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Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: a review

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Abstract

Listeria monocytogenes is an important food-borne pathogen and is widely tested for in food, environmental and clinical samples. Identification traditionally involved culture methods based on selective enrichment and plating followed by the characterization of *Listeria* spp. based on colony morphology, sugar fermentation and haemolytic properties. These methods are the gold standard; but they are lengthy and may not be suitable for testing of foods with short shelf lives. As a result more rapid tests were developed based on antibodies (ELISA) or molecular techniques (PCR or DNA hybridization). While these tests possess equal sensitivity, they are rapid and allow testing to be completed within 48 h. More recently, molecular methods were developed that target RNA rather than DNA, such as RT-PCR, real time PCR or nucleic acid based sequence amplification (NASBA). These tests not only provide a measure of cell viability but they can also be used for quantitative analysis. In addition, a variety of tests are available for sub-species characterization, which are particularly useful in epidemiological investigations. Early typing methods differentiated isolates based on phenotypic markers, such as multilocus enzyme electrophoresis, phage typing and serotyping. These phenotypic typing methods are being replaced by molecular tests, which reflect genetic relationships between isolates and are more accurate. These new methods are currently mainly used in research but their considerable potential for routine testing in the future cannot be overlooked.

Keywords: Listeria; Isolation; Identification; Food; Environment; Epidemiology; Culture; Serology; Molecular; Phenotyping

Contents

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1. Introduction

The genus *Listeria* is placed in the *Clostridium* subbranch of Gram-positive bacteria based upon the low G + C content of its genome. There are six species currently recognized: *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii*, *Listeria seeligeri*, *Listeria welshimeri* and *Listeria grayi*. Only two species of the genus are generally considered to be pathogenic, *L. monocytogenes* in humans and *L. ivanovii* in other mammals. However, there have been some reports of *L. seeligeri* and *L. ivanovii* [1,2] causing illness in humans. Pathogenic infection by *L. monocytogenes* results in listeriosis and usually affects individuals pre-disposed through an underlying disease affecting the immune system, such as cancer or AIDS, and also other susceptible individuals such as the elderly, pregnant women, newborn babies or fetuses. Symptoms of the disease are flu-like, yet may result in severe complications, such as meningitis, septicaemia, spontaneous abortion or listeriosis of the newborn [3]. The number of cases of listeriosis average 40–44 per year in Australia [4] and around 100 per year from 1993 to 1997 in the USA [5]. Although the incidence of listeriosis seems small compared to other food-borne diseases, the associated mortality is high at around 30% [3,6].

Although Murray [7] first suspected an oral route for the bacterial infection observed in animals in 1924, it was not until 1981 that for the first time an outbreak of listeriosis in Canada was linked to a contaminated food source [8]. Since the recognition of *L. monocytogenes* as a food-borne pathogen, there have been rapid advances in the development of suitable methods for isolation and identification [4]. Initial attempts to isolate *Listeria* from food based on clinical procedures such as direct plating onto blood agar were unsuccessful. Significant developments have occurred not only in selective culture enrichment procedures but also in the availability of many new and rapid detection methods based on antibody and molecular technologies. A major challenge for food testing has always been the interference of the tests by inhibitory food components and hence novel methods, such as immuno-capture, were developed to purify analytes from inhibitory food components as well as to increase sensitivity.

There has been a constant search for more rapid and sensitive methods, particularly in the food industry, where pressure from regulators to provide contaminant free food and the need to release perishable product onto the market prior to their expiry date are ever present. Culture and immunoassay methods are widely used because they are simple, inexpensive and allow a high sample throughput. Antibody-based tests target *L. monocytogenes*-specific proteins and utilization of culture tests using colorimetric or fluorescent substrates in media to detect virulence factor activity have been introduced to differentiate between pathogenic and non-pathogenic species. A comparison of the most widely used tests based on several key features is provided in Table 8 and a comprehensive overview of isolation, identification and typing methods for *Listeria* and *L. monocytogenes* in foods and environmental samples is provided in Fig. 1.

Many tests endorsed and regulated by government agencies such as the Food and Drug Administration (FDA) or US Department of Agriculture (USDA) in the United States, or the Australian and New Zealand Food Administration (ANZFA) in Australia, do not differentiate between *Listeria* species. From the perspective of food hygiene the presence of a non-pathogenic species such as *L. innocua* may indicate potential contamination with *L. monocytogenes*. However, epidemiological studies have revealed that only *L. monocytogenes* and only strains belonging to serotypes 1/2a, 1/2b and 4b were implicated in 90% of outbreaks of listeriosis [9]. It is unclear why only three of the 13 serotypes are

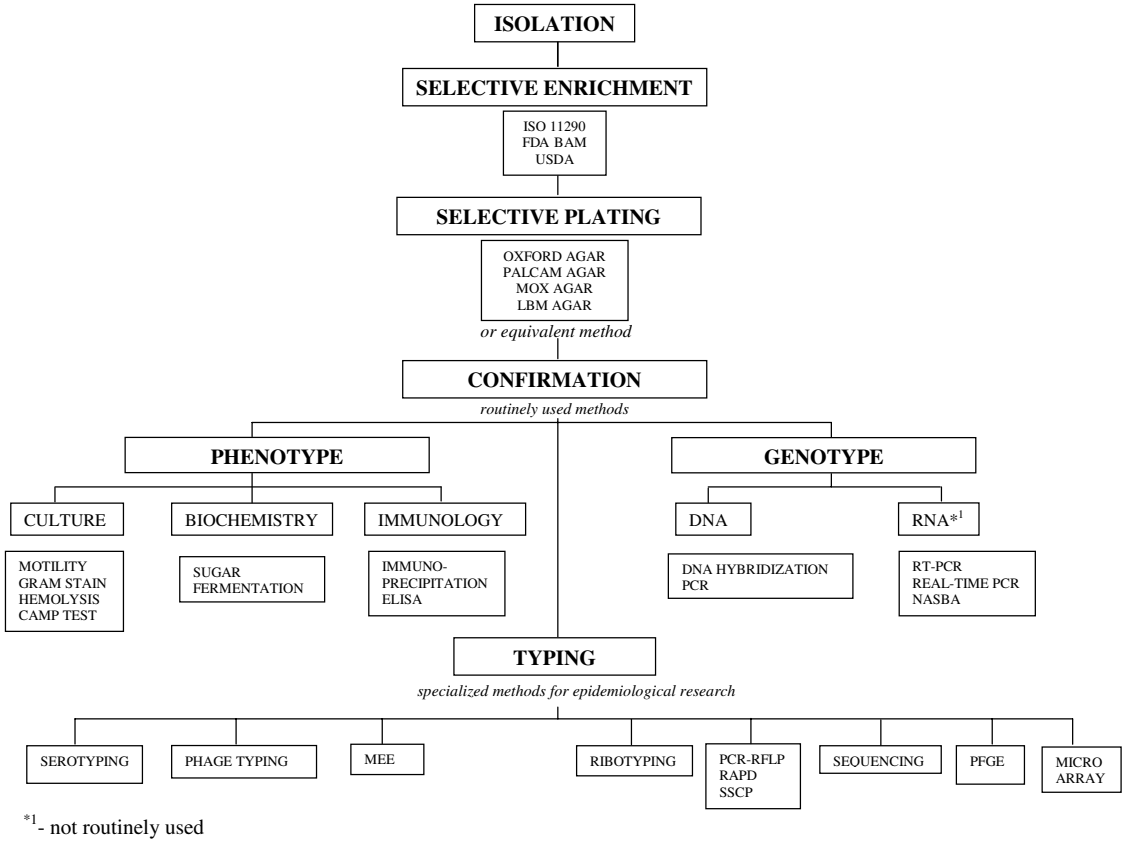


Fig. 1. Overview of isolation, identification and typing methods for Listeria and L. monocytogenes in foods and environmental samples.

implicated in food-borne outbreaks whilst other serotypes are also found as food contaminants. All serotypes possess the same virulence factors and hence have the same potential to cause disease. In light of these epidemiological data many food diagnostic tests have been developed to differentiate *L. monocytogenes* from the other species in food.

Although the majority of tests used for food testing are based on culture methods or antibodies, the trend in the food industry is towards the use of molecular methods. There are some disadvantages compared to culture methods such as equipment and reagent costs and the requirement of highly trained personnel. There is no regulatory approval for the majority of these tests, which prohibits their use in many food-testing laboratories. Recently, however, some molecular techniques such as PCR and DNA hybridization have become a feasible alternative to culture and serological techniques. The major advantage that molecular techniques offer over conventional methods is that these are based on differences within the genome and do not rely on the expression of certain antigenic factors or enzymes to facilitate identification. They are extremely accurate, reliable and some can be performed in the same time frame as immunoassay methods (Table 8). There is a wide range of molecular methods available for the identification and characterization of *Listeria*. Based on the multitude of publications that have appeared over the last two decades on molecular testing describing the adaptation of conventional PCR methods to the food testing laboratory, there is little doubt that many of these techniques will be applied routinely in the near future. The available technology in this area is both diverse and rapidly changing and here we review the most important developments for isolating and identifying *Listeria* spp. and *L. monocytogenes* (Table 8 and Fig. 1).

2. Methods of isolation

Historically, it has been challenging to isolate *Listeria* from food or other samples and this explains why it remained unnoticed as a major food pathogen until recently. In early studies it was noted that *Listeria* is able to grow at low temperatures and this feature has been used to isolate these bacteria from clinical samples by incubation for prolonged periods at 4 °C on agar plates until the formation of visible colonies. This method of isolation takes up to several weeks and usually does not allow for the isolation of injured *Listeria* cells, which will not survive and grow when stressed. These two key issues, enrichment/isolation time and the recovery of stressed *Listeria* cells must be addressed if methods

of enrichment and isolation are to provide meaningful results that can be used to control *Listeria* contamination and food-borne outbreaks.

Tests considered for approval by regulatory agencies must be able to detect one *Listeria* organism per 25g of food. Generally, this sensitivity can only be achieved by using enrichment methods in which the organism is allowed to grow to a detectable level of $\sim 10^4$ 10^5 CFU ml^{-1}. *Listeria* cells are slow growing and can be rapidly out-grown by competitors, and hence bacteriostatic agents, such as acriflavin and nalidixic acid that specifically act to suppress competing microflora, have been introduced into enrichment media or selective agar [10]. These two agents are incorporated into all standard methods used to isolate *Listeria* from food and environmental samples.

In the food industry, such standard culture procedures are used as reference methods for regulatory purposes and for validation of new technology. These methods are sensitive but often time consuming and may take 5–6 days before the result is available. Two of the most widely-used culture reference methods for detection of *Listeria* in all foods are the FDA bacteriological and analytical method (BAM) [11] and the International Organization of Standards (ISO) 11290 method. Both of these methods require enrichment of a 25 g food sample in a selective broth, designed to slow the growth of competing organisms, prior to plating onto selective agar and biochemical identification of typical colonies.

2.1. FDA BAM and ISO 11290 methods

For the FDA BAM the sample is enriched for 48 h at 30 °C in *Listeria*. Enrichment broth (LEB, FDA BAM formulation) containing the selective agents acriflavin, naladixic acid and the antifungal agent cycloheximide. Enriched broth is then plated onto selective agar (Oxford, PALCAM, MOX or LPM).

The ISO 11290 Method has a two-stage enrichment process: the food sample is first enriched in half Fraser broth for 24 h, then an aliquot is transferred to full strength Fraser broth for further enrichment. Fraser broth also contains the selective agents acriflavin and naladixic acid as well as esculin, which allows detection of β-D-glucosidase activity by *Listeria*, causing a blackening of the medium. Both the primary and secondary enriched broth are plated on Oxford and PALCAM agars.

Although selective agents are necessary to inhibit competitive organisms during enrichment, there have been many reports of the harmful effects of selective agents on stressed or injured *Listeria* cells [12,13]. The FDA BAM and ISO 11290 Method address this problem in different ways. In the FDA BAM method selective agents are added to the basal medium after 4 h incubation, allowing injured cells time to recover in a favourable environment. In the ISO 11290 Method the primary enrichment is in half Fraser broth, containing only half the concentration of selective agents. The large buffering capacity of both these enrichment media also enhances cell growth and repair.

2.2. USDA and Association of Analytical Chemists (AOAC/IDF) method 993.12 for the enrichment of Listeria from particular foods or environmental samples

Other reference methods are widely used for particular food groups. For example the USDA protocol [14] is often the method of choice for meat, eggs, poultry and environmental samples. This two-stage enrichment method uses a modification of University of Vermont Medium (UVM) containing acriflavin and naladixic acid for primary enrichment, followed by secondary enrichment in Fraser broth and plating onto Modified Oxford (MOX) agar containing the selective agents moxalactam and colistin sulphate.

The AOAC/IDF method 993.12 [15] is often the method of choice for dairy products and provides specific instructions for sample preparation of specific dairy foods. This method also uses selective enrichment in a broth containing acriflavin and naladixic acid for 48 h followed by plating onto Oxford agar.

3. Identification of isolated cultures

Enrichment methods, which usually take about 30–48 h, are followed by the identification of the enriched microorganisms. When selecting a method for enrichment and detection of *Listeria* in food or environmental samples it is important to consider the extent to which the method has been validated. While individual countries may have their own validation schemes, the AOAC in Washington is perhaps the most widely recognized authority. Reference to AOAC Official Methods will provide a variety of methods for *Listeria* testing including rapid methods, such as enzyme immunoassays and gene based tests, which have undergone collaborative validation by at least 10 laboratories. The AOAC Research Institute provides independent validation of commercial test kits by a single laboratory and performance testing certification. The AOAC Research Institute database (www.aoac.org) provides a useful reference for commercially available test kits. However, it is not exhaustive and not all commercial kits listed on this database have undergone AOAC validation (Table 8).

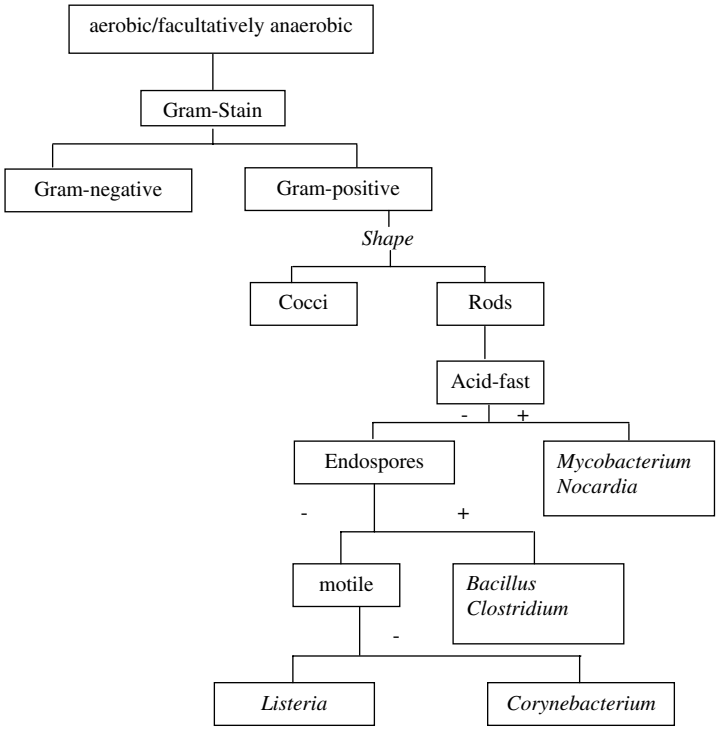
3.1. Cultural and biochemical confirmation

Early identification methods centered on biochemical and phenotypic markers, and these are still widely used for identification. Most selective agars for isolation and identification of *Listeriae* (Oxford, PALCAM, MOX)

rely on the esculinase reaction based on β-D-glucosidase activity to differentiate *Listeria* from other bacteria. Typical *Listeria* colonies appear black with a black zone in surrounding medium. These media also contain selective agents to inhibit the growth of most other organisms. However, some other organisms will grow on selective plates and some species, such as *Enterococcus* and *Bacillus* spp., also utilize esculin and may have a similar appearance. Further tests are required to identify *Listeria* colonies conclusively, but suspect colonies on selective agar must first be investigated for purity. Traditionally, suspect cultures were plated onto tryptose soy agar and colonies were examined by oblique lighting, a technique in which *Listeria* colonies appear reticulated with a distinct blue-green cast [10]. Currently, suspect bacteria are usually classified as *Listeria* if they display the following characteristics (Fig. 2): Gram-positive rods, aerobic and facultatively anaerobic, non-spore forming, catalase-positive (although there are reports of catalase negative *Listeria*), oxidase-negative, fermentative in sugars and producing acid without gas. Most strains are motile at 28 °C and non-motile at 37 °C [10]. Commercial identification kits are a widely-used alternative to traditional biochemical testing, which is time consuming and takes a week for differentiation of species using sugar utilization tests. Test strips such as API *Listeria* (bio-Merieux, Marcy-Etoile, France) and Micro-ID™ (Remel, USA) have been extensively validated and are now incorporated into standard methodology [11,14].

3.2. The Christie, Atkins, Munch–Petersen (CAMP) test

The CAMP test [16] *can be used to differentiate between hemolytic Listeria species*; *L. monocytogenes*, *L. ivanovii* and *L. seeligeri*. This test is carried out by streaking a β-hemolysin-producing *Staphylococcus aureus* strain and *Rhodococcus equi* parallel to each other on a blood agar plate. Suspect cultures are streaked at right angles in between (but not touching) the two streaks. Hemolysis by *L. monocytogenes* and to a lesser degree *L. seeligeri* is enhanced in the vicinity of *S. aureus*



		L. monocytogenes	*L. innocua*	*L. ivanovii*	*L. seeligeri*	*L. welshimeri*	*L. grayi*
Hemolysin		+	-	+	+	-	-
Catalase		+	+	+	+	+	+
Oxidase		-	-	-	-	-	-
Fermentation of:	L-Rhamnose	+	+/-	-	-	+/-	+/-
	D-Mannitol	-	-	-	-	-	+
	D-Xylose	-	-	+	+	+	-
	α-Methyl-Mannoside	+	+	-	-	+	+

Fig. 2. A methodological system for the phenotypic identification of *Listeriae*.

and hemolysis by *L. ivanovii* is enhanced in the vicinity of the *R. equi* streak. However, the CAMP test was examined for its applicability and it was shown that sometimes it cannot correctly differentiate between *L. monocytogenes* and *L. ivanovii* [17]. Therefore, a variation of the original CAMP test using commercially available β-lysin discs is recommended in the USDA method [11,14]. The identification of non-hemolytic species is based on differences in fermenting certain sugars (Fig. 2). Whilst these biochemical identification markers are useful, they are sometimes difficult to interpret because the colour reactions can be ambiguous.

3.3. Chromogenic substrates

A more recent development is the production and commercial availability of chromogenic media. Rapid identification of bacterial enzymes is provided by the use of chromogenic substrates, which are incorporated into plating media to allow direct identification of colonies by their characteristic colour. Phosphatidylinositol-specific phospholipase C (PIPL-C) is an enzyme that is produced only by *L. monocytogenes* and *L. ivanovii* [18,19] and activity of this enzyme is measured using chromogenic media. Many chromogenic media are commercially available and are gradually gaining acceptance by regulatory authorities [11]. These media offer many advantages over other tests. They are simple, cost effective, easy to interpret, allow a large sample throughput, highly sensitive and specific, and can be performed in the same time frame as ELISA methods (Table 8). A commercially available chromogenic agar for the detection of *L. monocytogenes* is the Rapid'L.mono® agar (BioRad, Marnes de la Coquette, France) on which PIPL-C-positive bacteria produce blue colonies. Differentiation of *L. monocytogenes* from *L. ivanovii* is accomplished by the fermentation of xylose (Fig. 2). The Rapid'L.mono test has been assessed extensively on a wide range of different samples [20,21]. Other commercially-available agar tests based on detection of PIPL-C positive bacteria are the BCM® chromogenic agar test (Biosynth International, Naperville, USA), the CHROMagar® *Listeria* test (Mast Diagnostics, Reinfeld, Germany), and ALOA (Biolife, Milan, Italy). However, none of these tests differentiate between *L. monocytogenes* and *L. ivanovii* [22–26]. Another bacterial enzyme used for the identification of *L. monocytogenes* is alanyl peptidase, which is produced by all *Listeria* species except *L. monocytogenes*. A simple colour reaction is used in which the substrates DL-alanine-β-naphthylamide and D-alanine-*p*-nitroanilide are hydrolyzed [27], and is the basis for the *Monocytogenes* ID Disc (Biolife, Milan, Italy).

3.4. Antibody-based tests

Immunoassay methods based on antibodies specific to *Listeria* have been applied in food testing for many years and they are popular because of their simplicity, sensitivity, accuracy and also because testing can be carried out directly from enrichment media without tedious sample preparation. Many of these immunoassays are available as commercial kits and are approved by regulatory authorities [15], Tables 1 and 8.

Table 1
Antibody-based commercial test kits for the detection of *Listeria* spp. in foods and environmental samples

Test	Manufacturer	Specificity	Sample types	Reference
Assurance *Listeria* EIA	BioControl Systems, Inc.	*Listeria* spp.	Variety of foods	[41,42]
Dynabeads anti-*Listeria*	Dynal Inc.	*Listeria* spp. *L. monocytogenes*	Variety of foods	[28,29,43]
EiaFoss Listeria ELISA kit	Foss Electric A/S	Listeria spp. (*except L. grayi*)	Poultry	[44]
*Listeria*Unique®	TECRA International	*Listeria* spp.	Food and environmental samples	N/A
Listeria-Tek™	Organon Teknika Corp.	*Listeria* spp.	Variety of foods	[45–48]
Listertest Lift Test	VICAM	*Listeria* spp.	Environmental surfaces, food	N/A
Oxoid *Listeria* rapid test	Oxoid Ltd.	*Listeria* spp.	Food and environmental samples	[49]
PATHATRIX *Listeria* species Test System	Matrix MicroScience Ltd	*Listeria* spp.	Variety of foods	N/A
PATHATRIX System for *Listeria*/*Salmonella* species	Matrix MicroScience Ltd	*Listeria*, *Salmonella* spp.	Variety of foods	N/A
Reveal® *Listeria*	Neogen Corporation	*Listeria* spp.	N/A	N/A
TECRA *Listeria* Visual Immuno Assay	TECRA International	*Listeria* spp.	Food and environmental samples	[24,47,50–52]
Transia Plate *Listeria*	Diffchamb AB	*Listeria* spp.	N/A	N/A
Transia Plate *Listeria* Monocytogenes	Diffchamb AB	*L. monocvytogenes*	N/A	N/A
VIDAS *Listeria* Assay	bioMerieux	*Listeria* spp.	Food and environmental samples	[51,53,54]
Vidas LMO	bioMerieux	L. monocvytogenes	Food	[54,55]
VIDAS *Listeria* Express	bioMerieux	*Listeria* spp.	Food and environmental samples	[56]
VIP for *Listeria*	BioControl Systems, Inc.	*Listeria* spp.	Food and environmental samples	[42]

3.4.1. Enzyme-linked immunosorbent assay (ELISA)

ELISA methods that use an antibody immobilized to a microtitre well for antigen capture in combination with a secondary antibody coupled to an enzyme (or another label) to detect the captured antigen, are the most widely applied methods because they combine ease of use with the generation of rapid test results. Furthermore, the ELISA methodology can be used with difficult sample matrices which makes these tests particularly well suited for food testing. A recent development is the availability of 'next day' *Listeria* tests for food and environmental samples. These tests claim equal sensitivity to traditional culture methods and enable a result within 30 h of sample receipt (*Listeria* Unique, TECRA International, Frenchs Forest, Australia and VIDAS *Listeria* Express, bioMerieux, Marcy Etoile, France) (Tables 1 and 8).

3.4.2. Immuno-capture

Immuno-capture is an elegant technique that uses magnetic beads (Dynal, Melbourne, Australia) or dip sticks (*Listeria* Unique®, TECRA International, Frenchs Forest, Australia) coated with specific antibodies to separate *Listeria* from competing microflora and inhibitory food components. Specific capture methods such as these also concentrate the target organism and increase the sensitivity of the test [28]. Capture techniques can be linked with molecular methods to increase the power of discrimination to a sub-species level [29–32].

Differentiation of *L. monocytogenes* from other *Listeria* species based on antibodies specific to virulence factors that are expressed by *L. monocytogenes* has met with variable success. Whilst there have been many reports of monoclonal and polyclonal antibodies directed against virulence factors [33], these have not, in most cases, been effective for testing. This is primarily because virulence factor expression in vitro was extremely variable [34–40]. However, an exception is the VIDAS LMO assay (bio-Merieux, Marcy-Etoile, France), which successfully targets a stable virulence antigen as the basis for the *L. monocytogenes* specific enzyme-linked fluorescent assay (EFLA).

3.5. Molecular tests

Identification of *Listeria* spp. and *L. monocytogenes* using molecular methods is becoming increasingly popular because these techniques are extremely accurate, sensitive and specific. Identification and differentiation of *L. monocytogenes* from other *Listeria* species to a sub-species level can be performed in the same time frame as ELISA-based assays.

3.5.1. DNA hybridization

DNA hybridization is the simplest molecular method used for the detection of *Listeria* and *L. monocytogenes* in foods (Table 2). The presence of a target sequence is detected using an oligonucleotide probe of complementary sequence to the target DNA sequence which contains a label for detection. Radioactive isotopes incorporated into an oligonucleotide sequence were previously used as labels for detection. More recently, biotinylated probes, probes incorporating digoxygenin (Boehringer–Mannheim), or fluorescent markers allow detection of target sequences with equivalent sensitivities to radioactive probes, without the biohazards associated with radioactivity. PCR combined with DNA hybridization in a microtitre plate is a convenient and highly sensitive and specific approach for detection of *Listeria* in a high-throughput 96-well format [57]. DNA hybridization tests aim primarily for the differentiation of *L. monocytogenes* from other *Listeria* species by targeting probes to virulence factor genes (Table 2). Commercially available DNA hybridization tests are routinely used for the testing of foods and have been extensively trialed for their sensitivity and accuracy [58]. These tests are robust and reliable. Accuprobe (Gen-Probe Inc., San Diego, USA) is a test based on the hybridization of labeled DNA probes to virulence factor mRNA, thereby ensuring that only viable cells are detected [59–61]. Another commercially available test is the GeneTrak DNA hybridization kit (Neogen Corporation, Lansing MI). A commercial test that recently has become available utilizes the vermicon identification technology (VIT®, Munich, Germany) and is based on in situ hybridization of fluorescently labeled oligonucleotide probes to intracellular target RNA. This test has been used so far only for the identification of *L. monocytogenes* in sewage and sludge samples [62].

Table 2
Molecular targets for DNA probes used for the identification of *Listeria* spp. by DNA hybridization

Probe target	Specificity	Reference
Delayed type hypersensitivity (*dth*)	*L. monocytogenes*	[63,64]
rRNA genes	*L. monocytogenes*	[65–68]
	L. ivanovii	[69]
	Listeria spp.	[32,70–73]
Hemolysin (*hly*)	*L. monocytogenes*	[74–79]
	Listeria spp.	[79,80]
Invasion-associated protein (*iap*)	*L. monocytogenes*	[57,74,79–82]
Putative transcriptional activator (*prfA*)	*L. monocytogenes*	[83]
Internalin A (*inlA*)	*L. monocytogenes*	[76,79,84]
Internalin B (*inlB*)	*L. monocytogenes*	[79]
Internalin C (*inlC*)	*L. monocytogenes*	[85]
Internalin D (*inlD*)	*L. ivanovii*	
Internalin related protein (*irpA*)	*L. monocytogenes*	[86]
Phospholipase A and B (*plcA, plcB*)	*L. monocytogenes*	[79]
Clp ATpase (*clpE*)	*L. monocytogenes*	[79]
Metalloprotease (*mpl*)	*L. monocytogenes*	[87]
Actin-polymerising protein (*actA*)	*L. monocytogenes*	[74]
Flagellin (*fla*)	*L. monocytogenes*	[74]

3.5.2. Polymerase chain reaction (PCR)

PCR [88] has had an immense impact on all molecular applications since its introduction. PCR is a technique whereby segments of DNA are amplified using a heat stable DNA polymerase and two primers (short DNA sequences specific to a particular gene) and the amplified fragments are then detected, usually using agarose gel electrophoresis. In contrast to DNA hybridization, where comparatively large amounts of target DNA or RNA are required to perform the test, PCR amplifies large amounts of DNA from minute amounts of target DNA. PCR is now established as a reliable and reproducible technique for identification of *Listeria* spp. And, more importantly, for the differentiation of *L. monocytogenes* from other *Listeria* species using primers targeting genes of virulence factors or RNA sub-unit genes (Table 3). Detection using PCR is carried out after selectively enriching samples for 24–48 h. It has been shown that direct testing of samples without prior enrichment gives unreliable results [77,89,90]. One major obstacle for the use of PCR in food and environmental samples directly after selective enrichment was that broths and food components contain inhibitors of the PCR which gave rise to false-negative results [91]. Several approaches can be taken to remove inhibitory factors after selective enrichments, such as sample treatments [92–96] or isolation procedures using magnetic beads, dip sticks or membranes to remove target DNA from reaction-inhibiting sample matrices [29–31,62,99–102].

Multiplex PCR, where multiple primer sets are used, allows the simultaneous detection of more than one pathogen in the same sample, such as *Listeria* and *Salmonella* [29,31] or *L. monocytogenes* and other *Listeria* species [101,103–106]. This approach is most attractive for food analysis, where testing time, reagents and labor costs are all reduced. Another adaptation of PCR that also uses multiple primer sets is nested PCR; however, these primer sets are used in sequential reactions and are designed against the same target. It is used to increase sensitivity and specificity and has been used for the identification of *L. monocytogenes* in clinical samples [57,107–110], environmental samples [78], and milk samples [97,111,112]. Commercially available PCR tests are the BAX® PCR system (Qualicon, Wilmington, DE, USA) and the Probelia® assay (Sanofi Diagnostic Pasteur, Marne la Coquette, France), which have been trialed in the field on a variety of different sample types [113–122].

Table 3
Primer targets for *Listeria* spp. and *L. monocytogenes*-specific PCR amplification

Target gene for oligo nucleotide primer	Reference
16S RNA	[67,69,72,106,109,123–125]
16S–23S RNA intergenic spacer region	[30,126–128]
23S RNA	[129,130]
Actin polymerizing protein (*act A*)	[114,131,132]
Aminopeptidase	[133]
Delayed type hypersensitivity factor (*dth*)	[134,135]
Fibrin binding protein (*fbp*)	[136]
Hemolysin (*hly*)	[77,96–98,106,110,114, 137–152]
Phospholipase (*plcA, plcB*)	[131]
Invasion-associated protein (*iap*)	[38,71,96,105,106,112, 147,153–155]
Metalloprotease (*mpl*)	[147,156]
L. monocytogenes antigen (*lma*)	[148]
Internalin A and B (*inlA, inlB, inlAB*)	[131,157–162]
Putative transcriptional regulator (*prfA*)	[95,147,162]
Sigma B factor (*sigB*)	[163]

4. Identification methods used in epidemiology

Detection of *Listeria* in food, environmental and clinical samples using biochemical and serological methods, as well as some molecular methods, generally identifies the contaminating *Listeria* to the species level. Only *L. monocytogenes* is regarded as a human pathogen and only three serotypes of *L. monocytogenes* are implicated in major food-borne listeriosis outbreaks. Therefore, epidemiological investigations must use techniques that are capable of discerning closely related *L. monocytogenes* strains in order to be able to confirm sources of outbreaks, establish patterns of transmission, and determine and monitor reservoirs of epidemic strains. Epidemiological investigations are based on the assumption that strains of the causal organism isolated from different sources are clonally related, and are similar or identical in their phenotypic and molecular characteristics. Therefore, these methods are based on species-specific proteins or genes that are relatively stable over time and are passed on from generation to generation. In addition, typing systems must have the capacity to correctly classify all epidemiologically related isolates from an outbreak according to clonal relationships (epidemiological concordance) [164]. Apart from these sensitivity and specificity requirements of the test, there are other considerations to take into account when choosing an appropriate test for use in epidemiology, such as the ease of use of the method and interpretation of results, labour and material costs, sample throughput ability, the time taken to perform the test, as well as the stability and reproducibility of the test itself.

4.1. Phenotypic typing methods

Typing of *L. monocytogenes* strains is either based on phenotypic markers, such as somatic O- and flagellar H-factors for serological typing, phage receptors (phage typing) or enzymes (multilocus enzyme electrophoresis),

or they are based on molecular variations within the bacterial genome (molecular typing methods).

4.1.1. Serological typing

Serological typing is based on antibodies that specifically react with somatic or "O" antigens and flagellar or "H" antigens (Table 4) of *Listeria* species. *Listeria* spp. are grouped into serotypes according to which O or H antigens are displayed. Serotypes are shared amongst *Listeria* species and antibodies directed against these antigens allow identification of the genus *Listeria* but do not differentiate *Listeria* species. Although, serological confirmation is not required for identification of *L. monocytogenes*, even for regulatory purposes, serology is often used to determine the prevalence of specific serotypes in epidemiological studies and for tracking environmental contamination [165,166]. A commercial kit for serotyping of *Listeria* isolates is available from Denka Seiken (Tokyo, Japan) and antisera can also be obtained from Difco (Difco Laboratories/Becton Dickinson and Co., Sparks, MD, USA).

4.1.2. Phage typing

Phage typing is a well-established typing method that has been intensively evaluated on a variety of samples (Table 5). It has been particularly useful in establishing that food is the primary vehicle for *Listeria* infections [167–170]. The method is based on the specific interaction of a particular bacteriophage with its *Listeria* host strain, resulting in host cell lysis. In 1985, the use of a set of defined bacteriophages (international set) with their respective host strains to standardize phage typing was proposed by Rocourt and colleagues [171]. A major disadvantage of the phage typing technique is that not all *L. monocytogenes* strains are typable. In particular *L. monocytogenes* serotype 1/2 strains have a low typability compared to other serotypes [172,173]. Since *L. monocytogenes* 1/2 strains are common food contaminants and one of the serotypes responsible for listeriosis outbreaks, this is a serious drawback in using phage-typing of *L. monocytogenes* isolates for epidemiological studies. It has also been noted that the typability of strains using the international set differs with geographical locations of isolates and this may contribute to the deficiencies of the typing system [173,174].

Table 4
Seeliger–Donker–Voet scheme for serological typing of the *Listeria* group (adapted from [89])

Species	Serovar designation	O-antigen	O-antigen
L. monocytogenes	1/2a	I II (III)	AB
	1/2b	I II (III)	ABC
	1/2c	I II (III)	BD
	3a	II (III) IV	AB
	3b	II (III) IV (XII) (XIII)	ABC
	3c	II (III) IV (XII) (XIII)	BD
	4a	(III) (V) VII IX	ABC
	4ab	(III) V VI VII IX X	ABC
	4b	(III) V VI	ABC
	4c	(III) V VII	ABC
	4d	(III) (V) VI VIII	ABC
	4e	(III) V VI (VIII) (IX)	ABC
	7	(III) XII XIII	ABC
L. ivanovii	5	(III) (V) VI VIII X	ABC
L. innocua	6a	(III) V (VI) (VII) (IX) XV	ABC
	6b	(III) (V) (VI) (VII) IX X XI	ABC
L. grayi		(III) XII XIV	E

Table 5
Summary of published studies employing phage typing for the subtyping of *L. monocytogenes* strains

Origin of isolates	No. of samples tested	No. of phages used	Reference
Clinical	823	20	[177]
N/A	142	11	[178]
Various	247	27	[174]
Clinical	186	27	[167]
Clinical	156	29	[171]
Clinical	475	28	[172]
Clinical	807	28	[179]
Sheep (clinical)	814	27	[180]
Various food	2470	28	[181]
Human, animal, food	3400	29	[169]
Dairy, food	511	16	[182]
Various	>1000	21	[183]
Food	227	27	[184]
Clinical	50	26	[185]
Environmental	44	29	[186]
Environmental, food, clinical	2679	35	[187]
Clinical	100		[188]
Various	80	33	[176]
Poultry	247		[189]
Clinical	395	33	[190]
Poultry	96	37	[173]

4.1.3. Multilocus enzyme electrophoresis (MEE)

MEE is also a well established typing technique (Table 6). It is based on the observation that variations in amino acid sequences of enzymes result in a different electrostatic charge of the protein, which in turn results in a different electrophoretic mobility. These variations in mobility are directly related to allelic variations of genes coding for these enzymes. Based on their electrophoretic mobility, isolates can be sorted into electrophoretic types. The procedure is simple and involves the preparation of enzyme extracts by lysis of bacterial cells, electrophoresis of the extracts using starch gels and the specific staining of enzymes [175].

Phage typing and MEE in general have proven to be reliable and convenient methods, which show good reproducibility when used in several WHO inter-laboratory studies [176]. These methods are easy to use, easy to

Table 6
Summary of published studies employing multilocus enzyme typing for the identification of sub-types of *L. monocytogenes*

Origin of isolates	No. of isolates	No. of enzymes tested	No. of electrophoretic types	Reference
Clinical	175	16	45	[191]
Clinical/food	390	16	82	[192]
Clinical	84	8	14	[193]
Wild-type/clinical	115		8	[194]
Clinical/environmental	305	16	78	[195]
Environmental/food	82	21	11	[196]
Clinical/food/environmental	85	14	45	[197]
Clinical/other	80	8–23	14–25	[198]
Clinical/food	219	10	59	[199]
Meat/environmental/waste	133	22	21	[200]
Seafood/environmental/clinical	305	21	40	[201]
Clinical/poultry	122	21	17	[202]

interpret, cost and labour efficient, and, most importantly, have been extensively tested in the field (Tables 5 and 6). These techniques were developed when many molecular methods were not available. Variations in the primary protein sequence are more accurately detected using molecular methods. In general molecular typing methods are superior to phenotypic typing methods both in terms of sensitivity and specificity. However, since many molecular tests are still in their infancy and inter-laboratory standardization of individual test parameters are often lacking, there is also a lack of analytical data for comparative studies. Therefore, current epidemiological investigations involve the use of several methods based both on phenotypic and molecular techniques to correctly classify implicated *Listeria* strains.

4.2. Molecular typing techniques

Molecular typing techniques are based on DNA hybridization, PCR, restriction enzyme analysis or direct sequencing of DNA. Direct sequencing of DNA is the most accurate way of comparing genetic differences or similarities. However, it is also the most expensive and time consuming method, and currently cannot be applied to high throughput testing. Hence, methods that give a relatively accurate reflection of genetic variation as well as a high sample throughput in a rapid timeframe were developed. These methods are aimed at establishing the degree of allelic variation of particular genes, which then forms the basis of measuring genetic relatedness of *Listeria* strains. Allelic variations can be measured as variations in the length of DNA fragments that can be generated, either by restriction digests or PCR, or as a change in conformation due to sequence differences (conformational polymorphism).

In order to interpret data with greater accuracy, electrophoretic techniques were developed to allow better resolution of DNA fragments, such as pulse-field gel electrophoresis (PFGE), which is primarily used in conjunction with restriction enzyme (endonuclease) digests of DNA. Denaturing gradient gel electrophoresis (DGGE) or capillary electrophoresis (CE) are electrophoretic techniques that are used in conjunction with single strand conformational polymorphism (SSCP) analysis to detect single nucleotide variations. Many of these molecular typing techniques continue to have problems that prevent routine use. These are primarily due to a lack of standardization of individual test parameters which can result in incorrect typing and poor inter-laboratory correlation of results [203]. Furthermore, some of these tests require specialized equipment, materials and reagents and testing is expensive when performed on a large scale that is necessary for most epidemiological investigations.

The most important molecular methods used for typing of *L. monocytogenes* are described in this review. Some are well-established techniques such as ribotyping, macrorestriction digests, PFGE, PCR-restriction fragment length polymorphism (RFLP) or random amplified polymorphic DNA (RAPD), which are used routinely. Other techniques such as SSCP or multilocus sequence typing (MLST) are currently becoming established as typing techniques for *L. monocytogenes* and show great promise. However, they are not used routinely and not many field data exist for these methods.

4.2.1. Ribotyping

Ribotyping is based on variations in ribosomal genes or proteins. This method was originally used to establish phylogenetic relationships and is the basis for modern systematics of prokaryotes. Relationships of organisms are best evaluated by how closely DNA sequences for a given gene match. The most useful gene for evaluation of phylogenetic relationships is the gene coding for ribosomal RNA because ribosomal genes are present in all organisms in multiple copies across the genome and ribosome function has been presumed to be constant over long evolutionary timescales. In general, ribotyping of *Listeria* isolates involves the restriction enzyme digestion of chromosomal DNA followed by DNA hybridization using an rRNA gene probe. Resulting banding patterns are used to sort *Listeria* isolates into ribotypes

and establish the relatedness of isolates [65,194,204,205]. Ribotyping has been extensively used in epidemiological studies [170,206–208] and automation has allowed the adaptation of this method for routine analysis [209–214]. Although the method is convenient, robust and gives excellent reproducibility the power of discrimination for *L. monocytogenes* typing is reported to be less than that of other molecular typing techniques [195,215].

4.2.2. Restriction enzyme analysis (REA)

This technique involves the use of restriction enzymes that recognize and cut particular sequences within DNA molecules. The digestion of DNA produces a banding pattern of fragments with varying sizes. Genetic relatedness is determined by comparison of the number and size of fragments which are separated and visualized using gel electrophoresis. REA of chromosomal bacterial DNA is referred to as macrorestriction analysis and the performance of the technique is significantly enhanced by combination with PFGE. In this type of electrophoresis, DNA molecules are subjected to periodic changes in the direction and/or the intensity of the current of the applied electrical field. DNA molecules can be separated according to their size because smaller molecules respond better to changes in the electrical field than larger molecules. Using conventional electrophoresis DNA molecules of up to 20 kb can be separated, whereas DNA molecules of up to 12 Mb can be separated and analyzed by PGFE [216]. The combination of macrorestriction digest with PFGE is considered the most effective typing method because it is simple, time and cost efficient, and highly discriminative (Table 7). With only minor adjustments this technique can be applied to almost any bacterium or strain. In comparative studies with other typing methods it was found to be the most discriminatory test, in particular for typing of *L. monocytogenes* serotypes 1/2 and 4 [65,158,203,214,217].

4.2.3. PCR-based typing methods

Two different PCR-based approaches can be used to type strains. One approach (RAPD) utilizes random primers to amplify DNA fragments randomly. The second approach is to amplify specific target sequences and analyze PCR products or their restriction enzyme digests, by comparing lengths of DNA fragments (RFLP) or conformational variations (SSCP) within these PCR products. In the RAPD technique genomic DNA is characterized based on the number and size of amplified DNA fragments generated by using a single random or universal primer in a PCR. Small changes in the genomic DNA will result in different sizes and numbers of amplified fragments. This technique has several advantages over conventional PCR. The primers used are typically 8–10 bp long and of random sequence, but prior knowledge of the target DNA sequence is not required. The same primers can be used for many different species of bacteria with only minor changes in the protocol. In general at least three universal primers are used in

Table 7
Summary of published studies using restriction enzyme analysis for the molecular typing of *L. monocytogenes* strains

Test	Target	Restriction endonuclease	Reference
Macrorestriction	Genomic DNA	*Nci*I	[218]
	Genomic DNA	*Eco*RI	[219]
	Genomic DNA	*Eco*RI, *Hae*III, *Hha*I, *Cfo*I	[220]
	Genomic DNA	*Hae*III	[202]
Macrorestriction/PFGE	Genomic DNA	*Apa*I, *Sma*I, *Not*I	[221]
	Genomic DNA	*Apa*I, *Sma*I	[213,215,222,225,227]
	Genomic DNA	*Apa*I, *Sma*I, *Xho*I	[223]
	Genomic DNA	*Sma*I	[224]
	Genomic DNA	*Apa*I, *Asc*I	[226]
	Genomic DNA	*Apa*I, *Not*I	[171]
	Genomic DNA	*Apa*I	[158,234]
	Genomic DNA	*Eco*RI	[199]
	Genomic DNA	*Apa*I, *Sma*I, *Ase*I	[228]
	Genomic DNA	*Asc*I, *Sma*I	[211]
	Genomic DNA	*Apa*I, *Asc*I, *Sma*I	[229]
PCR-REA (microrestriction)	*hly*, *iap*, *mpl*, *prfA*	Various	[147]
	inlA, *inlB*	*Alu*I	[157,158,161]
	iap	*Hin*dIII, *Rsa*I	[230]
	hly, *actA*, *inlA*	*Hha*I, *Hpa*II, *Sac*I, *Apo*I, *Hin*fI	[235]
	N/A	*Hin*dIII	[231]
	hly, *iap*	32 restriction enzymes	[232]
	actA, *hly*	*Sac*I, *Apo*I, *Hin*fI	[233]
	actA, *hly*	*Hha*I, *Hpa*II	[114]

Table 8
Comparison of methods for food, environmental and clinical testing for *Listeria* spp.

Test	Sensitivity[a]	Level of identification	Cost/test (~Au$)	Labour	Enrichment time (h)	Time[b]	Commercially available	Automation available	Regulatory approvals
Culture methods (e.g., FDA-BAM)	$\leqslant 10^4$ cells/mL	Genus *Listeria*, biochemical differentiation of species	$\leqslant$$1	High	~48	3–4 days up to 7 days for species	Dehydrated or prepared media	No	Yes standard methods
Chromogenic media	$\leqslant 10^4$ cells/mL	*L. monocytogenes*, no differentiation of other species	$1–$2	Medium	24–30	1–2 days	Yes	No	Some recently approved
Immunoassay methods, e.g., ELISA, EFLA	$\geqslant 10^5$ cells/mL	Genus *Listeria*. A few differentiate *L. monocytogenes* from *Listeria* sp.	$6	Low to medium	40–48 28[e]	1–2 h	Yes	Yes	Yes many approved methods
Immuno-capture/ELISA[c]	$\geqslant 10^5$ cells/mL	Genus *Listeria*. A few differentiate *L. monocytogenes* from *Listeria* spp.	$10	Medium	24–30	1–2 h	Yes	Yes[c]	No
Immuno-capture/PCR[d]	$\geqslant 10^5$ cells/ mL	Differentiation of all species, sub-species specific	$\geqslant$$10	Medium	24–30	$\geqslant$2 h	Yes	No	No
PCR[d]	$\geqslant 10^5$ cells/mL	Differentiation of all species, sub-species specific	$ 10	Low	24–30	$\geqslant$2 h	Yes	Yes	Yes some approved
DNA hybridization	$\geqslant 10^7$ cells/mL	Differentiation of all species, sub-species specific	$10	Low	40–48	2–4 h	Yes	Yes	Yes some approved

[a] Sensitivity of the test per ml of enriched sample. All approved tests are required to detect 1 cell per 25 g food sample; hence, all tests require culture enrichment.
[b] Approximate time it takes to perform the test excluding enrichment times.
[c] *Listeria* Unique™ (TECRA International, Frenchs Forest, Australia).
[d] Sensitivity is $\leqslant$5 cfu in pure culture, does not apply for food testing.
[e] VIDAS *Listeria* Express (bioMerieux, Marcy-Etoile, France).

separate reactions for finger-printing, and subsequent banding patterns are compared for each primer. The applicability of the technique for typing of *Listeria* species and sub-typing of *L. monocytogenes* strains has been documented in many studies from many different laboratories [205,236–241] and was also shown in an inter-laboratory trial using three universal primers [242]. RAPD typing is an excellent tool for epidemiological studies and has been extensively used to link *L. monocytogenes* strains isolated from listeriosis cases to foods that were implicated in outbreaks [238,243–250]. RAPD has been widely employed to type isolates from poultry plants [206,251], smoke houses [252–255], dairy environments [247], pork processing and slaughtering plants [158] and to either exclude or include implicated food products. The advantages of RAPD typing compared to other molecular methods are the simplicity of the test, the speed with which *Listeria* can be typed to a sub-species level and the ability to screen large sample numbers.

In contrast to RAPD typing, PCR-RFLP involves the PCR amplification of a particular target gene and digestion of the PCR product with a restriction enzyme. The restriction fragments are separated by electrophoresis and genetic relatedness is established by comparison of the number and size of fragments. Target genes are usually genes coding for ribosomal sub-units or virulence factors (Table 7). Whilst this approach is popular due to the fact that it allows a large sample throughput in a short time frame combined with ease of use and interpretation of results, the discriminatory power of PCR-RFLP is not high [164] and therefore its use in epidemiological investigations is limited.

Conformational polymorphism analysis is based on the assumption that a change in the nucleotide sequence of a PCR product will result in a change in the conformation of a single strand of that product. This conformational change can be detected by gel-electrophoresis because denatured DNA fragments of different sequence will migrate at different rates. Early studies of SSCP used non-denaturing polyacrylamide gels for analysis [256] but more recently denaturing (DGGE) or temperature gradient gel electrophoresis (TGGE) have been employed more effectively to separate DNA fragments that may differ in only a few nucleotides [257]. Electrophoresis is performed by introducing denaturing agents, such as urea and formamide, into a polyacrylamide gel (DGGE) or by applying a temperature gradient across the gel (TGGE) which partially denatures double stranded DNA. Differences in the nucleotide sequence will lead to different melting behaviour of the DNA and hence DNA fragments will migrate at a different rate through the gel. *L. monocytogenes* strains isolated from foods have been typed using this technique [153]. However, the most commonly applied gel electrophoresis in investigations using SSCP typing is capillary electrophoresis (CE). SSCP-CE has the capacity to differentiate DNA fragments, which differ by only one nucleotide. Separation of analytes is carried out in silica capillaries which are filled with a sieving matrix. DNA is injected into the sieving matrix and separated according to its size under high voltage. DNA fragments are detected under UV light when they pass a detector at the end of the capillary [258]. The process is fully automated and lends itself to high throughput screening. It is a useful and cost effective alternative to direct DNA sequencing, the only other method capable of detecting single nucleotide polymorphisms. *L. monocytogenes* has been typed using SSCP of the 16S rDNA [259] and the hemolysin (*hly*) and *iap* genes [260].

4.2.4. DNA sequencing

Direct sequencing of DNA is the most accurate method of evaluating genetic relationships of organisms, however it is expensive and time consuming. The most widely used sequencing method was first described by Sanger [261] and is based on the controlled interruption of DNA synthesis by incorporation of dideoxynucleotides during extension of DNA strands. Early sequencing efforts were hampered by the amount of starting material that was required for the reaction and so genes were targeted that are present in large copy numbers throughout the genome such as genes coding for RNA. Since the introduction of PCR the amplification of even minute amounts of target DNA is possible and hence sequencing has become a more applicable tool. The 16S RNA gene is the most extensively sequenced RNA gene [150,130,235,259], but 23S, 5S sequences and spacer regions have also been used for identification and typing purposes [127,129,204]. 16S RNA genes contain stretches of highly conserved regions but also regions that are variable. Primers designed to target conserved DNA sequences can be used to analyze a wide variety of different organisms using PCR amplification with subsequent sequencing of the PCR product [262], whilst highly diverse regions can be used to sub-type strains.

Sequencing of other genes has also been employed and this is the basis for multilocus sequence typing (MLST). House keeping genes or genes coding for virulence factors such as flagellin, (*fla*), *hly*, *actA*, *iap*, internalins (*inl*), the metalloprotease (*mpl*), *prfA* and other virulence-associated genes [263,264] are PCR amplified to generate internal gene fragments of approximately 450bp, which are then sequenced. Variations within the sequences of these genes are assigned alleles at these loci [265]. The discriminatory power of this approach is extremely high and accurate. MLST allows unambiguous typing of any strain and comparisons can be made based on sequence data which are easily accessible from appropriate data bases. The method was developed for typing of *L. monocytogenes* strains targeting several house keeping genes [266–269] however it cannot be

applied routinely and its application is limited to specialized laboratories.

Recently, the whole genome sequences of *L. monocytogenes* strain EGD and *L. innocua* were determined through a collaborative effort [270]. Since then numerous new *L. monocytogenes* genes and their functions have been elucidated and there are ever increasing sequence data compiled in databases such as the database of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) that are available for comparisons. The information that the complete sequence of the *L. monocytogenes* genome provides is invaluable and the impact that such information has had on all applications is huge. A commercial sequencing kit targeting bacterial 16S RNA genes is available from Applied Biosystems (Foster City, CA, USA) [89].

Epidemiological surveys on an international level are necessary to investigate the risks associated with *L. monocytogenes* contamination of foods and environmental samples. Recently, the WHO has been testing a new approach for assessing the risks that are associated with *L. monocytogenes* in ready-to-eat foods. These risk assessments are used to provide an estimate of the likelihood of illness from *L. monocytogenes* in a given population and are based upon epidemiological investigations from laboratories across the world [271]. Therefore, when comparing data from international sources it is of utmost importance that epidemiological tests are standardized and give good inter-laboratory results, which has not been achieved for most molecular typing methods to date. However, the trend of epidemiological testing is towards molecular methods and therefore measures must be taken to standardize these tests [271] because many laboratories use different reaction conditions (Tables 2 and 3) or restriction enzymes (Table 7) as well as different test parameters.

5. Future directions for the detection and identification of *L. monocytogenes*

The following section reviews novel methods for the detection of *Listeria* spp. in food, environmental and clinical samples. These methods are currently employed by specialized research laboratories only; but, because these methods offer significant advantages over conventional techniques, they may be used routinely for food pathogen testing in the future.

5.1. Tests targeting RNA

Testing of food or environmental samples for pathogenic *Listeria* should only target living organisms since only live *Listeria* cells can cause disease. The ability to form colonies on solid agar is the gold standard by which culture methods confirm the presence of live pathogens and this is the standard against which all other *Listeria* detection tests are compared. Tests that target DNA such as PCR have been criticized because dead organisms can give positive results due to the relatively high stability of DNA molecules. The choice of RNA or mRNA as a target for food pathogen testing has gathered increasing favour since the presence of mRNA is an indication of the living state of the cell [272,273]. mRNA is a labile molecule and readily degraded after cell death by RNases and environmental factors such as heat. Another advantage of testing for mRNA is that multiple copies of the target gene are present, which in turn enhances the sensitivity of the test. Instability of mRNA molecules, although an advantage, can also be a disadvantage because testing must be performed under conditions that prevent degradation of the target. Other disadvantages for using RNA-based technology are the high cost of equipment and reagents, and personnel must be highly trained to perform and evaluate the tests. Sensitivity and specificity of RNA tests are reported to be equivalent or better than standard PCR [274]. Many RNA-based tests are performed in a 96-well format which lends itself to high-throughput testing and automation, a factor that makes this technology attractive for future routine applications in food testing and epidemiological investigations.

5.1.1. Reverse transcription (RT)-PCR

RT-PCR involves a two-step reaction, with the first step employing a reverse transcriptase enzyme to translate mRNA into complementary DNA (cDNA). The reaction is usually initiated by random oligonucleotide primers. In the second step the cDNA is used as template for amplification of specific sequences by PCR using target specific oligonucleotide primers and a DNA polymerase to facilitate the reaction. *L. monocytogenes* has been detected using this method in artificially inoculated meat samples [275] by targeting mRNA transcripts of the *hly, prfA* and *iap* genes, in waste samples [276] by targeting the transcripts for rRNA genes, and also for the detection of heat-injured *L. monocytogenes* by targeting the *hly* transcript [277].

5.1.2. Real-time PCR

This technique is a modification of the RT-PCR method. The reaction mix contains a fluorescent marker (SYBR Green) that binds specifically to double stranded DNA. The increase of fluorescence after each successive cycle allows the direct quantitation of target DNA. This type of analysis requires specialized equipment and materials which substantially increase the cost of testing. This method has been used to identify and quantify *L. monocytogenes* in foods and clinical samples in several studies [74,154,278–281], and for the simultaneous detection of *Listeria* and *Salmonella* [151,152]. Establishing the actual number of contami-

nating *L. monocytogenes* cells in food and environmental samples is an important factor when investigating outbreaks of listeriosis. Real-time PCR is quantitative, which is a significant advantage over other molecular methods, and so this technology is extremely attractive for food testing and epidemiological investigations.

5.1.3. Nucleic acid sequence-based amplification (NASBA)

NASBA is an alternative to conventional PCR and is based on the action of three enzymes. In the first step a reverse transcriptase is used in combination with an oligonucleotide primer to produce a cDNA-RNA hybrid molecule. In the next step an RNase enzyme removes the RNA from the hybrid molecule allowing the reverse transcriptase to synthesise a double stranded cDNA molecule. This cDNA is then used as a template for the generation of RNA transcripts by a T7 polymerase. In contrast to PCR, NASBA yields single stranded RNA moleules which can be detected either by conventional agarose electrophoresis or by using specific labeled oligonucleotide probes for hybridization assays combined with colorimetric detection systems. Although NASBA is a method that shows great potential for routine analysis of food and environmental testing [273,282], there are some differences to conventional PCR methods particularly concerning sample preparation, which complicate this technique. Nucleic acids must be extracted prior to testing, because unlike PCR, the reaction is carried out at a constant temperature of 41 °C, which is not high enough for bacterial lysis and hence direct testing of enriched samples is not possible. On the other hand, since the reaction is performed at a constant temperature thermal cycling equipment is not required. NASBA has been used for the detection of *L. monocytogenes* in foods [283,284], and has been found to be of equivalent sensitivity, yet better specificity, than conventional culture and ELISA-based methods [284].

5.2. DNA microarrays

DNA microarrays are an exiting new technology, which is based on DNA or RNA hybridization. DNA microarrays are used to investigate microbial evolution and epidemiology and can serve as a diagnostic tool for clinical, environmental or food testing. They are essentially reverse dot-blots and are produced by spotting DNA (usually 100–200 μm in size 200–500 μm apart) onto a solid support matrix [285]. Microarrays are commercially available from many different suppliers (Applied Biosystems, Affymetrix, Qiagen and many others) and can be custom made for specific purposes. There are two main microarray formats, one is based on sequence specific oligonucleotides and the other employs specific PCR products.

5.2.1. PCR-based microarrays

To generate PCR-based microarrays it is necessary to amplify specific regions of interest using target-specific primers. Such microarrays are usually constructed to represent the whole bacterial genome and primers are designed to cover open reading frames as well as intergenic regions. PCR products are purified prior to spotting onto the support, usually coated glass slides or membranes, which are then used for hybridization studies with target DNA. The target DNA from the sample is generated from total bacterial RNA using random or specific primers and RT-PCR. These PCR products are subsequently labeled with a fluorochrome such as Cy3 or Cy5 for detection. For genomic investigations, genomic DNA is labeled prior to DNA hybridization. DNA fragments with complementary sequences will bind to immobilized PCR products and are visualized *via* the label. The generation of PCR based microarrays is labour and cost intensive and minute amounts of impurities in the PCR products can lead to cross-hybridization and ambiguous results.

5.2.2. Oligonucleotide-based microarrays

These arrays offer significant advantages over PCR-based microarrays. Use of synthetic oligonucleotides eliminates the need for RT-PCR amplification and product purification steps, and hybridization can be performed directly using total bacterial RNA, which is labeled prior to hybridization. There are other advantages including reduced cross-contamination and cross-hybridization [286].

Oligonucleotide microarrays based on the *iap* and *hly* genes have been used simultaneously to detect and discriminate between *Listeria* species [79]. In other studies, PCR-based genomic microarrays were constructed and used to sub-type *L. monocytogenes* strains [285,287]. Phylogenetic relationships of *L. monocytogenes* serotypes 1/2a, 1/2b, 1/2c and 4b have been examined using these methods [285,288,289] and portable systems for field testing have also been described [290,291]. The most attractive feature of this new technology is the capability of simultaneous identification and typing of *Listeria* strains in one test, a powerful feature that none of the other tests offer. However, the disadvantages are that high amounts of target DNA or RNA are required to perform the test and high-throughput testing is cost prohibitive.

6. Conclusion

Since food-borne transmission of *Listeria* was first established in 1981 there has been an explosion in the

development of new tests targeting *Listeria*, its reservoirs and sources of contamination. The main priority for testing is the identification of reservoirs of *Listeria* contamination in the food processing environment and the early detection of contaminated foods to prevent food-borne outbreaks and comply with regulatory requirements. There is continuing pressure for more rapid and sensitive methods, which results from the requirement of government organizations to protect the consumer, whilst the food industry is under pressure to release foods onto the market before they spoil. Since there is a 'zero tolerance' for the presence of *Listeria* in certain foods for human consumption in many countries, it is important to have rapid tests with high sensitivity. These tests should also be amenable to high sample throughput and be cost-effective. These criteria are best fulfilled to date by ELISA-based tests such as the "next day" *Listeria* tests for food and environmental samples (*Listeria* Unique, Tecra International, Frenchs Forest, Australia and VIDAS *Listeria* express. bio-Merieux, Marcy Etoile, France), or conventional PCR tests and tests based on DNA hybridization (Table 1 and 8).

There have been substantial improvements in culture methods, both in selectivity and ability to recover stressed organisms. These methods are used to enrich samples for testing and are routinely combined with antibody-based tests or more recently molecular tests based on *Listeria*-specific genes. Such culture and antibody-based tests are widely used in food industry laboratories because of their convenience, speed, automation, high sample throughput and cost effectiveness (Table 8). Their reliability, robustness and reproducibility, has been shown in numerous validation studies. Whilst these methods are important for the routine testing of food and environmental samples, these are not able to identify sub-types, which is a crucial parameter in the study of out-breaks of listeriosis.

Application of culture and serological methods in epidemiological studies is of limited value because of their low discriminatory power. Methods of higher discriminatory power, including molecular methods such as PCR and DNA hybridization tests, have been developed to differentiate between strains and identify subtypes of *L. monocytogenes* serotypes (Table 8). The first typing methods were based on phenotypic markers such as phage types and electrophoretic mobility of cytosolic enzymes. More recently, there has been a trend towards using molecular methods which also have significantly enhanced discriminatory power, are robust, reliable and give reproducible results. Standardization of methods is a key issue for routine application and can be achieved by automation of testing such as automated ribotyping, PCR and also real-time PCR. Given the high accuracy and speed with which molecular testing can be carried out, there is no doubt that most of these methods, if not already applied, will be adopted by regulatory authorities and in food testing laboratories in the future. There are exciting new developments in molecular technologies such as the microarray technology which have great potential as the basis for future routine testing.

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