

Review

Identification, subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen

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Listeria monocytogenes is an opportunistic intracellular pathogen that has become an important cause of human foodborne infections worldwide. Given its close relationship to other *Listeria* species and its tendency to produce non-specific clinical symptoms, the availability of rapid, sensitive and specific diagnostic tests for the differentiation of *L. monocytogenes* from other *Listeria* species is helpful for selecting appropriate treatment regimens. In addition, with *L. monocytogenes* comprising a diversity of strains of varying pathogenicity, the ability to precisely track the strains involved in listeriosis outbreaks and speedily determine their pathogenic potential is critical for the control and prevention of further occurrences of this deadly disease. Extensive research in recent decades has revealed significant insights regarding the molecular mechanisms of *L. monocytogenes* infection. This in turn has facilitated the development of laboratory procedures for enhanced detection and identification of *L. monocytogenes*, and has also contributed to the implementation of improved control and prevention strategies against listeriosis. The purpose of this review is to summarize recent progress in the species-specific identification, subtyping and virulence determination of *L. monocytogenes* strains, and to discuss future research needs pertaining to these important areas of listeriosis.

Introduction

The genus *Listeria* represents a group of closely related, Gram-positive, facultative anaerobic, non-spore-forming, rod-shaped bacteria 0.5 µm in width and 1–1.5 µm in length, and with a low G + C content. Taxonomically, it is divided into six species (i.e. *Listeria monocytogenes*, *Listeria ivanovii*, *Listeria seeligeri*, *Listeria innocua*, *Listeria welshimeri* and *Listeria grayi*), of which only *L. monocytogenes* and *L. ivanovii* are pathogenic (Robinson *et al.*, 2000). While *L. monocytogenes* infects both man and animals, *L. ivanovii* (previously known as *L. monocytogenes* serotype 5) is principally an animal pathogen that rarely occurs in man (Low & Donachie, 1997). Being tolerant to extreme pH, temperature and salt conditions (Sleator *et al.*, 2003; Liu *et al.*, 2005a), *Listeria* species are present in a variety of environments, including soil, water, effluents and foods. With manufactured ready-to-eat foods being consumed in increasing quantities, it is no surprise that *L. monocytogenes* has become recognized as an important opportunistic human foodborne pathogen.

Although *L. monocytogenes* is infective to all human population groups, it has a propensity to cause especially severe problems in pregnant women, neonates, the elderly, and immunosuppressed individuals. During the early stages of infection, human listeriosis often displays non-specific flu-like symptoms (e.g. chills, fatigue, headache, and

muscular and joint pain) and gastroenteritis. However, without appropriate antibiotic treatment, it can develop into septicaemia, meningitis, encephalitis, abortion and, in some cases, death (Vazquez-Boland *et al.*, 2001). Indeed, with mortality rates on average approaching 30%, *L. monocytogenes* far exceeds other common foodborne pathogens, such as *Salmonella enteritidis* (with a mortality of 0.38%), *Campylobacter* species (0.02–0.1%) and *Vibrio* species (0.005–0.01%) in terms of disease severity (Altekruse *et al.*, 1997; Mead *et al.*, 1999).

Given the close morphological and biochemical resemblances of *L. monocytogenes* to other *Listeria* species, and the non-specific clinical manifestations of listeriosis (Vazquez-Boland *et al.*, 2001), the availability of rapid, specific and sensitive diagnostic tests capable of distinguishing *L. monocytogenes* from other *Listeria* species is essential for the effective control of the disease. In addition, with *L. monocytogenes* comprising a diversity of strains (Kathariou, 2002), the development of subtyping procedures is critical to the epidemiologic investigation of listeriosis outbreaks. Furthermore, since *L. monocytogenes* demonstrates strain variations in virulence and pathogenicity (Liu *et al.*, 2003a; Roche *et al.*, 2003), the ability to determine accurately and rapidly the pathogenic potential of *L. monocytogenes* isolates is essential to limit the spread of listeriosis and reduce unnecessary recalls of food products.

Extensive research in recent decades has led to significant insights regarding *Listeria* species and listeriosis (Vazquez-Boland *et al.*, 2001). The establishment of animal models and *in vitro* cell culture systems for listeriosis has helped the delineation of key stages in *L. monocytogenes* infection and pathogenesis. The application of molecular techniques has facilitated the identification and characterization of major virulence-associated genes and proteins in *L. monocytogenes*. The development of serological and nucleic-acid-based detection procedures has enhanced the laboratory detection and differentiation of *Listeria* species. Since in-depth reviews on the epidemiology, pathogenesis and virulence determinants of *L. monocytogenes* are available (Low & Donachie, 1997; Vazquez-Boland *et al.*, 2001; Kathariou, 2002), this communication focuses on recent progress in the species-specific identification, subtyping and virulence determination of *L. monocytogenes*, and discusses future research needs in these areas.

Molecular characteristics

L. monocytogenes is a remarkable bacterium that has evolved over a long period to acquire a diverse collection of molecules, each with unique properties and functions, and each contributing to the success of *L. monocytogenes* as an intracellular pathogen. Upon ingestion by the host via contaminated food, *L. monocytogenes* withstands exposure to host proteolytic enzymes, the acidic stomach environment (pH 2.0), bile salts and non-specific inflammatory attacks, largely through the actions of several stress-response genes (*opuCA*, *lmo1421* and *bsh*) and related proteins (Sleator *et al.*, 2003).

Having survived this initial stage, *L. monocytogenes* adheres to and is internalized by host cells with the assistance of a family of surface proteins called internalins (Gaillard *et al.*, 1991). The most notable internalins are InlA and InlB. Whereas InlA (an 88 kDa protein encoded by *inlA*) interacts with E-cadherin to mediate *L. monocytogenes* entry into epithelial cells, InlB (a 65 kDa protein encoded by *inlB*) recognizes C1q-R (or Met) to facilitate *L. monocytogenes* entry into a much broader range of host-cell types, including hepatocytes, fibroblasts and epithelioid cells. Gaining entry to host cells enables *L. monocytogenes* to evade host immune surveillance functions (Vazquez-Boland *et al.*, 2001).

Following its uptake by host cells, *L. monocytogenes* is primarily located in single-membraned vacuoles. Two virulence-associated molecules are responsible for lysis of the primary single-membraned vacuoles and subsequent escape by *L. monocytogenes*: listeriolysin O (LLO) and phosphatidylinositol-phospholipase C (PI-PLC). LLO (a 58 kDa protein encoded by *hly*) is a pore-forming, thiol-activated toxin that is essential for *L. monocytogenes* virulence (Portnoy *et al.*, 1992). PI-PLC (a 33 kDa protein encoded by *plcA*), acting in synergy with phosphatidylcholine-phospholipase C (PC-PLC, a 29 kDa protein encoded by *plcB*), aids LLO in lysing the primary vacuoles (Vazquez-Boland *et al.*, 2001).

After lysis of the primary single-membraned vacuoles, *L. monocytogenes* is released to the cytosol, where it undergoes intracellular growth and multiplication. The intracellular mobility and cell-to-cell spread of *L. monocytogenes* require another surface protein, ActA (a 67 kDa protein encoded by *actA*), which is cotranscribed with PC-PLC and mediates the formation of polarized actin tails that propel the bacteria toward the cytoplasmic membrane. At the membrane, bacteria become enveloped in filopodium-like structures that are recognized and engulfed by adjacent cells, resulting in the formation of secondary double-membraned vacuoles. A successful lysis of the secondary double-membraned vacuoles signals the beginning of a new infection cycle, which is dependent on PC-PLC upon activation by Mpl (a 60 kDa metalloprotease encoded by *mpl*) (Vazquez-Boland *et al.*, 2001).

The genes encoding the virulence-associated proteins PI-PLC, LLO, Mpl, ActA and PC-PLC are located in a 9.6 kb virulence gene cluster (Gouin *et al.*, 1994), which is principally regulated by a pleiotropic virulence regulator, PrfA (a 27 kDa protein encoded by *prfA*). The *prfA* gene is situated immediately downstream of, and sometimes cotranscribed with, the *plcA* gene. PrfA activates the transcription of many *L. monocytogenes* virulence-associated genes. The genes encoding InlA and InlB are positioned elsewhere in the genome. As the *inlA* and *inlB* genes possess a transcription binding site similar to that recognized by PrfA, they may also be partially regulated by PrfA. In addition to these virulence-associated genes and proteins, several other genes, such as *iap* (encoding invasion-associated protein, or Iap), are also involved in *L. monocytogenes* virulence and pathogenicity (Vazquez-Boland *et al.*, 2001).

Species-specific identification

As *L. monocytogenes* is morphologically indistinguishable from other *Listeria* species, and often causes non-specific clinical symptoms, laboratory testing is required to differentiate *L. monocytogenes* from other *Listeria* species, and to diagnose listeriosis. The confirmation of *Listeria* species identity has clinical relevance, that is, the absence of *L. monocytogenes* in clinical specimens may render antibiotic therapy unnecessary, unless immunocompromised patients are involved. The earlier diagnostic methods for *L. monocytogenes* are largely phenotype-based, and characterize the gene products of *L. monocytogenes* through the measurement of biochemical, antigenic and bacteriophage properties. Since these properties may vary with changing external conditions, with growth phase and with spontaneous genetic mutations, the use of phenotypic tests may sometimes lead to equivocal results. Following recent advances in molecular genetic techniques, methods targeting unique genes in *Listeria* have been designed for the specific differentiation of *L. monocytogenes* from other *Listeria* species; these methods are intrinsically more precise and less affected by natural variation than the phenotypic methods.

Biochemical methods

Listeria species are closely related bacteria that share many morphological and biochemical characteristics. Apart from being catalase positive, and indole and oxidase negative, *Listeria* species can hydrolyse aesculin, but not urea. These common biochemical features have frequently been exploited for the differentiation of *Listeria* species from other bacteria. On the other hand, *Listeria* species also possess distinct biochemical properties that can be useful for species-specific identification. For instance, *Listeria* species show significant variations in their ability to haemolyse horse or sheep red blood cells, and in their ability to produce acid from L-rhamnose, D-xylose and α -methyl-D-mannoside (Robinson *et al.*, 2000).

Hence, *L. ivanovii* is differentiated biochemically from *L. monocytogenes* and other *Listeria* species by its production of a wide, clear or double zone of haemolysis on sheep or horse blood agar, a positive Christie–Atkins–Munch–Petersen (CAMP) reaction with *Rhodococcus equi* but not with haemolytic *Staphylococcus aureus*, and fermentation of D-xylose but not L-rhamnose (Rocourt & Catimel, 1985). In addition, *L. ivanovii* is distinguished from *L. monocytogenes* by its strong lecithinase reaction with or without charcoal in the medium, in comparison to *L. monocytogenes*, which requires charcoal for its lecithinase reaction (Ermolaeva *et al.*, 2003). Similarly, *L. innocua* is distinguished from *L. monocytogenes* on the basis of its negative CAMP reaction and its failure to cause β -haemolysis or to show PI-PLC activity on chromogenic media. *L. welshimeri* is differentiated from other *Listeria* species by its negative β -haemolysis and CAMP reactions, and by its acid production from D-xylose and α -methyl-D-mannoside (Robinson *et al.*, 2000).

Nevertheless, the identification of *Listeria* species by biochemical methods is a laborious process, involving primary isolation with selective and enrichment media, followed by Gram stain and multiple biochemical tests. For the isolation of *Listeria* that has been injured during food processing, a pre-enrichment in phosphate-buffered broth medium containing inhibitors is also needed. The incorporation of various biochemical procedures into a single testing platform has streamlined the diagnostic process for *Listeria* species (Rocourt & Catimel, 1985; Bille *et al.*, 1992). However, biochemical testing of *Listeria* species remains time-consuming (taking up to 6 days to finalize a result) and costly. Furthermore, as biochemical tests measure the phenotypic characteristics of *Listeria* bacteria, their performance can be influenced by external factors that affect bacterial growth and metabolic mechanisms.

Serological methods

Listeria species possess group-specific surface proteins, such as somatic (O) and flagellar (H) antigens that are useful targets for serological detection with corresponding monoclonal and polyclonal antibodies. While there are 15 *Listeria* somatic (O) antigen subtypes (I–XV), flagellar (H)

antigens comprise four subtypes (A–D) (Seeliger & Höhne, 1979; Seeliger & Jones, 1986), with the serotypes of individual *Listeria* strains being determined by their unique combinations of O and H antigens (Table 1). Through examination of group-specific *Listeria* O and H antigens in slide agglutination, at least 12 serotypes (i.e. 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e and 7) have been recognized in *L. monocytogenes*, several (e.g. 1/2a, 1/2b, 3b, 4a, 4b, 4c and 6b) in *L. seeligeri*, one (i.e. 5) in *L. ivanovii*, and a few (e.g. 1/2b, 6a and 6b) in *L. innocua*, *L. welshimeri* and *L. grayi* (Seeliger & Jones, 1986; Kathariou, 2002). Since slide agglutination is not easily adapted for high-throughput testing, an ELISA has recently been developed to improve efficiency (Palumbo *et al.*, 2003).

Besides their value for the differentiation of *Listeria* species, serotyping methods are also potentially useful for defining subtypes and clonal groups of *L. monocytogenes*. Indeed, it has been observed that *L. monocytogenes* serotypes 1/2a, 1/2b and 4b are responsible for 98% of documented human listeriosis cases, whereas serotypes 4a and 4c are rarely associated with outbreaks of the disease (Wiedmann *et al.*, 1996; Jacquet *et al.*, 2002). Furthermore, while *L. monocytogenes* serotype 4b strains are isolated mostly from epidemic outbreaks of listeriosis, serotypes 1/2a and 1/2b are linked to sporadic *L. monocytogenes* infection (Wiedmann *et al.*, 1996). On a note of veterinary relevance, *L. monocytogenes* isolates from sheep encephalitis are usually of serotypes 1/2b or 4b, and those from septicaemia and abortion cases are predominantly of serotype 1/2a (Low & Donachie, 1997).

However, due mainly to the high cost of acquiring subtype-specific antisera, serotyping methods are not routinely

Table 1. Compositions of somatic (O) and flagellar (H) antigens in *Listeria* serotypes

Based on Seeliger & Jones (1986).

Serotype	O antigens	H antigens
1/2a	I, II	A, B
1/2b	I, II	A, B, C
1/2c	I, II	B, D
3a	II, IV	A, B
3b	II, IV	A, B, C
3c	II, IV	B, D
4a	(V), VII, IX	A, B, C
4b	V, VI	A, B, C
4c	V, VII	A, B, C
4d	(V), VI, VIII	A, B, C
4e	V, VI, (VIII), (IX)	A, B, C
7	XII, XIII	A, B, C
5	(V), VI, (VIII), X	A, B, C
6a	V, (VI), (VII), (IX), XV	A, B, C
6b	(V), (VI), (VII), IX, X, XI	A, B, C

performed in clinical laboratories. With both *L. monocytogenes* and *L. seeligeri* containing serotypes 1/2a, 1/2b, 3b, 4a, 4b, 4c and 6b, the inability of serotyping methods to correlate serotypes directly with species identities further limits their potential for widespread clinical application. Moreover, as antigen sharing occurs frequently among various *L. monocytogenes* serotypes, with 1/2a and 3c both containing H antigens A and B; 4a–d, 1/2b and 3b all having H antigens A, B and C; 1/2c and 3a both possessing H antigens B and D; and multiple, common O antigens being present in different serotypes (Table 1), it can be a challenge to conclusively determine the serotype of some *L. monocytogenes* strains (Schonberg *et al.*, 1996; Liu *et al.*, 2006a). Like biochemical methods, serotyping methods are also liable to give occasional discrepant results because of their dependence on the phenotypic characteristics of *Listeria* bacteria. For these reasons, serotyping methods have largely been superseded by molecular procedures that are intrinsically more specific and sensitive for the identification and differentiation of *Listeria* species.

Molecular methods

Detection by gene probes. A gene probe is a stretch of specific single-stranded nucleic acid that is enzyme- or radiolabelled and employed for the identification and detection of a (usually membrane-bound) complementary nucleic acid sequence in a target organism. Being a first-generation nucleic acid detection technology, the detection of *L. monocytogenes* by gene probes is precise and relatively straightforward. *Listeria* DNA is spotted onto a supporting matrix (e.g. a nitrocellulose filter or nylon membrane), hybridized with an enzyme- or radiolabelled *Listeria* species-specific gene probe (derived from 16S rRNA, heat-shock protein P60 or other protein-coding gene), and subsequently detected with an appropriate substrate (enzyme label) or by autoradiography (radiolabel) (Klinger *et al.*, 1988; Kohler *et al.*, 1990). As this procedure exploits differences among *Listeria* species at the genetic level, it is more specific than biochemical and serological methods that are phenotype based. However, since it does not involve nucleic acid amplification, this technique has limited sensitivity, requiring at least 10^4 copies of target gene per microlitre for reliable detection without signal amplification, although improving to as few as 500 copies of target gene per microlitre with signal amplification; this is inadequate for most clinical samples. Since the introduction of nucleic acid amplification technology, the employment of gene-probe-based detection procedures has steadily declined.

Detection by nucleic acid amplification. *In vitro* amplification of nucleic acid is a more recent addition to the genetic detection methods for pathogen identification and diagnosis. Among several elegant approaches to nucleic acid amplification, PCR was the first and remains the most widely applied technique in both research and clinical laboratories. PCR employs two primers (usually 20–30 nucleotides long) that flank the beginning and end of a

specific DNA target, a thermostable DNA polymerase that is capable of synthesizing the specific DNA, and double-stranded DNA to function as a template for DNA polymerase. The PCR process begins at a high temperature (e.g. 94 °C) to denature and open the double-stranded DNA template into single-stranded DNA, followed by a relatively low temperature (e.g. 54 °C) to enable annealing between the single-stranded primer and the single-stranded template, and then a temperature of 72 °C to allow DNA polymerase copying (extension) of the template. The whole process is repeated 25–30 times so that a single copy of DNA template can turn into billions of copies within 3–4 h. As PCR has the ability to selectively amplify specific targets present in low concentrations (theoretically down to a single copy of DNA template), it offers exquisite specificity, unsurpassed sensitivity, rapid turnover, and ease of automation for laboratory detection of *L. monocytogenes* from clinical specimens, in addition to its value for identifying both cultured and non-cultivable organisms. The amplified DNA products can be separated by agarose gel electrophoresis and detected with a DNA stain, or alternatively detected via labelled probes, DNA sequencing, microarray and other related techniques (Wang *et al.*, 1993; Manzano *et al.*, 2000; Volokhov *et al.*, 2002). By exploiting molecular differences within 16S and 23S rRNA genes, intergenic spacer regions, *hly*, *inlA*, *inlB*, *iap* and other genes (e.g. delayed-type hypersensitivity gene, aminopeptidase gene and putative transcriptional regulator gene *lmo0733*), *L. monocytogenes* is rapidly and precisely differentiated from other *Listeria* species and common bacteria (Table 2) (Aznar & Alarcon, 2002).

The application of a multiplex PCR assay to selectively amplify a shared *iap* gene facilitates the differentiation of all six *Listeria* species in a single test (Bubert *et al.*, 1999). However, with assays relying on the selective amplification of a shared gene, extreme care is required to ascertain the sizes of the amplified products, which may show minute size differences among various *Listeria* species. For this reason, targeting *Listeria* genes unique to individual species is beneficial, as it provides an independent means of confirming the species identities (Gilot & Content, 2002; Liu *et al.*, 2003b, 2004b, 2004c, 2004d, 2005b). In cases where the co-presence of several *Listeria* species complicates the identification of *L. monocytogenes*, the availability of PCR assays for unique species-specific genes is desirable to help clarify the issue. The development of PCR-based serotyping procedures, such as the use of group-specific PCR primers, has provided additional tools for the identification and grouping of *L. monocytogenes* (Jinneman & Hill, 2001; Borucki & Call, 2003; Doumith *et al.*, 2004a). The adaptation of conventional PCR to the reverse transcription PCR (RT-PCR) format also permits the detection of viable *L. monocytogenes* organisms in specimens. Finally, by coupling PCR to the DNA sequencing analysis of *Listeria* 16S rRNA genes, it has been possible to further enhance the genetic speciation and phylogenetic study of *Listeria* bacteria.

Table 2. Identification of *Listeria* species by PCR-based procedures

Species	Target gene	Protein	Reference
<i>L. monocytogenes</i>	16S rRNA gene		Wang <i>et al.</i> (1992); Manzano <i>et al.</i> (2000)
	23S rRNA gene		Sallen <i>et al.</i> (1996); Paillard <i>et al.</i> (2003)
	16S/23S rRNA intergenic regions		Graham <i>et al.</i> (1996, 1997)
	<i>hly</i>	LLO	Furrer <i>et al.</i> (1991); Blais <i>et al.</i> (1997)
	<i>plcA</i>	PI-PLC	Volokhov <i>et al.</i> (2002)
	<i>plcB</i>	PC-PLC	Volokhov <i>et al.</i> (2002)
	<i>actA</i>	ActA	Longhi <i>et al.</i> (2003)
	<i>prfA</i>	Transcriptional regulator PrfA	Wernars <i>et al.</i> (1992)
	<i>inlA</i>	Internalin A	Poyart <i>et al.</i> (1996); Jung <i>et al.</i> (2003)
	<i>inlB</i>	Internalin B	Pangallo <i>et al.</i> (2001); Jung <i>et al.</i> (2003)
	<i>iap</i>	Invasion associated protein	Bubert <i>et al.</i> (1992, 1999)
	<i>lma/dth18</i>	LmA antigen/delayed-type hypersensitivity protein	Johnson <i>et al.</i> (1992)
	<i>fbp</i>	Fibronectin-binding protein	Gilot & Content (2002)
	<i>flaA</i>	Flagellin A	Gray & Kroll (1995)
	<i>pepC</i>	Aminopeptidase C	Winters <i>et al.</i> (1999)
	<i>clpE</i>	Clp ATPase	Volokhov <i>et al.</i> (2002)
<i>lmo0733</i>	Putative transcriptional regulator	Liu <i>et al.</i> (2004a)	
<i>L. ivanovii</i>	16S rRNA gene		Wang <i>et al.</i> (1993); Sallen <i>et al.</i> (1996); Manzano <i>et al.</i> (2000)
	23S rRNA gene		Sallen <i>et al.</i> (1996); Paillard <i>et al.</i> (2003)
	16S/23S rRNA intergenic regions		Graham <i>et al.</i> (1997)
	<i>iap</i>	Invasion associated protein	Bubert <i>et al.</i> (1992, 1999)
<i>liv22-228</i>	Putative <i>N</i> -acetylmuramidase	Liu <i>et al.</i> (2004c)	
<i>L. seeligeri</i>	16S rRNA gene		Sallen <i>et al.</i> (1996); Manzano <i>et al.</i> (2000)
	23S rRNA gene		Sallen <i>et al.</i> (1996); Paillard <i>et al.</i> (2003)
	16S/23S rRNA intergenic regions		Graham <i>et al.</i> (1997)
<i>L. innocua</i>	<i>iap</i>	Invasion associated protein	Bubert <i>et al.</i> (1992, 1999)
	<i>lse24-315</i>	Putative internalin	Liu <i>et al.</i> (2004d)
	16S rRNA gene		Manzano <i>et al.</i> (2000)
<i>L. welshimeri</i>	23S rRNA gene		Sallen <i>et al.</i> (1996); Paillard <i>et al.</i> (2003)
	16S/23S rRNA intergenic regions		Graham <i>et al.</i> (1997)
	<i>iap</i>	Invasion associated protein	Bubert <i>et al.</i> (1992, 1999)
	<i>lin0464</i>	Putative transcriptional regulator	Liu <i>et al.</i> (2003a)
	<i>lin2483</i>	Putative transporter	Rodríguez-Lázaro <i>et al.</i> (2004)
<i>L. grayi</i>	16S rRNA gene		Sallen <i>et al.</i> (1996); Manzano <i>et al.</i> (2000)
	23S rRNA gene		Paillard <i>et al.</i> (2003)
	16S/23S rRNA intergenic regions		Graham <i>et al.</i> (1997)
	<i>fbp</i>	Fibronectin-binding protein	Gilot & Content (2002)
<i>iap</i>	Invasion associated protein	Bubert <i>et al.</i> (1992, 1999)	
<i>lwe7-571</i>	Putative phosphotransferase system enzyme IIBC	Liu <i>et al.</i> (2004b)	
<i>L. grayi</i>	16S rRNA gene		Sallen <i>et al.</i> (1996); Manzano <i>et al.</i> (2000)
	23S rRNA gene		Paillard <i>et al.</i> (2003)
	16S/23S rRNA intergenic regions		Graham <i>et al.</i> (1997)
<i>iap</i>	Invasion associated protein	Bubert <i>et al.</i> (1992, 1999)	
<i>lgr20-246</i>	Putative oxidoreductase	Liu <i>et al.</i> (2005b)	

Subtyping

With *L. monocytogenes* consisting of a diversity of strains, the availability of subtyping procedures to track individual strains involved in listeriosis outbreaks, and to examine the epidemiology and population genetics of *L. monocytogenes* bacteria, is integral to control and prevention programmes aimed at listeriosis. Similar to species-specific identification, two major subtyping approaches are in common use: phenotypic and genetic (molecular or DNA) subtyping. The phenotypic subtyping approach includes serotyping, phage typing, multilocus enzyme electrophoresis (MLEE) and esterase typing. The genetic subtyping approach encompasses pulsed-field gel electrophoresis (PFGE), ribotyping, PCR-based subtyping techniques [e.g. random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), PCR-restriction fragment length polymorphism (PCR-RFLP) and repetitive element PCR (REP-PCR)] and DNA sequencing-based subtyping techniques [e.g. multilocus sequence typing (MLST)]. While the phenotypic subtyping approach occasionally suffers from low discrimination and reproducibility, the genetic subtyping approach is highly sensitive, discriminatory and reproducible. For improved subtyping discrimination, a combination of two or more subtyping techniques, be they gene or phenotype based, is often used in practice for epidemiologic investigation of *L. monocytogenes* outbreaks.

Phenotypic subtyping methods

Serotyping. By separating *L. monocytogenes* strains into 12 serotypes (i.e. 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e and 7) on the basis of serological reactions between somatic (O)/flagellar (H) antigens and their corresponding antisera (Table 1) (Seeliger & Höhne, 1979; Seeliger & Jones, 1986), serotyping may potentially be useful for tracking *L. monocytogenes* strains involved in disease outbreaks. Given that only three serotypes (1/2a, 1/2b and 4b) are commonly associated with human listeriosis, however, the value of serotyping in *L. monocytogenes* epidemiological investigations is somewhat limited. In addition, the inability of the serotyping procedures to give a fine distinction among serotypes 4a, 4b and 4c has further hampered their potential utility (Liu *et al.*, 2006a). In fact, whereas four serotype 4b strains belonging to lineage I strains as evaluated by *prfA* virulence gene cluster sequences reacted in PCR with serotypes 4b-, 4d- and 4c-specific ORF2110 virulence-specific *lmo1134* and *lmo2821* primers, all nine serotype 4b strains belonging to lineage III strains were negative by ORF2110 and *lmo1134* primers (Ward *et al.*, 2004). Based on their differential reactions in PCR and Southern blot, the four serotype 4b lineage I strains are unquestionably of serotype 4b; however, seven of the nine serotype 4b lineage III strains appear to be of serotype 4c and the other two of serotype 4a (Liu *et al.*, 2006a). Therefore, the serotyping procedure often plays an accessory role in the subtyping and tracking of *L. monocytogenes* epidemic strains.

Phage typing. Bacteriophages are viruses that occur naturally in *Listeria* and other bacteria. Being host specific, bacteriophages have the capacity to lyse closely related *Listeria* bacteria independently of the bacterial species and serovar identities. Through examination of bacteriophage-induced, host-specific lysis of *Listeria* bacteria on agar plates, *Listeria* strains can be separated into distinct phage groups and phagovars, which are useful for tracking the origin and course of listeriosis outbreaks (Audurier *et al.*, 1984). In a phage-typing study involving 16 selected phages, 57 *Listeria* reference strains and 454 food isolates, *Listeria* strains were classified into four phage groups, which in turn were divided into 41 distinct phagovars. On the basis of a *Listeria* strain being lysed by at least one phage at a 100 × routine test dilution, an overall typability of 84.5% was obtained (Loessner & Busse, 1990). By increasing the number of bacteriophages to 21, a typability of 89.5% was noted after analysis of 1087 *Listeria* strains (Loessner, 1991). Nonetheless, with close to 10% of *Listeria* strains being untypable (especially serovar 3 and *L. grayi* strains), the usefulness of phage typing as an independent tool for epidemiological investigations is severely constrained.

MLEE. MLEE is a protein-based, isoenzyme typing method that correlates specific protein band patterns with genotypes. For this method, soluble proteins (or bacterial lysates) from *L. monocytogenes* strains are separated by starch gel electrophoresis, polyacrylamide gel electrophoresis (PAGE), or isoelectrophoretic focusing under native conditions, followed by visualization with specific enzyme stains. Variations in the electrophoretic mobility of different enzymes (or electrophoretic types, ETs) enable differentiation of *L. monocytogenes* strains. Since multiple enzymes are present in *L. monocytogenes*, numerous ETs are often obtained. For instance, assessment of 305 *L. monocytogenes* strains by MLEE resulted in the detection of 78 ETs (Graves *et al.*, 1994). Based on the similar ETs detected in MLEE, *L. monocytogenes* serovars 1/2b, 3b and 4b are classified into one distinct division, and serovars 1/2a, 1/2c and 3a in another division (Bibb *et al.*, 1989; Piffaretti *et al.*, 1989). The detection of a large number of electrophoretic types in *L. monocytogenes* strains by MLEE necessitates careful optimization and standardization of the test procedure so that run-to-run variations are minimized.

Esterase typing. Esterases are a class of heat-stable enzymes that hydrolyse carboxylic acid esters. Being a variant of MLEE analysis, esterase typing measures the esterase activity from cell extracts of individual *L. monocytogenes* strains on starch gels following electrophoresis. Upon examination by esterase typing of 219 *L. monocytogenes* isolates from milk, non-dairy foods, and clinical and veterinary sources, Harvey & Gilmour (1996) detected 59 ETs. Like MLEE, esterase typing produces a high number of ETs that require careful documentation and standardization. Furthermore, as a phenotype-based procedure, the reproducibility of esterase typing is sometimes low.

Genetic subtyping methods

PFGE. PFGE is a highly reproducible, discriminatory and effective molecular typing method that is based on restriction fragment length polymorphisms (RFLPs) of bacterial DNA. In RFLP analysis, bacterial genomic DNA is digested with restriction enzymes to yield hundreds of fragments, which are then separated by conventional agarose gel electrophoresis to form distinctive banding patterns for individual strains. Given its complex band patterns, however, the interpretation of RFLP results is notably tedious and technically demanding. PFGE uses selected restriction enzymes to yield between 8 and 25 large DNA bands of 40–600 kb in size, and alternating currents to cause DNA fragments to move back and forth, resulting in a higher level of fragment resolution. For this method, *L. monocytogenes* bacteria are first placed in agarose plugs, where they are lysed, and the DNA is then digested with selected restriction enzymes. The plugs containing the digested DNA are transferred into an agarose gel and electrophoresed for 30–50 h with alternating currents. On the basis of distinct DNA band patterns, PFGE classifies *L. monocytogenes* into subtypes (or pulsotypes), providing sensitive subtype discrimination that is considered the reference standard (Brosch *et al.*, 1994, 1996; Graves *et al.*, 1994). Indeed, after a comparative examination of 35 *L. monocytogenes* strains by serotyping, esterase typing, ribotyping, RAPD and PFGE, PFGE along with ribotyping produced the most discriminatory outcomes for *L. monocytogenes* (Kerouanton *et al.*, 1998). However, due to its time-consuming nature (taking 30 h or longer to perform) and its requirement for special equipment, PFGE is not widely used outside reference laboratories.

Ribotyping. Ribotyping is a derivative of RFLP analysis that uses a ribosomal DNA (rDNA) probe to detect the restriction fragment patterns of chromosomal DNA digested with appropriate restriction enzymes, resulting in much simpler and more consistent band patterns. For this method, *Listeria* DNA is initially digested with restriction enzymes (e.g. *EcoRI*, *PvuII* and *XhoI*) into many pieces (>300–500) of small-sized fragments (1–30 kb). The resultant DNA fragments are then separated by agarose gel electrophoresis, transferred to a membrane (via Southern blot), and detected with a probe derived from the *Escherichia coli* gene that encodes rRNA (rDNA). Thus, only DNA fragments that contain rRNA genes are recognized (Graves *et al.*, 1994). In a study involving 1346 *L. monocytogenes* strains, Bruce *et al.* (1995) showed that 50 band patterns can be detected after digestion of *L. monocytogenes* DNA with *EcoRI* and detection with the *E. coli* *rrnB* rRNA operon.

On the whole, ribotyping is a robust, reproducible typing technique that has a similar discriminatory power to, but produces fewer bands than, PFGE. As ribotyping does not demand special equipment, it has become a practical tool that has been frequently employed for the tracking and subtyping of *L. monocytogenes* bacteria. Indeed, with an

automated ribotyping test becoming available commercially (RiboPrinter Microbial Characterization System, Qualicon, Wilmington, DE), it is envisaged that ribotyping will play an increasingly dominant role in the epidemiologic investigation of disease due to *L. monocytogenes*.

PCR-based subtyping techniques

RAPD and arbitrarily primed PCR (AP-PCR). Both RAPD and AP-PCR use low-stringency PCR amplification with a single primer of an arbitrary sequence to generate strain-specific arrays of anonymous DNA fragments. For the method, a single short random primer (usually 10 bases long for AP-PCR, and 10–15 bases long for RAPD) is used in PCR at a relatively low temperature (around 36 °C) to generate amplified products from *L. monocytogenes* DNA that form distinct band patterns after agarose gel electrophoresis. The possible mechanism behind RAPD and AP-PCR is that by reducing the stringency of the primer-annealing temperature, a random primer that shows no complete homology to a genome may have a perfect match of two to three nucleotides between the 3' end of the primer and the template strand to allow annealing and the priming of complementary strand synthesis by DNA polymerase, given that a putative three-nucleotide sequence can in principle be found once in each 64 nucleotide sequence (4^3 permutations). When two such annealing and priming events occur within a certain distance of each other and in proper orientation, the sequence between the matching sites can be amplified effectively. RAPD (or AP-PCR) is more economical and faster than other typing methods, and is particularly suitable for testing fewer than 50 strains. However, the discriminatory ability of RAPD and AP-PCR is sometimes inconsistent (Farber & Addison, 1994; O'Donoghue *et al.*, 1995). In a recent study, RAPD gave less robust results than PCR ribotyping for subtyping *L. monocytogenes* isolates involved in invasive and non-invasive listeriosis outbreaks (Franciosa *et al.*, 2001).

AFLP. AFLP is a modification of RFLP through the addition of adaptors to restriction enzyme-digested DNA, followed by PCR amplification and electrophoretic separation of PCR products. In this procedure, *L. monocytogenes* DNA is digested with two restriction enzymes, one with an average cutting frequency (e.g. *EcoRI*) and one with a higher cutting frequency (e.g. *MseI* or *TaqI*). The restriction fragments are ligated with double-stranded oligonucleotide adaptors (i.e. linkers and indexers), and then amplified by PCR with adapter-specific primers. The resultant PCR products are separated by PAGE to generate highly informative, polymorphic patterns of 40–200 bands for individual *L. monocytogenes* strains. Apart from differentiating among the *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri* and *L. grayi* species, AFLP is also useful for separating *L. monocytogenes* strains into different genotypes. Overall, AFLP is highly discriminatory, sensitive and reproducible, thus representing a valuable tool in the characterization of *L. monocytogenes* strains,

and also in the identification of *Listeria* species (Ripabelli *et al.*, 2000; Guerra *et al.*, 2002; Keto-Timonen *et al.*, 2003). An obvious shortcoming of AFLP is its requirement for the ligation of linkers and indexers to enzyme-digested DNA from individual strains, which apart from being another time-consuming step, adds an extra dimension of uncertainty to the testing procedure.

PCR-RFLP. In contrast to AFLP, in which *L. monocytogenes* DNA is digested with restriction enzymes, linked with adaptors to facilitate PCR amplification, PCR-RFLP undertakes PCR amplification of one or more *L. monocytogenes* housekeeping or virulence-associated genes (e.g. *hly*, *actA* and *inlA*), followed by digestion with selected restriction enzymes (e.g. *HhaI*, *SacI* or *HinfI*) and separation by agarose gel electrophoresis. Subsequent examination of the distinct band patterns permits differentiation of *L. monocytogenes* subtypes (Wiedmann *et al.*, 1997). As it involves nucleic acid amplification, PCR-RFLP requires only a small amount of starting DNA. It has the added advantage over AFLP of obviating the need to ligate linkers and indexers before PCR amplification. Used in combination with other subtyping procedures, PCR-RFLP provides a sensitive, discriminatory and reproducible method for tracking and epidemiological investigation of *L. monocytogenes* bacteria.

REP-PCR. Like other prokaryotic organisms, *L. monocytogenes* possesses a genome that contains randomly dispersed, repetitive sequence elements, such as repetitive extragenic palindromes (REPs) of 35–40 bp with an inverted repeat, and intergenic repeat units or enterobacterial repetitive intergenic consensus sequences (ERICs) of 124–147 bp with a highly conserved central inverted repeat. The REP and ERIC sequences represent useful primer binding sites for PCR amplification of the *L. monocytogenes* genome to achieve species and strain discrimination. Using REP-PCR, *L. monocytogenes* strains have been divided into four clusters that match the origin of isolation, each consisting of multiple subtypes (Jersek *et al.*, 1999). As it produces a similar level of discrimination to PFGE and ribotyping techniques, REP-PCR offers a valuable alternative for the rapid subtyping of *L. monocytogenes* strains.

DNA sequencing-based subtyping techniques. DNA sequencing of one or more selected genes is increasingly used for genetic subtyping of *L. monocytogenes*. MLST focuses on multiple genes or gene fragments (e.g. housekeeping or virulence-associated genes) to determine the subtypes and genetic relatedness of *L. monocytogenes* isolates. The availability of DNA sequencing data also aids the reconstruction of ancestral and evolutionary relationships among *L. monocytogenes* isolates. Compared to other typing methods, such as PFGE and ribotyping, MLST is less ambiguous and easier to interpret (Ward *et al.*, 2004). With the cost of DNA sequencing decreasing rapidly, MLST is poised to play a more important role in *L. monocytogenes* subtyping and phylogenetic studies.

Although each of the subtyping procedures above represents on its own an elegant approach to the tracking of *L. monocytogenes* strains, the combined use of two or more procedures is generally more discriminatory and powerful than each applied alone. In terms of technical simplicity and test reliability, ribotyping and PCR-RFLP stand out clearly. Indeed, by using ribotyping together with PCR-RFLP and other subtyping procedures, *L. monocytogenes* strains have been grouped into three genetic lineages (or divisions), with lineage I consisting of serotypes 1/2b, 3b, 4b, 4d and 4e; lineage II of serotypes 1/2a, 1/2c, 3a and 3c; and lineage III of serotypes 4a and 4c (Rasmussen *et al.*, 1995; Wiedmann *et al.*, 1997, 2002; Jeffers *et al.*, 2001; Nadon *et al.*, 2001; Gray *et al.*, 2004; Meinersmann *et al.*, 2004). Interestingly, *L. monocytogenes* isolates from sporadic and endemic human listeriosis mostly belong to lineages I and II, whereas those from animal and environmental specimens are of lineage III. This information has been invaluable for tracking and population genetic studies of *L. monocytogenes* strains involved in disease outbreaks.

While a general consensus on the compositions and divisions of lineages I and II exists, there is some uncertainty concerning the make-up and taxonomic status of lineage III. Within lineage III, three subsets (i.e. G8.1, H7.1; G5.8, H7.1; and E/G5.8, H7.1) are delineated through Southern blot analysis of *EcoRI*-digested *L. monocytogenes* DNA with a probe from the *rrnB* rRNA operon of *E. coli* (Bruce *et al.*, 1995). In turn, the subset G8.1, H7.1 is subdivided into seven ribotypes (dd0648, dd11903, dd8842, dd0652, dd11696, dd11698 and dd12388) by ribotyping *hly*, *inlA* and *actA* polymorphisms, with ribotype dd0648 being represented by ATCC 19114 (Wiedmann *et al.*, 1997). On the other hand, while the subset G5.8, H7.1 comprises a single ribotype, dd3823, the subset E/G5.8, H7.1 includes two ribotypes, dd6821 and dd6824. Since the ribotype dd0648 strain ATCC 19114 in the subset G8.1, H7.1 demonstrates only a 72 % DNA–DNA homology with the *L. monocytogenes* type strain (barely above the 70 % DNA–DNA relatedness required for the phylogenetic definition of a species) and a 54 % homology with the *L. innocua* type strain, it has been postulated that ATCC 19114 is a new subspecies (Wiedmann *et al.*, 1997). In addition, as strains in the subsets G5.8, H7.1 and E/G5.8, H7.1 possessing *inlA* and the G5.8 fragment are rhamnose negative, in contrast to the *L. monocytogenes* type strain, which is rhamnose positive, the existence of either two new subspecies or a new species has been also hypothesized (Wiedmann *et al.*, 1996, 1997).

After the recent examination of seven lineage III strains from subsets G8.1, H7.1, G5.8, H7.1 and E/G5.8, H7.1, as well as ribotypes DUP1061 and DUP10142, by using PCR, Southern blot and DNA sequencing techniques, the following have become apparent: (a) ribotype dd0648 strains (ATCC 19114 and FSL-J1-031) in the subset G8.1, H7.1, and ribotype dd3823 strain (FSL-X1-008) in the subset G5.8, H7.1, as well as ribotype DUP1061 strain (FSL-J1-168), are clearly of *L. monocytogenes* serotypes 4a

and 4c; (b) a close genetic relationship exists among three serotype 4c strains of ribotype dd0648 (FSL-J1-031) in the subset G8.1, H7.1, ribotype dd3823 (FSL-X1-008) in the subset G5.8, H7.1 and ribotype DUP1061 (FSL-J1-168) in comparison with the serotype 4a strain from ribotype dd0648 (ATCC 19114) in the subset G8.1, H7.1; (c) being rhamnose negative and possessing *inlA*, *lmo2672* and ORF2819, strains of ribotypes dd6821 (FSL-X1-009) and dd6824 (FSL-X1-010) in the subset E/G5.8, H7.1 are likely of serotype 7, which may represent a distinct genotype (or subspecies) within the species of *L. monocytogenes*; and (d) ribotype DUP10142 strain (FSL-J1-158), being related to ribotypes dd3823 (subset G5.8, H7.1), dd6821 and dd6824 (subset E/G5.8, H7.1) through a shared reaction with *lmo2672* primers, may constitute part of the genotype that encompasses ribotypes dd6821 and dd6824. Alternatively, because of its negative reaction with ORF2819 primers, ribotype DUP10142 strain (FSL-J1-158) may form another genotype (or subspecies) distinct from the one that covers ribotypes dd6821 and dd6824, which does not fit into the current serotype scheme for *L. monocytogenes* (Liu *et al.*, 2006b).

In addition, the transcriptional regulator *lmo2672* gene (Liu *et al.*, 2004a) has also proven valuable as a target for the specific identification of uncommon *L. monocytogenes* lineage III strains [e.g. ribotypes dd6821 (FSL-X1-009), dd6824 (FSL-X1-010) and DUP10142 (FSL-J1-158)], as these strains are undetected by the species-specific *lmo0733* primers (Liu *et al.*, 2003a). Therefore, a combination of *lmo0733* and *lmo2672* primers in a multiplex PCR format will facilitate the detection of all *L. monocytogenes* strains, including the rare lineage III isolates, in a rapid, sensitive and specific manner (Liu *et al.*, 2006b).

Virulence determination

Despite being pathogenic at the species level, *L. monocytogenes* is in fact made up of a spectrum of strains or genotypes with varying pathogenic potential. While many *L. monocytogenes* strains are highly pathogenic and sometimes deadly, others are relatively avirulent and cause little harm in the host. The availability of laboratory methods to accurately assess the pathogenic potential of *L. monocytogenes* strains is therefore vitally important to the effective control and prevention of listeriosis. Over the years, a variety of methods have been developed to gauge the virulence of *L. monocytogenes* strains. These include the mouse virulence assay, *in vitro* cell assays, and the detection of virulence-associated proteins and genes. These methods have not only contributed to the improved understanding of *L. monocytogenes* virulence and pathogenicity, but also helped devise appropriate control measures against listeriosis.

Mouse virulence assay

The mouse virulence assay was one of the first methods described for *L. monocytogenes* virulence assessment. Being capable of providing an *in vivo* measurement of all virulent determinants, the mouse virulence assay is regarded as the

gold standard for any newly developed tests for *L. monocytogenes* virulence (Nishibori *et al.*, 1995; Pine *et al.*, 1991; Roche *et al.*, 2001; Liu *et al.*, 2003a). In general, the mouse virulence assay is conducted by inoculating groups of mice with various doses of *L. monocytogenes* bacteria via the oral, nasal, intraperitoneal, intravenous or subcutaneous routes. The virulence of a given *L. monocytogenes* strain is determined by the mouse mortality resulting from infection, after estimation of c.f.u. by plate counts, and is commonly expressed as median lethal dose (LD₅₀) (Reed & Muench, 1938; Welkos & O'Brien, 1994). Alternatively, the virulence of a given *L. monocytogenes* strain can be determined by the number of *L. monocytogenes* bacteria that reach the spleen following experimental infection.

Although an essential step in LD₅₀ calculation, the estimation of c.f.u. by plate counts is a task requiring considerable attention and consistency, as it has a narrow margin of error. The observation that *L. monocytogenes* strains with varying levels of virulence often display vastly different growth rates on many selective media further exacerbates the problem (Gracieux *et al.*, 2003). Even on a non-selective medium (e.g. BHI agar), *L. monocytogenes* strains causing greater mouse mortality tend to yield higher numbers of c.f.u. than those causing less or no mouse mortality (Liu, 2004). Thus, the number of c.f.u. for a given *L. monocytogenes* strain may vary from run to run and from laboratory to laboratory, resulting in different LD₅₀ values for an identical test strain.

Recently, relative virulence (%) has been described as an alternative to LD₅₀ measurement for the practical and direct interpretation of the mouse virulence assay for *L. monocytogenes* (Liu, 2004). The relative virulence (%) is obtained by dividing the number of dead mice by the total number of mice tested for a particular strain, using a known virulent strain (e.g. *L. monocytogenes* EGD) as reference. Being independent of c.f.u. estimation, the relative virulence (%) requires fewer dosage groups, and appears to give a more accurate assessment of *L. monocytogenes* virulence. That is, while the LD₅₀ values provide a somewhat imprecise measure of *L. monocytogenes* virulence, the relative virulence (%) is much more direct and precise (Table 3). Nonetheless, given the high cost associated with animal experimentation, the mouse virulence assay is not routinely used for determining *L. monocytogenes* virulence.

In vitro cell assays

In vitro cell culture techniques have been explored as a low-cost alternative to the mouse virulence assay for assessing *L. monocytogenes* virulence. These methods measure the ability of *L. monocytogenes* to cause cytopathogenic effects in the enterocyte-like cell line Caco-2 (Pine *et al.*, 1991), to form plaques in the human adenocarcinoma cell line HT-29 (Roche *et al.*, 2001), or to cause death in chicken embryos (Olier *et al.*, 2002). Several other cell lines (e.g. hepatocyte Hep-G2, macrophage-like J774, epithelial Henle 407 and L2) have also been employed in various protocols to investigate *L. monocytogenes* ability to adhere, invade, escape

Table 3. Relative virulence of *L. monocytogenes* serotypes

ND, Not done; +, positive; -, negative.

Strain	Source	Serovar	<i>lmo2821</i> PCR*	LD ₅₀ †	Relative virulence (%)‡
ATCC 19112	Human	2	+	1.6 × 10 ⁹	30
ATCC 19114	Human	4a	-	1.9 × 10 ¹⁰	0
ATCC 19115	Human	4b	+	6.0 × 10 ⁸	70
ATCC 19116	Chicken	4c	+	2.6 × 10 ⁸	100
ATCC 19117	Sheep	4d	+	8.8 × 10 ⁸	40
ATCC 19118	Chicken	4e	+	7.8 × 10 ⁹	50
ATCC 15313§	Rabbit	1	+	> 1.2 × 10 ¹¹	0
EGD (NCTC 7973)	Guinea pig	1/2a	+	< 1.1 × 10 ⁷	100
HCC8	Catfish brain	1	+	< 7.0 × 10 ⁸	70
HCC25	Catfish kidney	4a	-	3.5 × 10 ¹⁰	0
874	Cow brain	4c	+	< 8.0 × 10 ⁷	100
1002	Pork sausage	ND	+	5.2 × 10 ⁸	60

*PCR was performed by using virulence-specific primers derived from *L. monocytogenes* internalin gene *lmo2821* (*inlJ*) (Liu *et al.*, 2003a; Liu, 2004; Sabet *et al.*, 2005).

†Determined by mouse virulence assay (Liu, 2004).

‡Relative virulence (%) is calculated by dividing the number of dead mice by the total number of mice tested for a particular strain, using virulent strain EGD as reference (Liu, 2004).

§Originating from an infected rabbit, ATCC 15313 was initially haemolytic, but later became non-haemolytic and avirulent after successive laboratory subculturing. Despite possessing intact *lmo2821* and many other virulence-specific genes, ATCC 15313 harbours a mutation in its *hly* gene (encoding LLO), rendering it avirulent in the mouse virulence assay.

from vacuoles, grow intracellularly and spread to neighbouring cells. In general, *L. monocytogenes* virulent strains tend to produce more severe cytopathogenic damage in Caco-2 cells, form more plaques with HT-29 cells and cause higher mortality in chicken embryos than avirulent strains. Furthermore, virulent strains are more capable of adhering and entering Caco-2 and other cells, and they are also more efficient in escaping from vacuoles, undergoing intracellular growth, and spreading to neighbouring cells. As a result, the pathogenic potential of *L. monocytogenes* can be evaluated without expensive animal experimentation. The main advantages of *in vitro* cell assays include their relatively low cost and ease of use. However, these tests often suffer from the drawbacks of being time-consuming, and occasionally variable (especially with isolates whose virulence lies between the virulent and avirulent extremes), which have prevented them from being adopted in clinical laboratories for determining *L. monocytogenes* virulence and pathogenic potential.

Detection of virulence-associated proteins and genes

Early attempts to determine *L. monocytogenes* virulence through the detection of virulence-associated proteins and genes were largely unsuccessful, since many of the target proteins and genes are present in both virulent and avirulent strains. While *in vitro* demonstration of LLO, PC-PLC and PI-PLC activities often provides general guidance on the pathogenic potential of *L. monocytogenes* strains, its reliability as a virulence indicator is by no means satisfactory.

Indeed, the association between haemolytic, PC-PLC and PI-PLC activities and *in vitro* virulence has not been convincingly established (Roche *et al.*, 2001; Olier *et al.*, 2002). Similarly, PCR detection of *L. monocytogenes* virulence-associated genes, such as *inlA*, *inlB*, *actA*, *hly*, *plcA* and *plcB*, and genetic lineage analysis has not resulted in a clear correlation between these genes and the underlying virulence of *L. monocytogenes* (Piffaretti *et al.*, 1989; Nishibori *et al.*, 1995; Rasmussen *et al.*, 1995; Wiedmann *et al.*, 1997; Jaradat *et al.*, 2002). Although some naturally virulence-attenuated *L. monocytogenes* strains (particularly those isolated from human carrier cases) often contain mutations in their *prfA*, *hly*, *actA* and *inlA* genes, resulting in the expression of truncated or non-functional PrfA, LLO, ActA and InlA proteins (Olier *et al.*, 2002; Roberts *et al.*, 2005; Roche *et al.*, 2005), targeting these gene mutations as a means of determining *L. monocytogenes* virulence does not constitute a sound option in practical terms. On the one hand, a screening assay for genetic alterations in multiple *L. monocytogenes* genes can be cumbersome and costly. On the other hand, many *L. monocytogenes* isolates will not be covered by the assay due to their lack of changes in the genes targeted. Therefore, an optimal strategy for *L. monocytogenes* virulence testing remains the detection of virulence-specific gene(s) that are present only in virulent strains, but absent in avirulent strains.

The recent completion of the whole genome sequences of several *L. monocytogenes* and *L. innocua* strains (Glaser *et al.*, 2001; Nelson *et al.*, 2004) has facilitated the identification of

novel virulence-specific genes with potential for improved determination of *L. monocytogenes* virulence and pathogenicity (Liu *et al.*, 2003a; Liu, 2004). Using PCR primers derived from the genes encoding putative transcriptional regulators (i.e. *lmo0833*, *lmo1116*, *lmo1134* and *lmo2672*), putative internalins (i.e. *lmo2821* and *lmo2470*) and unknown proteins (i.e. *lmo0834* and *lmo1188*), *L. monocytogenes* virulent strains could be readily differentiated from avirulent strains. In particular, PCR targeting the putative internalin gene *lmo2821* offers a rapid, sensitive and precise means of distinguishing virulent from avirulent *L. monocytogenes* strains (Table 3).

The virulence of *L. monocytogenes* as determined by PCR has subsequently been confirmed by a mouse model. Examination of the representative *L. monocytogenes* strains by a mouse virulence assay indicates that being the only serotype not recognized by *lmo2821* primers, serotype 4a strains (e.g. HCC23, HCC25 and ATCC 19114) are unable to produce mouse mortality, and are thus truly non-pathogenic, while other serotype strains are more or less virulent (using relative virulence as a criterion) (Table 3) (Liu *et al.*, 2003a; Liu, 2004). For instance, *L. monocytogenes* avirulent strains with a relative virulence of 0% are negative by PCR, whereas *L. monocytogenes* virulent strains with a relative virulence of 30–100% are positive by PCR targeting *lmo2821*. Recently, *lmo2821* has been confirmed to be a novel internalin gene (*inlJ*) directly involved in *L. monocytogenes* virulence (Sabet *et al.*, 2005). Being present in *L. monocytogenes* strains/serotypes that are capable of causing human listerial outbreaks and mouse mortality, but absent in avirulent, non-pathogenic strains/serotypes (Doumith *et al.*, 2004b; Liu, 2004), *lmo2821* (i.e. *inlJ*) represents the target of choice for laboratory differentiation of virulent from avirulent *L. monocytogenes* strains.

Future perspectives

Being an opportunistic intracellular pathogen capable of surviving various food manufacturing processes, *L. monocytogenes* has become recognized as a major cause of human foodborne infection during recent decades. As a consequence, a concerted research effort has been directed toward an improved understanding of the *L. monocytogenes* bacterium and its pathogenic mechanisms. This in turn has facilitated the development of laboratory procedures for enhanced identification, subtyping and virulence determination of *L. monocytogenes*, and contributed to the implementation of appropriate and effective control and prevention strategies against listeriosis.

As one of the earlier techniques developed, serotyping is a phenotype-based diagnostic technique that has made the early success in the detection and subtyping of *L. monocytogenes* possible. However, since serotyping measures the phenotypic characteristics of *L. monocytogenes*, it may sometimes give variable results (Schonberg *et al.*, 1996; Liu *et al.*, 2006a). Attempts have been made to design and apply PCR-based procedures for serotyping purposes, but these

methods fall short of achieving serotype-specific determination of *L. monocytogenes* (Borucki & Call, 2003; Doumith *et al.*, 2004a). Therefore, an improved understanding of the molecular mechanisms underlying the regulation of *L. monocytogenes* somatic (O) and flagellar (H) proteins is essential. Toward this goal, a novel *L. monocytogenes* serotype 4b-specific gene cassette (*gltA-gltB*) has been characterized (Lei *et al.*, 2001; Kathariou, 2002). Further research on the genes encoding other serotype-specific antigens will help illuminate the mechanisms behind the production and regulation of *L. monocytogenes* serotype proteins, and lead to the development of novel molecular tests for reliable and precise determination of *L. monocytogenes* serotypes. In addition, considering that three strains (FSL-X1-009, FSL-X1-010 and FSL-J1-158) from ribotypes dd6821, dd6824 and DUP10142, respectively, appear phenotypically and genetically distinct from other members within the species of *L. monocytogenes*, but are probably related to serotype 7, further study is required to verify the taxonomic status of these strains and serotype 7 strains (e.g. the possibility of their being either one or two separate novel subspecies).

The application of various genetic subtyping procedures has resulted in the classification of *L. monocytogenes* strains into three lineages. While *L. monocytogenes* isolates from sporadic and endemic human listeriosis are often in lineage I (comprising serotypes 1/2b, 3b, 4b, 4d and 4e) and lineage II (containing serotypes 1/2a, 1/2c, 3a and 3c), those from animal and environmental specimens are of lineage III (consisting of serotypes 4c and 4a). However, in spite of being in the same genetic lineage III, serotypes 4c and 4a demonstrate marked differences in the mouse virulence assay. That is, while serotype 4c strains (e.g. ATCC 19116 and 874) display a relative virulence equal to that of the control virulent strain EGD (i.e. 100%), serotype 4a strains (e.g. ATCC 19114 and HCC25) show a relative virulence of 0% (Table 3) (Liu, 2004). The fact that a negligible number of serotype 4c isolates originate from human listeriosis cases suggests the inability of serotype 4c strains to establish in human hosts. As *L. monocytogenes* serotype 4c strains lack many virulence-specific putative transcriptional regulator genes in comparison with strains of other serotypes (with the exception of serotype 4a) (Liu *et al.*, 2003a), it is possible that some key invasion-associated proteins are not produced by serotype 4c strains as a result. It appears that these proteins may be required for *L. monocytogenes* passage through the intestine and subsequent phases before the establishment of infection. Further experimentation is warranted to pinpoint the events at which *L. monocytogenes* serotype 4c strains fail to complete the infection cycle. This may involve characterization of the virulence-specific, putative transcriptional regulator genes and their protein products, as well as the identification of the genes controlled by these transcriptional regulators.

The internalin gene *lmo2821* (i.e. *inlJ*) appears to be an excellent target for determining *L. monocytogenes* virulence,

as strains capable of causing mouse mortality invariably harbour this gene (Table 3) (Liu *et al.*, 2003a; Liu, 2004). With InlJ playing a direct role in *L. monocytogenes* virulence (Sabet *et al.*, 2005), it is important to further examine the regulation of *lmo2821 (inlJ)* through identification of its possible regulator(s) and related proteins. In addition, while past research has largely been directed to elucidating the molecular mechanisms of *L. monocytogenes* virulence and pathogenicity, relatively little has been undertaken with regard to the inability of *L. monocytogenes* avirulent strains to cause disease. Given that both virulent and avirulent *L. monocytogenes* strains are equally resistant to acid (pH 3.0 or lower) (Liu *et al.*, 2005a), it is likely that avirulent strains are capable of surviving the acidic stomach environment once ingested by hosts. Therefore, the inability of *L. monocytogenes* avirulent strains to result in listeriosis is most likely due to their failure to go through other key stages of infection, such as internalization, escape from vacuoles, intracellular growth and cell-to-cell spread. Extending our research to this area may help provide a more comprehensive picture of *L. monocytogenes* virulence.

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