Spanish Journal of Agricultural Research (2006) 4(3), 235-247

# Review. Specific detection of *Listeria monocytogenes* in foods using commercial methods: from chromogenic media to real-time PCR

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## Abstract

Listeriosis is one of the most important food-borne diseases. A variety of culture and rapid methods are available for the detection of *Listeria* spp. in foods. Although the presence of *L. innocua* may indicate potential contamination with *L. monocytogenes*, only the latter species is pathogenic for humans. Therefore, the most adequate tests are those which specifically detect *L. monocytogenes*. Chromogenic media is currently the most common method used for the presumptive identification of *L. monocytogenes*. Some tests like those based on antigen detection are fast and easily applied, but only a few may specifically detect *L. monocytogenes*. Real-time polymerase chain reaction is increasingly applied in food diagnostics for the detection of *L. monocytogenes* due to the availability of different specific commercial test methods. Microarrays and biosensors are some examples of new technologies that might be used routinely for the detection of *L. monocytogenes* in foods in the future.

**Additional key words:** alternative rapid commercial methods, food-borne listeriae, human pathogenic species.

## Resumen

**Revisión. Detección específica de *Listeria monocytogenes* en alimentos mediante métodos comerciales: de los medios cromogénicos a la PCR a tiempo real**

La listeriosis es una de las enfermedades transmitidas por alimentos más importantes. Existen diferentes técnicas de cultivo y métodos rápidos para detectar *Listeria* spp. en los alimentos. Aunque la presencia de *L. innocua* puede indicar una contaminación potencial con *L. monocytogenes*, esta última especie es la única patógena para el hombre. Los métodos más adecuados son, por tanto, los que detectan específicamente *L. monocytogenes*. El aislamiento en medios cromogénicos es el método más utilizado actualmente para identificar *L. monocytogenes* de forma presuntiva. Los métodos rápidos basados en la detección de antígenos son de uso sencillo, pero existen muy pocos que detecten específicamente *L. monocytogenes*. La reacción en cadena de la polimerasa a tiempo real está cada vez más implantada en la industria alimentaria debido a que existen diferentes métodos comerciales específicos para *L. monocytogenes*. Los microarrays y los biosensores son algunos ejemplos de las nuevas tecnologías que se podrán utilizar en el futuro para detectar *L. monocytogenes* en los alimentos.

**Palabras clave adicionales:** especie patógena para el hombre, Listeria en alimentos, métodos comerciales rápidos alternativos.

## Introduction

*Listeria* is a genus of Gram-positive bacteria containing six species: *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, and *L. grayi.* Out of these, only *L. monocytogenes* is commonly associated with human listeriosis, while *L. ivanovii* is pathogenic for other mammals. *L. monocytogenes* has been largely studied in the past decades because of its importance as a food-borne human pathogen (Swaminathan, 2001; Ryser and Marth, 2004). Ongoing efforts are needed to further reduce the incidence of listeriosis, due to the manifestation of its high mortality rate (ILSI Research Foundation-Risk Science Institute, 2005).

The presence of *L. monocytogenes* has been widely observed in foods, and environmental and clinical samples. Its detection and identification in foods

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Received: 23-02-06; Accepted: 21-06-06.

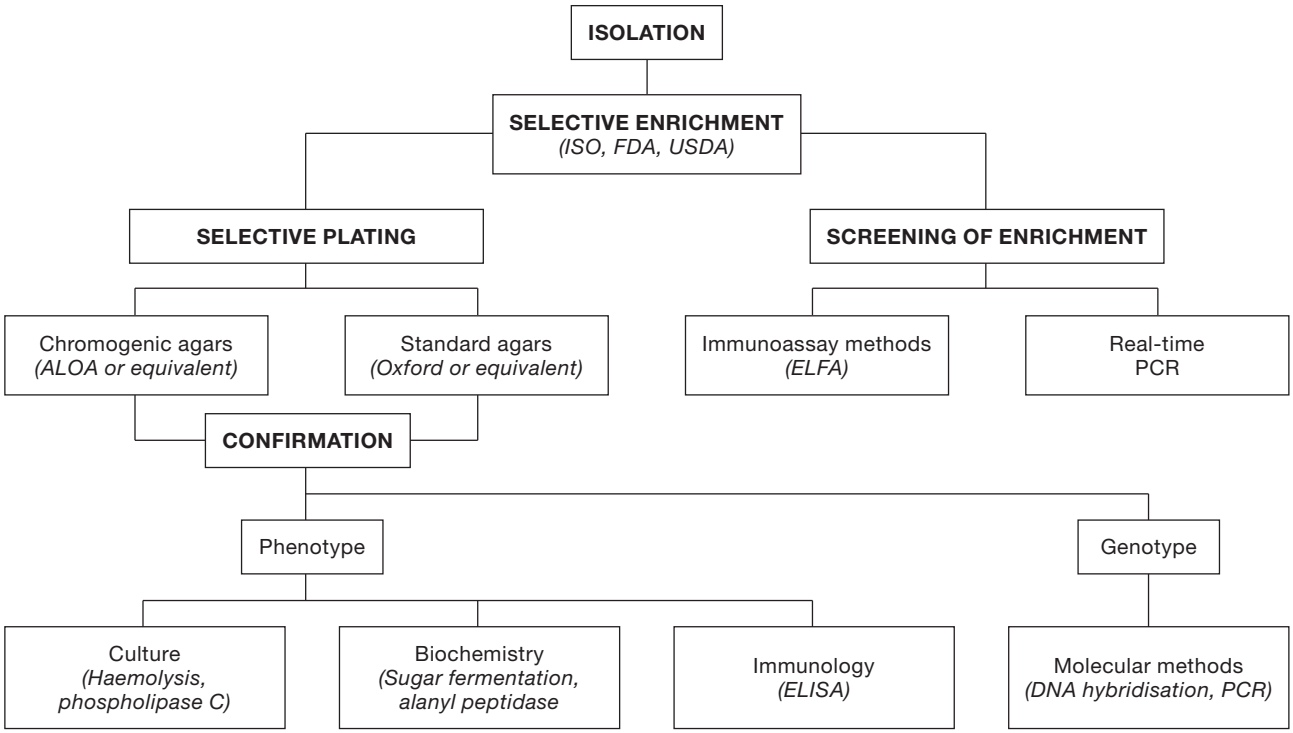


**Figure 1.** Overview of detection methods for *Listeria monocytogenes* in foods. Adapted from Gasanov *et al.* (2005).

traditionally involve culture methods based on selective pre-enrichment, enrichment and plating. This is followed by the characterization of *Listeria* spp. using colony morphology, sugar fermentation and haemolytic properties (Gasanov *et al*., 2005; Paoli *et al*., 2005) (Fig. 1; Table 1). Conventional microbiological methods are usually very sensitive and remain the «gold standard» as compared to other methods. These methods are particularly important when the bacterial culture is needed as the end result from positive samples. Although a negative result can be confirmed in 3-4 days, the time for a positive result is usually 5-7 days from sample collection (Paoli *et al*., 2005). As it is usually not possible to hold food products for 7 days prior to distribution, so the food industry desires faster methods for the detection of *L. monocytogenes*.

The detection of *L. monocytogenes* in foods is also hampered by the high population of competitive microflora, the low levels of the pathogen, and the interference of inhibitory food components (Norton, 2002).

As a result, in the last few years there has been a notable development of new culture media for the improved detection of *L. monocytogenes* in foods, and

**Table 1.** Main laboratory tests for the differentiation of *Listeria monocytogenes*[1]

| Species | Haemolysis[2] | Phosphatidylinositol phospholipase C[3] | Acid produced from | |
|---|---|---|---|---|
| | | | L-Rhamnose | D-Xylose |
| *L. monocytogenes* | + | + | + | – |
| *L. ivanovii* | + | + | – | + |
| *L. seeligeri* | + | – | – | + |
| *L. innocua* | – | – | V[4] | – |
| *L. welshimeri* | – | – | V | + |
| *L. grayi* | – | – | V | – |

[1] Adapted from Hitchins (2003) and Reisbrodt (2004). [2] Sheep blood agar sab. [3] On «Agar *Listeria* according to Ottaviani and Agosti» (ALOA) and similar media. [4] V: variable biotypes.

efficient methods based on antibodies or molecular techniques have also been developed (Fig. 1) (Rijpens and Herman, 2002; Gasanov *et al*., 2005; Paoli *et al*., 2005). While most of these new tests possess equal sensitivity, they are quicker and allow testing to be completed within 48 h.

Before making a decision about the selection of a new test it is important to acquire relevant details about the most adequate method according to the amount of samples processed daily or to the level of identification (genus or species) required. Most of these detection methods, such as the most common selective media, are unable to distinguish the different *Listeria* species. Within the genus *Listeria*, only *L. monocytogenes* is a human pathogen, which necessitates employing of species-specific analytical methodologies. Furthermore, not all *L. monocytogenes* strains are equally capable of causing disease in humans (McLauchlin, 1990; Farber and Peterkin, 1991). Of the 13 serovars of *L. monocytogenes,* only three i.e., 1/2a, 1/2b and 4b, cause more than 90% of the human cases (Gellin and Broome, 1989; Swaminathan, 2001). A multiplex polymerase chain reaction (PCR) assay has been developed to separate the four major *L. monocytogenes* serovars isolated from foods and patients (1/2a, 1/2b, 1/2c and 4b) into distinct groups (Doumith *et al*., 2004). This PCR test constitutes a rapid and practical alternative to laborious classical serotyping. In the future, groundbreaking methods such as microarrays will probably allow the screening of food contamination only by the virulent subtypes of *L. monocytogenes*, thus improving the prevention of food-borne human listeriosis (Call *et al*., 2003a).

This review comprises three sections. The first describes the standard detection of *L. monocytogenes* in foods based on culture methods. Secondly, quick tests based on biochemical, immunological, or molecular methods for the identification of this pathogen are reviewed. Finally, novel methods that could be applied in the future and different perspectives of *L. monocytogenes* detection are briefly discussed.

## Current standard methods

### Isolation

*Enrichment- and plating-based reference methods*

According to the most regulatory agencies, isolation methods must be capable enough to detect one *Listeria* organism per 25 g of food. This sensitivity can only be achieved by using enrichment methods. These methods employ antimicrobial agents that specifically suppress competing microflora, prior to plating onto selective agars and confirmation of cultures. The selective agents commonly used in enrichment broths are acriflavine, which inhibits the growth of other Gram-positive bacteria; nalidixic acid, which inhibits Gram-negative bacteria; and cycloheximide, which inhibits fungi. Other antimicrobials often used include the broad spectrum agents ceftazidime and moxalactam as well as lithium chloride. Another important characteristic of *Listeria* isolation media is the inclusion of esculin. All *Listeria* spp. hydrolyze esculin and the inclusion of esculin and ferric iron in enrichment or plating media results in the formation of an intense black color (Fraser and Sperber, 1988). This is due to the complexation of the ferric iron with 6,7-dihidroxycoumarin, the product of esculin cleavage by β-D-glucosidase, resulting in a black precipitate.

Several conventional methods for the isolation of *Listeria* spp. from foods have gained acceptance for international regulatory purposes. Depending on the nature of the sample, a particular method might be more suitable than the other. In general, food samples are homogenized and incubated in pre-enrichment and enrichment broth media for 24-72 h at 30-37ºC (ISO, 1996; USDA, 2002a; Hitchins, 2003).

The most commonly used culture reference methods world-wide for the detection of *Listeria* in foods are the ISO 11290 standards (ISO, 1996; EC, 1999). In the United States of America (USA) two main standards are used as reference methods to isolate *L. monocytogenes* from foods. One of the protocols was developed by the US Food and Drug Administration (FDA) to isolate *Listeria* spp. from dairy products, seafood, and vegetables (Hitchins, 2003). The US Department of Agriculture (USDA) developed another method to isolate the organism from meat and poultry products as well as from environmental samples (USDA, 2002a).

In all the enrichment methods other listerias can grow faster while hiding the presence of *L. monocytogenes*. Furthermore, before the 1990s, all commercially available isolation media for *Listeria* suffered from the lack of the ability to distinguish *L. monocytogenes* from non-pathogenic species. Even the selection of several suspect colonies could lead to the detection of only the non-pathogenic *Listeria* species, although a few *L. monocytogenes* colonies were present on the plate. Thus, the use of isolation media that allows the

identification of *L. monocytogenes* together with high numbers of other listerias is recommended.

*Chromogenic media*

Different chromogenic media have been developed to provide convenient management and identification of pathogenic *Listeria* spp. and/or *L. monocytogenes* (Table 2). This is done according to enzymes expressed by the pathogen and production of acids by the fermentation of sugars. Different antimicrobials are added to the media to obtain sufficient selectivity. Chromogenic media is the most used culture confirmation method because of its easy preparation and interpretation. Most of them are commercially available as ready-to-use plates. Using chromogenic agars, the presumptive identification of *L. monocytogenes* is possible after 24 h, compared with 3-4 days using Oxford and other conventional agars (Greenwood *et al*., 2005). 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 *plcA*, present on *L. monocytogenes*, *L ivanovii* and *L. seeligeri*, encodes the synthesis of a phosphatidylinositol-phospholipase C (PIPL-C) (Gouin *et al*., 1994) which is generally employed for the differentiation of haemolytic and non-haemolytic *Listeria* (Notermans *et al*., 1991). Cleavage of L-α-phosphatidylinositol (PI) by PI-PLC resulted in the production of water insoluble fatty acids and the formation of an opaque white halo-like zone of precipitation around the colonies of the haemolytic species. Ottaviani *et al*. (1997) combined this detection system with a chromogenic substrate (5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside, X-gluc) 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 the pathogenic species *L. monocytogenes* and *L. ivanovii* appeared surrounded with a distinct precipitation zone (Reissbrodt, 2004). Composition of ALOA is public (Hitchins, 2003; ISO, 2004) and can be prepared in-house; however, the preparation of media with so many different components is difficult to standardise (Reissbrodt, 2004). Nevertheless, it can

**Table 2.** Some commercial methods used for the specific detection or identification *Listeria monocytogenes*[1]

| Type of method | Analytical technique[2] | Test name | Company |
|---|---|---|---|
| Culture | Chromogenic medium | ALOA (also known under other names) | Several companies |
| | Chromogenic medium | Rapid'L.Mono® | Bio-Rad |
| Biochemical test | Biochemical tests | API® *Listeria* | bio-Mérieux |
| | Biochemical tests | Vitek® System | bio-Mérieux |
| | Enzyme reactions | MICRO-ID® *Listeria* | Organon Teknika |
| | Carbon source substrates | MicroLog® System | Biolog |
| | Carbohydrate use and microhaemolysis test | MICROBACT® 12L | Microgen |
| | Fatty acid patterns | Sherlock® Microbial Identification System | Microbial ID |
| Immunoassay | ELFA | VIDAS® LMO | bio-Mérieux |
| | ELISA | Transia® Plate *Listeria monocytogenes* | Diffchamb |
| Molecular method | Nucleic acid hybridization probe | Gene Trak® and GeneQuench® *Listeria monocytogenes* assays | Neogen |
| | Nucleic acid hybridization probe | AccuProbe® *Listeria monocytogenes* | Gen-Probe |
| | Real-time PCR | BAX® *L. monocytogenes* Detection System | DuPont-Qualicon |
| | Real-time PCR | Probelia® *Listeria monocytogenes* PCR System | Bio-Rad |
| | Real-time PCR | LightCycler® *Listeria monocytogenes* Detection Kit | Roche/Biotecon |
| | Real-time PCR | TaqMan® *Listeria monocytogenes* Detection Kit | Applied Biosystems |
| | Real-time PCR | GeneVision® Rapid Pathogen Detection System for *Listeria monocytogenes* | Warnex |

[1] Adapted from AOAC International (2000), USDA (2002a,b), Hitchins (2003), Ryser and Marth (2004), and Gasanov *et al.* (2005).
[2] ELFA: enzyme-linked fluorescent assay. ELISA: enzyme-linked immunosorbent assay. PCR: polymerase chain reaction.

be bought as a dehydrated medium, less expensive than the ready-to-use plates (or bottles) of most other chromogenic media for pathogenic *Listeria* spp.

Additional variations of ALOA medium have been developed and have received commercialization under different names such as BCM® chromogenic agar test, Biosynth International; CompassL.mono®, Biokar Diagnostics; BBL® CHROMagar® *Listeria,* Becton Dickinson Diagnostics; etc. (Table 2).

A different substrate for PI-PLC is 5-bromo-4-chloro-3-indolyl-myo-inositol-1-phosphate (X-IP) that leaves the bacterial colony blue when cleaved by PI-PLC (Restaino *et al*., 1999). Colonies of non-haemolytic *Listeria* appear white. Rapid'L.mono® agar (Bio-Rad) is based on X-IP for the detection of PI-PLC-positive *L. monocytogenes* and *L. ivanovii* which are differentiated by the inability of *L. monocytogenes* to metabolize D-xylose, resulting in the absence of a yellow halo (Lauer *et al*., 2005). The poor detection of species other than *L. monocytogenes* on Rapid'L.mono® agar is a disadvantage of this medium (Greenwood *et al*., 2005).

For the enumeration of *L. monocytogenes*, the ISO Standard 11290, part 2 (ISO, 1998) is applied, as well as optional protocols mentioned by the FDA and USDA methods (USDA, 2002a; 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 contamination, quantification of *Listeria* has traditionally been carried out by the most-probable-number (MPN) method (Hitchins, 2003). This technique is more sensitive than direct plating, but it needs 7 days to complete identification. Using chromogenic plating media after MPN enrichment, pathogenic *Listeria* or *L. monocytogenes* can be directly enumerated. On some chromogenic plating media such as ALOA, counts are higher than those observed with standard selective agars for *Listeria* spp. (Vlaemynck *et al*., 2000). This could be related to the suitability of ALOA for recovering injured cells of *L. monocytogenes* (Jantzen *et al*., 2006).

## Confirmation

Enrichment methods are followed by the isolation of the enriched microorganisms on specific plate media, and the identification or confirmation of the isolated bacterium. Though biochemical and other phenotypic characters are the most commonly used for species confirmation, it requires up to a week to complete the identification process. Rapid methods can not only be used for screening of enriched samples but also for culture confirmation (Fig. 1).

### *Genus* Listeria

In spite of the selective agents present in the media used for the isolation and identification of *Listeria* spp., other organisms can grow and some of them can also display a misleading colony morphology. Suspect colonies must be investigated for the typical characteristics of the genus *Listeria* (i.e. Gram-positive and non-spore forming rods, catalase-positive and oxidase-negative, and motile at 28ºC and non-motile at 37ºC) (Ryser and Marth, 2004).

### *Differentiation of all the species*

Fermentation of different sugars producing acid without gas allows differentiation of the species of *Listeria* (Table 1; Hitchins, 2003). The use of commercially miniaturized biochemical kits provides the identification of purified isolates in a simple and rapid way (Table 2). Furthermore, some of these kits have been extensively validated and are now incorporated into standard methodology (USDA, 2002a; Hitchins, 2003), such as API® *Listeria* (bio-Mérieux) and Micro-ID® (Organon Teknika) (Table 2).

### *Pathogenic species*

Pathogenic and non-pathogenic species can be differentiated by their haemolysin or PI-PLC activities. Haemolysis is the key character to distinguish the two species most frequently isolated, i.e. *L. monocytogenes* (haemolytic) and *L. innocua* (non-haemolytic). Confirmation of pathogenic *Listeria* species can also be based on their PI-PLC activity detected by most chromogenic media (Tables 1 and 2). Commercially available β-lysin discs are recommended (Hitchins, 2003) as a simple test to differentiate between haemolytic activities of *Listeria* species instead of the traditional CAMP (Christie-Atkins-Munch-Peterson) test (Christie *et al*., 1944; McKellar, 1994). D-xylose and L-rhamnose fermentation can also be used to differentiate *L. monocytogenes* (D-xylose-negative and L-rhamnose-positive)

from the other two haemolytic species *L. ivanovii* and *L. seeligeri* (D-xylose-positive and L-rhamnose-negative) (Table 1). Alanyl peptidase is an enzyme produced by all the *Listeria* species except for *L. monocytogenes*. The MonocytogenesID® discs (Biolife) use a simple colour reaction in which the substrates DL-alanine-β-naphthylamide and D-alanine-p-nitroanilide are hydrolyzed (Clark and McLauchlin, 1997). Similarly, the O.B.I.S.mono® (Oxoid Biochemical Identification System) has the ability to confirm within a very brief period of 10 min that suspect colonies are not *L. monocytogenes*.

## Alternative rapid methods for screening of enriched samples and/or culture confirmation

Most alternative methods still lack sufficient sensitivity and specificity for direct testing, and food samples need to be culture-enriched before analysis (Feng, 2001a,b). Immunological methods have relatively high limits of detection (Table 3) whereas nucleic acid amplification methods are much more sensitive (theoretically as little as a single cell is detectable). Molecular methods are often inhibited by components of food matrices and cannot distinguish living from dead cells. Thus, enrichment is needed to dilute inhibitors and to ensure that the detection of nucleic acids takes place from growing cells.

Before making a decision about the selection of a new test, it is important to investigate if it has been validated and approved. Private associations of methods regularization carry out subscriptions for manufacturers with the aim of providing independent validation of commercial tests. This regularization is according to a standardisation of the procedures, which simply does not mean that the method is recognized and approved by official government agencies. The International Organization for Standardization (ISO) is an organization which is known and recognized all over the world for standardising detection methods for *Listeria* spp. and for *Listeria monocytogenes* (ISO, 1996, 1998, 2004). The Association of Analytical Chemists (AOAC) in Washington is a widely recognized authority in validating methods. The AOAC Official Methods (AOAC International, 2000) include different alternative tests which have undergone collaborative validation and are indicated to be used for the specified food matrices according to the kit (Table 2). Some of these methods for screening enriched samples are also cited by the FDA (Hitchins, 2003).

### Immunoassay methods

There are various methods based on antibodies specific to *Listeria* available as commercial kits that have been applied in food testing for many years. However, only a few are available for the specific detection of *L. monocytogenes* (Table 2).

The enzyme-linked immunosorbent assay (ELISA) is the most common antibody assay format used for pathogen detection in foods. It is easily applied, generates rapid test results and allows the use of difficult sample matrices. The success of an ELISA depends on the specificity of the antibody. Using hybridoma technology,

**Table 3.** Comparison of commercial methods for food testing for *Listeria monocytogenes*[1]

| Type of method | Analytical technique[2] | Approximate time (h)[3] | Main use[4] and primary matrices | Sensitivity[5] (cells ml$^{-1}$) |
|---|---|---|---|---|
| Culture | Chromogenic medium | 24-48 | Isolation, food | $\leq 10^4$ |
| Immunoassay | ELFA | 1-2 | Screening, food | $\geq 10^5$ |
| Molecular method | Nucleic acid hybridization probe | 2-4 | Screening, food and environmental samples | $\geq 10^7$ |
| | Real-time PCR | $\geq 2$ | Screening, food | $\geq 10^5$ |

[1] Adapted from Gasanov *et al.,* 2005. [2] ELFA: enzyme-linked fluorescent assay. PCR: polymerase chain reaction. [3] Excluding enrichment times. [4] Screening tests are in various stages of validation and regulatory approvals, and when suitably validated they can be used to screen enrichments for *L. monocytogenes*. [5] 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 for 24-48 h. Sensitivity of PCR with pure cultures does not apply for food testing.

it has been possible to develop monoclonal antibodies that react only with *L. monocytogenes*.

There is a test which uses monoclonal antibodies that recognize the protein p60 (invasion-associated protein encoded by the *iap* gene) for identification of *L. monocytogenes* (Ky *et al*., 2004). By combining monoclonal antibodies recognized by this protein, it was possible to develop an ELISA system which can specifically identify *L. monocytogenes* or simply detect *Listeria* species. Similar tests for identification or confirmation have also been marketed. Transia® Plate *Listeria monocytogenes* (Diffchamb AB) (Bubert *et al*., 1994) and VIDAS® LMO (bio-Mérieux) (Vaz-Velho *et al*., 2000) are ELISA methods used to confirm *Listeria* isolates as *L. monocytogenes* (Table 2) (Hitchins, 2003).

Immunological tests for the specific screening of *L. monocytogenes* in enrichments are uncommon. The expression of the *prfA*-dependent virulence determinants in *L. monocytogenes* is thermo-regulated and it is achieved in a very low ratio in the extracellular environment (Ripio *et al*., 1996; Milenbachs *et al*., 1997; Shetron-Rama *et al*., 2003). Furthermore, antibody reactions have been shown to be significantly reduced in pathogens exposed to environmental stresses (Hahm and Bhunia, 2006). This is why detection of *L. monocytogenes* instead of *Listeria* spp. based on antibodies specific to virulence factors has been usually troublesome.

Nevertheless, there is a commercial test, the VIDAS® LMO assay (bio-Mérieux), which successfully targets a stable virulence antigen in an *L. monocytogenes*-specific, enzyme-linked fluorescent assay (ELFA) (Kerdahi and Istafanos, 2000; Vaz-Velho *et al*., 2000). The VIDAS® LMO II was validated in 2002 (USDA, 2002a) as a rapid method for all food products and for environmental samples. In this test, two complementary monoclonal antibodies directed to different antigenic sites of a specific *L. monocytogenes* virulence protein are used for the capture and detection process. It is a qualitative assay, but the higher the quantity of the antigen, the higher the intensity of the fluorescence captured. In a multilaboratory study conducted by Silbernagel *et al*. (2004a), VIDAS® LMO II immunoassay was evaluated. Five food types at three levels of contamination were analysed and it was demonstrated that from 1,152 samples assayed, 448 were positive by the VIDAS® LMO II assay and 457 by the standard culture methods. There is also a third method (VIDAS® *Listeria* DUO) for the simultaneous detection of *L. monocytogenes* and *Listeria* spp. in food products (bio-Mérieux).

## Nucleic acid-based methods

Detection of *L. monocytogenes* by molecular methods is very specific and can be as fast as the immunological assays.

### *DNA hybridisation*

DNA hybridisation tests have been extensively used for the differentiation of *L. monocytogenes* from other *Listeria* species by means of probes directed against specific genes. There are different commercially available kits for the testing of pure cultures or foods and environmental samples (Table 2) (Feng, 2001a,b; Hitchins, 2003). The GeneTrak® and GeneQuench® *Listeria monocytogenes* Test Kits (Neogen) are solution-based hybridisation assays for test-tubes or microtiter plates, respectively. They can be used to confirm *Listeria* isolates as *L. monocytogenes*, and also to screen food and environmental samples (Baylis, 2000). AccuProbe® *Listeria monocytogenes* Culture Confirmation Test (Gen-Probe), is also used for the identification of *L. monocytogenes* from culture, and it is based on the hybridisation of labeled DNA probes to specific ribosomal RNA sequences that are unique to *L. monocytogenes* (Ninet *et al*., 1992; USDA, 2002a). The labeled DNA:RNA hybrids are measured in a luminometer. Both DNA hybridization methods have been compared by Duvall and Hitchins (1997).

### *PCR*

The PCR is a rapid and specific nucleic acid amplification method useful for the detection of food-borne pathogens. A number of PCR assays have been described for the detection of *L. monocytogenes* in foods (Levin, 2003). PCR methods have superior sensitivity when compared to standard nucleic acid probes or immunoassays. However, complex sample preparation methods and the use of gel electrophoresis endpoint detection have hampered the transition of these methods from research to routine use in food microbiology laboratories. Nevertheless, factors influencing the performance of conventional PCR in foods continue to be investigated (Aznar and Alarcón, 2003) and standardised (D'Agostino *et al*., 2004).

In real-time PCR the amplified DNA can be quantitated by measuring the fluorescence with respect to

the binding of an intercalating dye or with respect to the binding of a fluorescent hybridisation probe. The increase in fluorescence can be monitored in real time, which allows accurate quantification over several orders of magnitude of the DNA or RNA target sequence. Results can be obtained in an hour or less, which is considerably faster than conventional PCR. Sensitivity and specificity of real-time PCR are equivalent to those of conventional PCR combined with Southern blot analysis. The method removes the manipulation of the PCR products after amplification, reducing the risk of false-positive results through cross-contamination between amplification products and subsequent test samples (Norton, 2002). All these characteristics combined with convenient applicability have made real-time PCR an alternative to conventional culture-based or immunoassay-based detection methods (Norton, 2002).

Different real-time PCR assays for the detection of *L. monocytogenes* in food have been described (Nogva *et al*., 2000; Hough *et al*., 2002; Koo and Jaykus, 2003; Rodriguez-Lázaro *et al.,* 2004a,b,c, 2005a,b; Rudi *et al*., 2005; Berrada *et al*., 2006; Oravcova *et al*., 2006). The commercial availability of real-time PCR reagents and kits (Table 2) makes it easier for food companies to adapt real-time PCR testing to their laboratories. They also facilitate the development of common testing protocols and standards so that proper collaborative studies can be performed (Silbernagel *et al*., 2004b). The USDA (2002b) has adopted the BAX® *L. monocytogenes* Detection System (DuPont-Qualicon) as a screening method for *L. monocytogenes* in enriched meat and poultry samples. It reduces the report out time for true negative samples by 24 h and reduces false-positive results, with a detection limit better than 1 cell $g^{-1}$ in a 25 g sample. In a collaborative study to compare the BAX® system and the standard cultural methods, the research concluded that, for the meat and milk products tested, this system performed well or better than the standard reference methods (Silbernagel *et al.*, 2004b).

Another PCR-based assay kit commercially available called Probelia® (Bio-Rad) was compared to the ISO method 11290-1 for the detection of *L. monocytogenes* in salmon samples by Wan *et al*. (2003). Results indicated that the Probelia® PCR method is as good as the ISO method.

The LightCycler® *Listeria monocytogenes* Detection Kit (Roche/Biotecon) (Junge and Berghof-Jager, 2006), the GeneVision® Rapid Pathogen Detection System for *Listeria monocytogenes* (Warnex) and the TaqMan® *Listeria monocytogenes* Detection Kit (Applied Biosystems) are some of the other PCR kits that can be used to qualitatively detect *L. monocytogenes* DNA in enriched samples of raw material and foods (Table 2). Most of these kits provide primers/probes, ready-to-use amplification and detection reagents, and a control template. Internal amplification controls are usually added to prevent misinterpretation of false-negative results due to amplification inhibition.

Currently, all these commercial methods are qualitative, but the application of the real-time PCR as a quantitative detection method, specific for *L. monocytogenes*, has also been developed (Nogva *et al*., 2000; Hough *et al*., 2002; Koo and Jaykus, 2003; Rodriguez-Lázaro *et al.,* 2004a,b,c, 2005a,b; Rudi *et al*., 2005; Berrada *et al*., 2006; Oravcova *et al*., 2006) and it shows good potential for routine analytical use. However, the direct quantification of *L. monocytogenes* in foods by PCR is difficult because the microorganism is generally present at very low levels. For example, in ready-to-eat food samples positive for *L. monocytogenes,* Gombas *et al*. (2003) found that 70% of the samples had levels of < 0.3 cells (MPN) $g^{-1}$. Therefore, sample enrichment is still necessary to achieve desired detection limits (Norton, 2002).

Nevertheless, PCR-based screening methods offer rapid and reliable results, and are ideal for testing pools of samples with a high probability of giving negative results for the presence of *L. monocytogenes*. In the future, better methods for template purification will facilitate the improvement of quantitative assays and the simultaneous detection of the main pathogens present in each kind of food by means of multiplex PCR (Kawasaki *et al*., 2005).

## Future trends of *Listeria monocytogenes* detection methods

Technology advances at a great pace and next generation assays that potentially have the capability for near real-time and on-line monitoring of multiple pathogens in foods are already being developed (Feng, 2001b). However, all detection methods suffer from one major drawback. The target analyte has to be found in the sample matrix before it can be presented to the detection method. Culturing of the organism in the sample may not be sufficient as the competing flora might outgrow

the target. Or, if the target is present in low numbers and a small volume of sample is taken (as many PCR methods only require 0.1 ml or less), there is a chance that this sub-sample may not include the target organism.

## Pre-detection methods to specifically concentrate *Listeria monocytogenes* cells

The use of antibody-coated immunomagnetic beads for the capture of *Listeria* from food matrices or enrichment cultures has received considerable attention (Jung *et al*., 2003). Magnetic beads coated with anti-*Listeria* antibodies are available from different companies (Paoli *et al*., 2005). In the future, methods to specifically concentrate *L. monocytogenes* will probably be developed. For example, a rapid and sensitive method for *L. monocytogenes* direct detection based on a magnetic capture hybridization procedure has been described. The amplification performed with the purified DNA could reliably identify 10 cells $ml^{-1}$, a detection level more sensitive than the PCR carried out with nucleic acids obtained using commercial nanoparticles. The method avoids the pre-enrichment and provides a rapid alternative to conventional microbiological detection methods (Amagliani *et al*., 2006).

## Tests targeting RNA

Culture-based methods for enrichment and enumeration of bacteria injured or stressed during food processing are not accurate because the selective media employed prevent their growth (Donnelly, 2002; Jantzen *et al*., 2006). In order to monitor bacterial viability, RNA-based methods must generally be used (Birch *et al*., 2001). Differentiation of living from dead bacteria by conventional PCR is difficult as DNA can be quite persistent in dead cells (Rudi *et al*., 2005). Amplification of *L. monocytogenes* mRNA by reverse transcription-PCR has been used to detect specifically viable bacteria contaminating meat (Klein and Juneja, 1997). However, sensitivity, difficulty and sample-to-sample variation preclude the extensive use of RNA methods (Keer and Birch, 2003; Navas *et al*., 2005). Specific amplification of RNA techniques has been developed including isothermal nucleic acid sequence-based amplification (NASBA) (Compton, 1991; Cook, 2003), but commercial kits for specific detection of *L. monocytogenes* RNA in foods are not available yet.

## Microarrays or biochips

DNA microarray technology has opened the way for the parallel detection and analysis of thousands of gene sequences in a relatively short time, and could be a fast and accurate method for testing common food-borne pathogenic microorganisms. DNA microarrays are composed of many discretely located probes on a solid substrate such as chemically modified glass slides. Each probe is complementary to a pathogen-specific gene sequence. PCR is used to amplify one or more genes, and subsequently the products are then hybridized to the array to identify species-specific polymorphism within one or more genes. This technology has been tested for the detection of *L. monocytogenes* in environmental samples (Call *et al.,* 2003b). The FDA microarray (Sergeev *et al*., 2004) was developed for the simultaneous detection of several food-borne pathogens and their virulence factors; therefore it has great potential in the food industry.

## Biosensors

Basically, a biosensor is a molecule of biological origin attached to a signal recognising material. When the sample comes in contact with the biosensor, the interaction will initiates a recognition signal which is reported in an instrument. The most frequently used recognition signals include electrochemical and optical (UV, bioluminescence, fluorescence, etc.) transducers.

There is an example of polyclonal antibodies and a new subtractive inhibition assay using a BIAcore 3000 biosensor, where *L. monocytogenes* cells and antibodies are incubated to allow the detection of the microorganism in less than 30 min (Leonard *et al.,* 2004). The free antibody is passed over an anti-Fab ligand-coated sensor chip surface with the generated response being inversely proportional to the inhibiting cell concentration. Another technique developed is the Multi-Analyte Array Biosensor (MAAB) with the goal of simultaneously detecting and identifying multiple target agents in complex samples with minimal user manipulation (Taitt *et al.,* 2004). Microbial sensors are particularly applicable in fluid systems with little organic substances, but this technique can present problems in its efficacy in food systems containing fats and proteins that coat the sensor and render it inoperable (Montville and Matthews, 2005).

## Conclusions

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 efficiently improved the isolation of *L. monocytogenes*. Food producers and distributors have great interest in more rapid methods, which has helped to bring about the desired changes in the available technology. The long-term goals in the development of any alternative method dictate that the test must be fast, simple, sensitive, accurate, and, for commercial purposes, inexpensive. Almost all alternative methods are designed to detect a single target, which makes them ideal for use in quality control programs to quickly screen large numbers of food samples. A positive result is considered presumptive and must be confirmed by standard methods. Even though several comparative studies have been reported, no single detection scheme appears to be so vastly superior so as to be adopted universally. Nevertheless, PCR-based screening methods allow to obtain efficient, reliable results and are ideal for monitoring the presence of *L. monocytogenes* in foods, offering results within two days after the sampling date.

## Acknowledgements

The work at the laboratories of the authors is supported by Nutreco Servicios, S. A., and the Spanish Ministry of Education Grants CAL03-027-C2-1, PTR1995-0789-OP, and RTA2005-00202-C02-02, as well as Fellowships of CNPq from Brazil (M.M. Jantzen) and INIA from Spain (J. Navas). We thank reviewers for their thoughtful comments and suggestions.

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