View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by University of East Anglia digital repository

Food Science & Technology
www.ifst.org
International Journal of Food Science and Technology 2012

Short communication

Evaluation of a novel *Listeria* enrichment broth combined with a real-time PCR diagnostics assay for the specific detection of *Listeria monocytogenes* in RTE pork products

Diego Gómez,[1,§] Sheila McGuinness,[2,§] Kate Reddington,[3] Justin O'Grady,[2,†] Javier Yangüela[1] & Thomas Barry[2,3]*

1 Animal Production and Food Science Department, Nutrition and Bromatology, Veterinary Faculty, University of Zaragoza, Zaragoza, Spain
2 Molecular Diagnostics Research Group, National Centre for Biomedical Engineering Science, National University of Ireland Galway, Galway, Ireland
3 Microbiology, School of Natural Sciences, National University of Ireland Galway, Galway, Ireland

(Received 5 June 2012; Accepted in revised form 19 October 2012)

Keywords Dry-cured meats, food borne outbreaks, *Listeria monocytogenes*, ONE Broth-Listeria, qPCR, RTE pork products.

Introduction

Foodborne outbreaks of listeriosis, human infection with *Listeria monocytogenes*, are relatively uncommon with approximately 0.1–1.1 cases /100 000 population globally, but high mortality rates of 20–30% are associated with infection (Siegman-Igra *et al*., 2002; Anonymous, 2012). Those at particular risk of infection include the immunocomprised, the elderly, neonates and pregnant women (Anonymous, 2011; Rocourt *et al*., 2003).

Since the 1990s, over 40% of foodborne listeriosis outbreaks have been associated with ready to eat (RTE) meat products (Warriner & Namvar, 2009). The most significant outbreak of recent times took place in Canada in 2008, with a total of 57 listeriosis cases associated with RTE deli meats which resulted in 23 deaths (Anonymous, 2010; Gilmour *et al*., 2010). Other significant outbreaks were related with meat frankfurters [USA, 108 cases and 14 deaths in 1998–99] (Anonymous, 1998, 1999; Mead *et al*., 2006); pâté [Australia, 11 cases and 6 deaths in 1990] (Kittson, 1992; Watson & Ott, 1990); turkey deli meats [USA, 54 cases and 8 deaths in 2002] (Anonymous, 2002; Gottlieb *et al*., 2006); 'Quargel', a brand of acid ripened curd cheese [Austria, Germany and Czech Republic, 34 cases and 8 fatalities in 2009/2010] (Fretz *et al*., 2010a,b). Listeriosis outbreaks such as these highlight the importance of surveillance in RTE foods. In spite of high salt content and low water activity (A_w), cured and ripened RTE meat products such as those examined in this study can also harbour *L. monocytogenes*. The organism is capable of growing in 10% NaCl with a water activity (A_w) of 0.90 which is typical of production conditions (AFSSA, 2000; Ryser & Donnelly, 2001).

Traditional methods for the specific detection of *L. monocytogenes* in food are both time consuming and laborious. Recently, a new culture-based method for the identification of *L. monocytogenes* from foods has been developed, namely the Listeria Precis method. This method uses a single enrichment in Oxoid Novel Enrichment Broth - Listeria (ONE Broth-Listeria) followed by selective plating on chromogenic agar [ALOA (Agar Listeria selon Ottaviani & Agosti) One Day®] and can provide presumptive results 48 h earlier than ISO 11290-1 (Anonymous, 2004a), a current standard method (Oxoid, 2010).

This initial study was performed to determine the feasibility of reducing turnaround time of the Listeria Precis method (considered the reference method used in this study) by incorporating a rapid molecular test after culture enrichment in the ONE Broth-Listeria. A previously described qPCR assay for the specific detection of *L. monocytogenes* in food (O'Grady *et al*., 2008, 2009) was combined with the ONE Broth-Listeria culture enrichment step (24 h) of the Listeria Precis method (considered the alternative method used in this study).

*Correspondent: Fax: +35391494598;
e-mail: thomas.barry@nuigalway.ie
†Present address: Centre for Clinical Microbiology, University College London (Royal Free Hospital campus), London NW3 2PF

§Both authors contributed equally to this work.

doi:10.1111/ijfs.12053
© 2012 The Authors. International Journal of Food Science and Technology © 2012 Institute of Food Science and Technology

One hundred and fifty samples of RTE pork products were tested for the presence of *L. monocytogenes* using this combined method and validated against the AFNOR approved Listeria Precis method (Oxoid, 2010).

Materials and methods

Samples

One hundred and fifty (150) RTE pork products (entire pieces in packaging) were collected from 19 small to medium-size processing plants located in Spain. The sample set included five different RTE meat products consisting of 18 morcillas (traditional Spanish cooked blood sausage), 114 raw cured products [70 chorizos (dry fermented sausage) and 44 salchichones (dry fermented-cured sausage)] and 18 dry-cured products [6 lomos embuchados (Spanish dry-cured pork loins) and 12 jamones curados (dry-cured hams)]. Food samples were transported to the laboratory in a cool box (temperature ≤ 4 °C), refrigerated and examined within 24 h.

Bacterial strains and culture conditions

Listeria species used in this study were grown in Tryptone Soya Broth (TSB; Oxoid) at 37ºC overnight (Table 1). For use as the Internal Amplification Control (IAC), *Candida albicans* was cultured in Sabouraud dextrose broth (SAB; Oxoid) at 37ºC overnight (Table 1).

Culture enrichment and microbiological examination of samples

Culture enrichment for both traditional and molecular methods was performed in the same manner. A representative sample from the entire piece of food (25 g) was added to 225 mL of ONE Broth-Listeria (Oxoid) in a sterile plastic stomacher filter bag (Seward) and homogenised in a stomacher (400 Lab System, Seward) for 2 min. After 1 h at room temperature, ONE Broth-Listeria Selective Supplement (Oxoid) was added to ONE Broth-Listeria. Flasks were incubated at 30 ºC for 24 h. Following incubation, 1.2 mL aliquots of ONE Broth-Listeria cultures were taken and stored at -80 °C for subsequent DNA isolation (i.e. for use in alternative method). At this same time point, the samples were subcultured onto ALOA (Microgen Bioproducts Ltd.) and RAPID *L. mono* (Bio-Rad Laboratories Inc.) agar plates using a 10μL microbiological loop and incubated at 37 ºC for 24–48 h (i.e. reference method). All agar plates were examined for typical blue/green with an opaque halo *L. monocytogenes* colonies, that is, suspect in ALOA agar and blue colonies in Rapid *L. mono* agar followed by confirmation using the Oxoid Biochemical Identification System (O.B.I.S.) mono test or bioMérieux's API® identification product, API Listeria (Marcy l'Etoile, France) to determine the presence or absence of *L. monocytogenes* in the enriched food.

Table 1 Bacterial and fungal strains used in this study

Bacteria	Strain designation	Serovar	Origin
Listeria monocytogenes	NCTC 5214	4a	Mammal, brain sheep circling disease
Listeria innocua	NCTC 12210	N/A	Plymouth / English produced cheese
Listeria welshimeri	NCTC 11857^T	6a	Compost; decaying vegetation
Listeria seeligeri	NCTC 11856^T	1/2b	Soil
Listeria invanovii subsp. *invanovii*	NCTC 11846^T	5	Mammal, sheep
Listeria grayi subsp. *grayi*	NCTC 10815	N/A	Cornstalks and leaves
Candida albicans var. albicans	CBS 562	N/A	Skin of man with interdigital mycosis

T = Type strain; N/A = Not applicable.

Table 2 Oligonucleotide primers and probes used in this study

Name	Type	Sequence (5′-3′)
L*ssr A* F	Forward assay primer	GCATCGCCCATGTGCTAC
L*ssr A* R	Reverse assay primer	TCTACGAGCGTAGTCACCG
IAC F	Forward composite primer for IAC generation	GCATCGCCCATGTGCTACATACCCAACTTGGAATG
IAC R	Reverse composite primer for IAC generation	TCTACGAGCGTAGTCACCGTCTTCACCAGAATAAAATTG
Hybprobe 1	*Listeria monocytogenes* hybridisation probe	CCATTCAGCTAGTCTGATTAAGCTCT-fluorescein
Hybprobe 2	*L. monocytogenes* hybridisation probe	LC Red 640-CTATTTAACCCCAGACGGAGA-phosphate
ALS1-FLU	IAC hybridisation probe	TGAATGTATCCCCTGGA-fluorescein
ALS1-LC	IAC hybridisation probe	LC Red 705-TGGCACTGGTACCATCTAA-phosphate

© 2012 The Authors
International Journal of Food Science and Technology © 2012 Institute of Food Science and Technology

Preparation of negative and positive control food samples

Chorizo sausages purchased in a local supermarket were chopped and mixed well a maximum of 15 min prior to performing the experiment. For negative control samples, 25 g portions of the prepared chorizo mixture were added to 225 mL of ONE Broth-Listeria in sterile plastic stomacher filter bags and homogenised in a stomacher for 2 min. For positive control samples, 25 g portions of the prepared chorizo sample were homogenised as above but in the presence of 1 mL broth containing $\sim$1000 CFU mL^{-1}, 100 CFU mL^{-1}, 10 CFU mL^{-1} or 1 CFU mL^{-1} of *L. monocytogenes* NCTC 5214 (approximate cell density of the test strain was previously established by plate counts in Tryptone Soya Agar (TSA; Oxoid)). Analysis was performed in triplicate, and microbiological and molecular-based methods were performed as described previously.

DNA isolation and quantification

For enriched food samples, genomic DNA was isolated from 1.2 mL of ONE Broth-Listeria cultures (thawed on ice) using the 'purification of total DNA from animal tissue' procedure in the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and eluted in 100 μL buffer AE. For *Listeria* species used as controls in real-time PCR, genomic DNA was isolated as above from 1.5–3.0 mL depending on culture density.

C. albicans DNA was isolated from 1.5 mL of culture using the 'Bacterial Genomic DNA Purification Kit' (Edge BioSystems, Gaithersburg, Maryland, USA), as per manufacturers' instructions.

Internal amplification control (IAC) for real-time PCR

Using conventional PCR and composite primers IAC F and IAC R [Table 2 (O'Grady *et al*., 2008, 2009)], a 213 bp long product from *Candida albicans* (CBS 562) was amplified. This chimeric DNA fragment (*Candida albicans* ALS1 partial sequence) was flanked on either side by the Listeria assay primer sequences (L*ssr A* F and L*ssr A* R; Table 2). The PCR product was cloned, as per manufacturers' guidelines, using the pCR 2.1-TOPO TA Cloning kit (Invitrogen). Plasmid DNA was extracted from a clone using the QIAprep Spin Miniprep Kit (Qiagen), and sequencing was performed externally (Sequiserve) using M13 primers to confirm that the sequence of interest was present.

Real-time PCR

Real-time PCR amplification was performed on the LightCycler 2.0 instrument using the 'LightCycler FastStart DNA Master HybProbe' kit (Roche Diagnostics). Real-time PCR was performed in a final volume of 20 μL as described by O'Grady *et al*. (2008, 2009). Positive controls, negative extraction and no template controls (NTC's) were included in each run. Genomic DNA extracted from all RTE pork products tested in this study were analysed in triplicate. Prior to sample analysis, a colour compensation file was generated on the instrument using a 'LightCycler Color Compensation Set' (Roche Diagnostics) following manufacturers' instructions. Analysis was performed in the 640 nm (*ssrA* gene target) and 705 nm (IAC) channels using the imported colour compensation file.

The terms of reference and formulae used in the calculation of relative accuracy, relative sensitivity and

Table 3 *Listeria monocytogenes* positive samples detected in RTE pork products by reference (culture) and alternative (qPCR based) methods

Sample type	Culture positive Sample no.	ONE broth/qPCR positive Sample no. (Tm value)
Morcilla	5	5 (60.18)
	6	6 (60.64)
	–	7 (58.61)
	9	9 (60.35)
	11	11 (58.36)
	12	12 (58.38)
	13	13 (62.41)
	–	14 (60.02)
Chorizo	–	45 (59.93)
	51	51 (59.69)
	53	53 (62.11)
	–	59 (61.04)
	–	60 (61.83)
	61	61 (61.53)
	67	67 (62.02)
	69	69 (61.15)
	–	70 (61.69)
Salchichón	89	89 (59.28)
	–	117 (60.69)
	–	118 (60.72)
	123	123 (62.24)
	–	128 (62.28)
Lomo embuchado	–	133 (62.25)
Total	**13 positives**	**23 positives**

Table 4 Summary of results for both reference (culture) and alternative (qPCR based) methods

	Reference positive	Reference negative	Total
Alternative positive	13	10*	23
Alternative negative	0	127	127
Total	13	137	150

*Positive deviation was observed in sample numbers: 7, 14, 45, 59, 60, 70, 117, 118, 128, 133.

© 2012 The Authors

International Journal of Food Science and Technology © 2012 Institute of Food Science and Technology

relative specificity are described in the ISO 16140:2003 (Anonymous, 2003).

Results

Artificial inoculation experiments revealed that the Listeria Precis method (reference method) and the novel diagnostic method developed in this study (alternative method) have the same limit of detection (LOD), that is, 1000 cells or genome equivalents of *L. monocytogenes*. Food samples spiked with $<10^3$ cells of *L. monocytogenes* were not detected by either method. Of the 150 RTE pork product samples tested, 13 were positive for *L. monocytogenes* (Table 3) by both methods. Additionally, 127 of the 150 samples yielded negative results for *L. monocytogenes* using both methods. Ten samples were positive for *L. monocytogenes* using the alternative method, but negative using the Listeria Precis reference method representing positive deviation (Table 4). Negative deviation (i.e. negative by the alternative test but positive by the reference method) was not observed in this study (Table 4). There was no evidence of PCR inhibition during analysis in the 127 negative pork samples, as the IAC was consistently detected. A variation in the melt peak melting temperatures (Tm) for *L. monocytogenes* positive samples is seen in Table 3. These data were collected from over ten runs, and the melt peak Tm shift observed was always reflected in the positive controls; the mean Tm value was 60.76 °C ± 1.29 °C. The alternative method presented in this study had a relative accuracy of 93.3%, a relative specificity of 92.7% and a relative sensitivity of 100% when compared to the Listeria Precis reference method for the detection of *L. monocytogenes* in RTE foods. The statistical significance between the reference and alternative methods was determined using the McNemar chi-squared test. The results were considered to be very statistically relevant (chi-square value of 8.100 with 1 degree of freedom, *P*-value (two-tailed) = 0.0044).

To identify the *Listeria* species detected by melt peak analysis, six control samples were included in each qPCR run, namely, *L. grayi*, *L. innocua*, *L. invanovii*, *L. monocytogenes,* *L. seeligeri* and *L. welshimeri*. Approximate melting Tm's of the control samples were as follows: *L. grayi* (51.37 °C), *L. innocua* (51.85 °C), *L. invanovii*/*L. seeligeri* (53.8 °C) and *L. monocytogenes* (59.99 °C). As demonstrated in Fig. 1 (representative data), when melt peak analysis was performed on the samples tested and compared with positive controls, the *L. monocytogenes* specific melt peak was observed in samples #12, #14 and #45 (circles). In sample #12, a melt peak (Tm 53.8 °C) was also observed indicating the presence of another *Listeria* species, possibly *L. invanovii* or *L. seeligeri*. Sample #45 also had an additional melt peak (Tm 51.85 °C), indicating the presence of *L. innocua*, which was confirmed by culture and biochemical testing (data not shown). Finally, samples #81 and #96 were negative

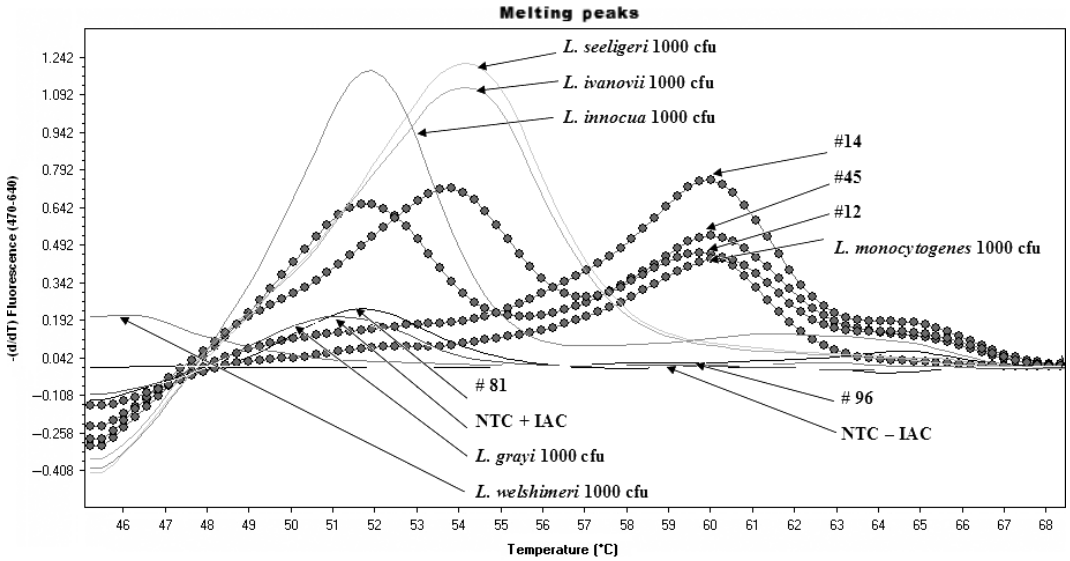

Figure 1 Melt peaks demonstrating *Listeria monocytogenes* control peak at 59.99 °C in addition to 3 RTE food samples (#12, 14 and 45) at similar Tm's indicating positive detection of the target organism. Melt peaks for additional control species: *L. seeligeri* at 53.87 °C, *L. invanovii* at 53.87 °C, *L. innocua* at 51.85 °C and *L. grayi* at 51.37 °C. Melt peak for #81 at 51.85 °C indicating the presence of species other than *L. monocytogenes*. No peak recorded for *L. welshimeri* or sample #96 (Lightcycler 2.0; 640 nm).

© 2012 The Authors
International Journal of Food Science and Technology © 2012 Institute of Food Science and Technology

for the presence of *L. monocytogenes* which was in agreement with the data generated using the Listeria Precis method (Table 3).

Discussion

Previously O'Grady *et al.* (2008) described a qPCR assay for the specific detection of *L. monocytogenes* in food samples. In this study, we have combined this qPCR assay with the Listeria Precis method after the 24 h ONE Broth-Listeria incubation step. This new approach shortens the Listeria Precis method by 24–48 h for a negative result and 48–72 h for a positive result.

Positive deviation was observed in ten samples analysed. A possible explanation for these discordant results is that DNA from dead or viable but nonculturable *L. monocytogenes* cells was detected by the alternative method in the food matrix. Alternatively, these positive deviations may in fact be true results, indicating that this new method is more sensitive than the Listeria Precis method for detecting *L. monocytogenes* in RTE pork products. Furthermore, negative deviation was not detected which demonstrates the robustness of the alternative method as food components such as organic compounds, calcium ions, glycogen and lipids have been demonstrated to inhibit PCR (Rodriguez-Lazaro & Hernandez, 2006).

The LOD was higher than expected at 1000 CFU/25 g for both alternative and reference methods in the artificial inoculation experiments. This may be due to the procedure involved in processing particulate samples such as sausages, where the sample is placed in a filter bag during homogenisation, and the bulk of the food matrix is not transferred to the flask for incubation. It is unclear whether the LOD of the methods was the same in the naturally contaminated meat product samples tested ($n = 150$), and this warrants further study.

The alternative method presented in this preliminary study requires further optimisation to improve the LOD in RTE meat products. The ONE Broth-Listeria step is the likely cause of the poor sensitivity, and this broth should be validated against the traditional ISO methods for the detection and enumeration of *Listeria monocytogenes* (ISO 11290-1 and ISO 11290-2) (Anonymous, 2004a,b) in RTE meat products. The ease of use and rapid turnaround to results of the alternative method developed in this study warrants further evaluation in other food types, where the sensitivity may be improved.

Acknowledgements

We thank the Consolider Programme (Programa Ingenio 2010), Carnisenusa (CSD 2007-00016) and the Aragon General Delegation (DGA): Consolidate Group of Research (ref: A01/2008) for financial support. D. Gómez was a beneficiary of a fellowship (CM13/10) from the DGA and the Caja de Ahorros de la Inmaculada. We also acknowledge the National University of Ireland, Galway, Ireland, which has funded K. Reddington with a postgraduate scholarship.

References

AFSSA (2000). Rapport de la Commission D'étude des Risques liès à Listeria monocytogenesAvailable at: http://www.afssa.fr. (acce ssed on 1/3/2011).

Anonymous (1998). Multistate outbreak of listeriosis – United States, 1998. *MMWR*, **47** 1085–1086.

Anonymous (1999). Update: multistate outbreak of Listeriosis – United States, 1998-1999. *MMWR*, **47**, 1117–1118.

Anonymous (2002). Public Health Dispatch: outbreak of Listeriosis – Northeastern United States, 2002. *MMWR*, **51**, 950–951.

Anonymous. (2003). *Microbiology of Food and Animal Feeding Stuffs - Protocol for the Validation of Alternative Methods* (ISO 16140:2003). Geneva, Switzerland: International Organisation for Standardisation.

Anonymous. (2004a). *Microbiology of Food and Animal Feeding Stuffs - Horizontal Method for the Detection and Enumeration of Listeria Monocytogenes - Part 1: Detection Method - Amendment 1: Modification of the Isolation Media and the Haemolysis Test, and Inclusion of Precision Data*. (ISO 11290-1:1996/Amd 1:2004). Geneva, Switzerland: International Organization for Standardization.

Anonymous. (2004b). *Microbiology of Food and Animal Feeding Stuffs - Horizontal Method for the Detection and Enumeration of Listeria monocytogenes* - Part 2: Enumeration Method - Amendment 1: Modification of the Enumeration Medium (ISO 11290-2:1998/Amd 1:2004). Geneva, Switzerland: International Organization for Standardization.

Anonymous (2010). *Update to 2008 Listeria Monocytogenes Case Numbers, Listeria Monocytogenes Outbreak*. Public Health Agency of CanadaAvailable at: http://www.phac-aspc.gc.ca/alert-alerte/listeria/listeria_20100413-eng.php (accessed on 16/2/2011)

Anonymous. (2011). The European Union summary report on trends and sources of Zoonoses, Zoonotic Agents and Food-borne outbreaks in 2009. *EFSA Journal*, **9**, 378.

Anonymous. (2012). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2010. *EFSA Journal*, **10**, 2597.

Fretz, R., Pichler, J., Sagel, U. *et al.* (2010a). Update: multinational listeriosis outbreak due to 'Quargel', a sour milk curd cheese, caused by two different L. monocytogenes serotype 1/2a strains, 2009-2010 *Euro Surveillance*, **15**(16), pii = 19543. Available at: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId = 19543. (accessed on 19/5/2011).

Fretz, R., Sagel, U., Ruppitsch, W. *et al.* (2010b). Listeriosis outbreak caused by acid curd cheese 'Quargel', Austria and Germany 2009. *Euro Surveillance*, **15**(5), pii = 19477. Available at: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId = 19477. (accessed on 20/5/2011).

Gilmour, M., Graham, M., Van Domselaar, G. *et al.* (2010). High-throughput genome sequencing of two *Listeria monocytogenes* clinical isolates during a large foodborne outbreak. *BMC Genomics*, **11** (1), 120.

Gottlieb, S.L., Newbern, E.C., Griffin, P.M. *et al.* , and Listeriosis Outbreak Working, G. (2006). Multistate outbreak of Listeriosis linked to Turkey Deli Meat and subsequent changes in US regulatory policy. *Clinical Infectious Diseases*, **42** (1), 29–36.

© 2012 The Authors

International Journal of Food Science and Technology © 2012 Institute of Food Science and Technology

Kittson, E. (1992). A case cluster of listeriosis in Western Australia with links to pâté consumption, Pp. 39–40, 11th International symposium on the problems of listeriosis, Copenhagen, Denmark.

Mead, P.S., Dunne, E.F., Graves, L. *et al*. (2006). Nationwide outbreak of listeriosis due to contaminated meat. *Epidemiology and Infection*, **134**, 744–751.

O'Grady, J., Sedano-Balbás, S., Maher, M., Smith, T. & Barry, T. (2008). Rapid real time PCR detection of *Listeria monocytogenes* in enriched food samples bassed on the *ssrA* gene, a novel diagnostic target. *Food Microbiology*, **25**, 75–84.

O'Grady, J., Ruttledge, M., Sedano-Balbás, S., Smith, T.J., Barry, T. & Maher, M. (2009). Rapid detection of *Listeria monocytogenes* in food using culture enrichment combined with real-time PCR. *Food Microbiology*, **26**, 4–7.

Oxoid. (2010). Listeria Precis. Folio No 1252/MS/11/10. Available at: http://www.oxoid.com/pdf/uk/25939_oxoid_listeria_precis.pdf. (accessed on 2/7/2011).

Rocourt, J., BenEmbarek, P., Toyofuku, H. & Schlundt, J. (2003). Quantitative risk assessment of *Listeria monocytogenes* in ready-to-eat foods: the FAO/WHO approach. *FEMS Immunology & Medical Microbiology*, **35**, 263–267.

Rodriguez-Lazaro, D. & Hernandez, M. (2006). Molecular methodology in food microbiology diagnostics: trends and current challenges. *IUFoST World Congress*, **IUFoST**, 1085–1099.

Ryser, E.T. & Donnelly, C.W. (2001). *Listeria*. In: *Compendium of Methods for the Microbiological Examination of Foods; Listeria* (edited by F.P. Downes & K. Ito). Pp. 343–356. Washington, DC: American Public Health Association.

Siegman-Igra, Y., Levin, R., Weinberger, M. *et al*. (2002). *Listeria monocytogenes* infection in Israel and review of cases worldwide. *Emerging Infectious Diseases*, **8**, 305–310.

Warriner, K. & Namvar, A. (2009). What is the hysteria with *Listeria*? *Trends in Food Science and Technology*, **20**, 245–254.

Watson, C. & Ott, K. (1990). *Listeria* outbreak in Western Australia. *Communicable Diseases Intelligence*, **24**, 9–12.

© 2012 The Authors
International Journal of Food Science and Technology © 2012 Institute of Food Science and Technology