and when *L. ivanovii* is cultured alongside *Rhodococcus equi* (NCTC 1621) [2, 4].

Materials and reagents

- 5% Sheep blood agar plates (Oxoid, Hampshire, UK).
- Standard cultures of *Staphylococcus aureus* (NCTC 1803) and *Rhodococcus equi* (NCTC 1621).
- Control cultures of *L. monocytogenes*, *L. ivanovii* and *L .seeligeri*.

Procedure

1. Prepare sheep blood agar plates by pouring a thin layer of 5% v/v blood agar made with washed sheep cells onto the surface of nutrient broth agar plates. Allow to set and dry before use.
2. Make parallel streaks of cultures of *S. aureus* and *R. equi* across each sheep blood agar plate.
3. Streak the test cultures at right angles to the *S.aureus* and *R.equi* leaving a minimum of 1-2mm between the cultures.
4. Incubate plates at 37°C overnight.
5. A separate sheep blood agar plate prepared using the known control cultures is recommended.

Reading and interpretation of results

Results are recorded as positive when an enhanced zone of haemolysis occurs between the test and standard culture. Table 10.2 shows the results obtained with the haemolytic species *L. monocytogenes* and *L. ivanovii* and the non-haemolytic species *L. innocua*.

Table 10.2 : CAMP reaction for L. monocytogenes, L. ivanovii and L. innocua.

	Rhodococcus equi	*Staphylococcus aureus*
L. monocytogenes	-	+
L. ivanovii	+	-
L. innocua	-	-

Note

• If using a commercial control strain of *L. monocytogenes*, strain NCTC 7973 should be used and not strain ATCC 15313 since the latter does not exhibit beta-haemolysis.

• Enhancement of the haemolysis of *L. monocytogenes* with *R. equi* may be apparent.

10.5.3.5 Further species identification criteria

In addition to the properties outlined in Table 10.1, all *Listeria* species produce acid within 24 hours from: amygdalin, cellobiose, esculin, fructose, glucose, mannose and salicin. Acid production from galactose, lactose, melezitose, alpha-methyl-D-mannoside, rhamnose, sorbitol, starch, sucrose, trehalose and xylose is variable both between species and in some cases within species. Acid is not produced from adonitol, dulcitol, erythritol, inulin, raffinose, sorbose, arabinose, or melibiose. Aesculin is hydrolysed. Litmus milk is acidified and slowly decolourised. The methyl-red reaction and Voges-Prokauer test are positive. The oxidase test is negative. There is no production of indole or urease and no growth in citrate. H_2S is not formed, and ornithine, lysine, glutamic acid and arginine decarboxylases are not produced, nor is an arginine dihydrolase present. Phosphatase is produced. Methylene blue is decolourised.

Tributyrinase is not formed. Lecithinase (phospholipase C) is produced, and hydrolysis of Tweens 20, 40, 60 and 80 takes place slowly.

Note

• When testing for the production of acid from carbohydrates a variety of basal media and pH indicators have been used. A peptone water medium with phenol red as indicator is recommended and where possible all carbohydrates should be sterilized by filtration and not autoclaving.

10.5.4 DNA analysis

The requirement for rapid and sensitive methods of detection of *L. monocytogenes* in human foods combined with recent advances in the genetic characterisation of the organism has led to the development of specific gene probes. Polymerase chain reaction (PCR) technology has been applied to these probes and PCR-based assays developed for detection of genes encoding haemolysin, a pleotrophic regulatory factor, phospholipases, invasin associated proteins, internalin, delayed type hypersensitivity proteins and rRNA sequences of *L. monocytogenes*. Such assays have proved to be rapid, sensitive and specific when carried out on pure cultures of *L. monocytogenes* but their effectiveness when applied to contaminated materials is influenced by the type of sample, the numbers of organisms and the presence of other microflora. Preliminary selective enrichment steps are advised and also eliminate the possibility of amplification of DNA from dead cells. Specific primers have subsequently been developed for *L. innocua*, a set of primers for L.ivanovii, *L. seeligeri* and *L. welshimeri*, and a set of primers for all members of the genus *Listeria* [5].

The use of such techniques in veterinary diagnosis has still to be evaluated.

10.5.5 Non-cultural methods of detection

In conjunction with the continued refinement of enrichment broths and selective media to isolate *Listeria* species many non-cultural methods including: nucleic acid hybridisation, immunochemical and flow cytometric techniques are being developed. Commercially available kits covering all these technologies are being marketed for use in the detection of *Listeria* in human foods. However, the suitability of such techniques and kits for use in the veterinary field have still to be evaluated. Indeed, many are directed towards genus specific rather than *L. monocytogenes* specific detection and are thus not strictly relevant to veterinary work.

10.6 INDIRECT DIAGNOSIS

10.6.1 Histopathology

Principle

A suitable method for histopathological preparation of tissue samples and haematoxylin and eosin staining is described in the chapter on Toxoplasmosis. Gram staining should also be carried out on dewaxed, hydrated samples as described in the chapter on Campylobacteriosis.

Reading and interpretation of results

Histologically, the foci found in the placental tissue show coagulative necrosis and infiltration to variable degrees by macrophages and neutrophils. Gram staining should demonstrate the presence of large numbers of Gram positive bacterial rods in affected areas.

The histological appearance of lesions in the foetal liver and spleen are of multiple, focal areas of necrosis with invasion by polymorphs and mononuclear cells. Gram staining should demonstrate the presence of large numbers of organisms consistent with the appearance of *Listeria* in the lesions.

10.7 FUTURE WORK

There have been major advances in recent years in the understanding of *Listeria*, its taxonomy and also a molecular definition of its mechanism of virulence. However, despite advances there remain major gaps in the understanding of the epidemiology and pathogenesis of listeric infections in farm animals. Improved isolation techniques and methods of bacterial typing have been used by a number of investigators but these studies are by no means complete since the diversity and ubiquitous nature of the organism require detailed study to determine the ecological niches in which strains may thrive and mechanisms by which the organism may spread.

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Chapter 11

LEPTOSPIROSIS

W.A. ELLIS & S.J. MCDOWELL

11.1 SUMMARY

Leptospirosis is a common zoonotic disease affecting most mammals throughout the world. It causes economic losses in cattle and swine production due to abortion, stillbirth, infertility, decreased milk production and death. Sheep and goats have been considered resistant to clinical leptospirosis but recent findings indicate they may be susceptible in late pregnancy with resulting abortion, stillbirth, birth of weakly lambs and kids and agalactia in ewes and does.

In cases of abortion, stillbirth or birth of weakly lambs and kids, direct diagnosis is based on the isolation of the organism from, or its demonstration by immunofluorescence, in the foetus and placenta. However, since leptospires are fastidious, slow growing organisms, most practical diagnostic attempts concentrate on indirect serological techniques. The most commonly used technique is the microscopic agglutination test (MAT).

11.2 INTRODUCTION

Leptospirosis is a zoonotic disease caused by members of the genus *Leptospira*. Relative to cattle and pigs, sheep and goats have always been considered resistant to leptospiral infections. While that view is still generally true, it is apparent that, under certain intensive management systems, leptospirosis can cause overt clinical disease in sheep during late gestation and in the immediate post-partum period. This can cause considerable loss to individual farmers on such management regimes.

Leptospires are thin, helical, motile, Gram negative organisms, which are often hooked at one or both ends. In a suitable liquid environment, they spin constantly on their long axis. They range in length from about 10-20μm, with an amplitude of approximately 0.1-0.15μm and a wavelength of about 0.5μm. Under adverse nutritional conditions, leptospires may be greatly elongated, while under conditions such as high salt concentration-sageing culture or in tissues, leptospires may form coccoid forms of about 1.5-2μm. They divide by binary fission.

The taxonomy of leptospires is in a period of change and this has caused considerable confusion. Until recently a single genus *Leptospira* was recognised in the family Leptospiraceae. Two groupings were recognised within the genus - those which are found in animal species (the parasitic strains) and in water (the saprophytic strains). These two groupings, which were referred to as the interrogans and biflixa complexes, can be differentiated by their growth requirements and biochemical reactions. Only the parasitic strains are of medical and veterinary interest. For taxonomic purposes and as an aid to epidemiological studies the parasitic leptospires were divided into serogroups on the basis of antigenic relationships as determined by cross agglutination reactions and further subdivided into serovars by agglutination-absorption patterns. There are some 23 serogroups recognised containing approximately 212 serovars.

The advent of genetic typing methods has provided rapid, reproducible typing protocols. The current recommendations on the taxonomy of leptospires [1] recognise eight species of pathogenic leptospires within the family Leptospiraceae. There are *Leptospira* interrogans, *L. borgpetersenii*, *L. inadai*, *L. kirschneri*, *L. noguchii*, *L. meyer*, *L. weilli*, and *L. santarosai*.

The species definition is based on a level of DNA-DNA homology of at least 70% and < 5% divergence in DNA relatedness. Taxonomy at the subspecies level continues to be based on serovars but other valid methods which give comparable results to conventional serotyping can be used for their identification. Such methods include monoclonal antibody agglutination profiles, factor analysis, analyses in which restriction fragment length polymorphisms or rRNA gene restriction patterns are used in pulsed field gel electrophoresis analyses. The term "type" is used to indicate strain differences at the subserovar level [1].

The epidemiology of leptospirosis in domestic animals is potentially very complicated because animals can be infected by any of the pathogenic serovars. However, only a small number of serovars are endemic in any particular region or country. Furthermore, leptospirosis is a disease which shows a natural nidality and each serovar tends to be maintained in specific maintenance hosts. Therefore, in any region an animal species will be infected by serovars maintained either in the same species or in other animal species maintained in the same area. The relative importance of these incidental infections is determined by the opportunities for contact and transmission of leptospires from other species to the target host species provided by prevailing social, management and environmental factors.

There is consistent serological evidence that hardjo infection is the major infection found in sheep worldwide. In some countries there is evidence that sheep may maintain this serovar without the presence of the established maintenance host - cattle. It has also been shown that transmission of hardjo can occur between sheep grazed together under experimental conditions and also that infected sheep urine can experimentally infect calves. However, despite widespread infection in England and Wales field investigations found no evidence to suggest that the prevalence of hardjo infection in sheep was dependent on broad sheep population parameters.

Seroprevalence data indicates that other leptospiral infections in sheep are uncommon and are incidental. The serovars involved in these incidental infections vary with the geographical region and reflect the common serovars found in other domestic and wild animals in those regions. Only the Pomona, Grippotyphosa, Australis and Ballum serogroups [2] have been implicated in incidental clinical infections in sheep. These are acquired from other hosts and there is no evidence that sheep populations are capable of maintaining them, for example, the purported transmission of pomona from pigs to sheep.

There is no cultural or serological evidence to suggest that goats act as maintenance hosts for leptospires. High seroprevalences have been reported in some countries but the predominant infecting serovar varies suggesting an incidental pattern of infection.

The vast majority of leptospiral infections are sub-clinical. Two groups of animals are most likely to experience clinical infections:

1. The very young animal infected by an incidental infection. This may give rise to severe illness characterised by jaundice, haematuria, haemaglobinuria, evidence of renal damage, meningitis and can be fatal. Such acute clinical events are very occasionally seen in older animals. Sporadic outbreaks of acute clinical disease have occasionally been reported in both sheep and goats.

2. Sexually mature lactating and/or pregnant females infected by either incidental or host maintained leptopsires. This can result in a) agalactia in sheep or b) reproductive wastage.

• Agalactia. An agalactia similar to that seen in cows has been observed in hardjo-infected sheep in Northern Ireland. Recently lambed ewes suddenly go off their milk giving rise to lamb deaths due to starvation. Udders of affected ewes feel soft, not hard as in mastitis and ewes return to milk after 3 to 4 days without treatment.

• Reproductive wastage in sheep. Reproductive wastage in sheep is seen as late-term abortion, stillbirth and the birth of weak lambs. In a study in Northern Ireland, approximately 17 per cent of 872 aborted lambs examined between 1981 and 1987 were positive for leptospires on immunofluorescent examination. Culture data indicated that in the majority of these the infecting strain was serovar hardjo while a small number were due to either pomona, ballum or bratislava [2]. In Scotland, it has been reported [3] that 7 out of 170 aborted sheep foetuses had antibody to serovar hardjo, while in a South Dakota study [4] 8 per cent of aborted foetuses had antibody to leptospires. Serovar pomona has also been implicated in abortions in Spain [5] and Albania [4] while grippotyphosa was deemed to be the cause of abortions in Hungary [6].

It has been observed [2] that hardjo abortion was rarely found in extensively managed flocks. It was largely confined to ewes which were bred in an extensive system and subsequently bought as replacements for intensively managed flocks with high stocking rates and where ewes are frequently housed indoors. Reproductive wastage and agalactia were seen in these groups in their first lambing season following introduction, but not in subsequent years.

• Reproductive wastage in the goat. There are few reports of leptospirosis causing reproductive wastage in goats. A report from Spain [5] attributed 2.7 per cent of goat abortions to leptospirosis - mainly pomona. In Israel an outbreak of acute grippotyphosa infection in goats has been described [7], a sequel to which was a high incidence of abortion. Abortion has also been reported in goats in Guyana [8] and in India [9].

• The main pathological changes are essentially the same in all species, with the primary lesion being damage to the membranes of the endothelial cells of small blood vessels.

In acute leptospirosis there are no pathognomonic gross changes. Many of the features recorded after death from leptospirosis are those which would occur in death from renal failure from other causes, accompanied by jaundice in some cases. There may be petechial or echymotic haemorrhages in the skin, conjunctivae, mucosal and serosal surfaces, in the subcutaneous tissues and fat and in the endocardium. The thoracic and peritoneal cavities may contain blood stained yellow fluid. There may be subpleural haemorrhages. Splenomegaly is occasionally a feature. The liver may be enlarged and tense or pale or yellow. There may be disruption of cord and lobular structure, which is sometimes accompanied by centrilobular necrosis. The liver cells become irregular, swollen and degenerate. The most significant lesions are in the kidneys, which may be swollen, and yellow-green in jaundiced patients, with subsurface haemorrhages. The constant histological feature is interstitial nephritis, and tubular necrosis. Haemorrhages are apparent particularly in the medulla.

In chronic leptospirosis in animals, lesions are confined to the kidneys and consist of scattered small grey/white foci, often surrounded by a ring of hyperaemia. Microscopic examination shows these lesions to be a progressive focal interstitial nephritis. The interstitial leucocytic infiltrations, which consist mainly of lymphocytes, macrophages and plasma cells, may be extensive in some areas. Focal damage may also involve glomeruli and renal tubules. Some affected glomeruli are swollen, some atrophic, and others are replaced by fibrosis. The Bowman's capsule may be thickened, containing eosinophilic granular material. Tubular changes involve atrophy, hyperplasia and presence of necrotic debris in the lumen in some areas. Occasionally, petechial haemorrhages may be present in interstitial spaces.

With the probable exception of hardjo infection in sheep, sheep and goats are always incidental hosts for infections maintained by other animals, thus interruption of transmission from these hosts to sheep and goats is the critical factor in the control. The following are key elements in achieving this and as many as are appropriate to the situation should be included in a strategy:

1) Identify the sources of infection: where transmission is occurring and what are the host species.

2) Control infection in the host species, if possible.

3) Control of rodent carriers e.g. rat control programs.

4) Reduce contact with carrier animals e.g. don't mix sheep and pigs.

5) Exclusion from known contaminated environments.

6) Vaccination. This option has been widely used in the former USSR for the control of leptospirosis in both sheep and goats. Vaccination with 1/4 a cattle dose of hardjo vaccine is the easiest method for controlling hardjo infection in sheep.

11.3 SAMPLES

All samples must be collected as aseptically as possible and transferred to the laboratory as quickly as possible, preferably maintained at a temperature of 4°C.

11.3.1 Direct diagnosis

11.3.1.1 Isolation

Isolation is best carried out on samples of tissue taken from the aborted foetus (kidney, liver, lung etc.), foetal fluids (aqueous humour, thoracic, peritoneal, pericardial etc.) and the placenta. Samples should be collected from the aborted foetus as quickly as possible after expulsion from the dam since the numbers of viable leptospires is likely to decrease rapidly as autolysis proceeds making recovery in culture more difficult.

For tissues, a 5-10g sample or 5-6 cotyledons from the placenta should be recovered aseptically and placed in a sterile container holding 100ml of 1% bovine serum albumin (BSA) diluent (1g BSA per 100ml 0.005M phosphate buffer [87 mg/l KH_2PO_4 + 664 mg/l Na_2HPO_4] containing 200µg/ml 5-fluorouracil). On arrival at the laboratory the tissue samples should be homogenised, one part tissue to nine parts 1% BSA diluent, using a suitable blender or laboratory "stomacher". Samples must be cultured immediately.

Foetal fluids should be collected aseptically by use of a sterile syringe and needle or an evacuated sterile glass tube and transferred to the laboratory in a suitable sterile container.

11.3.1.2 Demonstration of leptospire presence

Immunofluorescence can be used to detect the presence of leptospires in tissue homogenate smears and cryostat sections of aborted foetal tissues and the placenta.

Tissue samples for cryostat sections may be stored frozen at -70°C.

11.3.2 Indirect diagnosis

Serological analysis using the microscopic agglutination test (MAT) should be undertaken on sera recovered from aborted ewes. Blood samples should be collected from as many ewes as possible (minimum of 10) that have aborted as soon as possible after abortion. Samples of blood from the aborted foetuses may also contain antibodies to leptospires if infection occurred during the immunocompetent phase of development and should be collected for screening where possible. All blood samples should be collected in sterile, glass blood tubes containing no anticoagulant (e.g. Vacutainer). On arrival at the laboratory, blood samples should be centrifuged for 10 minutes at 3,000 xg and the sera removed from the clot. If not analysed immediately, sera can be stored frozen at -20°C.

11.4 RISKS TO HUMAN HEALTH

Leptospirosis is an occupational zoonosis of those who work in agriculture, meat processing and laboratory diagnosis. People must be made aware of the risks involved in handling both animals and potentially infected materials from the animal. Those working with potentially infected animals or materials must:

- use water-proof clothing to give a barrier to infection;
- be stringent with personnel hygiene;
- disinfect all areas and equipment after contact with potentially infected animals or materials;
- dispose of all infected material by incineration or sterilization.

11.5 DIRECT DIAGNOSIS

Direct diagnosis is based on the isolation of leptospires from, or their demonstration in the internal organs (such as kidney, liver, lung) and body fluids (aqueous humour, blood, cerebrospinal, thoracic and peritoneal fluid) of the aborted foetus and/or the placenta which provides a definitive diagnosis of leptospiral abortion and probable chronic infection of its mother.

11.5.1 Isolation

Isolation by bacteriological culture is expensive, difficult and time consuming (4-6 months may be required for a conclusive result) and is normally only conducted at specialist laboratories. Isolation is, however, the most sensitive method of demonstrating the presence of leptospires, providing that antibiotic residues are not present, that tissue autolysis is not advanced and that tissues for culture have been stored at a suitable temperature (4°C) since collection. Identification of isolated leptospires to serogroup level can be undertaken by cross agglutination reactions but these test are only available at large diagnostic laboratories and reference centres.

Principle

Culture should be carried out in semi-solid (0.1 to 0.2 per cent agar) bovine serum albumin medium: either Ellinghausen-McMullough-Johnson-Harris medium (EMJH) which is commercially available (Difco) or Tween 80/40 medium [10]. These should be supplemented by a small amount of fresh rabbit serum (0.4 to 2 per cent). Contamination may be controlled by the use of a variety of selective agents, e.g. 5-fluorouracil (100-400 µg/ml), nalidixic acid (10-20 µg/ml), fosfomycin (400 µg/ml) and a mixture of rifamycin (10 µg/ml), polymixin (0.2 µg/ml), neomycin (2 µg/ml), 5-fluorouracil (250 µg/ml), bacitracin (40 µg/ml) and actidione (100 µg/ml). The use of selective agents will reduce the chance of isolation where there are only small numbers of viable leptospires. The most useful technique to use is the dilution culture technique in which many aliquots of medium are used. This technique is time consuming and is not really a practical consideration for the routine diagnostic laboratory. It has, however, been shown to be very useful in culturing leptospires from aborted foetuses, even those in advanced states of autolysis.

Materials and reagents

- 1% BSA diluent (1g BSA per 100ml 0.005M phosphate buffer [87 mg/l KH_2PO_4 + 664 mg/l Na_2HPO_4]).
- Modified semi-solid (0.1 to 0.2% agar) bovine serum albumin medium (EMJH or Tween 80/40 medium).
- Positive control cultures.
- Microscope suitable for darkground examination.

Procedure

1. Make ten-fold dilutions of samples of homogenised tissue or foetal fluids in 1% BSA diluent to cover the range 10^{-1} to 10^{-4}.
2. Inoculate 50µl aliquots of each of the sample dilutions and positive control cultures into six 7.5ml volumes of culture medium in plastic tissue culture tubes.
3. Incubate cultures at 29°C for at least 12 weeks.
4. Examine cultures by darkground microscopy every 1-2 weeks.

Interpretation of results

Organisms with typical leptospiral morphology can be seen in positive samples.

Note

• Visualisation of very small numbers of leptospires present in positive cultures can be helped by diluting drops of semi solid culture media with liquid medium to give a flatter, less dense preparation for microscopic examination and to make movement of the organisms more discernible.

11.5.2 Demonstration of leptospires

Leptospires do not stain satisfactorily with the aniline dyes and silver staining techniques lack sensitivity and specificity. Dark-ground microscopy of foetal fluids has been used in the diagnosis of leptospirosis abortion and can be useful if conducted by an experienced diagnostician. However, many tissue artefacts can be mistakenly identified as leptospires. The demonstration of leptospires by immunochemical staining techniques is more suited to most laboratory situations although these tests are 'number of organism dependent', lack the sensitivity of culture and require access to antiserum to the specific serovar(s) being detected. The immunochemical methods which have been used for diagnosis include immunofluorescence, immunoperoxidase, avidin-biotin and immunogold techniques.

11.5.2.1 Immunofluorescence

Immunofluorescence has been the most widely used immunochemical technique and has the advantage of giving the best contrast between the leptospires and the tissue background. This is particularly important since leptospires are small and filamentous which makes them difficult to differetiate from some connective tissue elements and cilia. The technique, however, requires a long inoculation regime in rabbits to produce the good quality polyclonal antisera required,full details of which have been given by Ellis et al (1982) [11]. With suitable antisera the following method can be carried out on foetal tissue homogenates:

Materials and reagents

- Fluorescent microscope equipped for transmitted and incident light investigations.
- Acetone.
- Conjugate: Fluorescein isothiocyanate (FITC) labelled rabbit antiserum of known titre.
- 0.1M Phosphate buffer (pH 7.4).
- Eriochrome black solution (1:60 aqueous solution).
- Buffered glycerol solution (pH 8.0).

Procedure

1. Make smears of tissue homogenates on microscope slides and air dry.
2. Fix smears in acetone at 4°C for 10 minutes and air dry. Smears can be stored frozen at -20°C for up to two weeks if not analysed immediately.
3. Cover smears with diluted conjugate and incubate at 37°C for 45 minutes.
4. Wash smears in 0.1M phosphate buffer for two 20 minute periods.
5. Counterstain for 10 seconds in eriochrome black solution.
6. Rinse smears rapidly three times in phosphate buffer.
7. Mount in buffered glycerol solution.
8. Examine samples by microscope.

Interpretation of results

Fluorescing organisms with typical leptospiral morphology can be seen in positive samples.

Note

- Immunofluorescent techniques can also be undertaken on cryostat sections cut in a freezing microtome from blocks of foetal kidney, lung, liver, brain and cotyledon tissue which has been snap frozen in isopentane and liquid nitrogen.
- It is generally easier to see distinct organisms in tissue homogenate smears than in cryostat sections but care must be taken to prevent smears being washed off glass slides during staining.

11.6 INDIRECT DIAGNOSIS

Indirect serological testing is the most widely used method for diagnosis of leptospirosis. Leptospiral antibodies appear within a few days of the onset of acute infection and persist for weeks or months. Abortions may occur any time between 1 and 12 weeks after infection, the actual period varying with the causal serovar. Blood samples taken from ewes at the time of, or just after, abortion are likely to have either high or declining titres. It is therefore critical that diagnosis is carried out on a herd basis rather than in the single animal to confirm *Leptospira* as the cause of the observed abortions. The presence of antibody in foetal serum is diagnostic of leptospiral abortion and has been shown to be useful in the investigation of sheep abortions.

11.6.1 Microscopic Agglutination Test (MAT)

Among the various serological tests which have been described, the microscopic agglutination test (MAT) using live antigens is generally accepted as the reference test against which all other serological tests are evaluated. The test can, however, be time-consuming and present a degree of risk to laboratory workers due to its use of live antigens. Formalin-killed antigens have been used by some laboratories but titres tend to be lower and cross-reactivity higher compared to the results obtained with the use of live antigens.

Principle

The MAT is used for the detection of agglutinating antibodies. It involves the use of live antigen derived from an undiluted culture which has been sub-cultured at least 5 days before use. The MAT is largely serogroup specific and therefore for optimum sensitivity representative strains of all the serogroups known to exist in the country or preferably, strains representing all the known serogroups should be used.

N.B. All manipulations of live leptospires or material which may contain live leptospires should, when ever possible, be carried out in a safety cabinet. The microscope stage and controls must be swabbed with 70% alcohol after use.

Materials and reagents

- U-well microtitre plates.
- Dark ground microscope (dry x 200 magnification).
- Physiological saline (0.85 % NaCl (w/v)).
- Positive control sera of known titre.
- Negative control sera.

Procedure

1. Make 1 in 12.5 dilutions of serum in sterile physiological saline.
2. Place 100µl volumes of each sample in the first row of the microtitre plate.
3. A positive and negative control sample should be included in each test batch.
4. Make serial four fold dilutions of each sample and controls in subsequent rows of the plate.
5. Add 100 µl of live antigen to each well.
6. Incubate plates at 29°C for 2 hours.
7. Determine the antibody level by dry examination of drops taken from individual wells and placed on a microscope slide.

Interpretation of the results

The degree of agglutination is assessed in terms of the proportion of free leptospires present, e.g., at 100% (++++) agglutination there are no free leptospires visible, at 50% (++) agglutination about half the leptospires remain free when compared with a negative control. The titre of a serum sample is taken as the highest dilution in which there is agglutination of 50% or more of the organisms.

Note

• Culture used as antigens in the MAT should be actively motile, free from contamination and at the late log phase of growth with no sign of breakdown. Cultures are incubated at 29°C but if on microscopic examination, a culture appears to be growing more quickly than required, it may be held at room temperature in the dark.

• Despite its universal use and numerous attempts by various investigators to standardise the MAT, it is difficult to obtain consistent results between laboratories. There are varying opinions as to what is a diagnostic titre and no one single titre can be considered diagnostic for all cases.

11.7 FUTURE WORK

Currently detection of leptospires as the cause of abortion in small ruminants is undertaken by a combination of culture isolation, demonstration by immunofluorescence and serology. None of these techniques provides a definitive diagnosis and all tend to be time consuming, expensive and inconvenient for use in routine diagnosis in non-specialised laboratories. Future improvements in diagnosis may include:

1) Improved media for culture.

2) Improved ELISA techniques for more rapid, sensitive, specific and automated serological analysis. Attempts have been made to develop an ELISA technique to detect leptospiral antibodies and although the method has been found to have technical advantages over the MAT it still presents the problem that cross-reactivity among heterologous serovars is greater than in the MAT.

3) Application of gene probe and polymerase chain reaction (PCR) techniques. A variety of PCR methodologies for the detection of leptospires have been published [e.g. 12] but these have not gained acceptance as diagnostic tests due to problems with inhibitors interfering with test sensitivity.

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