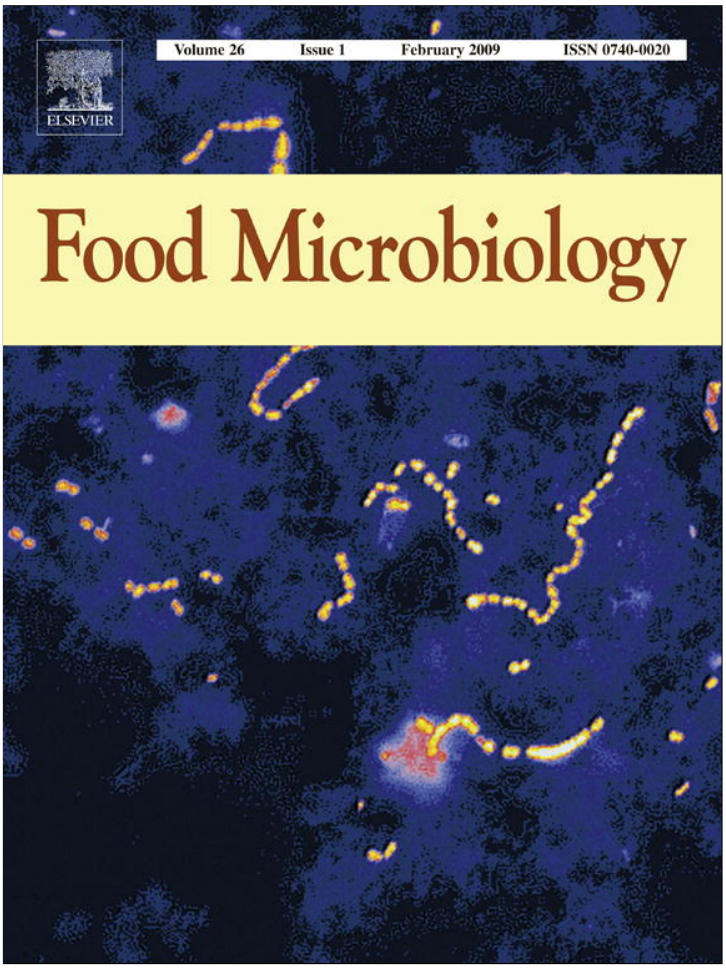

Food Microbiology 26 (2009) 4–7

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Short communication

Rapid detection of *Listeria monocytogenes* in food using culture enrichment combined with real-time PCR

Justin O'Grady [a,*], Margaret Ruttledge [b], Sara Sedano-Balbás [b], Terry J. Smith [c], Thomas Barry [a], Majella Maher [b]

[a] Molecular Diagnostics Laboratory, Department of Microbiology, National University of Ireland Galway, University Road, Galway, Ireland
[b] National Diagnostics Centre, National University of Ireland Galway, University Road, Galway, Ireland
[c] National Centre for Biomedical Engineering Science, National University of Ireland Galway, University Road, Galway, Ireland

ARTICLE INFO

Article history:
Received 4 March 2008
Received in revised form 27 August 2008
Accepted 28 August 2008
Available online 13 September 2008

Keywords:
Listeria monocytogenes
Real-time PCR
Detection
Food
ssrA gene/tmRNA
Internal amplification control

ABSTRACT

A rapid method for the detection of *Listeria monocytogenes* in foods combining culture enrichment and real-time PCR was compared to the ISO 11290-1 standard method. The culture enrichment component of the rapid method is based on the ISO standard and includes 24 h incubation in half-Fraser broth, 4 h incubation in Fraser broth followed by DNA extraction and real-time PCR detection of the *ssrA* gene of *L. monocytogenes*. An internal amplification control, which is co-amplified with the same primers as the *L. monocytogenes* DNA, was also included in the assay. The method has a limit of detection of 1–5 CFU/25 g food sample and can be performed in 2 working days compared to up to 7 days for the ISO standard. A variety of food samples from retail outlets and food processing plants ($n = 175$) and controls ($n = 31$) were tested using rapid and conventional methods. The rapid method was 99.44% specific, 96.15% sensitive and 99.03% accurate when compared to the standard method. This method has the potential to be used as an alternative to the standard method for food quality assurance providing rapid detection of *L. monocytogenes* in food.

1. Introduction

Rapid, cost-effective and automated food-borne pathogen detection and identification continues to be of major concern to the food industry and public health laboratories worldwide (Malorny et al., 2004). PCR and more recently real-time PCR technologies have become powerful diagnostic tools for the analysis of micro-organisms in food and can potentially fulfil the requirements of the industry. Validation of PCR and real-time PCR based methods for pathogen detection in food is essential if such new technologies are to be adopted by the food testing industry on a large scale (Malorny et al., 2003).

Listeria monocytogenes is a food-borne pathogen widely distributed in nature. Those most at risk of infection are pregnant women, neonates, newborns, immunocompromised persons and the elderly (Schuchat et al., 1991). Infection has been associated with a variety of foods, including cheese, meat, milk, vegetables and fish (Davies et al., 1984; Tham et al., 2000; de Valk et al., 2001; Lunden et al., 2004; Makino et al., 2005). Conventional methods for the detection of *L. monocytogenes* in food are labour intensive and time consuming, involving selective culture enrichment with subsequent culturing on selective media, followed by serological and/or biochemical tests (Cox et al., 1998). Although direct PCR based detection methods have been described for *L. monocytogenes*, pre-enrichment procedures are still necessary to ensure the detection of low numbers of viable *L. monocytogenes* in foods (Norton, 2002; O'Grady et al., 2008).

In a recent study by O'Grady et al. (2008), a rapid method for the detection of *L. monocytogenes* in food was described combining culture enrichment and a real-time PCR assay targeting the *ssrA* gene and including an internal amplification control (IAC). The enrichment procedure was based on the ISO 11290-1 standard method (Anon, 2004) and results were obtained within two working days (<30 h). The method was tested on a small number of naturally and artificially contaminated samples and a detection limit of 1–5 CFU/25 g food sample was determined. All artificially contaminated samples tested positive for the presence of *L. monocytogenes* and all natural samples tested negative. The results obtained correlated with those obtained using the Roche "LightCycler *Listeria monocytogenes* Detection Kit" and the presence or absence of typical *Listeria* colonies on PALCAM agar plates.

In this study, 206 food samples and controls, comprising a large variety of food matrices, were tested for the presence of *L. monocytogenes* using the method developed by O'Grady et al. (2008) and results compared to the ISO 11290-1 reference method (Anon, 2004).

* Corresponding author. Tel.: +353 9 151 2325.
E-mail address: justin.ogrady@nuigalway.ie (J. O'Grady).

0740-0020/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fm.2008.08.009

2. Methods

2.1. Culture enrichment of food samples

Culture enrichment of food samples and controls was performed by the Food Testing Laboratory, University College Hospital, Galway, Ireland ($n = 164$; 133 food samples, 16 spiked positive controls, 15 negative controls) and Complete Laboratory Solutions (CLS), Connemara, Galway, Ireland ($n = 42$) according to the ISO 11290-1 standard method (Anon, 2004). Various food sample types were prepared according to standard methods ISO 8261:2002 (milk and milk products), ISO 6887-1:1999 (preparation of test samples for microbiological examination), ISO 6887-2:2003 (meat and meat products), ISO 6887-3:2003 (fish and fishery products) and ISO 6887-4:2003 (products other than milk and milk products, meat and meat products and fish and fishery products) (Anon, 1999, 2002, 2003a, b, c). Food samples (25 g/25 ml) were added to 225 ml of half-Fraser broth (Oxoid, Hampshire, UK), in sterile plastic Seward Stomacher filter bags (Norfolk, UK) and homogenized in a stomacher (Seward Stomacher 400 Lab System, Norfolk, UK) where necessary for 1 min. For swabs (Sodibox, La Forêt-Fouesnant, France), 225 ml of half-Fraser broth was added to the sterile plastic bag containing the swab and homogenized in a stomacher (Seward Stomacher 400 Lab System, Norfolk, UK) for 1 min. The mixture was poured into sterile containers and incubated at 30 °C. After 24 h ($n = 164$ samples) primary enrichment in half-Fraser broth or 24 h primary enrichment in half-Fraser and 4 h secondary enrichment at 37 °C in Fraser broth ($n = 42$ samples), 10 ml aliquots of culture were removed. Samples grown for 24 h in half-Fraser broth only were sub-cultured in Fraser broth for 4 h according to the standard method (100 μl half-Fraser broth culture inoculated into 10 ml Fraser broth incubated at 37 °C). Post-enrichment 1.5 ml aliquots of the culture-enriched samples were combined with *Escherichia coli* cells with the IAC containing plasmid (mean 98 CFU, range 60–150 CFU) for DNA extraction.

2.2. Preparation of stressed (frozen) L. monocytogenes culture and "spiked" positive control food samples

Serial 10-fold dilutions of an overnight culture of *L. monocytogenes* were performed in nutrient broth containing 10% glycerol. Plate counts were performed to estimate the cell numbers of *L. monocytogenes*. One-millilitre aliquots of 10^{-6} dilution ($\sim$100 CFU/ml) were prepared and frozen at −80 °C as a method of stressing the cells. Food samples (25 g/25 ml food) previously shown to be *L. monocytogenes* negative using ISO 11290-1 were added to 225 ml of half-Fraser broth and homogenized as described above. To generate the "spiked" positive control food samples for this study, an aliquot of frozen *L. monocytogenes* culture was thawed and 100 μl ($\sim$10 CFU) was inoculated into the homogenized sample prior to incubation.

2.3. DNA isolation and quantification

Genomic DNA was isolated from 1.5 ml of enriched food cultures combined with *E. coli* cells with the IAC containing plasmid using the "Bacterial Genomic DNA Purification Kit" (Edge BioSystems, Gaithersburg, Maryland, USA) according to the manufacturer's instructions and resuspended in 50 μl nuclease free dH_2O (Ambion, Austin, TX, USA). DNA samples were stored at −20 °C.

2.4. L. monocytogenes real-time PCR assay

Real-time PCR amplification was performed on the LightCycler using the "LightCycler FastStart DNA master hybridization probes" kit (Roche Diagnostics, Mannheim, Germany). PCR was performed in a final volume of 20 μl including 2 μl of template DNA in 10× LightCycler hybridization buffer with $MgCl_2$ adjusted to 5 mM concentration. PCR primers (0.5 μM concentration) and FRET hybridization probes for *L. monocytogenes* and IAC targets (0.2 μM concentration) were added to the reaction mixture and the volume was increased to 20 μl by addition of nuclease free dH_2O. The cycling parameters consisted of: 95 °C incubation for 10 min for enzyme activation and DNA denaturation, followed by 45 PCR amplification cycles consisting of 95 °C for 10 s, 55 °C for 20 s and 72 °C for 10 s. The temperature transition rate for all cycling steps was 20 °C/s. Fluorescence acquisition was at the end of the annealing stage of each cycle. The thermocycling program was followed by a melting program of 95 °C for 1 min (denaturation), 45 °C for 30 s (annealing), and then 45–80 °C at a transition rate of 0.1 °C/s with continual monitoring of fluorescence. All subsequent analysis was carried out in the F2/BackF1 (*ssrA* gene target) and F3/BackF1 (IAC) channels with colour compensation using the second derivative maximum option of the LightCycler software (version 3.5). A no-template negative control was included in each run.

2.5. Sequencing of real-time ssrA gene PCR products

For food samples negative for *L. monocytogenes* by the standard method which yielded a positive result with the rapid method, the *ssrA* gene PCR product generated in the rapid method for these samples was sequenced (Sequiserve, Vaterstetten, Germany).

3. Results and discussion

In this study 175 food samples including a large variety of food types and 31 control samples (16 spiked foods, 15 negative controls), were tested for the presence of *L. monocytogenes* with the ISO 11290-1 standard method and a previously developed rapid method combining culture and real-time PCR (O'Grady et al., 2008). The assay targets the *ssrA* gene, includes an IAC and has a detection limit of 1–10 cell equivalents per PCR reaction. The assay is capable of detecting 1–5 *L. monocytogenes* CFU/25 g in a variety of food types in <30 h. The assay, which specifically detects *L. monocytogenes* based on melt-peak analysis, is also capable of detecting *L. innocua*, *L. ivanovii*, *L. seeligeri and L. grayi* based on melt-peak analysis.

The rapid real-time PCR based method performed very well compared to the conventional method (Table 1). PCR inhibition, monitored by the IAC, was not observed in any of the samples tested. Of the 206 food and control samples tested, 26 samples were positive for *L. monocytogenes* and 180 samples were negative for *L. monocytogenes* by both conventional and rapid methods. One false positive and one false negative result were obtained during the study. *Listeria* spp. (not *L. monocytogenes*) were detected by the rapid method for the false negative sample. One possibility for this result is that the enriched food sample contained more *Listeria* spp. cells than *L. monocytogenes* and as the rapid method can also detect *Listeria* spp. the *Listeria* spp. DNA present in the extracted sample may have out-competed the *L. monocytogenes* DNA for primers and been preferentially amplified in the rapid method. In the case of the false positive result, *L. monocytogenes* was detected by the rapid method while *Listeria* spp. were detected by microbiological culture and although the result of the rapid method was incorrect there was *Listeria* contamination present in the food sample. Overall, the developed method was 99.44% specific, 96.15% sensitive and 99.03% accurate in comparison to the standard method. The low numbers of *L. monocytogenes* positive samples ($n = 26$) skew the sensitivity results slightly as only one false negative was observed and the developed method detected the presence of *Listeria* spp. in that sample.

Table 1
Food types tested and results obtained for rapid method and ISO standard method.

Food type	Real-time PCR based method			Total number of samples	ISO 11290 standard method	
	Listeria spp. NEGATIVE	*L. monocytogenes* POSITIVE	*Listeria* spp. POSITIVE (not *L. monocytogenes*)		*L. monocytogenes* POSITIVE	*L. monocytogenes* NEGATIVE
Beef type 1: Cooked/roast beef	5	0	0	5	0	5
Beef type 2: Minced beef	1	0	0	1	0	1
Beef type 3: Beef stew	1	0	0	1	0	1
Beef type 4: Roast beef with mustard and salad	1	0	0	1	0	1
Black pudding	5	0	0	5	0	5
Cake type 1: Boiled fruit cake	1	0	0	1	0	1
Cake type 2: Various cream cakes	3	0	0	3	0	3
Cake type 3: Coffee cake (iced cake)	1	0	0	1	0	1
Cake type 4: Cheese cake	1	0	0	1	0	1
Coleslaw	5	0	0	5	0	5
Dairy type 1: Fresh cream	4	0	0	4	0	4
Dairy type 2: Ice cream	17	0	0	17	0	17
Dairy type 3: Flavoured ice cream	4	0	0	4	0	4
Dairy type 4: Soft ice cream	12	0	0	12	0	12
Dairy type 5: Ice cream base	1	0	0	1	0	1
Dairy type 6: Flavoured milk	6	0	0	6	0	6
Egg type 1: Egg salad sandwich	1	0	0	1	0	1
Egg type 2: Quiche	1	0	0	1	0	1
Egg type 3: Custard	1	0	0	1	0	1
Fish type 1: Fish swab	7	9[b]	0	16	8	8
Fish type 2: Smoked salmon	12[a]	2	7[c]	20	3	17
Fish type 3: Cooked salmon	1	0	0	1	0	1
Fish type 4: Tuna sandwich with various fillings	2	0	0	2	0	2
Fish type 5: Fish base sauce	1	0	0	1	0	1
Garlic sauce	1	0	0	1	0	1
Gravy	1	0	0	1	0	1
Ham type 1: Cooked ham	9	0	1	10	0	10
Ham type 2: Ham salad sandwich	2	0	0	2	0	2
Ham type 3: Picnic shoulder	1	0	0	1	0	1
Lasagne	3	0	0	3	0	3
Noodles: Sweet chilli noodles	1	0	0	1	0	1
Pasta: Roasted pepper pasta	1	0	0	1	0	1
Pork type 1: Roast pork	1	0	0	1	0	1
Pork type 2: Pork pie	1	0	0	1	0	1
Poultry 1: Cooked chicken	4	0	0	4	0	4
Poultry 2: Cajun chicken	1	0	0	1	0	1
Poultry 3: Chicken sandwiches various	5	1	0	6	1	5
Poultry 4: Chicken curry	1	0	0	1	0	1
Poultry 5: Cooked turkey	3	0	0	3	0	3
Rice type 1: Cooked rice	1	0	0	1	0	1
Rice type 2: Risotto	1	0	0	1	0	1
Salad type 1: Salad leaves (various)	9	0	0	9	0	9
Salad type 2: Trio bean salad	1	0	0	1	0	1
Salad type 3: Pasta salad	1	0	0	1	0	1
Salad type 4: Potato salad	2	0	0	2	0	2
Salad type 5: Potato salad with bacon	1	0	0	1	0	1
Sliced onions	1	0	0	1	0	1
Surface swab	5	0	0	5	0	5
Trifle type 1: Black forest trifle	1	0	0	1	0	1
Trifle type 2: Sherry trifle	1	0	1	2	0	2
Trifle type 3: Fruit cocktail trifle	0	0	1	1	0	1
Vegetable soup	1	0	0	1	0	1
Controls:						
Spiked food type 1: Coleslaw	0	2	0	2	2	0
Spiked food type 2: Cooked beef	0	2	0	2	2	0
Spiked food type 3: Cooked ham	2	2	0	4	2	2
Spiked food type 4: Potato salad	0	6	0	6	6	0
Spiked food type 5: Trifle	0	1	0	1	1	0
Spiked food type 6: Tuna	0	1	0	1	1	0
Negative controls (uninoculated broth)	15	0	0	15	0	15
Total	171	26	10	206	26	180
Agreement with standard method	99.44% specific, 96.15% sensitive, 99.03% accurate					

[a] One of the smoked salmon samples produced a false negative result.
[b] One of the fish swab samples produced a false positive result.
[c] *L. monocytogenes* and *Listeria* spp. detected in one smoked salmon sample.

Of the 26 *L. monocytogenes* positive samples, 14 were spiked food sample positive controls. Stressed (frozen) *L. monocytogenes* (approx. 10 CFU) were spiked into various food samples, one per batch of food samples tested. For two batches, the positive control gave a negative result in both the rapid and standard methods. In these batches, one food sample tested positive for *L. monocytogenes* validating the testing methods for that batch. The most likely explanation for the failure of the positive controls is that the *L. monocytogenes* cells used for inoculation were dead. Nine food samples which were negative for *L. monocytogenes* by the standard method were positive with the rapid method for *Listeria* species. The *ssrA* gene PCR products generated for these samples were sequenced and the sequence data was analysed using the Basic Local Alignment Search Tool (BLAST). Six samples were identified as *L. innocua*, 2 as *L. seeligeri* and 1 as *L. ivanovii*. These results correlated with the melt-peak data obtained in the real-time PCR assay for these samples.

A PCR based method for the detection of pathogens in food should fulfil a number of criteria, such as analytical and diagnostic accuracy, high detection probability, high robustness (including an IAC), low carryover contamination and accessible user-friendly protocols for its application and interpretation (Malorny et al., 2004). The rapid method evaluated in this study meets these requirements.

The rapid method allows fast and sensitive detection of *L. monocytogenes* in various food matrices and could be used as a screening method. The method is based on the ISO 11290-1 standard, facilitating its integration in routine diagnostics laboratories (Rossmanith et al., 2006).

Acknowledgements

The authors wish to acknowledge the Food Testing Laboratory, University College Hospital, Galway, Ireland, and Complete Laboratory Solutions (CLS), Connemara, Galway, Ireland for kindly providing enriched food samples and results for the conventional *L. monocytogenes* detection method. We would also like to acknowledge the Food Institutional Research Measure Programme funded through The Department of Agriculture and Food under the National Development Plan 2000–2006 for funding the project.

References

Anon, 1999. Microbiology of food and animal feeding stuffs—Preparation of test samples, initial suspension and decimal dilutions for the microbiological examination—Part 1: General rules for the preparation of the initial suspension and decimal dilutions (EN ISO 6887-1:1999). International Organization for Standardization, Geneva.

Anon, 2002. Milk and milk products—General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination (EN ISO 8261:2002). International Organization for Standardization, Geneva.

Anon, 2003a. Microbiology of food and animal feeding stuffs—Preparation of test samples, initial suspension and decimal dilutions for the microbiological examination—Part 2: Specific rules for the preparation of meat and meat products (EN ISO 6887-2:2003). International Organization for Standardization, Geneva.

Anon, 2003b. Microbiology of food and animal feeding stuffs—Preparation of test samples, initial suspension and decimal dilutions for the microbiological examination—Part 3: Specific rules for the preparation of fish and fishery products (EN ISO 6887-3:2003). International Organization for Standardization, Geneva.

Anon, 2003c. Microbiology of food and animal feeding stuffs—Preparation of test samples, initial suspension and decimal dilutions for the microbiological examination—Part 4: Specific rules for the preparation of products other than milk and milk products, meat and meat products and fish and fishery products (EN ISO 6887-4:2003). International Organization for Standardization, Geneva.

Anon, 2004. Microbiology of food and animal feeding stuffs. Horizontal method for the detection and enumeration of Listeria monocytogenes. Part 1: Detection method (EN ISO 11290–1:1996/A1:2004). International Organization for Standardization, Geneva.

Cox, T., Frazier, C., Tuttle, J., Flood, S., Yagi, L., Yamashiro, C.T., Behari, R., et al., 1998. Rapid detection of *Listeria monocytogenes* in dairy samples utilizing a PCR-based fluorogenic 5′ nuclease assay. Journal of Industrial Microbiology and Biotechnology 21, 167–174.

Davies, J., Ewan, E., Varughese, P., Acres, S., 1984. *Listeria monocytogenes* infections in Canada. Clin. Investi. Med 7, 315–320.

de Valk, H., Vaillant, V., Jacquet, C., Rocourt, J., Le Querrec, F., Stainer, F., Quelquejeu, N., et al., 2001. Two consecutive nationwide outbreaks of listeriosis in France, October 1999–February 2000. Am. J. Epidemiol 154, 944–950.

Lunden, J., Tolvanen, R., Korkeala, H., 2004. Human listeriosis outbreaks linked to dairy products in Europe. J. Dairy Sci. 87, E6–E12.

Makino, S., Kawamoto, K., Takeshi, K., Okada, Y., Yamasaki, M., Yamamoto, S., Igimi, S., 2005. An outbreak of food-borne listeriosis due to cheese in Japan, during 2001. Int. J. Food Microbiol. 104, 189–196.

Malorny, B., Paccassoni, E., Fach, P., Bunge, C., Martin, A., Helmuth, R., 2004. Diagnostic real-time PCR for detection of Salmonella in food. Appl. Environ. Microbiol. 70, 7046–7052.

Malorny, B., Tassios, P.T., Radstrom, P., Cook, N., Wagner, M., Hoorfar, J., 2003. Standardization of diagnostic PCR for the detection of foodborne pathogens. Int. J. Food. Microbiol. 83, 39–48.

Norton, D., 2002. Polymerase chain reaction-based methods for detection of *Listeria monocytogenes*: toward real-time screening for food and environmental samples. J AOAC Int 85, 505–515.

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

Rossmanith, P., Krassnig, M., Wagner, M., Hein, I., 2006. Detection of Listeria monocytogenes in food using a combined enrichment/real-time PCR method targeting the prfA gene. Res. Microbiol. 157, 763–771.

Schuchat, A., Swaminathan, B., Broome, C.V., 1991. Epidemiology of human listeriosis. Clin. Microbiol. Rev. 4, 169–183.

Tham, W., Ericsson, H., Loncarevic, S., Unnerstad, H., Danielsson-Tham, M., 2000. Lessons from an outbreak of listeriosis related to vacuum-packed gravad and cold-smoked fish. Int. J. Food Microbiol. 62, 173–175.