Review Article
Detection of *Listeria monocytogenes* in foods

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Abstract: *Listeria monocytogenes* is a gram positive, facultative intracellular pathogen with the capacity to cause food poisoning outbreaks as well as severe illness in vulnerable human population groups. It can cause a rare but serious disease called listeriosis with high fatality rates (20–30%) compared with other foodborne microbial pathogens. Although *Listeria monocytogenes* is infective to all human population groups, it is more likely to cause severe problems among pregnant women, immunocompromised individuals, the elderly and neonates. There are a variety of phenotyphic and genotyphic methods for the detection of *Listeria monocytogenes* in foods. Recent technological advances have increased the ability of scientists to detect *Listeria monocytogenes*. The purpose of this review is to discuss molecular characteristics of the *Listeria monocytogenes* pathogen, standard detection methods of this pathogen in foods based on culture methods, confirmation of species and subtyping based on phenotypic and genotyphic methods.

Keywords: *Listeria monocytogenes*, **molecular characteristic, phenotypic, genotyphic, detection**

Introduction

Listeria are Gram-positive, facultative anaerobic, non-spore-forming, rod-shaped bacteria with a low G+C content. The genus consists of six species i.e. *Listeria monocytogenes*, *Listeria ivanovii*, *Listeria seeligeri*, *Listeria innocua*, *Listeria welshimeri* and *Listeria grayi*, of which only *L. monocytogenes* is the primary human pathogen although there have been rare reports of illnesses caused by *L.seeligeri, L.ivanovii* and *L.innocua* (Perrin *et al*., 2003; Gasanov *et al*., 2005)

Listeria monocytogenes is an opportunistic intracellular pathogen that has become an important cause of human foodborne infections worldwide (Liu,2006). Foodborne listeriosis, caused by the pathogen *Listeria monocytogenes*, is a relatively rare but serious disease with high fatality rates (20–30%) compared with other foodborne microbial pathogens, such as *Salmonella*. (FAO/WHO, 2005). While *L. monocytogenes* causes a relatively mild gastroenteritis in healthy adults, the illness can be severe in susceptible individuals. Basically, *L. monocytogenes* most often affects those with a severe underlying disease or condition (e.g.

immunosuppression, HIV/AIDS, chronic conditions such as cirrhosis that impair the immune system); pregnant women; unborn or newly delivered infants; and the elderly. Symptoms range from flu-like illness to severe complications including meningitis, septicaemia, spontaneous abortion or listeriosis of the newborn (FAO/WHO, 2005).

The *Listeria* species are tolerant to extreme conditions such as low pH, low temperature and high salt conditions (Sleator *et al.,* 2003; Liu *et al.,* 2005). Therefore they can be found in a variety of environments, including soil, sewage, silage, water, effluents and foods. With globalization and increased consumption of manufactured ready-to-eat foods throughout the world, it is hardly surprising that *L. monocytogenes* has become recognized as an important opportunistic human foodborne pathogen.

Molecular characteristics

L. monocytogenes is a facultative intracellular pathogen that induces its own uptake into phagocytic and non-phagocytic cells and spreads from cell to cell using an actin-motility process. It enters the host primarily through the intestine when food

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contaminated with this pathogen is consumed. Here, the *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).

L. monocytogenes then 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, important virulence genes mediating adhesion and invasion of eukaryotic cells. The internalins trigger entry of the bacteria into non-phagocytic cells such as epithelial cells, hepatocytes and fibroblasts cells. Gaining entry to host cells enables *L. monocytogenes* to evade host immune surveillance functions (Vazquez-Boland *et al.*, 2001). Following internalization, *L. monocytogenes* is primarily located in single-membraned vacuoles. The cytolysin, listeriolysin O (LLO), a pore-forming, thiol-activated toxin that is essential for *L. monocytogenes* virulence (PI-PLC) 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) and allow the release of *L. monocytogenes* cells to the cytosol (Portnoy *et al.*, 1992).

Here, the cells undergo intracellular growth and intracytoplasmic multiplication. The intracellular mobility and cell-to-cell spread of *L. monocytogenes* is mediated by another surface protein, ActA which polarizes the actin into comet 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 (Vazquez-Boland *et al.*, 2001). In this way, *L. monocytogenes* disseminate in host tissues sheltered from the humoral arm of the immune system.

Over the last fifteen years, a number of virulence factors involved in key steps of this intra-cellular life cycle have been identified. Most of the genes encoding listerial virulence determinants are clustered located in a 9.6 kb region (Gouin *et al.*, 1994), and form a regulon tightly controlled by a pleiotropic virulence regulator, PrfA. 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* are also involved in *L. monocytogenes* virulence and pathogenicity (Vazquez-Boland *et al.*, 2001).

Current standard methods

Isolation through enrichment and plating-based reference methods

Most regulatory agencies stipulate that isolation must be capable of detecting one *Listeria* organism per 25 g of food. This can only be achieved through enrichment methods that employ antimicrobial agents to suppress competing microflora, prior to plating onto selective agars and confirmation of cultures. The selective agents that are normally used in enrichment are acriflavine which inhibits growth of other gram positive bacteria; nalixidic acid which inhibits gram negative bacteria and cycloheximide which inhibits fungi. Listeria isolation media also contains esculin as all Listeria spp. hydrolyze esculin and the inclusion of esculin and ferric ion in enrichment or plating media results in the formation of an intense black colour (Fraser and Sperber, 1988). This is due to the formation of a complex between ferric iron with 6,7-dihydroxycoumarin, the product of esculin cleavage by β-D-glucosidase, resulting in a black precipitate.

The most commonly used culture reference methods for the detection of *Listeria* in foods are the ISO 11290 standards (ISO, 1996; EC,1999); FDA-BAM method to isolate *Listeria* spp. from dairy products, seafood and vegetables (Hitchins, 2003); USDA Standard method to isolate *Listeria* spp. for meat and poultry products as well as from environmental samples (USDA, 2002).

In all the enrichment methods, other listeria can grow faster while hiding the presence of *L. monocytogenes*. Thus, the use of isolation media that allows the identification of *L. monocytogenes* together with high numbers of other listeria is recommended.

Isolation through chromogenic media

Different chromogenic media have been developed to enable identification of pathogenic *Listeria* spp and *L. monocytogenes* based on enzymes produced by the pathogen and acids produced due to fermentation of sugars. Different antimicrobials are added to the media to obtain sufficient selectivity. Chromogenic media is the most popular culture confirmation method because of its easy preparation and interpretation. It enables presumptive identification of *L. monocytogenes* after 24 hours. Most of these media have been tested on a wide range of different foods (Reissbrodt, 2004) and are now included in most protocols and standards (Hitchins, 2003; ISO, 2004).

The virulence gene *plc A*, present on *L. monocytogenes, L. seeligeri* and *L. welshimeri* encodes the synthesis of phosphatidylinisitol phospholipase C (PIPL-C), which is generally employed for differentiation of haemolytic and non-haemolytic *Listeria*. A white halo-like zone of precipitation is formed around the haemolytic species. Ottaviani *et al*. (1997) combined this detection system with chromogenic substrate for β-D-glucosidase activity. In this medium, referred to as 'Agar Listeria according to Ottaviani and Agosti' (ALOA), all *Listeria* spp. produced turquoise colonies and pathogenic species *L.monocytogenes* and *L.ivanovii* appeared surrounded by a distinct precipitation zone (Reissbrodt, 2004).

For enumeration of *L.monocytogenes*, the ISO Standard 11290, part 2 (ISO 1998) is applied, as well as optional protocols mentioned by FDA and USDA methods (USDA, 2002; Hitchins, 2003). The initial enrichment broth can thus be quantified, before starting incubation, by direct spread plate count on chromogenic media, but for low level of contamination, quantification of *Listeria* has traditionally been carried out by the most-probable-number (MPN) method (Hitchins, 2003). Using chromogenic media after MPN enrichment, *L. monocytogenes* can be directly enumerated.

Confirmation of bacterial species

Enrichment methods are followed by the isolation of the enriched microorganisms on specific plate media, and the identification and confirmation of the isolated bacteria. This can be carried out using phenotyphic and genotyphic methods.

Species-specific identification

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 (Liu, 2006).

Biochemical methods

Listeria species vary in their ability to haemolyse horse or sheep red blood cells, and produce acid from L-rhamnose, D-xylose and α-methyl-D-mannoside (Robinson *et al.*, 2000). *L. ivanovii* can be 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 and Catimel, 1985). *L. ivanovii* can be 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 *ß*-haemolysis or to show PI-PLC activity on chromogenic media. *L. welshimeri* is differentiated from other *Listeria* species by its negative *ß*-haemolysis and CAMP reactions, and by its acid production from D-xylose and α-methyl-D-mannoside (Robinson *et al.*, 2000).

Serological methods

Strains of *Listeria* species are divided into serotypes on the basis of specific heat-stable somatic (O) and heat-labile flagellar (H) antigens. There are 15 *Listeria* somatic (O) antigen subtypes (I–XV) whereas flagellar (H) antigens comprise four subtypes (A–D) (Seeliger and Höhne, 1979; Seeliger and Jones, 1986). The serotypes of individual *Listeria* strains are determined by their unique combinations of O and H antigens. At least 13 serotypes (i.e. 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 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 and Jones, 1986; Kathariou, 2002) through examination of group-specific *Listeria* O and H antigens in slide agglutination. An ELISA has recently been developed to improve efficiency since slide agglutination is not easily adapted for high-throughput testing (Palumbo *et al.*, 2003)

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). 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). Serotype designations are universal unlike those of other sub-typing methods.

Phage typing

Bacteriophages are viruses that attach to bacterial cells and can cause lysis of the cell. Being host-specific, they are able to lyse closely related Listeria bacteria independently of the bacterial species and serovar identities. Phage typing refers to the use of characterized bacteriophages to type bacteria beyond the species and serotype level. Through examination of bacteriophage-induced, host-specific lysis of Listeria bacteria, it is possible to differentiate Listeria strains into distinct phage groups and phagovars which are useful for tracking for epidemiological purposes. However, with 10% of Listeria strains being untypable, the usefulness of phage typing is constrained.

Molecular methods

Detection by nucleic acid probes

Nucleic acid probes are segments of DNA or RNA labelled with radioisotopes, enzymes or chemiluscent reporter molecules that bind to complementary nucleic acids with high degrees of specificity. Synthetic oligonucleotides of <50 nucleotides are commonly incorporated into commercial kits. The detection of *L. monocytogenes* by nucleic acid 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 nucleic acid probe and subsequently detected with an appropriate substrate (enzyme label) or by autoradiography (radiolabel) (Klinger *et al.*, 1988; Kohler *et al.*, 1990). This method is more specific than biochemical and serological methods that are phenotype based as this procedure exploits differences among *Listeria* species at the genetic level. However, it has limited sensitivity as this technique does not involve nucleic acid amplification.

Detection by nucleic acid amplification

All target amplification systems share certain fundamental characteristics. They use enzyme-mediated processes in which a single enzyme or multiple enzymes synthesize copies of target nucleic acids. There are several approaches to nucleic acid amplification. However, polymerase chain reaction (PCR) was the first and remains the most widely applied technique in both research and clinical laboratories. PCR is a simple way to quickly amplify specific sequences of target DNA from the indicator organism to an amount that can be viewed by the human eye with a variety of detection devices. 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.

Gel electrophoresis is typically used to detect the amplified product. As the primers bind to specific sites surrounding the target DNA, the size of the amplified product can be anticipated and detected with a DNA stain, as a band of known size on gel 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 and Alarcon, 2002).

Multiplex PCR is a way to amplify two or more amplicons in a single PCR reaction. Multiplex PCR assay has been used to selectively amplify a shared *iap* gene and facilitate the differentiation of all six *Listeria* species in a single test (Bubert *et al.*, 1999). However, caution should be exercised to ascertain the sizes of the amplified products, which may show minute size differences among various *Listeria* species. Thus, targeting *Listeria* genes unique to individual species may be better, as it provides an independent means of confirming the species identities (Gilot and Content, 2002; Liu *et al.*, 2003b, 2004a, 2004b, 2004c, 2005b). If 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 confirm species identities. Multiplex assays are very useful for identification and characterization of bacterial isolates. 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 and Hill, 2001; Borucki and Call, 2003; Doumith *et al.*, 2004a).

Subtyping

Characterization of *L. monocytogenes* by subtyping procedures is essential to track individual strains involved in listeriosis outbreaks, and to examine the epidemiology and population genetics of *L. monocytogenes* bacteria. 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 (Liu, 2006).

Phenotypic subtyping methods

Serotyping

By separating *L. monocytogenes* strains into 13 serotypes (i.e. 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b,4ab, 4c, 4d, 4e and 7) on the basis of serological reactions between somatic (O)/flagellar (H) antigens and their corresponding antisera (Table 1)(Seeliger and Höhne, 1979; Seeliger and 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. Bacteriophages have the capacity to lyse closely related *Listeria* bacteria independently of the bacterial species and serovar identities. The use of characterized bacteriophages to type bacteria beyond the species and serotype level is well-known. 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). Through 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 100x routine test dilution, an overall typability of 84.5 % was obtained (Loessner and 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). However, 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 (Liu *et al.*, 2006).

Multi-enzyme Enzyme Electrophoresis (MLEE)

MLEE represents a robust genetic fingerprinting method which relies on phenotypic polymorphism. It is based on separation of intracellular enzymes by electrophoresis under non-denaturing conditions.

Differences in migration patterns for the variants of the enzyme are directly dependent on its amino-acid sequence and therefore on the nucleic acid sequence at the corresponding genetic locus. This allows the differentiation of many subtypes within a species and the subsequent assessment of the genomic relatedness among isolates. Thus, 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 and 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

Pulsed field gel electrophoresis (PFGE)

*P*FGE is an effective molecular typing technique that allows the comparison of genetic material from different isolates of the same bacteria. Chromosomal DNA from bacterial isolates is isolated and cut into several pieces by restriction enzymes. These pieces of DNA are separated by agarose gel electrophoresis. Electrophoresis allows separation of the DNA pieces by size. Bacterial genomic DNA can yield hundreds of fragments, which are then separated by conventional agarose gel electrophoresis to form distinctive banding patterns for individual strains. The bands are usually complex and the interpretation of

RFLP results is tedious and technically demanding. For *L. monocytogenes,* the bacteria are first placed in agarose plugs and lysed, then the DNA is digested with selected restriction enzymes. The plugs with 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). PFGE is particularly useful for subtyping serotype 4b isolates, which are not typed by most other typing methods.

Ribotyping

Ribotyping is a derivative of RFLP analysis that utilizes the transfer of electrophretically separated genome restriction fragments onto nitrocellulose or nylon membranes followed by hybridization with specific DNA or RNA probes. This results in much simpler and more consistent band patterns. For *Listeria,* the DNA is initially digested with restriction enzymes (e.g. *Eco*RI, *Pvu*II and *Xho*I) 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 *Eco*RI 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

PCR-based subtyping techniques

RAPD and arbitrarily primed PCR (AP-PCR)

Arbitrary amplification of polymorphic DNA sequences is used as a method of genetic characterization of microorganisms (Farber and Addison, 1994; Learn-Han et al., 2009; Zulkifli et al., 2009). Both RAPD and AP-PCR use low-stringency

PCR amplification with a single primer of an arbitrary sequence to generate a genetic profile of anonymous DNA fragments. Here, a single short random primer is used at a relatively low temperature which allows for mismatches and thus permits arbitrary primer sequences to bind non-specifically as well as specifically to the DNA template. Distinct band patterns are formed from *L. monocytogenes* DNA after agarose gel electrophoresis. 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. 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 and AP-PCR are more economical and faster than other typing methods.

However, it is not as discriminatory as some genotypic methods such as PFGE (Farber and 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).

Amplified fragments length polymorphism (AFLP)

AFLP is a similar to RFLP but involves the addition of adaptors to restriction enzyme-digested DNA, followed by PCR amplification and electrophoretic separation of PCR products. Here, *L. monocytogenes* DNA is digested with two restriction enzymes which are ligated with double-stranded oligonucleotide adapters 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. It can differentiate among the *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri* and *L. grayi* species as well as separate *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). It however requires ligation of linkers and indexers to enzyme-digested DNA from individual strains, which is time-consuming step and adds uncertainty to the testing procedure.

Poplymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

PCR-RFLP requires a target containing suitable polymorphism to allow discrimination within the species. The primer set is therefore dependent on the DNA target. The amplified DNA fragment is then subjected to digestion by a specific restriction endonuclease or combination of nucleases and run on agarose gel to view the resulting polymorphism. 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. *Hha*I, *Sac*I or *Hin*fI) and separation by agarose gel electrophoresis. Subsequent examination of the distinct band patterns permits differentiation of *L. monocytogenes* subtypes (Wiedmann *et al.*, 1997). It obviates 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.

Repetitive extragenic palindrome-polymerase chain reaction (REP-PCR)

Known conserved regions can be amplified using a single DNA primer set in a way that gives rise to polymorphic DNA fingerprints. Repetitive DNA sequence are particularly amenable to this approach. *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. *L. monocytogenes* strains have been divided into four clusters that match the origin of isolation, each consisting of multiple subtypes using REP-PCR (Jersek *et al.*, 1999). REP-PCR offers an alternative for the rapid subtyping of *L. monocytogenes* strains since it produces a similar level of discrimination to PFGE and ribotyping techniques,.

DNA sequencing-based subtyping techniques

Multi-locus sequence typing (MLST)

MLST is a subtyping method to be based on DNA sequencing rather than DNA fragment sizes. It is increasingly used for genetic subtyping of *L.*

monocytogenes. MLST focuses on multiple genes or gene fragments to determine the subtypes and genetic relatedness of *L. monocytogenes* isolates. The availability of DNA sequencing data also enables an understanding of the evolution of the microorganism or strain. 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.

Multi-virulence-locus sequence typing (MLVST)

MLVST is a subtyping approach that differentiates *L.monocytogenes* based on sequence differences in three virulence genes and three virulence associated genes (Zhang *et al*., 2004). The method is able to differentiate strains that had indistinguishable PFGE patterns. An abbreviated version of MLVST was later reported in which a multiplex PCR assay first identified *L.monocytogenes* serotypes 1/2a and 4b and then two PCR products were sequenced to provide interstrain discrimination (Zhang *et al*., 2005).

Conclusion

As an opportunistic intracellular pathogen that is able to survive under extreme pH, osmolarity and temperature, *L. monocytogenes* has been detected in a variety of processed foods. The current reference methods for the detection of *L. monocytogenes* allow the recovery of this pathogen from a variety of foods with relative ease. The introduction of chromogenic media has efficiently improved the isolation of *L. monocytogenes.* Molecular diagnostic techniques have also greatly contributed to the detection and identification of this pathogen since it is highly sensitive, precise and fast. Although there are many subtyping procedures that can be used for tracking of *L. monocytogenes* strains, the combined use of two or more procedures is generally more discriminatory and powerful than each applied alone.

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