AUSTRALIAN STANDARD DIAGNOSTIC TECHNIQUES FOR ANIMAL DISEASES

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Listeriosis

Pathology, Bacteriology and Serology

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Contents

Listeriosis

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1. Introduction

Listeriosis has been recognised as an animal disease since 1911 and a human disease since 1917. *Listeria monocytogenes* was first described as a cause of disease in rabbits and guinea pigs in 1926 (Murray *et al.*, 1926). Since then listeriosis has been described in some 43 species of mammals and 22 species of birds while *L. monocytogenes* has been recovered from reptiles, fish, crustaceans, leeches, snails and some terrestrial arthropods. The organism has also been isolated from sewage, effluent, water, mud, fodder, silage, soil, vegetable and faecal matter. It is likely that a natural cycle exists between the soil–plant environment and animals with transient or persistent carriage in the intestines. Decaying moist vegetation appears to favour the maintenance of *Listeria* in the environment. Effluent treatment also favours the growth of *Listeria* in sewage with the organism surviving for months in sludge (up to 10^3–10^4/mL) resulting in significant risk where effluent is used as fertiliser on edible crops or pasture. Similar risks arise with the use of uncomposted poultry litter, meatplant effluent and sheep manure as fertiliser. Most of the studies have been done in countries with a temperate climate and little intensive epidemiological investigation has been done in tropical or subtropical countries. Epizootic and enzootic animal disease has been described in Europe, Australia, New Zealand and North America with common manifestations being meningoencephalitis, abortion/perinatal loss or septicaemia (Blood and Radostits, 1989).

Listeriosis often evokes only a mild influenza-like syndrome in humans and animals and in some cases, infections may be subclinical. Faecal carriers are detected much more frequently than clinical cases of disease with asymptomatic human carriage of 3.3–8% being reported. *L. monocytogenes* has been isolated from the mesenteric lymph nodes of healthy cattle, pigs and sheep. Domestic poultry under intensive systems of management are also reported to be a common source of the organism. The distribution of *L. monocytogenes* is so widespread that it must be considered an environmental organism, an opportunistic pathogen rather than a primary parasite of humans or animals. Nonetheless, rural workers may be at an increased risk of zoonotic infection as evidenced by a report of conjunctivitis in poultry workers due to *L. monocytogenes* and cutaneous listeriosis in veterinarians investigating abortion and stillbirths in dairy cows (Hird, 1987). Intercurrent disease, immunodeficiency disorders, malignant cancer and pregnancy appear to be important factors in activating human listeriosis particularly in pregnant women, infants and the elderly with similar predisposing factors evident in animals. Damage to respiratory, oral or alimentary mucosal surfaces appears to facilitate the entry of the organism.

In cold and temperate regions the greatest incidence of carriers and clinical disease in cattle tends to occur in late winter and early spring. This may be related to inadequate nutrition, feeding of contaminated fodder, stress of calving or other stressful influences such as cold weather. The dose of *Listeria* needed to cause disease in humans or animals has not been defined either in normal individuals or those at increased risk.

Writers in Russia and eastern Europe have demonstrated that *L. monocytogenes* is carried by a wide range of arthropods but the relevance of this finding to Australia is unclear. Gross environmental contamination, however, has a much greater potential for spread of infection. *Listeria* have been isolated from sewage sludge used as fertiliser and from clear sewage effluents. The organisms survive over a wide range of ambient temperatures and can multiply in soil at 18–20°C. Silage feeding has been associated with outbreaks of listeriosis. Poor quality or poorly cured silage (pH>5.6) provides a good substrate for multiplication. Feeding of visibly spoiled silage, particularly from the edge of the silage mass, should be avoided.

In those animals that become infected orally, ingestion of *L. monocytogenes* leads to localisation of the organism in the Peyer's patches of the intestinal wall, with subsequent haematogenous spread to liver and other organs including the uterus in pregnant animals. In contrast encephalitis is considered to follow local invasion. Contamination of the nasopharynx can lead to localisation of the organism in the ethmoturbinate regions and entry to the brain via the first cranial nerve. It is also considered that the *Listeria* can penetrate minor buccal abrasions and reach the brain via branches of the trigeminal nerve resulting in central nervous system (CNS) infection (Hyslop, 1975; Sullivan, 1985). Some workers have reported that it is rare to find the meningoencephalitic and the visceral forms (abortion or septicaemia) of listeriosis together in one outbreak (Blood and Radostits, 1989; Jubb *et al.*, 1985).

Recently in developed countries, sporadic and epidemic listeriosis following consumption of contaminated dairy products (e.g. soft cheeses, pasteurised milk), seafood, raw vegetables and coleslaw has emerged as a major human health problem. The increased medium- to long-term storage of fresh food under refrigeration in preference to more traditional methods of preservation has been associated with an apparent increase in the prevalence of listeriosis in humans. *Listeria*, initially present at a low level of contamination continue to multiply at storage temperatures which inhibit other bacteria. Alternatively, contamination can occur at the various stages of preparation prior to retail sale. Studies in many countries have commonly shown *Listeria* contamination rates for raw meats of 20–70% with poultry usually showing a

much higher rate compared to beef and pork. The reduced use of preservatives in such foods, in response to consumer demand for food free of additives, has decreased another means of controlling microbial growth. *Listeria* can tolerate low and high pH, high levels of salt, nitrates, smoking and may on occasions survive standard pasteurisation (Pociecha, 1990).

Contamination of processing plants can be widespread. In a survey of all 156 milk-processing plants in California, where the drains and condensation on hard surfaces were sampled, *L. monocytogenes* was isolated from 31 plants (19.9%). This supports the view that listeria in milk products are probably environmental contaminants introduced during or after processing of milk (Charlton *et al.*, 1990).

Recent reports from Australia include three human cases of listeriosis (Weinstein and Cameron, 1991), *L. monocytogenes* as a contaminant of food in South Australia (Hobson *et al.*, 1991) and human listeriosis following consumption of smoked mussels in Tasmania (Mitchell, 1991; Misrachi *et al.*, 1991).

For a comprehensive review of the characteristics of *Listeria* spp., human listeriosis, strain typing, mechanisms of virulence, listeria in foods and methods of detection in foods, see Farber and Peterkin (1991).

2. Bacteriology

2.1. Description

The taxonomic position of the genus *Listeria* has been clarified (Seeliger and Jones, 1986). Veterinary workers have long tended to regard *L. monocytogenes* as the sole species within the genus but evidence now supports the recognition of at least one other pathogenic species (*L. ivanovii*). There are three other apparently apathogenic species (*L. innocua, L. seeligeri* and *L. welshimeri*) which may be widespread in nature having been isolated from soil and decaying vegetation and in the case of some strains from animal, human or bird faeces. Evidence suggests that *L. seeligeri* is also pathogenic (Seeliger and Jones, 1986), which would then include all three haemolytic strains as pathogenic and the non-haemolytic strains as apathogenic. Two other apathogenic species, *L. grayi* (isolated from the faeces of chinchillas, natural habitat unknown) and *L. murrayi* (isolated from decaying corn leaves, natural habitat probably soil and vegetation) which are antigenically identical to each other (but antigenically distinct from members of the genus *Listeria*) are of uncertain generic status. They should probably be considered subspecies of the one species within the proposed genus *Murraya*. The organism referred to as *L. denitrificans* is no longer considered a member of the *Listeria* genus (Seeliger and Jones, 1986). Other than a general description of the characteristics of the genus *Listeria*, I will emphasise those features which are useful in differentiating *L. monocytogenes* and *L. ivanovii*.

So far as intergeneric relationships are concerned, *Listeria* are phenotypically closely related to *Erysipelothrix, Streptococcus, Lactobacillus, Gemella* and in particular *Brochothrix* sp. (formerly *Microbacterium thermosphactum*). Refer to Kandler and Weiss (1986) for a more comprehensive description.

Colonies on solid media show variation in size and haemolytic activity. Classically surface colonies after 24–48 hours incubation on blood agar (sheep, rabbit, horse, ox or human) are 0.5–2.0 mm in diameter, smooth, round, low convex with an entire margin, translucent with a dew-drop appearance, and are usually surrounded by a zone of complete haemolysis. *L. monocytogenes* produces narrow zones of beta haemolysis which are sometimes only visible following removal of the colony. *L. ivanovii* strains show multiple zones of haemolysis; in early stages of growth there is a zone of complete haemolysis surrounded by a dark ring which later clears to a zone of incomplete haemolysis. This second zone is not seen on horse blood agar plates and is most pronounced on ox blood agar plates. A weak or doubtful beta haemolysis can usually be resolved by use of the CAMP test (Christie *et al.*, 1944). A wide zone of complete haemolysis (a positive CAMP test, see 6.1.) occurs following incubation of beta haemolytic *Staphylococcus aureus* streaked in proximity to *L. monocytogenes* or *L. seeligeri* on sheep blood agar. In contrast, *L. ivanovii* is CAMP positive with *Rhodococcus equi* but not *Staph. aureus* (Seeliger and Jones, 1986). Presence of antibodies to *Listeria* (particularly in sheep blood) may occasionally interfere with haemolysis. This can be overcome by using washed red cells.

Oblique illumination of *Listeria* colonies on clear media such as Tryptose agar reveals a characteristic iridescent bluish sheen on colonies with a finely textured surface. Refer to Kandler and Weiss (1986) for more details.

Dissociation into different colony types occurs frequently in cultures incubated for several days, with older colonies (three to seven days) being 3–5 mm in diameter with a more opaque centre. Rough colony forms may occur after variable periods of incubation. They are characterised by an irregular surface and are difficult to emulsify. Smears from rough colonies contain long filamentous organisms.

Listeria grow between 1 and 45°C with optimal growth between 30 and 37°C but do not survive heating at 60°C for 30 min. Growth occurs between pH 6 and pH 9, with cultures tending to be non-viable below pH 5.5 making subculture from acid fermentation media generally unsuccessful.

Listeria may be preserved for some months by stab inoculation into nutrient agar in screw capped containers after overnight incubation at 37°C. It is essential that the caps are tightly sealed to ensure the medium does not dry out. Plastic containers are generally unsuitable due to gradual loss of moisture. The containers should be stored at room temperature or preferably at 4°C. They can be stored for longer periods (at least seven years) by freezing on glass beads at -70°C or by freeze drying (Seeliger and Jones, 1986).

The antigenic structure of *Listeria* is complex. They have been divided into 16 serovars on the basis of somatic (O) and flagellar (H) antigens with *L. grayi* and *L. murrayi* comprising a single and serologically distinct category. However, from a practical viewpoint in the routine laboratory, comprehensive serotyping appears of little benefit due to the complex antigenic interactions between serovars and the occurrence of some individual serovars in a range of *Listeria* species. In contrast, confirmation of cultures as *Listeria* sp. by the detection of a specific antigen or combination of antigens common to all serovars offers greater practical advantage. This approach has been utilised in the Tecra Listeria Visual Immunoassay, a sandwich ELISA technique [available from Bioenterprises Pty Ltd, 28 Barcoo St, Roseville, NSW 2069. Tel. (02) 417 0271; Fax (02) 417 7858]. The procedure uses monoclonal antibodies for the rapid detection of *Listeria* in food and environmental samples following enrichment in selective broth using a two-step enrichment technique. An antigenic scheme for typing *Listeria, L. grayi* and *L. murrayi* appears in 6.2.

2.2. *Isolation of* Listeria

Most *Listeria* grow readily on simple laboratory media (e.g. blood agar or tryptose agar) in air but growth is enhanced by the addition of glucose, blood or serum. *L. ivanovii* are more fastidious and grow best on 7.5% ox blood agar incubated in 10% carbon dioxide (CO_2) in air for primary isolation (Cooper and Dennis, 1978). Incubation in a microaerophilic environment may help to inhibit strict aerobes such as *Bacillus* spp. and *Pseudomonas* spp.

The approach to culture varies with different samples depending on the anticipated numbers of *Listeria* organisms present, the level of contamination by other bacteria and compliance with the routine operations and aims of the laboratory undertaking the examination. Most veterinary diagnostic laboratories culturing sporadic cases of suspect listeriosis would use simple cultural techniques (e.g. direct plating on non-selective media with or without cold storage enrichment depending on the expected numbers of *Listeria*) to isolate pathogenic *Listeria* from clinical material with an expected low level of contamination. The use of media containing blood enables observation of haemolysis, a useful tool in detection of *Listeria*. In contrast, the use of solid media without blood enables the use of oblique lighting as an aid to detection of *L. monocytogenes*.

Animal diagnostic investigations in situations where listeriosis is a common occurrence, studies on the intestinal carrier state in animals, and culture of environmental or food samples would justify plating onto selective medium with or without prior enrichment in selective broth. The selective media now available are very effective in inhibiting the growth of bacteria other than *Listeria*.

Direct culture on solid media such as the following will indicate the numbers of *Listeria* present in pathological material including faeces.

(a) Palcam.
(b) Listeria selective agar (LSA) — Oxford formulation (OXA) (recommended by the International Dairy Federation for isolation from milk and milk products).
(c) Modified Oxford agar (MOX).
(d) McBride medium.
(e) Modified McBride (McBride medium with cycloheximide added at 200 mg/L); or
(f) LPM agar (lithium chloride–phenylethanol —moxalactam) (McBride Medium with moxalactam added at 40 mg/L.

Enrichment broths such as the following, although more sensitive in detecting *Listeria* from food and environmental samples, may give misleading positive results in disease investigations involving culture of pathological material due to contamination by small numbers of *Listeria* from the intestinal tract, soil or vegetable matter.

(a) Listeria enrichment broth (LEB).
(b) Listeria enrichment broth modified (LEBM) (LEB with acriflavine reduced from 15 mg/L to 10 mg/L — recommended by the International Dairy Federation).
(c) Fraser broth or Fraser broth modified.
(d) University of Vermont Medium (UVM) (a two-step Listeria selective enrichment media — recommended by USDA–FSIS for isolation from meat and poultry products); or
(e) Buffered listeria enrichment broth.

Details of these media are listed in 6.3.

2.2.1. Abortion–Septicaemia Material

The high numbers of organisms present in this type of material coupled with the characteristic appearance of *Listeria* colonies on routine media, such as blood agar, makes it easy to pick out the organisms from plates even where there is a heavy growth of 'contaminants'. In cases of suspected septicaemic listeriosis, routine culture of internal organs such as liver, lung, heart blood, spleen or mesenteric lymph nodes using a swab or Pasteur pipette should be satisfactory. Tissue should also be retained for cold enrichment or inoculation of enrichment broth should isolation be unsuccessful, particularly where localisation

of infection such as hepatic abscessation is evident. In abortion or stillbirth cases foetal stomach, liver and placenta should be cultured. In the latter case select a relatively uncontaminated cotyledon showing evidence of lesions for routine culture. If there is gross contamination by soil and vegetable matter then individual cotyledons can be washed in sterile normal saline prior to culture. The isolation rate may be increased by thorough maceration of tissues in broth to ensure release of intracellular organisms.

2.2.2. Central Nervous System Lesions

Listeria may be difficult to isolate from CNS material and diagnosis may be profitably based on the histological picture. Despite the wide range of selective enrichment media available one of the best methods of isolation still appears to be incubation at 4°C for an extended period and subculturing weekly. A 10% suspension of brain (midbrain and medulla oblongata, include the trigeminal nerve nuclei plus any sections of brain with visible lesions) in brain–heart infusion or tryptose-phosphate broth appears to be the most satisfactory approach. Thorough maceration of the tissue is important. The underlying mechanism of this method appears to be the ability of *Listeria* to multiply at 4°C while other contaminating organisms (which are generally mesophiles) are generally present in small numbers and gradually die out. At the same time there is a gradual breakdown of inhibitory factors initially present in brain material. One additional reason for the difficulty in isolating *Listeria* from CNS disease is that the organisms are more localised and present in small numbers in contrast to the large numbers usually found in the more widespread lesions of listeric abortion or septicaemia.

2.2.3. Environmental, Food and Faecal Samples

The use of selective media is probably mandatory with environmental samples (e.g. silage, effluent, pasture), food samples (e.g. meat, vegetables and dairy products) and faeces because *Listeria* may be present in low numbers and are readily overgrown by psychrophylic organisms capable of growth at 4°C which are likely to be present in high numbers. Despite the reporting of a variety of selective enrichment broths for use in association with selective plates, many workers appear to favour cold enrichment (4°C) in brain–heart infusion broth (Difco), tryptose broth (Difco) or tryptose-phosphate broth, sometimes in parallel with selective broths, followed by subculturing onto selective plates. Cold enrichment is rather slow (often taking weeks or months), so that the most satisfactory technique would seem to be the use of selective enrichment broths and selective plating media. This is particularly important when testing food samples, which often have a limited shelf life, prior to sale.

Potassium tellurite (K_2TeO_3) and potassium thiocyanate (KSCN) which were commonly included in earlier described isolation media are now known to be inhibitory to some strains of *Listeria*. Some examples of media and isolation procedures are listed in 6.3.

Listeria, which are often isolated from meat and meat products, may be confused with species of *Kurthia* and *Brochothrix*, non-pathogenic saprophytes also common in this habitat. *Listeria* can be readily differentiated from *Kurthia* as the former is a facultative anaerobe while the latter is a strict aerobe. *Brochothrix* has many characteristics similar to *Listeria* but can be separated by differences in motility, the inability of the former to grow at 37°C and the range of sugars fermented (Kandler and Weiss, 1986).

2.3. Detection of Listeria *in Smears*

2.3.1. Gram Stain

Coccoid forms are frequently seen in smears from infected tissue or from liquid cultures but are rarely seen in smears from colonies on solid media. These coccoid forms may be mistaken for *Streptococcus* sp. but can be differentiated by production of catalase by *Listeria* on suitable media (Seeliger and Jones, 1986).

2.3.2. Immunofluorescence

L. monocytogenes can be detected readily by this technique (Khan *et al.*, 1977). There is specific fluorescence according to serotype. Cross reactions with streptococci and staphylococci occur and, although morphology of the organisms in the smears can assist in differentiation, positive immunofluorescence results should be followed up by culture where possible. A polyvalent fluorescent antiserum against *L. monocytogenes* types 1–4 is produced commercially (Difco 2469-56-3, Bacto FA Listeria Poly, fluorescein conjugated) for use in a direct fluorescent procedure. It is used in parallel with a fluorescein labelled rabbit globulin control (Difco product 2379-56-2, Bacto FA Rabbit Globulin) which detects non-specific staining of some organisms, particularly *Staphylococcus*. These products are not listed in the Australian Difco catalogue and must be specially imported. Immunofluorescent screening of enrichment broths prior to subculture of the latter onto selective plates could be of assistance where large numbers of samples are being examined. Immunofluorescent staining of histopathological sections would also be of value in providing a speedy screening for listeriosis where culture was unsuccessful or not undertaken.

2.4. Identification of Listeria

Colony morphology and conventional biochemical techniques (see 6.1.) are satisfactory (Bortolussi *et al.*, 1986; Kandler and Weiss, 1986; Seeliger and Jones, 1986). Previously, animal inoculation techniques such as the Anton test

(rabbit or guinea pig conjunctival sac inoculation), rabbit inoculation (mononucleosis) or mouse inoculation tests were used. There are no indications for their use now.

Rapid slide agglutination tests can be used for presumptive identification but require confirmation by biochemical techniques due to the presence of cross reactions with other genera of bacteria. Type 1, type 4 and polyvalent antisera (types 1 plus 4) are available commercially (Difco 2300, 2301 and 2302, respectively) as are reference type 1 and 4 antigens for use in the slide test, although use of live reference cultures would also be appropriate. Complete serological typing is not currently available in Australia although a full range of absorbed O-somatic (against antigens I–XV) and H-flagellar (against antigens A, B, C and D) antisera are being prepared by Dr E. Russell (Department of Microbiology, Monash Medical Centre, 246 Clayton Rd, Clayton, Vic. 3168, Australia) and a serotyping reference service is available. Where complete serological typing has been utilised in other countries there has been no demonstrable correlation between serotypes occurring in animals and those associated with disease in humans. Nonetheless complete serotyping is potentially a very useful epidemiological tool.

Commercially available antisera are of very high titre and may be used in the slide test. Agglutination is detected after mixing a loopful of antiserum with a suspension of organisms taken directly from plate culture and emulsified in 0.85% saline, although the Difco Manual (1984) advises heating the organism suspension at 80°C for one hour in a water bath, centrifuging the suspension, removing the bulk of the supernatant fluid and testing the organism after resuspension of the bacteria in the remaining fluid.

Antisera may be prepared in rabbits by the user but these tend to be of lower titre (particularly following cross absorption) and will generally require performance of a macroscopic tube test. This involves adjusting the density of a heated (80°C for one hour) suspension of broth or agar culture in Bacto FA buffer (Difco 2314-33-8) [with 0.3% formaldehyde solution (CH_2O) added] to approximate the density of a McFarland barium sulfate ($BaSO_4$) standard opacity tube No. 3. The antigen is then titrated against doubling dilutions of antiserum from 1:20 to 1:2560, incubated at 50°C for two hours then refrigerated overnight. The titre is the highest dilution with 50% of cells showing clumping. Commercial antisera should be expected to yield an homologous titre exceeding 1:320.

The H-flagellar antigens A, B, C and D are confirmed in a tube agglutination test using specific absorbed antisera titrated against formalised broth cultures of *Listeria*.

Serotyping of human strains isolated in Australia has confirmed that *L. monocytogenes* types 1 and 4 are the only recognised pathogenic strains involved in clinical disease (E. Russell, pers. comm. 1991). No information is available on serotyping of strains from diseased animals in Australia other than that *L. monocytogenes* is by far the most common pathogenic species while *L. ivanovii* (characterised by haemolytic pattern and biochemical characteristics) has been isolated only rarely. Gray and Killinger (1966) reported that typing of available *Listeria* in the United States confirmed 32.8% as type 1, 65.3% as type 4 and less than 1% as types 2 or 3. Murray and Cameron (1991) in a survey of foodborne *Listeria* found type 1 common from meats and type 4 common in the dairy products and the dairy environment while there were a high number of strains isolated from salads that could not be typed.

Phage typing schemes have been devised for use in epidemiological studies but this facility is only currently available from one centre, in Tours, France. Because of the complexity of phage typing it has proven of limited value, nonetheless it has been effective in characterising isolates. About 50 Australian isolates, mainly from human sources, sent for phage typing (E. Russell, pers. comm., 1991) showed a similar pattern of phage types to those reported from other countries. Should phage typing be desired isolates could be referred through Dr E. Russell to the Listeria Reference Laboratory, Monash Medical Centre, 246 Clayton Road, Clayton, Vic. 3168. Australia. Tel. (03) 03 550 1111; Fax (03) 550 4533.

3. Serology

It has recently become clear that resistance to *L. monocytogenes* depends principally on cell-mediated immunity. Hence, it is not surprising that difficulties have been encountered in attempting to develop reliable serological tests for diagnosis of listeriosis. Unfortunately there appear to be no commercially available tests for cell mediated immune responses generally applicable as diagnostic tests. *Listeria* reference antigens type 1 and type 4, for use in the tube agglutination test, are available from Difco; however, there is little value in pursuing such testing because of the following.

(a) *L. monocytogenes* cross reacts with a wide range of bacteria, particularly staphylococci, Group D streptococci and some Gram-negative organisms including coliforms.

(b) There is a lack of correlation between serological and cultural diagnosis of listeriosis. *L. monocytogenes* has often been isolated from serologically negative patients. High titres persist for a long time. In endemic areas, 'positive' agglutinin titres have been detected in 84% of cattle, 43% of swine, 35% of sheep and 40% of the human population (Hyslop, 1975). Various surveys have

demonstrated a high distal alimentary tract carrier rate in many species, including humans, with no correlation between faecal isolation and serological titre.

The development of serological tests to detect antibodies against listerial toxins such as haemolysin is an area of current research which may yield a more specific test.

The types of serological tests that have been examined for the diagnosis of listeriosis include (reviewed by Gray and Killinger, 1966):

(a) Agglutination test O+H antigens (Seeliger, 1958);
(b) Precipitation test (Seeliger, 1958);
(c) Complement fixation test (CFT) (Patocka *et al.*, 1956; Seeliger, 1958);
(d) Haemagglutination test (Neter *et al.*, 1960);
(e) Antigen fixation test (Njoku-Obi, 1962);
(f) 'Wachstumsprobe' growth test (Potel and Degen, 1960); and
(g) Passive immunohaemolysis (Bind *et al.*, 1975).

Some workers have described using serological tests in association with listerin-induced delayed hypersensitivity tests, e.g. agglutination and CFT, before and after the skin test where a rise in CFT may be significant (reviewd by Gray and Killinger, 1966).

4. Pathology

The reader is referred to Sullivan (1985) and Ladds *et al.* (1974) for a more comprehensive description.

4.1. Central Nervous System

4.1.1. Sheep

L. monocytogenes has a definite predilection for the brain stem, with the most severe lesions in the medulla and the pons with less severe lesions anteriorly in the thalamus and posteriorly in the cervical spinal cord. This distribution of lesions is not typical of haematogenous spread and is probably the result of local invasion from conjunctival, nasal or facial tissue with strong evidence for transmission along nerves, particularly the trigeminal nerve, to medullary centres. This often results in unilateral lesions and associated trigeminal neuritis.

There is often no gross change. Occasionally the medullary meninges are thickened by greenish gelatinous oedema with greyish foci of softening in cross sections of the medulla. Slight clouding or pinpoint grey–white foci of the meninges may also be seen. Sometimes the meninges may appear congested or oedematous with an increase in the amount of cerebrospinal fluid which may be cloudy.

Two types of lesions are described as follows.

4.1.1.1. Focal parenchymatous lesions

Lesion foci are composed of loose clusters of cells resembling microglial macrophages and containing few organisms. Some more advanced lesions show parenchymal necrosis and myelin breakdown, with neutrophils as well as macrophages and larger numbers of *L. monocytogenes*. In some cases the process goes further, resulting in the formation of non-encapsulated abscesses with a central caseated area surrounded by a wide band of polymorphs then a zone of Gitter cells and a zone in which perivascular cuffing is evident.

4.1.1.2. Vaso meningeal lesions

These lesions are commonly in the basal region (particularly ventrally from the medulla to the optic chiasma) with pronounced haemorrhagic exudation and subsequent purulent leptomeningitis. Cellular infiltrates form perivascular cuffs in the parenchyma. Most infiltration cells consist of macrophages and lymphocytes with few neutrophils.

4.1.2. Cattle

Listeric CNS infections in cattle are more chronic than the disease in sheep. Lesions are rarely as extensive in cattle, perivascular cuffs are smaller and focal lesions are limited to oedema and small collections of microglia and lymphocytes.

4.1.3. Pigs

The distribution of lesions is the same as in ruminants. In chronic cases ependymitis may develop.

4.1.4. Horses, Dogs and Cats

Meningoencephalitis has been reported in these species.

4.1.5. Chickens

CNS lesions have been reported.

4.2. Uterine Infection

4.2.1. Sheep

Uterine infection is probably haematogenous with the bacteraemic phase asymptomatic, localisation probably only occurring in the uterus. Infection of the uterus can be readily established following oral or intravenous inoculation of pregnant animals. Death of the foetus and consequent abortion usually occurs during the later stages of pregnancy. Gross placental lesions include white or light coloured segmental or focal necrosis of the cotyledons, as well as ulceration, oedema, thickening and red–brown discolouration of the intercotyledonary area of the chorioallantois. Mummified foetuses have dehydrated, brown, leathery placentas.

Microscopic placental lesions include extensive oedema, diffuse leucocyte infiltration and vasculitis of the chorioallantois, necrosis and desquamation of the chorionic epithelium. In some cases there is fibrinoid necrosis and thrombosis of the septal vessels of the foeto–maternal interdigitation.

Macroscopic lesions are not always present in aborted foetuses or dead neonates. Intra-uterine autolytic changes which occur prior to abortion may mask the minor foetal lesions that occur. Changes described include discrete pale foci up to 3 mm diameter on the skin of hairless foetuses; subcutaneous oedema with excessive clear or blood-stained fluid in body cavities; mummification; haemorrhage in the subcutis, lungs, epicardium, kidneys and mesenteric lymph nodes; miliary grey–white foci in the liver which may be soft and bronze–brown in colour. Microscopic skin lesions have been described in foetuses which consist of microcolonies of *Listeria* in the epidermis, sometimes extending into the dermis and primordial hair follicles. Microscopic liver lesions vary from necrotic foci without reactive processes to distinct granulomata. Lesions are often associated with blood vessels and may contain clumps of *Listeria* with a few or many neutrophils.

4.2.2. Cattle

Abortion due to *L. monocytogenes* usually occurs in the seventh month of pregnancy, sometimes as early as the fourth month. No gross lesions characteristic of *Listeria* infection are seen in aborted foetuses which usually die several days before expulsion. Histological examination of placentas may reveal villous necrosis and neutrophilic infiltration. Bacterial colonies may be observed in the crypts of foeto-maternal interdigitation. Foetal livers and spleens may show foci of necrosis and *Listeria* may be present without tissue reaction.

4.2.3. Other Species

Listeric abortion has been reported in the horse but not in the pig, dog or cat.

4.3. Septicaemia

4.3.1. Ruminants

Acute listeric septicaemia is largely confined to newborn lambs and calves, probably a sequel to intrauterine infection. Hepatic necrosis and gastroenteritis appear to be the most consistent lesions.

4.3.2. Pigs

Septicaemia in the first few weeks of life, characterised by focal hepatic necrosis is the common form of listeriosis.

4.3.3. Horse

Septicaemia with focal hepatic necrosis and encephalitis has been described.

4.3.4. Dogs and Cats

Systemic listeriosis associated with distemper has been described in dogs. Listeria septicaemia with hepatomegaly, hyperplastic enlargement of the spleen, subepicardial petechiae and swelling of the medulla of mesenteric lymph nodes has been reported in cats.

4.3.5. Chickens

Young birds are more susceptible and the disease most frequently seen is septicaemia with areas of severe myocardial degeneration. Focal lesions are sometimes seen in the spleen and lungs.

4.4. Other Lesions

4.4.1. Cattle

Mastitis was associated with the isolation of *L. monocytogenes* serotype 1 from one quarter only of a cow derived from a 72-cow herd. The herd bulk milk had tested positive for this organism. The mastitis did not respond to antibiotic therapy (Fedio *et al.*, 1990).

4.4.2. Pigs

Skin lesions resembling swine pox have been attributed to *L. monocytogenes*.

4.4.3. Horses

Tarsitis in a foal has been reported.

4.4.4. Rats

Suppurative peritonitis has been reported with multiple necrotic white foci in liver and spleen.

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Table I. Identification key for *Listeria* (from Seeliger and Jones,1986)

	L. mono-cytogenes	*L. ivanovii*	*L. seeligeri*	Non-haemolytic *Listeria*
Beta-haemolysis	+	+[1]	+	–
CAMP test — *Staph. aureus*	+	–	+	–
CAMP test — *Rhodococcus equi*	–	+	–	–
Motility 20–25°C	+	+	+	+
Growth at 35°C	+	+	+	+
Catalase	+	+	+	+
Voges–Proskauer	+	+	+	+
Nitrate reduction	–	–	–	d
Hydrogen sulfide production	–	–	–	–
Acid from:				
L-arabinose	–	–		
D-galactose	d	d		
Glucose	+	+	+	+
Lactose	d	+		
D-mannitol	–	–	–	d
L-rhamnose	+	–	–	d
Sorbitol	d	–		
Sucrose	–	d		
D-xylose	–	+	+	d
Pathogenicity for mice	+	+	–	–

d: 11–89% of the strains are positive.
[1] A very wide zone or multiple zones of haemolysis are exhibited by *L. ivanovii*.

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Table 2. Serovars of the genus *Listeria* (from Seeliger and Jones, 1986)

Designation		*O Antigens*											*H Antigens*	
1	/2a	I	II	(III)									AB	
	1/2b	I	II	(III)									ABC	
2	1/2c	I	II	(III)									B	D
3	3a		II	(III)	IV								AB	
	3b		II	(III)	IV						(XII)	(XIII)	ABC	
	3c		II	(III)	IV						(XII)	(XIII)	BD	
4	4a			(III)		(V)		VII		IX			ABC	
	4ab			(III)		V	VI	VII		IX	X		ABC	
	4b			(III)		V	VI						ABC	
	4c			(III)		V		VII					ABC	
	4d			(III)		(V)	VI		VIII				ABC	
	4e			(III)		V	VI		(VIII)	(IX)			ABC	
	5			(III)		(V)	VI		(VIII)		X		ABC	
	7?(4bx)			(III)								XII XIII	ABC	
	6a(4f)			(III)		V	(VI)	(VII)		(IX)			XV ABC	
	6b(4g)			(III)		(V)	(VI)	(VII)		IX	X	XI	ABC	
L. grayi/ L. murrayi				(III)								XII	XIV	E

6. Appendixes

6.1. Appendix 1 — Identification Key for Listeria (See Table 1).

6.2. Appendix 2 — Serovars of the genus Listeria *(from Seeliger and Jones, 1986)* (See Table 2).

6.3. Appendix 3 — Selective Media for Listeria

N.B. Extreme care should be exercised when handling the ingredients used in producing selective media. A face mask and gloves should be worn when dispensing dry materials to avoid inhalation of airborne particles. Due to the risk of infection pregnant staff should not handle *Listeria* cultures. Broth cultures present a higher risk than plate cultures.

Readers are referred to the appropriate technical manuals and literature produced by Oxoid Australia Pty Ltd [104 Northern Rd, West Heidelberg, Vic. 3081. Tel. (03) 458 1311, (008) 331 163; Fax (03) 458 4759], Difco (FSE Pty Ltd 149 Arthur St, Homebush, NSW 2140) and BBL (Becton Dickinson Pty Ltd, 15 Orion Rd, Lane Cove, NSW 2066). Selection of the most appropriate media will depend on the sample type and whether enrichment is required. In general the more recently reported media are likely to be superior. A single medium may not support all strains of *Listeria*.

6.3.1. Listeria Selective Agars

A range of solid media are suitable for isolation of *Listeria* from contaminated samples.

6.3.1.1. Listeria Selective Agar (LSA)

Oxford Formulation (OXA, Oxoid Listeria Selective Agar Base CM856 with supplement SR140, or Difco 0225 + 0214. This medium is recommended by the International Dairy Federation for isolation from milk and milk products (Curtis *et al.*, 1989*a*). It is suitable for the isolation of *Listeria* from clinical and food specimens.

Formula (g/L):

Columbia blood agar base	39.0
Aesculin	1.0
Ferric ammonium citrate	0.5
Lithium chloride, LiCl	15.0

pH 7.0

The Listeria Selective Supplement (Oxford) added to 500 mL of medium contains:

Cycloheximide	200 mg
Colistin sulfate	10 mg
Acriflavine	2.5 mg
Cefotetan	1.0 mg
Fosfomycin	5.0 mg

The agar medium is sterilised by autoclaving at 121°C for 15 min and is then cooled to 50°C. The supplement is reconstituted in 5 mL of ethanol (C_2H_5OH) /sterile distilled water (1:1) and added aseptically to the agar medium. Plates may be stored for up to 10 days at 4°C in the dark.

L. monocytogenes hydrolyses aesculin producing black zones around the colonies. Gram negative bacteria are completely inhibited, most unwanted Gram positive bacteria are suppressed; however, some enterococci grow and exhibit weak aesculin reactions after 40 hours of incubation. Some staphylococci may grow as aesculin negative colonies. Typical *L. monocytogenes* colonies are usually visible after 24 hours or after 48 hours with slow growing strains (Oxoid Manual, 1990).

Faeces and biological material can be inoculated directly; however, better results may be obtained if the sample is first homogenised in 0.1% peptone water (1 part to 9 parts peptone water). Inoculate 0.1 mL of suspension onto the plate and incubate at 35°C for up to 48 hours. The sample may be pre-enriched in a suitable selective broth at 30°C and 0.1 mL of broth inoculated onto the agar medium after 24 hours, 48 hours and seven days incubation.

6.3.1.2. Modified Oxford Agar (MOX)

Oxoid CM856 with selective supplement SR157, or Difco 0225 + 0218, or BBL 12397 + 12402 — McClain and Lee (1989). The selective supplement to make 500 mL of agar contains:

Colistin	5.0 mg
Moxalactam	7.5 mg

This formulation comprises part of the United States Department of Agriculture — Food Safety Inspection Service (USDA–FSIS) (McClain and Lee, 1989) recommended method for the isolation and identification of *L. monocytogenes* from processed meat and poultry products. It has a lower concentration of moxalactam than LPM agar (see 6.3.1.5.). as the higher levels are inhibitory to some strains of *Listeria*.

The moxalactam and colistin inhibit *Staphylococcus, Proteus* and *Pseudomonas* which may be present as part of the normal flora of the specimen. Moxalactam is considered more effective than cefotetan against methicillin-resistant *Staph. aureus*. *Listeria* appear black in and around the colony.

The selective supplement is dissolved in 2 mL sterile distilled water and added to the autoclaved agar base (cooled to 50°C) then dispensed into sterile petri dishes. The prepared medium is stable for up to two weeks when stored at 2–8°C.

Primary or secondary enrichment broth (after 24 and 48 hours incubation) is inoculated onto MOX plates and incubated aerobically at 35°C for 24 hours (*Listeria* usually visible) and 48 hours (for slow-growing strains). Suspect *Listeria* isolates should be confirmed by biochemical and serological testing (Curtis *et al.*, 1989*b*).

6.3.1.3. McBride medium

Oxoid CM819, or Difco 0922 — McBride and Girard (1960). This medium is one of the original selective media for *Listeria*.

Formula (g/L):

Peptone	10.0
'Lab-Lemco' powder	3.0
NaCl	5.0
LiCl	0.5
2-Phenylethanol, $C_4H_6N_2O_2$	2.5
Glycine anhydride	10.0
Agar	15.0
pH 7.3	

6.3.1.4. Modified McBride medium

Oxoid CM819 with cycloheximide added at 200 mg/L — Lovett *et al.* (1987). Note that cycloheximide is a hazardous compound.

6.3.1.5. LPM agar

McBride Medium Oxoid CM819 with moxalactam added at 40 mg/L, or Difco 0221 + 0216 — McClain and Lee (1988), Lee and McClain (1986).

6.3.1.6. Palcam agar

Oxoid CM877 with supplement SR150 — Van Netten *et al.* (1989).

Formula (g/L):

Columbia blood agar base	39.0
Yeast extract	3.0
Glucose	0.5
Aesculin	0.8
Ferric ammonium citrate	0.5
Mannitol, $C_6H_{14}O_6$	10.0
Phenol red	0.08
Lithium chloride, LiCl	15.0
pH 7.2	

The Palcam selective supplement added to 500 mL of Palcam Agar contains:

Polymixin B	5 mg
Acriflavine hydrochloride	2.5 mg
Ceftazidime	10 mg

Palcam is suitable for the isolation of *Listeria* from foods and has the advantage of a double indicator system. Hydrolysis of aesculin by *Listeria* is indicated by formation of a black halo around the colony while fermentation of mannitol by contaminants such as enterococci and staphylococci results in yellow colonies whereas *Listeria* which do not ferment mannitol yield red colonies. Incubation under microaerophilic conditions tends to inhibit strict aerobes such as *Bacillus* spp. and *Pseudomonas* spp. Colonies presumptively identified as *Listeria* must be confirmed by biochemical and serological testing (Bortolussi *et al.*, 1986).

The agar base is suspended in distilled water and gently brought to the boil to dissolve completely before being sterilised by autoclaving at 121°C for 15 min. It is cooled to 50°C before the supplement (reconstituted in 2 mL of distilled water) is added aseptically. The prepared plates may be stored for up to four weeks at 4°C in the dark. The addition of 2.5% (v/v) egg yolk emulsion (Oxoid SR47) to the medium may aid recovery of damaged *Listeria*. Prior enrichment of samples in LEBM (for dairy products) or UVM broth (for meats and poultry, see 6.3.2.4.) may be required.

6.3.2. Listeria Selective Enrichment Broths

6.3.2.1. Listeria enrichment broth

LEB (Oxoid CM862 with supplement SR141, or Difco 0222) (Lovett *et al.*, 1987).

Formula (g/L):

Tryptone soya broth	30.0
Yeast extract	6.0
pH 7.3	

Listeria selective enrichment supplement for 500 mL of broth:

Nalidixic acid	20.0 mg
Cycloheximide	25.0 mg
Acriflavine hydrochloride	7.5 mg

Reconstitute the supplement with 2 mL of sterile distilled water and add aseptically to the sterilised broth cooled to 50°C.

LEB is suitable for the selective enrichment of *Listeria* from food, milk and milk products and has been shown to recover an inoculum of

<10 colony forming units (CFU)/mL from raw milk. Isolation is improved by subculture onto LSA plates after one, two and seven days. Either 25 g or 25 mL samples are added to 225 mL of LSEB (homogenise if necessary), incubated at 30°C for seven days and subcultured onto LSA plates (either McBride medium, Oxford medium or Modified McBride medium) after one, two and seven days either by direct plating or by vortex mixing 1 mL of broth with 9 mL of 0.5% (0.09 mol/L) potassium hydroxide (KOH) prior to plating. This method is generally referred to as the FDA method.

Store prepared medium away from light as acriflavine can photooxidise to form compounds inhibitory to *Listeria*.

6.3.2.2. Listeria enrichment broth modified LEBM (Oxoid CM862 with supplement SR149 with acriflavine reduced from 15 mg/L to 10 mg/L — recommended by the International Dairy Federation).

6.3.2.3. Fraser broth

(Oxoid CM895 with supplement SR156, or Difco 0219 + 0211, or Fraser Broth Modified BBL 12395 + 12401) — McClain and Lee (1987), Fraser and Sperber (1988).

Formula (g/L):

Protease peptone	5.0
Tryptone	5.0
'Lab-Lemco' powder	5.0
Yeast extract	5.0
NaCl	20.0
Potassium dihydrogen phosphate, KH_2PO_4	1.35
Disodium hydrogen phosphate, Na_2HPO_4	12.0
Aesculin	1.0
LiCl	3.0
Nalidixic acid	20 mg

Autoclave at 121°C for 15 min, cool immediately after removal from autoclave and dispense into 10 mL tubes. Just before use add the following filter sterilised reagents:

Acriflavine (2.5 mg/mL)	0.1 mL
Ferric ammonium citrate (5% in distilled water)	0.1 mL

Fraser broth can be used as an alternative to LEB for the isolation of *Listeria* from food and environmental samples when used as a secondary enrichment medium in the USDA–FSIS method (see 6.3.2.4.) where UVM 1 is used as the primary enrichment broth. Fraser broth is a modification of the UVM 2 second step enrichment broth and contains ferric ammonium citrate and lithium chloride. Blackening of the broth due to hydrolysis of aesculin to aesculetin which reacts with ferric ions resulting in blackening is presumptive evidence of the presence of *Listeria*. Cultures which do not blacken after 48 hours can be assumed to be *Listeria* free, eliminating the need to subculture to agar plates. There is also evidence that ferric ions enhance the growth of *Listeria* (Cowart and Foster, 1985). Lithium chloride is included to inhibit enterococci which can also hydrolyse aesculin.

The medium is sterilised by autoclaving at 121°C for 15 min, cooled to 50°C and the supplement (reconstituted with 5 mL of 1 part ethanol:1 part sterile water) added aseptically before dispensing into sterile containers. The prepared medium may be stored at 2–8°C for up to two weeks.

The Fraser broth (10 mL) is inoculated with 0.1 mL of the primary enrichment broth (UVM 1) which has been incubated for 20–24 hours. After incubation of the Fraser Broth for 26±2 hours in air the medium is compared with uninoculated broth against a white background. Tubes that darken or turn black are subcultured onto either Oxford Medium, MOX or Palcam medium. Tubes that retain their original yellow colour are recorded as negative. It is essential that the Fraser broth is incubated for a minimum of 24 hours to permit development of the black colour. Where presence of *Listeria* is strongly suspected the broth should be incubated for another 24 hours and once again subcultured.

6.3.2.4. Listeria selective enrichment media — UVM formulation

(University of Vermont Media Oxoid CM863 with step one UVM 1 supplement SR142 and step two UVM 2 supplement SR143, or Difco 0223 — recommended by USDA–FSIS for isolation from meat and poultry products) — Donnelly and Baigent (1986) and McClain and Lee (1988).

Formula (g/L):

Proteose peptone	5.0
Tryptone	5.0
'Lab-Lemco' powder	5.0
Yeast extract	5.0
NaCl	20.0
Na_2HPO_4	12.0
KH_2PO_4	1.35
Aesculin	1.0

pH 7.4

Sterilise at 121°C for 15 min and cool to 50°C.

6.3.2.4.1. Listeria primary selective enrichment supplement (UVM 1). Oxoid SR142 for 500 mL of broth:

Nalidixic acid	10.0 mg
Acriflavine hydrochloride	6.0 mg

Add supplement to 2 mL sterile distilled water, invert gently to dissolve, then add aseptically to 500 mL of UVM broth, mix well and dispense into sterile bottles.

6.3.2.4.2. Listeria secondary selective enrichment supplement (UVM 2). Oxoid SR143 for 500 mL of broth:

Nalidixic acid	10.0 mg
Acriflavine hydrochloride	12.5 mg

Prepare as for UVM 1.

The two-step enrichment procedure (USDA–FSIS method) results in a higher detection rate of *L. monocytogenes* in meat products and has the added advantage of taking only three to four days.

Either 25 g or 25 mL of sample is added to 225 mL of UVM 1 and homogenised in a Stomacher for two minutes. The broth is incubated in the Stomacher bag at 30°C, and after four hours incubation 0.2 mL is inoculated onto LSA plates. After 24 hours incubation 0.1 mL is transferred to 10 mL of UVM 2 which is incubated at 30°C. At the same time 1 mL is added to 4.5 mL of potassium hydroxide solution (next paragraph), vortex mixed and subcultured within one minute onto LSA. The UVM 2 after 24 hours incubation is cultured onto LSA (0.2 mL) and also 1 mL added to 4.5 mL potassium hydroxide, vortex mixed and subcultured within one minute onto LSA.

The potassium hydroxide solution is prepared by dissolving 2.5 g of potassium hydroxide and 20 g of NaCl in 1 L of distilled water, sterilising at 121°C for 15 min and cooling to 18–25°C before checking that the pH is >12.0 prior to use.

The listeria selective agar (LSA) recommended for use in the USDA method is LPM plating medium (see 6.3.1.5.); however, Oxford formulation (see 6.3.1.1.) has also been shown to be satisfactory (Oxoid manual). The prepared medium should be stored at 2–8°C.

6.3.2.5. Buffered Listeria Enrichment Broth

BLEB (Oxoid CM897 and supplement SR141) — Lovett *et al.* (1987).

Formula (g/L):

Tryptone soya broth	30.0
Yeast extract	6.0
KH_2PO_4	1.35
Na_2HPO_4	9.60

pH 7.3

Supplement SR141 is as used in the LEB (see 6.3.2.1.). The BLEB is a buffered modification of LEB to enhance the isolation of *Listeria* from fermented products. The medium is prepared by sterilising the broth base at 121°C for 15 min, cooling to 50°C, aseptically adding the supplement dissolved in 2 mL of sterile distilled water, mixing well and dispensing into sterile containers.

The medium is inoculated with 25 g or 25 mL of sample to 225 mL of BLEB, homogenised if necessary then incubated at 30°C for 48 hours. The broth is subcultured directly onto solid medium (Oxford Medium, MOX or Palcam medium) after 24 and 48 hours of incubation. At the same time 1 mL of broth is added to 9 mL of 0.5% potassium hydroxide (0.09 mol/L), vortex mixed and plated out immediately.