CHAPTER 3.9.6.

LISTERIA MONOCYTOGENES

SUMMARY

A wide variety of animal species can be infected with Listeria monocytogenes*, but clinical listeriosis is mainly a ruminant disease, with occasional sporadic cases in other species. The main clinical manifestations of animal listeriosis are encephalitis, septicaemia and abortion, and the disease is often associated with stored forages, usually silage. Post-mortem findings and histopathology depend on the clinical presentation.*

Listeriosis is one of the most important food-borne diseases of humans. The disease manifestations include septicaemia, meningitis (*or meningoencephalitis*) *and encephalitis. In pregnant women, intrauterine or cervical infections may result in spontaneous abortion or stillbirths, and may be preceded by influenza-like signs, including fever.* Listeria monocytogenes *has also been associated with gastroenteric manifestations with fever. Although the morbidity of listeriosis is relatively low, the mortality of the systemic/encephalitic disease can be very high, with values in the vicinity of 20–30%. In Europe, the hospitalisation rate is estimated at more than 95%. The elderly, pregnant women, newborns and the immunocompromised are considered to be at high risk of contracting the disease.*

A number of molecular and cellular determinants of virulence have been identified for this intracellular pathogen, and although there is evidence of polymorphism among different strains of L. monocytogenes *for some of these virulence determinants, this heterogeneity cannot be correlated with the ability or inability of the organism to produce disease. New information from whole genome sequencing may change this view in the coming years. Therefore, all* L. monocytogenes *strains are considered to be potentially pathogenic.*

Identification of the agent: *A variety of conventional and rapid methods are available for the detection and identification of* L. monocytogenes *in primary production, feed and food samples and specimens from animal listeriosis. Conventional methods remain the 'gold standard' against which other methods are validated. These methods use selective agents and enrichment procedures to reduce the number of contaminating microorganisms and allow multiplication of* L. monocytogenes*. Development of chromogenic media has allowed more reliable detection of this microorganism. Immunohistochemical detection of* L. monocytogenes *antigens is a useful tool for the diagnosis of the encephalitic form of the disease.*

Although not required for regulatory purposes, different levels of subtyping L. monocytogenes *strains are available, including serotyping by classical agglutination or polymerase chain reaction grouping, and pulse-field gel electrophoresis*). *The structure of population and phylogeny may be studied by multi-locus sequence typing. Subtyping tests are standardised at the international level by the World Health Organization collaborative trials and the PulseNet International Network*.

Serological tests: *Serological tests for the detection of antibodies have not been traditionally used for the diagnosis of listeriosis. A number of formats have been tried and they have all been found to be largely unreliable, lacking sensitivity and specificity. Experimental serological assays based on the detection of anti-listeriolysin O have been used in some epidemiological investigations and as support for the diagnosis of culture-negative central nervous system infections.*

Requirements for vaccines: *It has proven very difficult to develop effective vaccines against* L. monocytogenes *which, as an intracellular organism, requires effector T cells for an effective immune response. Experimental vaccines in laboratory animals are being explored to confer protection to* L. monocytogenes *infection by a number of different approaches, including immunisation with plasmid DNA, CD40 signalling along with heat-killed* L. monocytogenes*,*

listeriolysin O-deficient mutants inoculated along with liposome-encapsulated listeriolysin O, and immunisation with listerial antigens and IL-12.

A. INTRODUCTION

A wide variety of animal species can be infected by *Listeria monocytogenes,* including mammals, birds, fish and crustaceans (Table 1), although most clinical listeriosis cases occur in ruminants; pigs rarely develop disease and birds are generally subclinical carriers of the organism. Most infections in animals are subclinical, but invasive disease can occur either sporadically or as an outbreak. In addition to the economic impact of listeriosis in ruminants and other animal species, ruminants may play a role as a source of infection for humans, primarily from consumption of contaminated animal products. Direct transmission from infected animals, especially during calving or lambing (Wesley, 2007) can occur but these infections are very rare. The relative importance of the zoonotic transmission of the disease to humans is not clear, and contamination from the food-processing environment is of greater public health importance (Roberts & Wiedmann, 2003).

Table 1. *Species with reported isolation of* Listeria monocytogenes

		Mammals		
Cattle	Cats	Rabbits	Sheep	Deer
Guinea-pigs	Goats	Raccoons	Chinchillas	Pigs
Rats	Skunks	Horses	Mice	Mink
Dogs	Lemmings	Ferrets	Foxes	Voles
Moose	Humans			
		Birds		
Canaries	Ducks	Owls	Chaffinches	Eagles
Parrots	Chickens	Geese	Partridges	Cranes
Hawks	Pheasants	Doves	Lorikeets	Pigeons
Seagulls	Turkeys	Whitegrouse	Whitethroat	Woodgrouse
		Others		
Frogs	Crustaceans	Ticks	Fish	Ants
Flies	Snails			

The clinical manifestations of listeriosis in animals include rhombencephalitis (or in some cases more disseminated encephalitic changes), septicaemia and abortion, especially in sheep, goats and cattle. During an outbreak within a flock or herd, usually only one clinical form of listeriosis is encountered. The rhombencephalitic form is referred to as 'circling disease' because of a tendency to circle in one direction, and it is the most common manifestation of the disease in ruminants. Also, it is amongst the most common causes of neurological disease in ruminants. The clinical signs include depression, anorexia, head pressing or turning of the head to one side and unilateral cranial nerve paralysis. The latter is due to involvement of cranial nerves and their nuclei within the brainstem. Abortion is usually late term (after 7 months in cattle and 12 weeks in sheep) (Hird & Genigeorgis, 1990; Walker, 1999). The septicaemic form is relatively uncommon and generally, but not invariably, occurs in the neonate. It is marked by depression, inappetence, fever and death. Bovine and ovine ophthalmitis have also been described (Walker & Morgan, 1993). Rarely, mastitis of ruminants has been associated with *L. monocytogenes* infection. Gastrointestinal infections can occasionally occur in sheep (Clark *et al.*, 2004). When listeriosis occurs in pigs, the primary manifestation is septicaemia, with encephalitis reported less frequently, and abortions rarely. Although birds are usually subclinical carriers, sporadic cases of listeriosis have been reported, most frequently septicaemia and far less commonly meningoencephalitis. Avian listeriosis may be the result of a secondary infection in viral disease conditions and salmonellosis (Wesley, 2007).

The post-mortem findings and histopathology in animal listeriosis depend on the clinical presentation. In the encephalitic form, the cerebrospinal fluid may be cloudy and the meningeal vessels congested. Gross lesions are generally subtle and characterised by vascular congestion and mild tan discoloration of the brainstem. On occasion, the medulla shows areas of softening (malacia) and abscessation. Characteristic histopathological changes consist of foci of intraparenchymal neutrophils and macrophages (microabscesses) in the brainstem with adjacent perivascular mononuclear cuffing. The microabscesses often affect one side more severely. More extensive malacic pathology may occur. The medulla and pons are most severely involved. In the septicaemic form, multiple foci of necrosis in the liver and, less frequently the spleen, may be noted. Aborted fetuses of

ruminants show very few gross lesions, but autolysis may be present if the fetus was retained before being expelled (Low & Donachie, 1997; Walker, 1999).

The evidence indicates that animal listeriosis is frequently associated with stored forage and with the environment as the main source of contamination. In the environment, this saprophytic microorganism can live in soil, water, and decaying vegetables from which it could contaminate animal feed. Silage is the most frequent source (Fenlon, 1985; Wiedmann *et al.*, 1997a). In septicaemic/abortive listeriosis, the intestinal mucosa is the main route of entry after oral ingestion. The incubation period can be as short as 1 day. In rhombencephalitis, *L. monocytogenes* likely invades the brainstem via cranial nerves after breaching of the oral mucosa. The incubation period is significantly longer than in the septicaemic form, usually 2–3 weeks. The course of the disease is usually acute in sheep and goats, 1–4 days (Roberts & Wiedmann, 2003), although it can be more protracted in cattle.

Although *L. monocytogenes* has been recognised as an animal pathogen for many years, its significant role as a food-borne human pathogen became evident only in the 1980s, when a documented report of a Canadian listeriosis outbreak, traced to contaminated coleslaw, was published (Schlech *et al.*, 1983). Data from this outbreak and the level of the contaminated coleslaw were used several years later to establish the microbiological criteria at 100 colony-forming units/g as the *Codex Alimentarius* level. Today, *L. monocytogenes* is considered to be one of the most important agents of food-borne disease. Eighty outbreaks worldwide have been reported in the literature. Although outbreaks have been reported from several countries, the majority of human cases are sporadic. Possible explanations for the emergence of human food-borne listeriosis as a major public health concern include major changes in food production, processing and distribution, increased use of refrigeration as a primary preservation means for foods, changes in the eating habits of people, particularly towards convenience and ready-to-eat foods, and an increase in the number of people considered to be at high risk for the disease (elderly, pregnant women, newborns, immunocompromised) (Rocourt & Bille, 1997; Swaminathan & Gerner-Smidt, 2007). If *L. monocytogenes* has been reported in several countries, its incidence depends on eating habits, cooking practices, use of refrigeration and food importation.

The invasive forms of listeriosis in humans include septicaemia, meningitis (or meningoencephalitis), and encephalitis (rhombencephalitis). Gastroenteric manifestations with fever also occur. Although the morbidity of listeriosis is relatively low, the mortality can reach values around 30%. In pregnant women, infection may result in abortion, stillbirth or premature birth and may be preceded by influenza-like signs including fever (Painter & Slutsker, 2007; Rocourt & Bille, 1997).

Listeria monocytogenes is a Gram-positive rod and is responsible for almost all infections in humans; although rare cases of infection caused by *L. ivanovii* have been reported. In animals, *L. monocytogenes* is responsible for the majority of infections, but *L. ivanovii* and *L. seeligeri* infections have also been recorded. *Listeria ivanovii* has been associated with abortions and has been reported very occasionally to cause meningoencephalitis in sheep (Table 2).

Although *L. monocytogenes* has definite zoonotic potential, it is also one of the main environmental contaminants of public health significance.

Subtyping of *L. monocytogenes* strains by a variety of methods is available for epidemiological investigations, but the fundamental question of whether all strains of *L. monocytogenes* are capable of causing disease remains unanswered (Graves *et al.*, 2007; Jacquet *et al.*, 2002; Lopez *et al.*, 1992; 1993).

Several molecular virulence determinants have been identified that play a role in the cellular infection by *L. monocytogenes* and the unravelling of their mechanism of action has made of *L. monocytogenes* one of the most exciting models of host–pathogen interaction at the cellular and molecular levels. These virulence determinants include, among others, the internalins, listeriolysin O (LLO), ActA protein, two phospholipases, a metalloprotease, Vip protein, a bile exclusion system (BilE) and a bile salt hydrolase (Cabanes *et al.*, 2005; Cossart & Toledo-Arana, 2008; Dussurget *et al.*, 2002; Sleator *et al.*, 2005). Although there is polymorphism among different strains of *L. monocytogenes* for some of these virulence determinants, it cannot be correlated with the ability or inability of the organism to produce disease (Jacquet *et al.*, 2002). Nevertheless, internalins A or B mediates entry of *L. monocytogenes* into some human-cultured cell lines and crossing of the intestinal in gerbil or transgenic mice expressing its receptor, human E-cadherin, in enterocytes. Some *L. monocytogen*es isolates express a truncated nonfunctional form of internalin. Jacquet *et al.* (2004), found that clinical strains from humans expressed full-length internalin far more frequently (96%) than did strains recovered from food products (65%). Inside these clinical strains, serovar 4b, the most frequently implicated serovar in human listeriosis, expressed full-length internalin. Thus, a critical role of internalin in the pathogenesis of human listeriosis is established, and the expression of internalin is recognised as a marker of virulence in humans (Bonazzi *et al.*, 2009; Jacquet *et al.*, 2004).

Table 2. *Virulence of* Listeria *species*

***Listeria* species**	**Virulence in humans**	**Virulence in animals**
L. monocytogenes	+	+
L. ivanovii subsp. *ivanovii*	–[a]	+
L. ivanovii subsp. *londoniensis*	–	+
L. innocua	–[b]	–
L. welshimeri	–	–
L. seeligeri	–[b]	+
L. grayi subsp. *grayi* *L. grayi* subsp. *murrayi*	–[b]	–
L. marthii	–	–
L. rocourtiae	–	–
L. weihenstephanensis	–	–
L. fleischmannii subsp. *fleischmanii*	–	–
L. fleischmannii subsp. *coloradensis*		

a: only 11 human cases of infection reported; b: only 1 human cases of infection reported.

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

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

There is a variety of conventional and rapid methods currently available for the detection and identification of *L. monocytogenes* in samples from the food chain (primary production samples, feed, food samples, and environmental samples) and specimens from animal listeriosis. As low levels of *L. monocytogenes* could be difficult to detect, methods could also target *Listeria* spp. that have been used as bioindicators of the presence of *L. monocytogenes* in food and plant environmental samples. For animals and humans, conventional bacteriological methods are important for various reasons: their use results in a pure culture of the organism, which is useful for regulatory, epidemiological surveillance and outbreak management purposes. They remain the 'gold standards' against which other methods are compared and validated. These methods are usually very sensitive and they do not require sophisticated and expensive equipment, allowing widespread use. Some of the disadvantages of this group of methods include the relatively long period of time that the protocols require for completion, several 'hands-on' manipulations, the requirement for many different chemicals, reagents and media, the possibility of contaminating microorganisms masking the presence of the target ones, including overgrowth, the potential overlook of atypical variants of the target organism and the relative subjectivity involved when interpreting bacterial growth on selective and differential agar plates (Andrews, 2002).

The isolation and identification of *L. monocytogenes* from samples from the food chain and specimens from animal listeriosis require the use of selective agents and enrichment procedures that keep the levels of contaminating microorganisms to reasonable numbers and allow multiplication of *L. monocytogenes* to levels that are enough for detection of the organism. In the early days of listerial clinical bacteriology, cold enrichment (Gray *et al.*, 1948) was regularly used to this end, exploiting the ability of the organism to multiply at refrigeration temperatures (around 4°C), whereas contaminating bacteria would not multiply under these conditions. However, this procedure requires very long incubation times, often months, making it unacceptable for current investigations of food-borne outbreaks and sporadic cases, as well as for the implementation of effective hazard analysis critical control points (HACCP) programmes in food production and processing establishments. A number of selective compounds that allow growth of *L. monocytogenes* at normal incubation temperatures have been incorporated into culture media, shortening the time required for selective growth of the organism. Examples of these selective compounds include cycloheximide, colistin, cefotetan, fosfomycin, lithium chloride, nalidixic acid, acriflavine,

phenylethanol, ceftazidime, polymixin B and moxalactam. Development of chromogenic media has allowed better isolation of this microorganism in samples from the food chain.

Bacteriological diagnosis of animal listeriosis has traditionally involved direct plating of specimens on blood agar or other enriched media and concomitant use of the 'cold enrichment' technique, with weekly subculturing for up to 12 weeks (Gray *et al.*, 1948; Quinn *et al.*, 1999; Walker, 1999). Immunohistochemical detection of *L. monocytegenes* antigens in formalin-fixed tissue has proven to be more sensitive than direct plating and cold enrichment bacterial culture for the diagnosis of the encephalitic form of the disease in ruminants (Campero *et al.*, 2002; Johnson *et al.*, 1995). This is also the case for diagnosis of rhomboencephalitis in humans. Nevertheless, in contrast to human medicine, in animals it is very difficult or not possible to isolate the microorganism from the cerebrospinal fluid or to identify the microorganism by PCR in the cerebrospinal fluid. At present, therefore, confirmative diagnosis of listeric rhomboencephalitis in the living animal is not possible and is only achieved post-mortem by finding characteristic histopathological lesions or immunohistochemistry, bacterial isolation from the brainstem, or PCR on the brainstem.

The introduction of alternative enrichment procedures and selective agents for the isolation of *L. monocytogenes* from food and environmental samples has opened up the possibility of using some of these techniques for the bacteriological analysis of samples from animal listeriosis. Nevertheless, it should be stressed that performance characteristics cannot be ensured when these last methods are used outside the scope of their validation.

In spite of advances made in the selective isolation of *L. monocytogenes* from samples from the food chain, there is still room for improvement in a number of areas. No single procedure can be credited with being sensitive enough to detect *L. monocytogenes* from all types of food (Donnelly & Nyachuba, 2007). In addition, sublethally injured *L. monocytogenes* cells can be found in processed food resulting from freezing, heating, acidification and other types of chemical or physical treatment. These sublethally injured and viable but not cultivable bacteria require special culture conditions for damage repair, before being able to be detected in culture.

1.1. Isolation methods

Conventional methods for the isolation of *L. monocytogenes* from samples from the food chain that have gained acceptance for international regulatory purposes include the United States Food and Drug Administration (FDA) method (Hitchins & Jinneman, 2011), the Association of Official Analytical Chemists (AOAC) official method (AOAC, 2012), the European Committee for standardization (CEN, EN) and the International Organization for Standardization (ISO) (ISO, 1996; 1998; 2005a; 2005b), the Nordic Committee on Food Analysis (NMKL) method (NMKL, 2007) and the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) method (USDA-FSIS, 2013a; 2013b).

The EN ISO, FDA, USDA and AOAC methods shall be used according to their scope but cover a large variety of food matrices. Food samples intended for analysis must be representative from the food, including the outer surface and the interior. The conventional culture methods include an enrichment procedure based on the use of liquid culture media containing selective agents. The AOAC method calls for different selective enrichment containing different selective agents.

Depending on the nature of the sample, a particular method might be more suitable than others. The ISO Technical Committee ISO/TC 34, Agricultural Food Products, Subcommittee SC 9, Microbiology, in agreement with the EN Technical committee CEN/TC275, Food analyses, Working group 6, Microbiology from the food chain, claim that the EN ISO Standard 11290, parts 1 and 2 (ISO, 1996; 1998; 2005), can be used for the detection of *L. monocytogenes* in a large variety of food and feed products but also in primary production and environmental samples. Although they recognise that this standard might not be appropriate in every detail in certain instances, they recommend that every effort should be made to apply this horizontal method as far as possible.

The principle of the EN ISO 11290 Part 1 amended method for the detection of *Listeria monocytogenes* (ISO, 2005a), covering all food chain and primary production samples, is given. Briefly, after preparation of the test portion and initial suspension, the first stage is inoculation of a selective primary enrichment medium containing one volume of lithium chloride and half a volume of both acriflavine and nalidixic acid (half-Fraser broth), which is also used as a dilution fluid for the test portion. Incubate the test portion at 30±1°C for 24±3 hours. The second stage is inoculation of the full-strength secondary liquid enrichment medium (Fraser broth) with a culture obtained in the first stage. Incubate the Fraser broth at from 35±1°C to 37±1°C for 48±3 hours. In the third stage, samples from the cultures obtained in the first and second stages are plated out on the two selective solid media: "agar *Listeria* according to Ottaviani and Agosti" (ALOA®) and ALOA®-like agar, which contains lithium chloride, nalidixic acid, ceftazidime, polymyxin B and amphotericin B (or cycloheximide), and any other solid selective medium

at the choice of the laboratory, such as Oxford or PALCAM (polymixin-acriflavine-lithium chloride-ceftazidime-esculin-mannitol agar). Incubate the agar *Listeria* according to Ottaviani and Agosti at 37±1°C and examine after 24±3 hours to check for the presence of characteristic colonies that are presumed to be *L. monocytogenes*. Typical colonies of *L. monocytogenes* in agar *Listeria* according to Ottaviani and Agosti are green-blue surrounded by an opaque halo (ISO, 2005a). Oxford agar contains lithium chloride, cycloheximide, colistin, acriflavine, cefotetan and fosfomycin as selective agents, and typical colonies of *Listeria* spp. are small, black and surrounded by a black halo. Incubate the second selective medium at the appropriate temperature and examine after the appropriate time. Subculture the presumptive *L. monocytogenes* and confirm by means of appropriate morphological, physiological and biochemical tests described in the standard. For the enumeration method described in EN ISO 11290 Part 2, only "agar *Listeria* according to Ottaviani and Agosti" shall be used (ISO, 2005b).

There are two general groups of chromogenic media for *Listeria*. The first group of media employs a chromogen that detects β-D-glucosidase activity, which is indicative of *Listeria* species, and the formation of a distinct halo, indicative of the organism's lecithin use, surrounding the colony is used to identify *L. monocytogenes* and *L. ivanovii*. Media in this group include ALOA® and ALOA®-like media. In the second group, a chromogenic substrate is used to detect phospatidylinositol-specific phospholipase C (PI-PLC) activity. With this group of agars, *L. monocytogenes* and some *L. ivanovii* cleave the chromogen and the remaining *Listeria* species remain white. In some media of this last group, sugar as xylose has been added to the media to distinguish between *L. monocytogenes* and *L. ivanovii* by the presence of a yellow halo surrounding the *L. ivanovii* colonies. *Listeria monocytogenes* develops blue colonies (PI-PLC positive) without a yellow halo (xylose negative) and *L. ivanovii* produces greenish-blue colonies (PI-PLC positive) with a yellow halo (xylose positive). Other *Listeria* spp. colonies are white (PI-PLC negative). No xylose and PI-PLC negative *L. monocytogenes* has been reported.

For the FDA method (Hitchins & Jinnemans, 2011) described in chapter 10 of Bacteriological Analytical Manual (BAM), which can be accessed online, the buffered *Listeria* enrichment broth (BLEB) is the base enrichment. The Tryptone soya broth with yeast extract base has been supplemented with monopotassium phosphate to improve the buffering capacity, and pyruvic acid is added to aid in the recovery of stressed or injured cells. Analytical portions are pre-enriched in BLEB for 4 hours at 30°C, selective agents, acriflavin HCl (10 mg/litre), nalidixic acid (40 mg/litre) and cyclohexamide (50 mg/litre) are added and the enrichment is continued at 30°C for 48 hours. Enriched samples are streaked at 24 and 48 hours to selective/differential agar plates that contain esculin and ferric iron such as Oxford or a modification, MOX agar (MOX), or lithium chloride/phenylethanol/moxalactam (LPM) supplemented with Fe^{3+}. The option of a secondary chromogenic agar is also recommended. Subculture the presumptive *L. monocytogenes* and confirm by means of appropriate morphological, physiological and biochemical tests described in the method.

The USDA-FSIS method uses two enrichment steps (USDA-FSIS, 2013a; 2013b): the 'primary' enrichment is done in University of Vermont medium (UVM) containing nalidixic acid and acriflavine, and the 'secondary' enrichment is carried out in Fraser broth, containing nalidixic acid, lithium chloride and acriflavine or morpholine-propanesulfonic acid-buffered *Listeria* enrichment broth (MOPS-BLEB). Incubation conditions are described in this method and distinct depending on the matrix chosen for the enrichment step. After selective enrichment, cultures are then plated on MOX agar that contains lithium chloride, colistin and moxalactam. Subculture the presumptive *L. monocytogenes* and confirm by means of appropriate morphological, physiological and biochemical tests described in the method.

For the NMKL method (NMKL, 2007), primary enrichment in half-Fraser broth at 30°C for 24 hours, is followed by a secondary enrichment in Fraser broth at 37°C for 48 hours. The cultures obtained from both the enrichment steps are plated out on a *L. monocytogenes*-specific isolation medium, agar ALOA® or *Listeria monocytogenes* blood agar medium (LMBA) or chromogenic *Listeria* agar medium, which is basically like ALOA® and on another solid selective isolation medium; the latter is optional. Subculture the presumptive *L. monocytogenes* and confirm by means of appropriate morphological, physiological and biochemical tests described in the standard.

All culture media prepared should be subjected to quality control, such as according to ISO 11133 standards (ISO, 2003; 2009).

The original and traditional procedure for the isolation of *L. monocytogenes* from animal tissues has been direct plating of specimens on sheep blood agar or other rich culture media and concomitant use of the 'cold enrichment' technique, with weekly subculturing for up to 12 weeks (Gray *et al.*, 1948; Quinn *et al.*, 1999; Walker, 1999). The cold enrichment technique is not currently performed. Isolation of the organism by direct plating is relatively easy when numbers are large in a normally sterile site, such as in the case of the septicaemic form of the disease, but isolation is difficult when the organism

is present in low numbers, as in the case of the encephalitic form or when samples are heavily contaminated.

In the case of animal listeriosis, the samples should be chosen according to the clinical presentation of the disease: material from lesions in the liver, kidneys or spleen, in the case of the septicaemic form; spinal fluid, pons and medulla in the case of the rhombencephalitic form; and placenta (cotyledons), fetal abomasal contents or uterine discharges in the case of abortion. Refrigeration temperatures (4°C) must be used for handling, storing and shipping specimens. If the sample is already frozen, it should be kept frozen until analysis.

The protocol recommended for isolation of *L. monocytogenes* from animal necropsy material is described below as originally published (Eld *et al.*, 1993).

1.1.1. Isolation procedure from animal necropsy material

i) Inoculate 10–25 g or ml of sample (depending on the amount of sample available) into 225 ml *Listeria* enrichment broth. When dealing with samples from animal listeriosis, the size of the sample for inoculation may be limited and less than that recommended for food samples (25 g or ml). If that is the case, as much sample material as possible (aim at 10–25 g or ml) should be inoculated (Eld *et al.,* 1993). (*Listeria* enrichment broth base: 30 g Oxoid tryptone soya broth; 6 g Difco yeast extract; 1 litre water; selective agents: 2.3 mg Acriflavine; 9.2 mg nalidixic acid; 11.5 mg cycloheximide; add selective agents to 225 ml of the broth base).

ii) Incubate broth at 30°C for 48 hours.

iii) Spread 0.1 ml of the enrichment broth culture onto Oxford agar plates.

iv) Incubate plates at 37°C. Examine bacterial growth after 24 and 48 hours.

v) Test five colonies (or all when fewer available) with typical appearance of *L. monocytogenes* for cell shape, Gram reaction, haemolytic activity on blood agar (defibrinated horse blood), tumbling motility at 20°C, fermentation of glucose (+), rhamnose (+) and xylose (–), hydrolysis of esculin and production of catalase.

1.1.2. Alternative protocol

Alternative protocols exist at the national level for veterinary laboratories; here is one example:

i) Check that the sample has not been contaminated by the environment. If there is a doubt, sterilise with a Bunsen burner or cauterise with a brand, for example in the case of brain sample contaminated during extraction from skull. The test portion is homogenised in buffered-peptone water with a crusher to give a consistent initial suspension. Any sample that has not yet been crushed is stored at 2–8°C.

ii) The initial suspension is inoculated in enrichment broth such as brain–heart broth or Rosenow broth. In parallel, it is spread, for direct observation, on modified Palcam and a Columbia sheep blood agar with nalidixic acid (15 mg/litre) and colistine sulphate (10 mg/litre), if it is presumed that the sample is not contaminated. The Palcam base is modified as follows: a supplement (containing 100,000 International Units of Polymyxin B sulphate, 20 mg ceftazidin, 5 mg acriflavin chlorhydrate, 200 mg of cycloheximide, and 10 ml of sterile water) is prepared, sterilised by filtration and 10 ml is added to 1000 ml of Palcam base medium.

iii) Incubate at 37±1°C for 24 hours for liquid culture and 24–48 hours for solid media.

iv) After 24 hours, if colonies presumed to be *Listeria* appear on the Petri plates, select them for further confirmation tests. If none is present, incubate the plates again in the same conditions for 24 hours. Enrichment broth is streaked on Palcam and Columbia sheep blood agar with nalidixic acid (15 mg/litre) and colistine sulphate (10 mg/litre), and incubated at 37±1°C for 24 hours. On Palcam and modified Palcam, expose the plates in the air for 1 hour to allow the medium to regain its pink to purple colour. After 24 hours, *Listeria* spp. grow on these last media as small or very small greyish green or olive green colonies, 1.5–2 mm in diameter, sometimes with black centres, but always with black halos. After 48 hours, *Listeria* spp. appear in the form of green colonies about 1.5–2 mm in diameter, with a central depression and surrounded by a black halo. On Columbia sheep blood agar with nalidixic acid and colistine sulphate, *Listeria* spp. grow as grey and flat colonies and *L. monocytogenes* presents a small haemolysis zone that could be observed after removing the colony. *Listeria ivanovii* presents a weak haemolytic activity around the colony.

v) At 48 hours and 72 hours, if colonies presumed to be *Listeria* appear on Petri plates, select them for further confirmation tests. If there are five presumed *Listeria* colonies on the plate, select them all. If more than five presumed *Listeria* colonies are on the plate, pick five colonies only.

For faeces and silage, and placental envelop, there are two modifications to this last protocol.

For faeces and silage, a 1/10 suspension (25 g in 225 ml) is performed in half-Fraser broth and incubated at 30±1°C for 24 hours. At 24 hours, this suspension is streaked on modified Palcam and a subculture in Fraser broth at 0.1 ml in 10 ml is performed. Media are incubated at 37±1°C for 24 hours. At 48 hours, this incubated Fraser broth is streaked on modified Palcam and Petri plates are incubated at 37±1°C for 24–48 hours. Fraser broth is re-incubated at 37±1°C for 24 hours before to be streaked on modified Palcam.

For placental envelop, the test portion is diluted at 1/2 and 1/5 in buffered-peptone water and directly isolated on selective media. The Palcam is replaced, in this case, by modified Palcam.

ALOA® and other chromogenic media for *Listeria* allow the growth of most *Listeria* spp. and can be used in clinical microbiology to screen human faeces.

1.2. Conventional identification methods

Typical *Listeria* spp. colonies, on the above selective/differential agar plates, are then selected for further identification to the species level, using a battery of tests. The tests include the Gram-staining reaction, catalase, motility (both in a wet mount observed under phase-contrast microscopy and by inoculation into semi-solid motility agar [0.2–0.4% agar] or U/Graigie's tube), haemolysis and carbohydrate use (Tables 3 and 4).

To observe of tumbling motility, a hanging drop preparation is made from a young broth culture, such as tryptone soya yeast extract broth, and incubated at room temperature for 8–24 hours. When semi-solid motility agar is used after stab inoculation (about 1 cm) and incubation at 20–28°C, listeriae swarm through the medium, which becomes cloudy. At about 0.5 cm below the surface of the agar, a characteristic layer of increased growth is observed, like an umbrella. This occurs because of the better development of *Listeria* under aerobic conditions as opposed to strictly anaerobic conditions.

For haemolysing activity, horse and sheep blood-containing agar plates shall be used. After incubation at 37°C for 24 hours and inoculation by piercing the medium, *L. ivanovii* exhibits a wide zone of haemolysis. The haemolysis zone of *L. monocytogenes* is narrow, frequently not extending much beyond the edge of colonies. In this case, removal of the colonies could help interpretation. Rare strains of *L. monocytogenes* are not haemolytic.

The Christie–Atkins–Munch–Peterson (CAMP) test is a very useful tool to help identify the species of a *Listeria* spp. isolate. It is used in the ISO and some AOAC protocols and it is considered to be optional in the FDA and USDA-FSIS methods. The test is simple to perform and easy to read. It consists of streaking a ß-haemolytic *Staphylococcus aureus* (ATCC™ strain 49444® or 25923®, NCTC™ strain 7428® or 1803®) and *Rhodococcus equi* (ATCC™ strain 6939®, NCTC™ strain 1621®) in single straight lines in parallel, on a sheep blood agar plate or a double-layered agar plate with a very thin blood agar overlay. The streaks should have enough separation to allow test and control *Listeria* strains to be streaked perpendicularly, in between the two indicator organisms, without quite touching them (separated by 1–2 mm). After incubation for 24–48 hours at 35–37°C (12–18 hours if using the thin blood agar overlay), a positive reaction consists of an enhanced zone of ß-haemolysis, at the intersection of the test/control and indicator strains. *Listeria monocytogenes* is positive with the *S. aureus* streak and negative with *R. equi*, whereas the test with *L. ivanovii* gives the reverse reactions (Quinn *et al.*, 1999).

Within the genus *Listeria*, ten species have been taxonomically described: *Listeria monocytogenes, L. ivanovii* subsp. *ivanovii* and subsp. *londoniensis, L. welshimeri, L. innocua, L. seeligeri, L. gra*yi subsp. *grayi* and subsp. *murrayi, L. marthii, L. rocourtiae, L. weihenstephanensis, L. fleischmannii* subsp. *fleischmannii* and susbp. *coloradensis.* New species (*L. rocourtiae, L. marthii, L. weihenstephanensis, L. fleischmannii* subsp. *fleischmannii* and susbp. *coloradensis*) are mostly isolated from environmental samples and are rare (den Bakker *et al.*, 2013). *Listeria fleischmannii* could be isolated in primary production sample and soil of plants or cellar.

Table 3. *Principal characteristics of the main* Listeria *species*

Test	***Listeria* spp. reaction**
Gram stain	Positive
Cell morphology	Short (0.4-0.5 µm × 0.5-2.0 µm) nonspore forming rod with a few peritrichous flagella
Growth conditions	Aerobic and facultative anaerobic
Motility	Positive tumbling motility or in umbrella in motility agar at 20–28°C, negative at 37°C
Catalase	Positive
Oxidase	Negative
Aesculin hydrolysis	Positive
Indole	Negative
Urease	Negative

Table 4. *Differentiation of main* Listeria *species*

Species	**β-haemolysis**	**Production of acid from**			**Christie, Atkins, Munch-Petersen (CAMP) reaction on sheep blood with**	
		L-Rhamnose	D-Xylose	D-Mannitol	*S. aureus*	*R. equi*
L. monocytogenes	+	+	–	–	+	–
L. innocua	–	V	–	–	–	–
L. ivanovii subsp. *ivanovii*	+	–	+	–	–	+
L. ivanovii subsp. *londoniensis*	+	–	+	–	–	+
L seeligeri	(+)	–	+	–	(+)	–
L. welshimeri	–	V	+	–	–	–
L. grayi subsp. *grayi*	–	–	–	+	–	–
L. grayi subsp. *murrayi*	–	+	–	+	–	–

V: variable; (+): weak reaction; +: >90% positive reactions; –: no reaction.

Serology, DNA macrorestriction, multi-locus sequence typing (MLST) and the animal model pathogenicity assay are considered to be optional methods. Liu *et al.* (2007) reviewed the virulence test for *L. monocytogenes* and tried to define its virulence.

1.3. Rapid identification methods

The following protocols include conventional and nonconventional commercially available tests, and nucleic acid assay kits, to help in the identification of *L. monocytogenes* (Jadhav *et al.,* 2012). Polymerase chain reaction (PCR), targeting the *hly* gene, has been found to be a sensitive and rapid technique for confirmation of the identification of suspect *L. monocytogenes* isolated on selective/differential agar plates (Gouws & Liedemann, 2005).

Alternative commercially available methods for identification have been validated by one or more recognised formal validation systems, such as AOAC, MicroVal, Nordval International and Afnor Certification (Afnor certification, 2013; AOAC, 2012; Lombard & Leclercq, 2011; Microval, 2013; NordVal international, 2013). The list is growing steadily as new technologies are exploited for application to the needs of laboratories. Regular updates of these alternative methods are published online on the websites of validation bodies, together with key references and scope, validation status and certification of the method.

Chromogenic confirmation media for identification of *L. monocytogenes* have been developed. They are mostly based on detection of PI-PLC activity and fermentation of L-rhamnose. A presumed *L. monocytogenes* colony is selected and spread on a form of band (2 cm). *Listeria monocytogenes* shows a PI-PLC activity and a yellow zone of L-rhamnose fermentation. Rare strains of *L. monocytogenes* are rhamnose negative.

A system is commercially available for the presumptive identification of *Listeria* species isolated from samples from the food chain. It provides an alternative to conventional biochemical testing of *Listeria* spp. isolates by the reference methods. It is based on testing miniaturised microtubes on a strip or a card that give reactions by fermentation, utilisation or enzymatic activity, which can be detected after 24 hours at 37°C. For biochemical identification, differentiation of *Listeria* species is based on a code derived after adding the numerical values for each group of several tests and, an additional test such as the reactions obtained from the CAMP test and haemolysis characteristics, which are assayed separately. A commercial method based on the presence or absence of arylamidase, distinguishes between *L. monocytogenes* and *L. innocua* without the need for further tests for haemolytic activity.

Identification can be done by sequencing the 16S rDNA or *iap* genes (Bubert *et al.*, 1999). After extraction of DNA with commercial kits, an end point PCR for 16S rDNA or *iap* genes is performed. PCR products are purified and sequenced with a sequencer in the laboratory. The sequence is compared with DNA database accessible via the internet using blast. Recently, a quantitative PCR assay for the identification of isolates has been added to the online BAM manual of FDA methods at: http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm279532.htm.

An alternative method for the rapid identification of *Listeria* species is the matrix-assisted laser desorption ionisation–time of flight mass spectrometry (MALDI-TOF MS), which is increasingly being used in microbiology laboratories. MALDI-TOF MS identification systems are based on the comparison of the tested isolate mass spectrum with reference databases. Several databases and identification strategies have been developed. For *Listeria* isolates, the genus could be accurately identified but not the species (Farfour *et al.*, 2012).

1.4. Alternative methods of classical *Listeria* detection

A number of methods based on immunological tests or on nucleic acid recognition have been developed to detect *L. monocytogenes* in samples from the food chain (Jadhav *et al.,* 2012). Some of them have been validated by one or more recognised formal validation systems, such as AOAC, MicroVal, Nordval International and Afnor Certification (Afnor certification, 2013; AOAC, 2012; Dunbar *et al.*, 2003; Lombard & Leclercq, 2011; Microval, 2013; NordVal international, 2013; Sewell *et al.*, 2003). A regular update of these alternative methods is published online together with key references and the validation status and certification of the method.

Target DNA sequences for diagnostic purposes include the *hly* gene, the *iap* gene and 16S rDNA gene in a PCR and qPCR format. The USDA-FSIS method MLG8A.05[1] describes the use of PCR to screen foods for *L. monocytogenes* and is based on the use of BAX assay for Screening *Listeria monocytogenes*.

Alternative methods based on PCR for the detection of *Listeria* shall be validated and used with quality control according to OIE guidelines described in the OIE Terrestrial manual and/or rules or guidelines described in regional or national regulation.

1.5. Antimicrobial susceptibility testing

Listeria monocytogenes is intrinsically resistant to cephalosporins (cefazolin, ceftiofur, cefpirome), quinolones (nalidixic acid and early fluoroquinolone such as ofloxacine), fosfomycine and clindamycine. Acquired resistance has been rarely identified. Most of the isolates are susceptible to Penicillin G, amoxicilline, aminoglycosides (gentamycin), tetracyclines, phenicols, trimethoprim and sulfonamides, rifampin, glycopeptides (vancomycin) (Granier *et al.*, 2011; Troxler *et al.*, 2000). At very low frequencies, resistance to tetracycline has been identified from various sources: beef meat, beef processing plants, pork cheeks, and sheep. Resistance to erythromycin was also identified in environmental and food samples. Remarkably, no resistance to penicillins has been evidenced to date (Granier *et al.*, 2011).

Susceptibility testing is usually indicated for bacterial pathogens when they induce infection requiring antimicrobial treatment and if organism identification is not enough to reliably predict the outcome of this treatment.

For treatment of *L. monocytogenes* infection, susceptibility to antimicrobial agents is still predictable, and therapy is widely deployed on an empirical basis. Nevertheless, susceptibility test can be a valuable tool for epidemiological studies or to evaluate new antimicrobial agents. Moreover, as *L. monocytogenes* is considered a fastidious organism to grow, methodologies for antimicrobial susceptibility tests and interpretation used to be poorly standardised and the rare published studies might have sometimes been questionable.

1 Available from Qualicon-Dupont, Wilmington, USA.

Recently, those methodology issues have been addressed by two different institutions. In Europe, Eucast (www.eucast.org) proposed in 2011 a methodology for *L. monocytogenes* susceptibility testing by disc diffusion. In the USA, two documents from the Clinical and Laboratory Standards Institute (www.clsi.org), M31-A3 regarding susceptibility tests of bacteria from animals and M45-A2 regarding susceptibility tests of fastidious bacteria, provide guidelines and interpretation criteria to assess susceptibility of *L. monocytogenes* by the broth microdilution method.

1.6. Subtyping methods

Most regulatory detection of *L. monocytogenes* does not require any specific subtyping of the isolates. However, subtyping schemes can be useful in outbreak investigations, environmental tracking and public health surveillance.

Listeria monocytogenes can be subtyped by a number of different approaches including serotyping, phage typing, DNA restriction enzyme analysis (either using high-frequency cutting enzymes and conventional gel electrophoresis to separate fragments, or using rare-cutting enzymes and pulse-field gel electrophoresis [PFGE] to separate fragments), and nucleic acid sequencing-based typing, microarray analysis.

Because of the requirement for specific reagents, stringent quality assurance procedures and some sophisticated equipment, it is recommended that subtyping of *L. monocytogenes* isolates be referred to the appropriate reference centre. These reference laboratories could be at the national, regional or international level. At the international level, there is only a World Health Organization (WHO) Collaborating Centre for foodborne listeriosis (Institut Pasteur, Paris).

1.6.1. Serotyping and genoserotyping (PCR group)

Strains of *L. monocytogenes* can be assigned to 13 different serovars (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e, and 7), based on their combination of somatic (O) and flagellar (H) antigens, according to the Seeliger and Höhne protocol (1979). Serotyping antigens are shared among *L. monocytogenes, L. innocua, L. seeligeri* and *L. welshimeri*. There is only one commercial kit with all this antifactor sera (Denka Seiken, Tokyo, Japan). Although all of them are considered to be potentially pathogenic, most (>95%) human clinical isolates belong to three serovars 1/2a, 1/2b, and 4b. Compared with other subtyping methods, serotyping has poor discriminatory power, but can provide valuable information to facilitate the ruling out of isolates that are not part of an outbreak or an investigation on a human sporadic case. Isolates from foods and from environmental sources are frequently nontypable using commercial standard antifactor sera and require additional sera. In this case, typing could be performed at the WHO Collaborating Centre for foodborne listeriosis (Institut Pasteur, Paris).

Because serotyping is not cost-effective, necessitates technical expertise and antisera, it is now often substituted by a quick and reproducible PCR-based method, developed by Doumith *et al.* (2004), which targets the four DNA fragments *prs, ORF2110, ORF2819, lmo1118, lmo0737*. This last genoserotyping method is now internationally recognised and validated. All *Listeria* species but *L. rocourtiae* possess an amplifiable *prs* gene fragment. PCR serogroup IIa comprises strains of serovars 1/2a and 3a (amplification of *prs* and *lmo0737* DNA fragments); PCR serogroup IIb comprises strains of serovars 1/2b, 3b, and 7 (amplification of the *prs* and *ORF2819* DNA fragments); PCR serogroup IIc comprises strains of serovars 1/2c and 3c (amplification of *prs, lmo0737* and *lmo1118* DNA fragments); PCR serogroup IVb comprises strains of serovars 4b, 4d and 4e (amplification of *prs, ORF2819* and *ORF2110* DNA fragments). Finally, PCR serogroup L comprises strains of other serovars of *L. monocytogenes* and other species, except *L. rocourtiae*.

1.6.2. Lineage

After serotyping, *L. monocytogenes* can be classified into three lineages, of which lineage I encompasses serovars 1/2b, 3b, 4b, 4d and 4e; lineage II includes serovars 1/2a, 1/2c, 3a, and 3c; and lineage III comprises serovars 4a, 4c and atypical 4b, according to Wiedmann *et al.* (1997b). The lineage status of serovars 4ab and 7 remains unclear due to limited availability of such strains. Within the lineage III, three genetically distinct subgroups (IIIA, IIIB, and IIIC) have been identified after comparative analysis of *actA* and *sigB* gene sequences. Phenotypically, lineage IIIa strains be-have like typical *L. monocytogenes* in their ability to ferment rhamnose, whereas lineages IIIB and IIIc strains are notably deficient in rhamnose utilisation. Lineages I and II are involved in the documented human listeriosis cases and lineage III are rarely associated with outbreaks despite their frequent isolation from food and environmental specimens. Lineage I and II isolates seem to be similarly prevalent in animals.

1.6.3. Chromosomal DNA restriction endonuclease analysis

Restriction endonuclease analysis (REA) of chromosomal DNA is a useful subtyping method for *L. monocytogenes*. As these enzymes are highly specific in recognising nucleotide sequences, the resulting DNA digestion fragments, of different size and electrophoretic mobility, reflect genomic differences, resulting in specific 'fingerprints' among otherwise related strains. Because of the restriction endonuclease specificity, the method is highly reproducible. Of the restriction endonucleases tested on *L. monocytogenes* in a WHO Multicentre study, *Hae*III, *Hha*I and *Cfo*I were the most useful (Bille & Rocourt, 1996; Graves *et al.*, 2007). However, because of a potentially large number of enzyme recognition sites in the bacterial genome, sometimes complex fingerprints evolve, with overlapping or poorly resolved bands that are difficult to interpret. The technique is therefore not adequate for comparing a large number of strain patterns or for building dynamic databases (Graves *et al.*, 2007).

By combining REA with Southern hybridisation, using chromosomal-labelled probes, only the particular restriction fragments associated with the corresponding chromosomal loci are detected, thereby significantly reducing the number of DNA fragments to be analysed. This technique is known as restriction fragment length polymorphism (RFLP) analysis. When ribosomal RNA/DNA probes are used, only the particular restriction fragments associated with the chromosomal loci for rRNA are detected. This technique is known as ribotyping and it has been widely used for subtyping *L. monocytogenes*, mainly through the use of the restriction endonuclease *Eco*RI. However, the technique was found to be less discriminating than phage typing, REA or multilocus enzyme electrophoresis (MEE). Qualicon has designed an automated ribotyping system, the RiboPrinter, which generates, analyses and stores riboprint patterns of bacteria, including *Listeria*.

When restriction endonuclease enzymes that cut infrequently are used to digest unsheared chromosomal DNA, such as *Apa*I, *Sma*I, *Not*I and *Asc*I, very large fragments are obtained. Because of their size, these large fragments do not separate when run under conventional agarose gel electrophoresis. However, by periodically changing the orientation of the electric field across the gel, through pulses, the large fragments can 'crawl' through the agarose matrix and are separated according to size differences. This technique is known as pulsed-field gel electrophoresis (PFGE) and has revolutionised the precise separation of DNA fragments larger than 40 kilobases. PFGE has been applied to the subtyping of *L. monocytogenes* and has been found to be a highly discriminating and reproducible method. PFGE is particularly useful for subtyping serotype 4b isolates, which are not satisfactorily subtyped by most other subtyping methods. The main disadvantages of PFGE are the time required to complete the procedure (2–3 days), the large quantities of expensive restriction enzymes required, and the need for specialised, expensive equipment (Graves *et al.*, 2007). The Centers for Disease Control and Prevention (CDC) in the USA has established PulseNet, a network of public health and food regulatory laboratories at the national or international levels that routinely subtype food-borne pathogenic bacteria by PFGE. PulseNet laboratories use highly standardised protocols for PFGE of *Listeria* with endonuclease enzymes *Apa*I and *Asc*I, and can quickly compare PFGE patterns from different locations via the Internet. *Listeria monocytogenes* was added to PulseNet in 1999 (Graves & Swaminathan, 2001) and the last protocol published in 2009 (Graves *et al.,* 2001, Halpin *et al.,* 2009). In Europe, the European Centre for Disease Prevention and Control (ECDC) and the European Food Safety Authority (EFSA) are building databases with PGFE profiles of *L. monocytogenes* isolated from human cases and food and veterinary sources, respectively, with the aim of investigating transnational or cross-border outbreaks.

1.6.4. Nucleic acid sequence-based typing

Although there have been some reports on the sequence analysis of single genes as a means to type *L. monocytogenes* strains, determination of allelic variation of multiple genes, has been recently introduced as a very promising subtyping methodology for this microorganism. This approach has been reported for a handful of other microorganisms and it is known as multi locus sequence typing (MLST) (Chenal-Francisque *et al.,* 2011; Ragon *et al.*, 2008; Salcedo *et al.*, 2003; Spratt, 1999). Direct amplification and nucleotide sequencing has been used with good discrimination between the strains analysed. Because MLST is based on nucleotide sequence, it is highly discriminatory and provides unambiguous results. MLST allowed definition of a clonal complex, which gives a view of the structure of the population and phylogeny inside a population of isolates. Some of this clonal complex has been implicated in outbreaks or could be linked to clinical forms that give the risk manager additional information on isolates. New information form whole genome sequencing that could be used in research and molecular surveillance of *Listeria* may change this view in the coming years (den Bakker *et al.*, 2010).

2. Serological tests

Serological tests for the detection of antibodies have not been traditionally used for the diagnosis of listeriosis. They have been largely unreliable, lacking sensitivity and specificity. A number of formats, including enzyme-linked immunosorbent assay (ELISA), dot-blot and microagglutination (Gruber-Widal reaction) have been largely unsuccessful in the diagnosis of culture-proven human listeriosis, even in the absence of immunosuppression. Considerable cross-reactivity with antigenic determinants of other Gram-positive organisms has been observed. On the other hand, *L. monocytogenes* is a ubiquitous organism, and regular exposure of animals and humans to this microorganism is very common. Many healthy individuals are intestinal carriers (2–6%) and anti-*L. monocytogenes* serum antibody prevalence as high as 53% have been reported in humans. Carriage rate for animals is similar to that of humans, with some differences depending on the species and a slightly higher rate during the indoor season, as compared to animals on pasture (Husu, 1990; Iida *et al.*, 1991).

The discovery that the *L. monocytogenes* haemolysin, listeriolysin O (LLO), is a major virulence factor and that it can stimulate an antibody response, has recently renewed interest in the possibility of using serological tests for the diagnosis of listeriosis, particularly in central nervous system patients, with sterile blood and cerebrospinal fluid, and in perinatal listeriosis. An indirect ELISA based on the detection of anti-LLO was used for the diagnosis of experimental listeriosis in sheep (Low *et al.*, 1992). However, LLO is antigenically related to a number of cytolysins, including streptolysin O (SLO) from *Streptococcus pyogenes*, pneumolysin from *S. pneumoniae* and perfringolysin from *Clostridium perfringens*. Problems of cross-reactivity of anti-LLO antibodies with these cytolysins, particularly SLO and pneumolysin, have hampered the development of specific reliable serological tests based on the detection of anti-LLO antibodies. In addition, anti-LLO antibodies have been found in a proportion of healthy individuals and patients with other bacterial, fungal or viral infections (27%, all combined), although at lower titres than in patients with listeriosis. Absorption of diagnostic antisera with SLO is only partially effective in eliminating all cross-reactivity. These experimental assays have been used in some epidemiological investigations and as support for the diagnosis of culture-negative central nervous system infections. Recombinant forms of LLO have been explored as alternatives to wild LLO as a diagnostic antigen in dot-blot assays (Lhopital *et al.*, 1993). Full validation of these serological tests for the diagnosis of listeriosis is needed but a sera biobank needs to be developed.

C. REQUIREMENTS FOR VACCINES

It has proven very difficult to develop effective vaccines against *L. monocytogenes* which, as an intracellular organism, requires effector T cells for an effective immune response. Experimental vaccines in laboratory animals are being explored to confer protection to *L. monocytogenes* infection by a number of different approaches, but these are still far from becoming available for human and farm animal use. These experimental approaches include immunisation with plasmid DNA, CD40 signalling along with heat-killed *L. monocytogenes*, LLO-deficient mutants inoculated along with liposome-encapsulated LLO, and immunisation with listerial antigens and IL-12.

Genetically modified *L. monocytogenes* is also being considered as an effective vaccine vector for the expression, secretion and intracellular delivery of foreign antigens for the induction of potent immune responses against viral antigens and tumour cells.

However, the most feasible and practical means to reduce the risk of listeriosis in humans is through dietary and food preparation measures that not only decrease the risk of acquiring listeriosis, but also contribute to the prevention of other common food-borne infections such as those caused by *Escherichia coli* O157:H7, *Salmonella* and *Campylobacter*. These preventive measures include thorough cooking of raw food of animal origin, keeping uncooked meats separate from vegetables, cooked foods, and ready-to-eat foods, thoroughly washing raw vegetables before eating, washing hands, knives, and cutting boards after handling uncooked foods, and avoiding unpasteurised milk or products made from it. Immunocompromised and elderly persons, pregnant women and other groups at increased risk of listeriosis should avoid foods that have been epidemiologically linked to this disease, e.g. soft cheeses, cold smoked fish and pâté. These individuals should also avoid other ready-to-eat foods, unless they are heated until steaming hot before being consumed.

The food industry and public health agencies play a pivotal role in the prevention of food-borne listeriosis by developing and implementing effective HACCP programmes to reduce the presence of *L. monocytogenes* at all critical points in the food production and distribution chain (from the farm to the market).

Likewise, the lack of well designed and tested vaccines for animal use, means that control of listeriosis in animals is most feasible by preventing the environmental conditions that favour its presentation. There is a well-established linkage between silage feeding and listeriosis and, as *L. monocytogenes* is widely distributed in nature, with animals and birds acting as carriers, contamination of silage is not uncommon. Emphasis should therefore be placed on reducing the likelihood of the multiplication of the organism, which occurs more frequently at pH values greater than 5, particularly where ineffective fermentation has occurred and where there is

concomitant growth of moulds. Every effort should be made to produce silage of good quality, with early cutting of grass, minimal contamination with soil or faeces and ensuring optimal anaerobic fermentation, which will insure that the pH falls below 5.0; at that level, growth of *Listeria* spp. is inhibited. The best silage for feeding should be selected, especially in the case of sheep, discarding material that has obvious signs of contamination with mould. Material a few centimetres from the top, front and sides of an opened bale or bag, should also be discarded. Leftover silage should be removed (Low & Donachie, 1997).

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2 AFNOR: Association Française de Normalisation
3 AOAC: Association of Official Analytical Chemists

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5 Microval: European Validation and Certification Organisation
6 NMKL: Nordic Committee on Food Analysis
7 USDA-FSIS: United States Department of Agriculture – Food Safety and Inspection Service

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NB: FIRST ADOPTED IN 2004; MOST RECENT UPDATES ADOPTED IN 2014.