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ARTICLE

Comparison of Culture, Conventional and Real-time PCR Methods for *Listeria monocytogenes* in Foods

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Abstract

We compared standard culture methods as well as conventional PCR and real-time PCR for the detection of *Listeria monocytogenes* (*L. monocytogenes*) in milk, cheese, fresh-cut vegetables, and raw beef that have different levels of background microflora. No statistical differences were observed in sensitivity between the two selective media in all foods. In total, real-time PCR assay exhibited statistically excellent detection sensitivity ($p<0.05$) and was less time consuming and laborious as compared with standard culture methods. Conventional culture methods showed poor performance in detecting *L. monocytogenes* in food with high levels of background microflora, generating numerous false negative results. While the detection of *L. monocytogenes* in fresh cut vegetable by culture methods was hindered only by *L. innocua*, various background microflora, such as *L. innocua, L. welshimeri, L. grayi,* and *Enterococcus faecalis* appeared on the two selective media as presumptive positive colonies in raw beef indicating the necessity of improvement of current selective media. It appears that real-time PCR is an effective and sensitive presumptive screening tool for *L. monocytogenes* in various types of foods, especially foods samples with high levels of background microflora, thus complementing standard culture methodologies.

Keywords: *Listeria monocytogenes*, culture method, profiling of false-positive colonies, conventional PCR, real-time PCR

Introduction

Listeria monocytogenes is an emerging bacterial foodborne pathogen responsible for listeriosis, an illness characterized by meningitis, encephalitis, and septicemia (Churchill *et al.*, 2006). Most countries have a zero tolerance policy toward the presence of *L. monocytogenes* in ready-to-eat (RTE) foods owing to the possible severe consequences (Berrada *et al.*, 2006; Jadhav *et al.*, 2012; Yang *et al.*, 2007). As such, the capability to detect *L. monocytogenes* in low numbers in food samples is essential.

Various methodologies, including conventional culture, molecular biological, biochemical, and immunological techniques, have been implemented for the rapid and specific detection of *L. monocytogenes* (Almeida and Almeida, 2000; Amagliani *et al.*, 2006; Klein and Juneja, 1997; Manzano *et al.*, 1998; Solve *et al.*, 2000; Wang and Hong, 1999). However, all methods are not well suited for routine use (Amagliani *et al.*, 2006). The most commonly used reference methods for the detection of *L. monocytogenes* in foods worldwide are the ISO 11290 standards, which use conventional culture methods with selective and chromogenic media, Oxford agar, polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol (PALCAM) agar, and Agar *Listeria* Ottaviani Agosti (ALOA) (Churchill *et al.*, 2006; ISO, 1996; Janzten *et al.*, 2006). These methods can be used to detect *L. monocytogenes* at the level of 5-100 colony-forming units (CFU)/25 g of food; however, the presence of competing microflora such as *Listeria innocua* leads to false-negative results (Churchill *et al.*, 2006; Scotter *et al.*, 2001). Rapid and sensitive screening tests have been recommended for coupling with conventional culture methods to overcome this drawback (Han *et al.*, 2008).

In this study, we compared the sensitivities and selectivities of 4 methods (culture on Oxford agar, culture on PALCAM agar, conventional polymerase chain reaction

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[PCR], and real-time PCR) of detecting *L. monocytogenes* to determine whether PCR assays could be used as an alternative rapid screening tool for *L. monocytogenes* in food samples. To determine the effect of background microflora on the detection of *L. monocytogenes*, we used food matrices composed of foods that have different background microflora levels and have been most commonly implicated in human listeriosis (Churchill *et al.*, 2006; Gugnani, 1999; Meng and Doyle, 1997). In addition, we identified false-positive colonies that most commonly appeared on the 2 selective media, in order to obtain background information for the future development of improved culture media.

Materials and Methods

Bacterial strains

Twenty *L. monocytogenes* strains were used in this study. Most strains were originally obtained from the Food and Drug Administration (College Park, USA), and five standard strains were acquired from the American Type Culture Collection (ATCC). All *L. monocytogenes* strains were grown in tryptic soy broth (Difco Laboratories, USA) containing 0.6% yeast extract (Difco) for 18 h at 37°C. In total, 42 non-*L. monocytogenes* spp. were streaked onto nutrient agar (Difco) for 2 passages and incubated in tryptic soy broth (Difco) for 24 h at 37°C. All strains used in this study are listed in Table 1. For artificial inoculation into food samples, viable *L. monocytogenes* counts were obtained by serially diluting (10-fold) the overnight cultures in phosphate-buffered saline (PBS, pH 7.2, Difco) and plating 100 μL of the dilutions on tryptic soy agar (Difco) containing 0.6% yeast extract.

Sample preparation and inoculation of *L. monocytogenes*

Milk, cheese, fresh-cut vegetables, and raw beef with different matrices and background microflora levels were used to determine differences in the detection capabilities of culture methods through conventional and real-time PCR. All samples were purchased from a local retail market in Seoul, Korea. A mesophilic aerobic plate count was performed for uninoculated food samples to enumerate the background microflora in experimental food samples according to a previously described method (Chon *et al.*, 2010).

L. monocytogenes ATCC 51776 was used in experimental inoculation testing. One milliliter of the inoculum was prepared via serial dilution of the overnight culture grown in 225 mL *Listeria* enrichment broth (Difco). The inoculum was then evenly inoculated into 500 g (mL) of bulk samples via pipetting to generate partial positives and partial negatives for statistical comparison after division into subsamples. The inoculum levels ranged from 43 to 1,040 CFU of *L. monocytogenes* for bulk samples. The inoculated bulk samples were subsequently divided into 20 subsamples of 25 g each. Two additional food samples (25 g each) were used as positive and negative controls. A positive control was prepared by spiking 25 g of the sample with approximately 10^7 CFU/mL of *L. monocytogenes* ATCC 51776. As a negative control, uninoculated food (25 g) and sterilized PBS (1 mL) were prepared.

Culture methods

The detection of *L. monocytogenes* in the food samples by using culture was performed according to the methods described in ISO 11290-1 (ISO, 1996). After sample preparation and artificial inoculation of *L. monocytogenes* ATCC 51776, twenty-five grams of food was placed in 225 mL *Listeria* enrichment broth (Difco), homogenized in a BagMixer stomacher (Interscience, France) for 2 min, and incubated at 30°C for 24 h. Aliquots (100 mL) of these primary enrichments were transferred to 10 mL of a secondary enrichment Fraser broth (Difco) and incubated at 37°C for 24 h. Enrichment broths were inoculated on Oxford agar (Oxoid, UK) and PALCAM agar (Oxoid) and incubated at 37°C for 24-48 h. One typical gray-green colony with a black halo on Oxford and PALCAM agar on each plate was selected for biochemical confirmation using the Vitek 2 system (bioMerieux, France).

DNA isolation

Bacterial DNA templates were extracted as described by Seo and Brackett (2005) with some modifications. One-milliliter samples from pure cultures in PBS or food samples in secondary enrichment broth were centrifuged at 14,000 rpm for 3 min. The pellets were washed in 1 mL of PBS and centrifuged at 14,000 rpm for 3 min and then resuspended in 200 μL of PrepMan Ultra Reagent (Applied Biosystems, USA) and boiled for 10 min. The samples were centrifuged at 14,000 rpm for 3 min. The supernatant was used for conventional and real-time PCR.

Conventional PCR

Specific primers derived from conserved sequences of the *hlyA* gene were used to test conventional PCR methods (Pagotto *et al.*, 2002). The primer sequences were 5'-

Table 1. Bacterial strains used in sensitivity and specificity tests

Listeria monocytogenes strains	Non-*Listeria monocytogenes* strains
Poly O Type 4 isolated from brie cheese	*Listeria innocua* isolated from meats (n=6)
Serovar 4b isolated from clinical sample	*Listeria welshimeri* isolated from meats (n=6)
Poly O Type 1 isolated from ground veal	*Staphylococcus aureus* ATCC 6538[1)]
Poly O Type 1 isolated from flounder	*Staphylococcus aureus* isolated from beef
Poly O Type 1 isolated from hamburger	*Staphylococcus aureus* isolated from chicken
Poly O Type 1 isolated from sausage	*Staphylococcus aureus* isolated from pork (n=2)
Poly O Type 1 isolated from monkfish	*Enterobacter sakazakii* FSM 145
Poly O Type 4 isolated from bovine tissue	*Enterobacter sakazakii* FSM 261
ATCC 51776[1,3)]	*Enterobacter sakazakii* FSM 262
ScottA	*Enterobacter sakazakii* FSM 265
FSL-C1-109	*Enterobacter sakazakii* FSM 270
299056-A	*Enterococcus faecalis* isolated from beef (n=2)
TS29/F2365	*Enterococcus faecalis* isolated from pork (n=2)
FSL-C1-122	*Enterococcus faecalis* isolated from chicken
457778-1A	*Salmonella* Enteritidis D1 Serotype 3
FSL-J1-177	*Salmonella* Enteritidis D1 Serotype 13A
FSL-C1-056	*Salmonella* Enteritidis D1 Serotype 24
CU-BR 1/93	*Salmonella* Enteritidis Serotype 97
FSL-M1-004	*Salmonella* Enteritidis D1 SerotypeS132
908260	*Escherichia coli* O157:H7 (n=5)[2)]
	Serratia odorifera I
	Serratia marcesens
	Enterobacter aerogenes ATCC 13048[1)]
	*Citrobacter freundii*ATCC 8090[1)]
	Proteus mirabilis ATCC 7002[1)]
n=20	n=42

[1)] Obtained from American Type Culture Collection (ATCC).
[2)] Obtained from Centers for Disease Control and Prevention (CDC).
[3)] This strain was used in experimental inoculation testing.
Strains with no superscripts were obtained from the Food and Drug Administration.

CATTAGTGGAAAGATGGAATG-3' (primer A) and 5'-GTATCCTCCAGAGTGATCGA-3' (primer B) and were used to amplify a 730-bp fragment. PCR was performed with the Takara Taq™ Hot Start Version (Takara Bio Inc., Japan), using a Biometra T-Personal thermal cycler (Biometra GmbH, Germany). The reaction was performed at 94°C for 8 min for the initial denaturation, followed by 30 cycles each at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and then a final extension at 72°C for 2 min. In total, 5 μL of amplified PCR product was analyzed with electrophoresis on a 1.5% agarose gel containing 50 μL SafeView™ (Applied Biological Material Inc., Richmond, Canada) per liter. The amplified sequences were examined under ultraviolet light using a BioRad Molecular Imager® GelDoc™ XR (BioRad Laboratories, USA).

Real-time PCR

The *iap* gene was targeted using the primers and probe according to the method described by Hein *et al.* (2001). The *L. monocytogenes* probe was labeled with 6-carboxyfluorescein (FAM, the reporter dye) and 6-carboxytetramethylrhodamine (TAMRA, the quencher dye). The sequences for *iap* (amplicon size, 175 bases) were as follows: forward primer, 5'-CTA AAG CGG GAA TCT CCC TT-3'; reverse primer, 5'-CCA TTC TCT TGC GCG TTA AT -3'; and probe, 5'-FAM CCT CTG GCG CAC AAT ACG CTA GCA CT-3' TAMRA. The extracted DNA fluids (5 μL) were transferred into 20 μL of PCR mix consisting of 12.5 μL TaqMan Universal PCR Master Mix (Applied Biosystems), forward primer (2.5 μL, 900 nM), reverse primer (2.5 μL, 900 nM), and TaqMan probe (2.5 μL, 250 nM). The 96-microwell plate was sealed with optical adhesive covers (Applied Biosystems) and was placed in an ABI-Prism 7500 sequence detector (Applied Biosystems). The reaction was run at 50°C for 2 min and then at 95°C for 10 min, followed by 40 cycles each of 95°C for 15 s and 60°C for 60 s.

Sensitivity and specificity of 3 detection methods

To determine the sensitivity and specificity of each test,

a total of 62 strains − 20 *L. monocytogenes* and 42 non-*L. monocytogenes* − were streaked onto Oxford and PALCAM agar. Plates yielding any colonies were considered positive regardless of the color and morphological features of the colonies. In parallel, conventional and real-time PCR were examined for pure cultures of these strains.

Detection limits

Detection limits were determined as described by Chon *et al.* (2012), with modification. To determine the detection limits of conventional and real-time PCR in PBS, we extracted genomic DNA from diluted overnight cultures containing 10^8 CFU/mL, as described above. The extracted DNA was then serially diluted (10-fold) in PBS. A total of 5 μL of amplified PCR product was analyzed with electrophoresis as described earlier, and the cycle threshold value of the dilutions was measured with real-time PCR. The detection limits of conventional and real-time PCR were also measured in all foods used in this study. Inocula (1 mL each) containing 7.2×10^1-1.2×10^8 CFU of *L. monocytogenes* ATCC 51776 were serially inoculated into 10 g of food samples to yield a final *L. monocytogenes* concentration range of 7.2×10^0-7.2×10^7 CFU/g. Each inoculated sample was transferred into 90 mL of 0.85% saline water and homogenized for 1 min using a stomacher. Conventional and real-time PCR were performed with genomic DNA extracted from 1 mL of each diluted sample (7.2×10^0-7.2×10^7 CFU/mL) as previously described. The lowest bacterial count that yielded a positive reaction was considered the detection limit of conventional and real-time PCR.

Statistical analysis

The number of positives was compared in pairs using the McNemar test with SPSS Statics (ver 18.0, SPSS Inc., USA), and statistical differences were determined. Significant difference was reached when the *P* value was less than 0.05.

Results and Discussion

Sensitivity and specificity of detection methods for various strains

Data describing the sensitivity and specificity of 2 selective media, conventional PCR assay, and real-time PCR assay are presented in Table 2. Conventional and real-time PCR assays revealed no positive reaction with non-*L. monocytogenes* strains, providing sensitivity and specificity for the detection of *L. monocytogenes* at the species level. In contrast, *L. innocua*, *Listeria welshimeri*, and *Enterococcus faecalis* grew on the 2 selective media. All non-*L. monocytogenes* strains (6 each of *L. innocua* and *L. welshimeri*) and 3 of 5 *E. faecalis* strains (Table 2) grew on both selective media, forming the typical gray-green colony with a black halo. Firstenberg-Eden and She-

Table 2. Comparison of sensitivity (inclusivity) and specificity (exclusivity) of selective media and PCR methods using pure cultures of *Listeria monocytogenes* and non- *L. monocytogenes* strains

Strain		No. of positive strains / total No. of strains tested (%)			
		Culture method		Conventional PCR	Real-time PCR
		Oxford agar	PALCAM agar		
Listeria monocytogenes [1)]		20/20 (100)[a]	20/20 (100)[a]	20/20 (100)[a]	20/20 (100)[a]
Non-Listeria monocytogenes strains[2)]	*Listeria innocua*	6/6 (100)	6/6 (100)	0/6 (0)	0/6 (0)
	Listeria welshimeri	6/6 (100)	6/6 (100)	0/6 (0)	0/6 (0)
	Staphylococcus aureus	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
	Cronobacter spp.	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
	Enterococcus faecalis	3/5 (60)	3/5 (60)	0/5 (0)	0/5 (0)
	Salmonella spp.	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
	Escherichia coli O157:H7	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
	Serratia odorifera	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)
	Serratia marcesens	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)
	Enterobacter aerogenes	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)
	Citrobacter freundii	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)
	Proteus mirabilis	0/1 (0)	0/1 (0)	0/1 (0)	0/1 (0)
	Non-LM Total[2,3)]	15/42 (35.7)[c]	15/42 (35.7)[c]	0/42 (0)[d]	0/42 (0)[d]

[1)]Different letters (a, b) within a row indicate a significant difference ($p<0.05$) in sensitivity.
[2)]Different letters (c, d) within a row indicate a significant difference ($p<0.05$) in specificity.
[3)]Total number of non-*L. monocytogenes* strains.
PALCAM, polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol; PCR, polymerase chain reaction.

lef (2000) first reported that certain enterococci formed typical colonies on PALCAM, thus demonstrating their capability to hydrolyze esculin. Marlene *et al.* (2001) have also demonstrated that PALCAM and Oxford media do not accommodate the differentiation of *L. monocytogenes* colonies and those of other *Listeria* species. Altho- ugh ALOA was not used in this study, the detection rate of *L. monocytogenes* on ALOA is also affected by the presence of *L. innocua* (Scotter *et al.* 2001). Our results correspond with those of these previous studies and suggest that rapid screening for *L. monocytogenes* should include PCR-based methodologies, which precisely differentiate *L. monocytogenes* from non-*L. monocytogenes Listeria* species (Table 2).

Detection limits of PCR assays

The detection limits of conventional and real-time PCR assays in pure culture and food samples are shown in Table 3. In pure cultures, more than 7.2×10^3 CFU and 7.2×10^2 CFU of bacteria were required to achieve a positive reaction with conventional and real-time PCR, respectively (Table 3). Furthermore, for all food samples, more than 7.2×10^4 CFU of bacteria was required for a positive reaction with conventional and real-time PCR (Table 3). The detection limits of conventional and real-time PCR have been reported to be influenced by the matrix or background microflora level of foods (Lee *et al.*, 2010; McLauchlin *et al.*, 2000; Tamarapu *et al.*, 2001). However, in this study, the detection limits of *L. monocytogenes* with conventional and real-time PCR were identical in all foods studied.

Identification of presumptively positive colonies on the 2 selective media

The levels of background microflora and the confirmation of presumptive positive colonies on Oxford and PAL CAM agar obtained using the Vitek 2 system are presented in Table 4. As determined with aerobic plate counts, milk and cheese had less than 2 Log CFU/mL or g of background microflora. In the case of fresh-cut vegetables and raw beef, the counts of background microflora were 6.81±0.28 Log CFU/g and 4.00±0.17 Log CFU/g, respectively. In the case of milk and cheese, all suspicious *L. monocytogenes* colonies on both Oxford and PALCAM agar were confirmed as *L. monocytogenes* (29 of 29 in milk; 26 of 26 in cheese). Both media apparently detected

Table 3. Detection limits of *Listeria monocytogenes* ATCC 51776 with conventional and real-time PCR assays in pure culture and experimentally inoculated food samples without enrichment

Number of cells (CFU/mL)	Pure culture		Food samples							
	PBS		Milk		Cheese		Vegetable salad		Raw beef	
	PCR	Real-time PCR	PCR	Real-time PCR	PCR	Real-time PCR	PCR	Real-time PCR	PCR	Real-time PCR
7.2×10^7	+	+	+	+	+	+	+	+	+	+
7.2×10^6	+	+	+	+	+	+	+	+	+	+
7.2×10^5	+	+	+	+	+	+	+	+	+	+
7.2×10^4	+	+	+	+	+	+	+	+	+	+
7.2×10^3	+	+	−	−	−	−	−	−	−	−
7.2×10^2	−	+	−	−	−	−	−	−	−	−
7.2×10^1	−	−	−	−	−	−	−	−	−	−
7.2×10^0	−	−	−	−	−	−	−	−	−	−

ATCC, American Type Culture Collection; CFU, colony-forming units; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

Table 4. Identification of presumptively positive colonies on selective media using the Vitek 2 system

Food samples	No. of background microflora[1)]	No. of presumptively positive plates / total No. of samples tested	No. of plates confirmed by Vitek 2 system / No. of presumptively positive plates (%)				
			Listeria monocytogenes	*Listeria innocua*	*Listeria welshimeri*	*Listeria grayi*	*Enterococcus faecalis*
Milk	< 2	29/40	29/29 (100)	0/29 (0)	0/29 (0)	0/29 (0)	0/29 (0)
Cheese	< 2	26/40	26/26 (100)	0/26 (0)	0/26 (0)	0/26 (0)	0/26 (0)
Vegetable salad	6.81±0.28	40/40	15/40 (37.5)	25/40 (62.5)	0/40 (0)	040/ (0)	0/40 (0)
Raw beef	4.00±0.17	40/40	23 or 24[2)] /40 (57.5)	8/40 (20)	4/40(10)	1/40 (2.5)	4 or 3[3)]/40 (10)

[1)]log colony-forming units (CFU)/g.
[2)]23 on Oxford agar and 24 on polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol (PALCAM) agar.
[3)]4 on Oxford agar and 3 on PALCAM agar.

L. monocytogenes effectively in foods with low levels of background microflora. However, for fresh-cut vegetables, only 15 of 40 suspicious colonies (37.5%) on both media were confirmed to be of *L. monocytogenes* and 25 of 40 suspicious colonies (62.5%) on both media were of *L. innocua*. In the case of raw beef, only 23 of 40 suspicious colonies (57.5%) were confirmed as *L. monocytogenes*; 8 (20%) were of *L. innocua*, 4 (10%) were of *L. welshimeri*, 4 (10%) were of *E. faecalis*, and 1 (2.5%) was of *Listeria grayi*. Our results indicate that the detection of *L. monocytogenes* could be highly hindered by other *Listeria* spp. and *Enterococcus* spp. in food samples with high levels of background microflora. Although the count of background microflora in raw beef was lower than that in fresh-cut vegetables, a wider variety of non-*L. monocytogenes* colonies was notably observed on both Oxford and PALCAM media in fresh beef samples.

Of all meat products, raw minced meat has been reported to have the highest incidence of *Listeria* spp. - more than 86% positivity - which can be attributed either to fecal contamination during evisceration or to food handling (Fenlon *et al.*, 1996; Yucel *et al.*, 2005). In addition, fresh-cut vegetables are commonly contaminated with *Listeria* spp., which hinders the selective detection and isolation of *L. monocytogenes* with selective culture media (Little *et al.*, 2007). The most prevalent background microflora in this study was *L. innocua*, which is known to produce a bacteriocin-like substance that inhibits the growth of *L. monocytogenes* during enrichment culture (Yokoyama *et al.*, 1998). *Listeria innocua* also has a higher growth rate in selective liquid media than that of *L. monocytogenes,* resulting in a high number of false-negative results on PALCAM and Oxford media (Curiale and Lewus, 1994; MacDonald and Sutherland, 1994). In addition, *E. faecalis* strains, which are ubiquitous and can hydrolyze esculin, grew and formed a typical *L. monocytogenes*-like colony on Oxford and PALCAM agar in this study (Table 2) (Robin *et al.*, 1997). While examining food samples with high levels of background microflora, random selection of presumptive positive colonies leads to a high chance of missing coexisting *L. monocytogenes* (Curiale and Lewus, 1994; MacDonald and Sutherland, 1994; Petran and Swanson, 1993).

We clearly showed that the standard culture methods present challenges for the detection of *L. monocytogenes*, especially in foods with high levels of background microflora. The culture methods should be improved by inhibiting the growth of non-*L. monocytogenes* strains such as *L. innocua*, *L. welshimeri*, *E. faecalis*, and *L. grayi*, especially at the secondary enrichment step. No enrichment medium that selects *L. monocytogenes* over other *Listeria* spp. is currently available (Vlaemynck *et al.*, 2000). Alternatively, other rapid and selective screening methods performed with the enrichment broth are required to reduce the risk of listeriosis.

Comparison of detection methods for *L. monocytogenes* in various food samples

A comparison of the performance of the culture methods as well as that of conventional and real-time PCR in food samples is shown in Table 5. No positive reactions were obtained in the negative controls with the culture methods, conventional PCR, or real-time PCR, whereas all positive controls were detected as positives with all detection methods. Therefore, we conclude that samples used in the experiments were not naturally contaminated by *L. monocytogenes*. Significant differences were seen between real-time PCR and culture methods ($p<0.05$) in the overall results (Table 5). Real-time PCR appears to have a higher detection capability than culture methods and conventional PCR assays for *L. monocytogenes* in foods, regardless of the matrix and count of background microflora.

In particular, conventional and real-time PCR assays provide more positives in the case of food samples such as fresh-cut vegetables and raw beef, which have high background microflora levels (Table 5). In fresh-cut vegetables, although both media revealed only 15 positives in 40 samples, conventional and real-time PCR yielded 18 and 22 positives, respectively, in 40 samples. This tendency was also found in the raw beef, in which 23 and 24 positives were found in 40 samples with Oxford and PAL CAM agar, respectively, and 25 and 27 positives with conventional and real-time PCR, respectively.

In food samples with high levels of background microflora, *L. monocytogenes* seemed likely to be partly missed by the culture methods, thus resulting in false negatives. To overcome this disadvantage, Vlaemynck *et al.* (2000) have suggested that using additional confirmation techniques immediately for the enriched broth might reduce the number of false negatives. Many novel techniques have been applied for the detection and screening of *L. monocytogenes* in food, including immuno-based method, molecular method, on-site analysis method (loop-mediated isothermal amplification), and biosensor-based techniques to date (Jadhav *et al.*, 2012; Suh *et al.*, 2014). PCR-based method, however, is still one of the most powerful screening methods for *L. monocytogenes* in food.

Table 5. Comparison of culture methods, conventional PCR, and real-time PCR for the detection of *Listeria monocytogenes* in artificially inoculated food samples

Food samples	Trial No.	Inoculum (CFU/500g of sample)	No. of positive samples / No. of samples tested (%)			
			Culture method		Conventional PCR	Real-time PCR
			Oxford agar	PALCAM agar		
Milk	1	43	11/20 (55)	11/20 (55)	11/20 (55)	12/20 (60)
	2	107	18/20 (90)	18/20 (90)	18/20 (90)	19/20 (95)
	Total		29/40 (72.5)	29/40 (72.5)	29/40 (72.5)	31/40 (77.5)
Cheese	1	46	9/20 (45)	9/20 (45)	9/20 (45)	11/20 (55)
	2	98	17/20 (85)	17/20 (85)	17/20 (85)	18/20 (90)
	Total		26/40 (65)	26/40 (65)	26/40 (65)	29/40 (72.5)
Vegetable salad	1	485	6/20 (30)	6/20 (30)	8/20 (40)	10/20 (50)
	2	1040	9/20 (45)	9/20 (45)	10/20 (50)	12/20 (60)
	Total		15/40 (37.5)	15/40 (37.5)	18/40 (45)	22/40 (55)
Raw beef	1	450	13/20 (65)	13/20 (65)	14/20 (70)	15/20 (75)
	2	113	10/20 (50)	11/20 (55)	11/20 (55)	12/20 (60)
	Total		23/40 (57.5)	24/40 (60)	25/40 (62.5)	27/40 (67.5)
Grand total			93/160 (58.13)*	94/160 (58.75)*	98/160 (61.25)	109/160 (68.13)

*Significantly difference compared with real-time PCR (McNemar test, $p<0.05$).
PALCAM, polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol; PCR, polymerase chain reaction.

Recently, there appears to have been an increasing interest in the improvement of the real-time PCR assays by enhancing sensitivity and reducing test time and cost (Gattuso *et al.*, 2014; Rodriguez-Lazaro *et al.*, 2014). In addition, the validation of real-time PCR in various food samples including soft cheese and pork has been conducted by comparing with ISO standard methods (Gattuso *et al.*, 2014; Gianfranceschi *et al.,* 2014). In these studies, real-time PCR methods showed higher performance in detecting *L. monocytogenes* compared to standard method. These results correspond with our study, suggesting that PCR assays, especially real-time PCR, are useful screening tools for the detection of *L. monocytogenes* in food samples, especially with high levels of background microflora.

In conclusion, our results indicate that more sophisticated and precise selective media should be developed for the detection of *L. monocytogenes* in foods with high levels of background microflora, especially fresh vegetables and meats. The results also suggest that real-time PCR could be an effective and sensitive presumptive screening tool for detecting *L. monocytogenes* in those food matrices. The limitations of the current study include lack of internal amplification controls (IAC) in PCR assays to rule out false negatives that might occur as a result of PCR inhibitors in the food matrix and samples of naturally contaminated foods. Therefore, further validation of PCR assays with IACs using more naturally contaminated foods is necessary in future studies.

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