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Comparison of selective and nonselective primary enrichments for the detection of *Listeria monocytogenes* in cheese

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Abstract

A completely selective enrichment procedure was compared with two partially nonselective ones for the detection of *Listeria monocytogenes* in cheeses. After enrichment for approximately 48 h, the enrichment media were streaked on selective agars and presumptive *Listeria* colonies were confirmed using PCR. In some cases, PCR was also performed directly on the enrichment broth. The conventional, completely selective enrichment procedure was not always the best choice for the detection of stressed *L. monocytogenes* in cheeses. Especially in the case of semi-hard cheeses from pasteurized milk and soft cheeses of the blue veined and the red smear types, the methods that incorporated a nonselective enrichment step gave better results than the completely selective method. For mold ripened, soft cheeses, the results were highly dependent on the brand of cheese and time of sampling, but the best results were obtained using the completely selective enrichment procedure.

Keywords: *Listeria monocytogenes*; Stressed; Nonselective enrichment; Cheese

1. Introduction

There is no consensus regarding a standard method for the detection of *Listeria monocytogenes* in food products as various methods have been proposed by different organizations. These various procedures all specify a total enrichment period of 48 h, usually in two steps of 24 h each, but they differ with respect to the types of enrichment broth(s) and the selective plating media used. Independent of the method used, a positive identification of *L. monocytogenes* takes from 7 to 12 days when confirmation of presumptive *Listeria* colonies is performed biochemically. Many rapid methods, which the enrichment steps have been modified and/or biochemical identification procedures have been replaced with immunological or nucleic acid techniques (Olsen et al., 1995; Hill, 1996; Scheu et al., 1998), were developed and commercialized in order to speed up diagnosis by replacing the biochemical identification and/or part of the enrichment process. However, several studies have shown that using multiple enrichment procedures for each sample will increase the isolation rate of *Listeria* in foods (Heisick et al., 1989; Lammerding and Doyle, 1989; Hayes et al., 1992; Slade, 1992; Dever et al., 1993; Vlaemynck and Moermans, 1996; Duarte et

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al., 1999; Pritchard and Donnelly, 1999; Donnelly, 2002).

It has been demonstrated that *L. monocytogenes* can be sublethally stressed by environmental challenges and that many of the selective agents used in selective enrichment media interfere with the process of repair in such cells (Speck and Ray, 1977; Gnanou Besse, 2002). Also, different authors have shown that *L. monocytogenes* has a significantly greater generation time than *Listeria innocua* in certain selective media (Petran and Swanson, 1993; MacDonald and Sutherland, 1994; Curiale and Lewus, 1994; Sheridan et al., 1994; Jackson et al., 1993). This favored growth of *L. innocua* may lead to overgrowth of *L. monocytogenes* when both species are present (Jackson et al., 1993; Petran and Swanson, 1993; Curiale and Lewus, 1994). Consequently, in some cases, the *L. monocytogenes* isolation rate from food products was higher using a short, nonselective enrichment rather than a longer, selective enrichment step (Sheridan et al., 1994; Beumer et al., 1996; Walsh et al., 1998). In other studies, however, no benefit from a nonselective enrichment was found (In 't Veld et al., 1995). Overgrowth by uninjured background microflora or inhibition by selective agents might variously explain these conflicting results.

To identify which type of procedure would be appropriate to use for cheeses, a completely selective enrichment procedure with two partially nonselective ones for the detection of *L. monocytogenes* in different types of cheeses was compared with or without the cheese being artificially contaminated with stressed *L. monocytogenes* cells.

2. Materials and methods

2.1. Procedures for the detection of *L. monocytogenes* in cheese

The nine procedures used for the recovery of L. monocytogenes from cheeses are detailed in Fig. 1.

2.2. Analysis of cheeses artificially contaminated with 5 CFU of stressed *L. monocytogenes*

Enrichment procedures A*/A^{SP}, B*/B^{SP} and C*/C^{SP} were used for the detection of L. monocytogenes in Gouda-type cheese purchased at retail stores on six occasions. These cheeses were contaminated with a commercially available reference material (In 't Veld et al., 1995). This reference material is prepared at the RIVM/SVM (National Institute of Public Health and Environment, Bilthoven, Netherlands) by spray drying a mixture of a *L. monocytogenes* suspension and pasteurised full fat milk, and subsequently mixing the highly contaminated milk powder with sterile milk powder. As a consequence of this spray drying process, the *L. monocytogenes* cells can be considered as stressed by heat and desiccation (In 't Veld et al., 1995).

A capsule containing reference material with an average of 5 CFU sublethally injured *L. monocytogenes* ALM 92 (RIVM/SVM) (In 't Veld et al., 1995) was added to 25 ml of heated buffered peptone water (BPW, Oxoid, Basingstoke, UK) or to half strength Fraser broth containing 500 mg/l ammonium ferric citrate, 10 mg/l nalidixic acid and 12.5 mg/l acriflavine (Fraser selective supplement code SR166G, Oxoid) and was incubated at 37 °C until dissolved (~ 30 min), to obtain reference material solutions AB or C, respectively. Cheese (25 g) was stomached for 2 min in 200 ml of BPW (flask AB) or half strength Fraser broth (flask C), added to the solution of reference material (AB or C) and subsequently incubated at 37 or 30 °C, for 5 h (flask AB) or 20–24 h (flask C). After 5 h of incubation, half of the BPW-broth (flask AB) was added to 125 ml of half strength Fraser broth further supplemented with ammonium ferric citrate to a final concentration of 1000 mg/l (flask B), and flasks A (remains of flask AB) and B were incubated for 15–19 h at 37 and 30 °C, respectively. After this first enrichment period, 0.1 ml of the contents of flask A was added to 9.9 ml of half strength Fraser broth (tube A) while 0.1 ml of the contents of each of flasks B and C was added to 9.9 ml of Fraser broth (tubes B and C). All tubes were incubated for 20–24 h at 35 °C and subsequently streaked on Oxford agar plates (Oxoid), which were incubated at 37 °C for 48 h. Presumptive *Listeria* colonies were individually subjected to the polymerase chain reaction (PCR) analysis (samples A^{SP}, B^{SP}, C^{SP}). Additionally, immunomagnetic separation was performed on 1 ml of the contents of tubes A, B and C as described below and the resulting pellets (samples A*, B*, C*) were used for PCR.

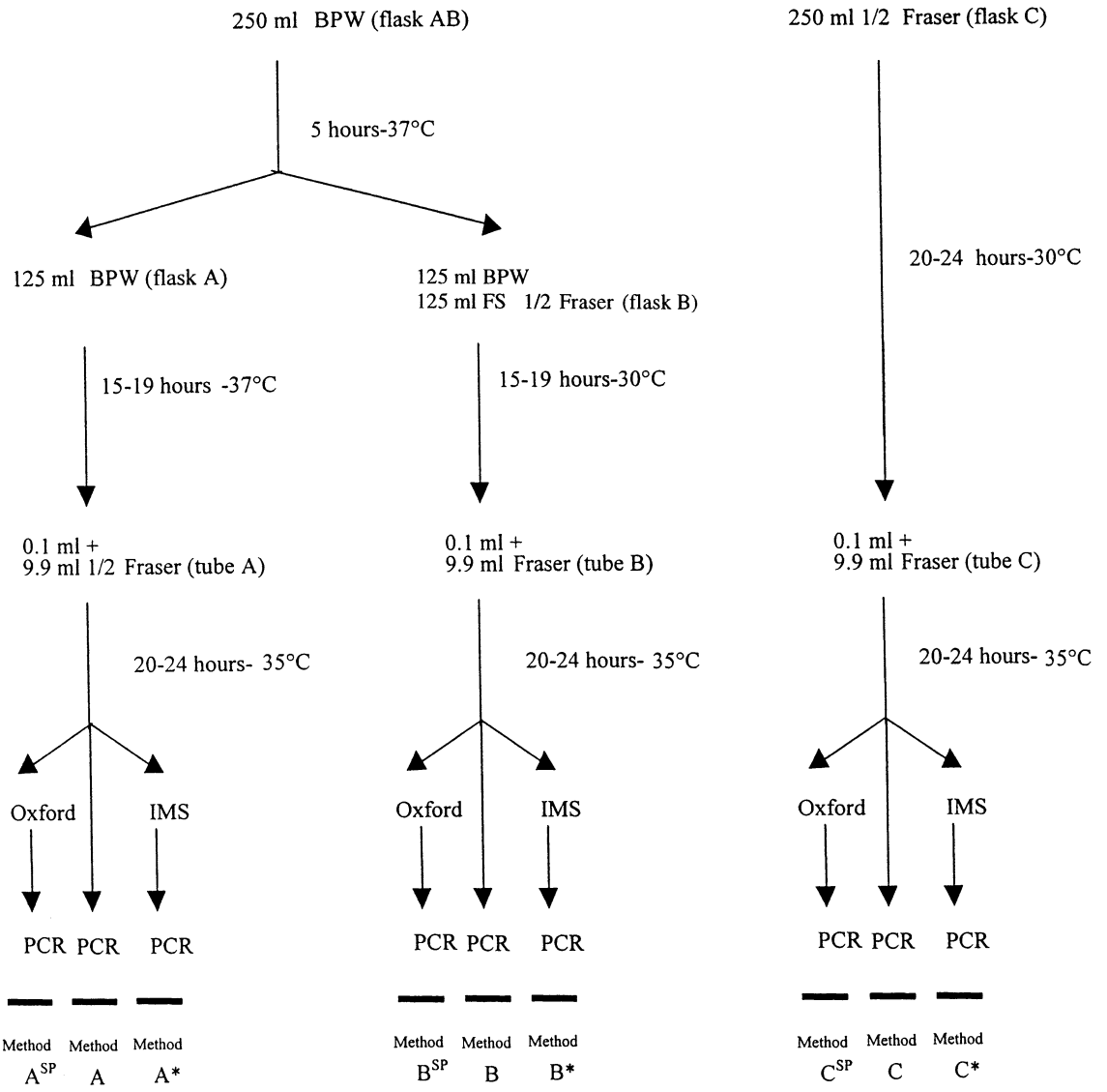

Fig. 1. Schematic presentation of the enrichment procedures used in this study. BPW: Buffered peptone water; 1/2 Fraser: half strength Fraser broth; FS 1/2 Fraser: further supplemented 1/2 half strength Fraser broth; IMS: immunomagnetic separation; PCR: polymerase chain reaction.

2.3. Analysis of cheeses artificially contaminated with 50 CFU of stressed *L. monocytogenes*

Procedures A/A*/A^{SP}, B/B*/B^{SP} and C/C*/C^{SP} were compared for the detection of *L. monocytogenes* in mold ripened, red smear, and blue veined, soft cheeses made from raw or pasteurized milk, purchased at retail stores on 23 occasions.

A capsule of reference material containing an average of 5000 CFU of sublethally injured L. monocytogenes was added to 25 ml of BPW, which was warmed to 25 °C, then incubated at room temperature until the capsule was dissolved. A 25-g portion of each cheese was stomached for 2 min with 225 ml of BPW (flask AB) or half strength Fraser broth (flask C). A 250-μl portion of the solution of reference material was

added to each flask. The flasks were subsequently incubated at 37 or 30 °C, for 5 h (flask AB) or 20–24 h (flask C). After 5 h of incubation, half of the BPW-broth (flask AB) was added to 125 ml of half strength Fraser broth. Further supplemented with ammonium, ferric citrate (flask B) and flasks A (remains of flask AB) and B were incubated for 15–19 h at 37 and 30 °C, respectively. After this first enrichment period, 0.1 ml of the contents of flask A was added to 9.9 ml of half strength Fraser broth (tube A) while 0.1 ml of the contents of each of flasks B and C was added to 9.9 ml of Fraser broth (tubes B and C) and all tubes were incubated for 20–24 h at 35 °C.

Subsequently, these samples were streaked on Oxford agar plates, which were incubated at 37 °C for 48 h. Presumptive *Listeria* colonies were subjected to PCR analysis (samples A^{SP}, B^{SP} and C^{SP}). Additionally, 1 ml of the contents of each of tubes A, B and C was centrifuged for 5 min at 13,000 × g, and the resulting pellet was washed with 100 μl of water and subsequently used for PCR (samples A, B and C). Also, immunomagnetic separation was performed on 1 ml of the contents of each of the tubes A, B and C as described below, and the resulting pellets (samples A*, B* and C*) were used for PCR.

2.4. Immunomagnetic separation (IMS)

Twenty microliters of Dynabeads anti-*Listeria* (Dynal, Oslo, Norway) was added to 1 ml of enrichment broth and this mixture was rotated for 10–15 min at room temperature. The magnetic particles were separated from the broth by applying a magnet at the outside of the microcentrifuge tube for 5–10 min. The magnetic beads were washed twice with 1 ml of phosphate buffered saline (PBS; 138 mM NaCl, 2.7 mM KCl, 0.05% Tween-20, pH 7.4). The cells were pelleted by centrifugation for 5 min at 13,000 × g.

2.5. Preparation of crude cell lysates and PCR

Crude cell lysates were prepared for all methods by suspending the pellets obtained from enrichment broths in 50 μl of 50 mM NaOH and 0.125% of sodium dodecyl sulphate (SDS) and heating for 17 min at 90 °C. PCR was performed on each of these samples in 50 μl of a reaction mixture containing 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.5% Tween-20, 0.01% gelatine, 200 μM of each dNTP, 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer), 100 pmol of primers, and 5 μl of crude cell lysate. As primers, LM1 (5′ CCTAAGACGCCA-ATCGAA 3′) and LM2 (5′ AAGCGCTTGCAACT-GCTC 3′) directed at the listeriolysin O gene *hlyA* (Mengaud et al., 1988) were used. The reaction mixture was subjected to 30 cycles of amplification in a thermal cycler (Cetus 9600; Perkin Elmer, Norwalk, CT, USA). The first cycle was preceded by initial denaturation for 1 min at 95 °C. Each cycle consisted of denaturation for 15 s at 95 °C, annealing for 15 s at 50 °C, and extension for 30 s at 72 °C. The last cycle was followed by a final extension step for 8 min at 72 °C. The PCR products were analysed on a 1.5% (w/v) Seakem agarose gel (FMC BioProducts, Rockland, ME, USA).

Presumptive *Listeria* colonies on Oxford agar plates were individually picked and washed with 100 μl of water, suspended in 50 μl of 50 mM NaOH and 0.125% SDS, and heated for 17 min at 90 °C. PCR was performed as described above except that only 1.5 U of AmpliTaq DNA polymerase, 50 pmol of each primer and 1 μl of crude cell lysate were used.

3. Results

3.1. Analysis of cheeses samples artificially contaminated with 5 CFU of stressed *L. monocytogenes*

With samples of soft cheese, artificially contaminated with 5 CFU of stressed *L. monocytogenes*, the recovery rates ranged from 0 to 14% depending on the method used (results not shown). Method A- and B-based protocols (both including a nonselective enrich-

Table 1
Detection of *L. monocytogenes* in 25 g samples of semi-hard cheeses inoculated with 5 CFU of stressed *L. monocytogenes*

Sample type	N[a]	Number of positive samples (% recovery)					
		Detection method in enrichment broth[b]			Detection method on agar plates[b]		
		A*	B*	C*	A^{SP}	B^{SP}	C^{SP}
Semi-hard cheese	30	27 (90)	18 (60)	19 (63)	25 (83)	19 (63)	17 (57)

[a] *N*, number of samples analysed.
[b] A*, B*, C*, A^{SP}, B^{SP}, C^{SP}. The methods are described in Fig. 1.

ment step in BPW) tended to give better recoveries than the method C-based protocols (not including a BPW-step).

When samples of semi-hard cheeses of the Gouda type made from pasteurized milk were artificially contaminated with 5 CFU of *L. monocytogenes*, the organism was recovered from 57% to 90% of the samples, depending on the method used (Table 1). Procedure A* and A^{SP} gave better results (83% to 90%) than either of the other methods (57% to 63%). There was little difference in the results obtained with the methods incorporating a short nonselective en-

Table 2
Detection of *L. monocytogenes* in 25 g samples of soft cheeses inoculated with 50 CFU of stressed *L. monocytogenes*

Type of cheese	Brand	N[a]	Number of positive samples								
			Method of detection in enrichment broth[b]						Method of detection on agar plates[b]		
			A	B	C	A*	B*	C*	A^{SP}	B^{SP}	C^{SP}
Soft, mould ripened, from raw milk	1	5	0	0	1	0	0	1	0	0	1
	2	10	0	0	0	0	0	0	0	0	0
	Total	15	0	0	1	0	0	1	0	0	1
Soft, mould ripened, from pasteurized milk	3	5	4	3	1	1	5	5	4	5	5
	4	5	2	2	1	0	3	2	2	3	3
	5	5	0	0	1	0	0	0	0	0	2
	6	5	ND[c]	ND	ND	ND	ND	ND	ND	5	3
	Total brands 3–5	15	6	5	3	1	8	7	6	8	10
	Total brands 3–6	20								13	13
Soft, red smear, from pasteurized milk	7	5	0	0	0	0	0	0	0	1	0
	8	5[d1]	ND	ND	ND	ND	ND	ND	0	0	0
	9	5[d2]	ND	ND	ND	ND	ND	ND	4	3	1
	10	5[d3]	ND	ND	ND	ND	ND	ND	ND	0	0
	11	5	ND	ND	ND	ND	ND	ND	ND	0	0
	12	5[d4]	ND	ND	ND	ND	ND	ND	ND	4	2
	13	5	ND	ND	ND	ND	ND	ND	ND	5	0
	14	5[d5]	ND	ND	ND	ND	ND	ND	ND	0	0
	15	5	ND	ND	ND	ND	ND	ND	ND	0	1
	16	5	ND	ND	ND	ND	ND	ND	ND	4	1
	17	5	ND	ND	ND	ND	ND	ND	ND	3	2
	18	5	ND	ND	ND	ND	ND	ND	ND	0	0
	Total brands 7–9								4	4	1
	Total brands 7–18									20	7
Soft, blue veined, from raw milk	19	5	2	5	1	4	5	2	4	5	2
	20	5[d6]	0	4	2	3	3	2	5	5	2
	Total	10	2	9	3	7	8	4	9	10	4
Soft, blue veined, from pasteurized milk	21	5	5	5	2	5	5	2	5	5	2
	22	5	2	3	0	4	3	0	5	4	0
	Total	10	7	8	2	9	8	2	10	9	2

[a] *N*, number of samples analysed.
[b] A, B, C, A*, B*, C*, A^{SP}, B^{SP}, C^{SP}. The methods are described in Fig. 1.
[c] ND, not determined.
[d1] Four out of five blanks were positive for *Listeria* spp.
[d2] Five out of five blanks were positive for *Listeria* spp.
[d3] Three out of five blanks were positive for *L. innocua*.
[d4] Two out of five blanks were positive for *L. innocua*.
[d5] Three out of five blanks were positive for *L. innocua*.
[d6] Three out of five blanks were positive for *L. monocytogenes*.

richment step (5 h) (B*, B^{SP}) or with the completely selective methods (C*, C^{SP}). With the short PCR-based method A*, 27 out of 30 samples (90%) were positive, but only 25 out of 30 samples (83%) were positive when a fraction of the final enrichment broth was streaked on selective agar.

3.2. Analysis of soft cheeses artificially contaminated with 50 CFU of stressed *L. monocytogenes*

When the different methods including a selective plating step (A^{SP}, B^{SP}, C^{SP}) were examined, the success of these different procedures was highly dependent on the cheese brand as well as the type of cheese (Table 2).

For the mold ripened, raw milk cheeses only 1 out of 15 samples was found positive, solely with the method that involved only selective enrichment. For the mold ripened, pasteurized milk cheeses, the results were highly dependent on the cheese brand. The results obtained with the short nonselective enrichment procedure (B^{SP}) and the completely selective enrichment procedure (C^{SP}) were similar, and better than the results with a long nonselective pre-enrichment.

For the soft, blue veined type of cheese made from raw and pasteurized milk, the methods that incorporated a nonselective enrichment step gave better results than the conventional, completely selective, method.

For the cheeses of the red smear type, all made from pasteurized milk, 5 out of 12 cheese brands were initially contaminated with *Listeria* species that were not *L. monocytogenes*. For three of these cheese brands, the *Listeria* spp. was identified as *L. innocua*. For 7 out of 12 cheese brands of this type, it was very difficult to resuscitate the stressed *L. monocytogenes* cells. It was clear that it was much easier to resuscitate stressed *L. monocytogenes* cells when a nonselective enrichment step (A- and B-based methods) was incorporated in the procedure.

In general, independent of the cheese type, immunomagnetic separation (methods A*, B*, C*) gave better results as a sample preparation for PCR than did centrifugation (methods A, B, C) and better results were obtained when PCR was performed starting with colonies from solid selective media rather than with enrichment broths.

4. Discussion

For this study, samples of cheese artificially contaminated with stressed *L. monocytogenes* cells were used because the incidence of *L. monocytogenes* in cheeses on the Belgian market (approximately 2.8%) is very low (De Reu et al., 2002), and because *Listeria* may be non-uniformly distributed in the food product so that all samples of a given cheese may not give the same result. The results obtained from a study of naturally contaminated cheeses may therefore reflect variable contamination rather than differences in rates of recovery by various methods.

The results show that the incorporation of a non-selective enrichment step, for either 5 or 24 h, in the standard enrichment procedures improved recovery of *L. monocytogenes* from some cheese types, particularly semi-hard cheeses and soft cheeses of the red smear or blue veined types.

The semi-hard cheeses and the red smear soft cheeses that were examined were all made from pasteurized milk. It is possible that the findings cannot be extended to cheeses made from raw milk, as these may contain a large background flora that might overgrow *L. monocytogenes* during nonselective enrichment. However, with soft cheeses of the blue veined type made from raw and pasteurized milk, the methods that incorporated a nonselective enrichment gave much better results than the completely selective procedures. For the cheeses of the red smear type, 5 out of 12 cheese brands were initially contaminated with *Listeria* species. It is well known that this cheese type often becomes contaminated during ripening (Breer and Schopfer, 1988; Terplan et al., 1986; Loncarevic et al., 1995; Rudolf and Scherer, 2001), so this result was not unexpected.

The advantage of using a nonselective primary enrichment was obvious for those cheeses in which *L. innocua* was initially present. This result is in agreement with reports showing that *L. monocytogenes* has a significantly greater generation time than *L. innocua* in certain selective media but not in nonselective media (Petran and Swanson, 1993; MacDonald and Sutherland, 1994). It also confirms the suggestion of Jackson et al. (1993) that such favored growth of *L. innocua* may lead to overgrowth of *L. monocytogenes* when both species are present. The results of this study differ from those reported by

Duffy et al. (2001) who found that the presence or absence of L. innocua in meat containing L. monocytogenes did not significantly affect the growth of L. monocytogenes in or its recovery from selective or nonselective media. On the other hand, those authors also found that selective enrichment may offer no advantage over nonselective enrichment media for the recovery of L. monocytogenes from minced beef samples.

Conflicting results obtained with different kinds of food samples are not unexpected. Walsh et al. (1998) noticed that the nature of the food sample differentially influenced the efficiency of different enrichment protocols. These differences can be explained by a difference in inherent pH buffering capacity, which is high for meat (Warburton et al., 1992) and low for dairy products (Lammerding and Doyle, 1989). With the latter types of food, the use of a highly buffered, nonselective enrichment medium may be advisable (Walsh et al., 1998).

At least in the case of cheeses, the combined use of a selective and a nonselective primary enrichment broth might be the best option to ensure a sensitive detection of possibly stressed L. monocytogenes cells. To our knowledge, the U.S. Food and Drug Administration (FDA) protocol (Hitchins, 1995) is the only standard method which prescribes such a combined procedure.

The recovery of L. monocytogenes from some soft cheeses of the mold ripened and red smear types was remarkably difficult. A European collaborative study has also indicated that the isolation of stressed L. monocytogenes from soft cheeses is difficult (In 't Veld et al., 1995). It is possible that the cells used for inoculating cheeses in this study were more stressed than the L. monocytogenes cells that usually occur naturally in soft cheeses. Nevertheless, it is also possible that the methods currently used are not adequate for the screening of soft cheeses of certain types.

For the retail cheeses and the semi-hard cheeses made from pasteurized milk, the results of PCR performed on the 44-h enrichment medium were in good agreement with PCR performed after selective plating, independent of the enrichment protocol used. This was, however, not the case for the soft cheeses that were artificially contaminated with 50 CFU of stressed L. monocytogenes. In the latter case, more reliable results were obtained when PCR was performed after selective plating. That is, for identification rather than for detection. The higher sensitivity achieved by performing PCR on colonies picked from selective agar as opposed to performing PCR on enrichment media may in some circumstances compensate for the extra 1 or 2 days required for the analysis.

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