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Two-Stage Enrichment Procedures for Isolating *Listeria monocytogenes* from Raw Milk

PETER J. SLADE and D.L. COLLINS-THOMPSON*

Department of Food Science/Environmental Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

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ABSTRACT

Recovery of two strains of *Listeria monocytogenes* (Scott A and V7) inoculated into raw milk, and of strains indigenous to milk was investigated. Isolation of the organisms from the milk was attempted using pre-enrichment broths [nutrient broth no. 2 (NB2) and tryptose broth (TB)] at 1:5 and 1:10 dilutions of milk. The broths were incubated at 4°C for 0, 7, 14, 21, and 28 d. Recoveries were compared by direct plating onto acriflavine-nalidixic acid agar (AN), McBride *Listeria* agar (MLA) and tryptose agar (TA), and after selective enrichment in thiocyanate-nalidixic acid broth, with and without acriflavine. Favorable recoveries were obtained using a two-stage protocol consisting of cold enrichment of the sample diluted 1:10 in TB, followed by plating 0.1 ml of this pre-enrichment to MLA, and transfer of 1 ml to 9 ml Thio-Nal-Acri broth. Selectively-enriched cultures streaked to MLA and TA yielded optimal isolations after 7-14 of cold enrichment.

Recent foodborne outbreaks of fatal listeriosis (*5,7,9,10*) have prompted several investigations into isolation of the causative microorganism, *Listeria monocytogenes,* from foods (*1,3,6*). No standard methods for isolation of *L. monocytogenes* from raw milk have been established, but a protocol involving 1:5 dilution of the milk, primary non-selective cold enrichment, then secondary selective enrichment and plating onto McBride *Listeria* agar (MLA) has been proposed (*6*). Dilution of the sample before direct plating onto MLA has been used with nonfat dry milk (2). Conclusions of a study on three methods for the isolation of *L. monocytogenes* re-emphasized the possible benefits of a two-stage enrichment (*3*).

Use of thiocyanate-nalidixic acid broth for selective enrichment following pre-enrichment has been advocated in the examination of sewage and water (*11,12*). The addition of acriflavine to this medium facilitated recovery of the organism from silage and bird feces (*4*). Tryptose agar (TA) and MLA are, by tradition, the plating media of choice; the latter often used in direct plating, following dilution or enrichment of the sample. Solid media containing acriflavine have been developed (*8*), but their use with milk has not been critically evaluated.

This research reports on recovery of strains from naturally contaminated milk, and of two strains of *L. monocytogenes* inoculated into raw milk, following dilution of the milk to two different concentrations in each of two non-selective broths for cold enrichment. At intervals, the efficacy of direct plating onto selective media is compared with plating onto the same media after enrichment in selective broths.

MATERIAL AND METHODS

Raw milk samples

These were obtained from the University of Guelph herd, and from farm bulk tanks in southwest Ontario, as supplied by the Ontario Ministry of Agriculture and Food (OMAF) Central Milk Testing Laboratory, Guelph. Milk samples from 15 bulk tanks were composited for use as raw material in the inoculation studies. In addition, samples from 5 bulk tanks, each previously found to contain wild strains of *L. monocytogenes,* were mixed for the naturally contaminated study.

Cultures

Two strains of *L. monocytogenes,* Scott A (clinical isolate, serotype 4b) and V7 (milk isolate, serotype 1a), were obtained from M.P. Doyle, University of Wisconsin, Madison, WI. Cultures were grown in tryptose broth (TB) for 24 h at 37°C. The level of growth was determined by plating appropriate dilutions onto tryptose agar (TA) and incubating 48 h at 37°C. (All media are Difco Labs, Detroit, MI 48232, unless otherwise stated.)

Inoculation of milk

Two hundred-ml quantities of composited raw milk were inoculated with appropriate amounts of one of each *L. monocytogenes* strain to give a final concentration of approximately 10^2 organisms/ml. A 200-ml portion of milk was the uninoculated control.

Twenty ml of each milk (control, SA and V7 inoculated) were diluted in: (*a*) 180 ml of nutrient broth no. 2 (NB2) (Oxoid Ltd., Basingstoke, Hants, England) - dilution ratio 1:10, (*b*) 80 ml of NB2 - dilution ratio 1:5, (*c*) 180 ml of TB - dilution ratio 1:10, and (*d*) 80 ml of TB - dilution ratio 1:5. All dilutions were performed in duplicate.

Naturally contaminted samples

The combined naturally contamined samples were diluted as above.

Pre-enrichment

All diluted milks were stored at 4°C (cold enrichment) and examined after zero time (0 d), 7, 14, 21 and 28 d.

Direct plating

At the intervals stated above, 0.1 ml from each pre-enrichment was directly (spread) plated onto: (*a*) acriflavine-nalidixic acid (AN) agar containing g/L: tryptose agar (TA) 41.0; acriflavine (Sigma Chemical Co., St. Louis, MO 63178) 0.01; nalidixic acid (Sigma) 0.04 prepared according to Höhne (*8*); (*b*) McBride *Listeria* agar (MLA) (Maknur Labs, Houston, TX 77242), but with the addition of 5 g agar/L to the manufacturer's original 10 g/L); (*c*) tryptose agar (TA). All agars were incubated 24-48 h at 37°C.

Selective enrichment

Concurrent with direct plating, 1 ml from each pre-enrichment was inoculated into: (*a*) 9 ml of thiocyanate-nalidixic acid broth (Thio-Nal) containing g/L: NB2 (Oxoid) 25.0; potassium thiocyanate (Aldrich Chemical Co. Inc., Milwaukee, WI 53233) 37.5; nalidixic acid (Sigma) 0.1 prepared according to Watkins and Sleath (*12*); (*b*) 9 ml of thiocyanate-nalidixic acid broth + acriflavine (Thio-Nal-Acri) prepared like Thio-Nal, but with addition of 0.1 ml of 0.25% aqueous solution of acriflavine (Sigma) to each tube before inoculation (final concentration 25 mg/L) as according to Fenlon (*4*). Inoculated broths were incubated 24 h at 37°C.

Isolation procedures and confirmatory tests

A standard 3-mm loopful of each selective enrichment culture was streaked onto AN agar, MLA and TA, which were incubated 24-48 h at 37°C. All plates were examined using Henry's oblique transillumination. Suspect colonies from each presumptive streak were restreaked onto TA and incubated 24-48 h at 37°C. Colonies were confirmed using gram stain, catalase and hemolysis tests, the latter on blood agar (BA) containing 5% defibrinated sheep blood (Woodlyn Labs, Guelph, Ont., N1H 6H9). If necessary, isolates were further characterized based upon umbrella-shaped growth in motility medium after 5 d at 22°C, MR-VP at 22°C, fermentation pattern of glucose, mannitol, rhamnose and xylose in bromocresol purple broth within 48 h at 37°C, and urea and nitrate broth tests.

RESULTS

Inoculated milk

Raw milk from the Guelph herd had an average aerobic plate count (APC) of 2.3×10^4 CFU/ml, and the OMAF composite milk 3.4×10^4 CFU/ml. Milks were inoculated with 2.1×10^2 CFU/ml of either Scott A or V7 strains of *L. monocytogenes*. Tables 1-5 show the populations of *L. monoctyogenes* isolated on plating media at various periods using combinations of pre-enrichment, dilution, direct plating, and plating from two selective enrichment broths.

Direct plating

With direct plating at 0 d, limited recovery was seen on all three agars. No major differences were observed between strains, dilutions or media used (Table 1). After 7 d at 4°C (Table 2), only MLA was capable of providing isolated colonies of *L. monocytogenes*. Both AN medium and TA were overgrown, making it difficult to screen for *Listeria*. At 14 d (Table 3), MLA was still the only medium capable of recovering *Listeria* isolates. No substantial differences were observed between the two strains, dilution, or pre-enrichment broth. Following 21 d at 4°C (Table 4), colonies of *L. monocytogenes* were seen on MLA mainly from NB2 pre-enrichment. Again, heavy growth of competing flora was noted on both AN and MLA media, making it difficult to discern colonies of *L. monocytogenes*. Finally, after 28 d at 4°C (Table 5), AN agar showed improved selection of *Listeria* isolates from both pre-enrichment broths. MLA allowed isolation of more colonies, particularly following NB2 pre-enrichment.

Selective enrichment techniques

At 0 d (Table 1), the Thio-Nal-Acri enrichment broth allowed greater growth of *Listeria* than the Thio-Nal broth. It was also observed that pre-enrichments gave better recoveries than direct plating. Dilution in TB followed

TABLE 1. *Estimate*[a] *of* L. monocytogenes *growth after 0 d at 4°C from various enrichment combinations.*

Inoculated strain	Pre-enrichment broth	Dilution ratio	Selective-enrichment/Plating medium								
			Direct			Thio-Nal			Thio-Nal-Acri		
			AN	MLA	TA	AN	MLA	TA	AN	MLA	TA
SA	NB2	1:10	-[b]	-	+	++	-	+	+++	+	+++
		1:5	+	+	+	+	-	-	++	-	+
	TB	1:10	+	-	+	+	-	-	+++	+	+++
		1:5	+	+	+	++	-	+	+++	+	+++
V7	NB2	1:10	+	+	-	+	-	+	++	+	++
		1:5	+	+	+	+	-	+	+	-	+
	TB	1:10	+	+	+	++	-	-	++	+	++
		1:5	+	+	-	+	-	-	++	++	+++

[a]Average of three trials, each with duplicate plating.

[b]The density of growth was estimated by scoring the number of well-separated colonies observed: + = <10 colonies/plate, ++ = 10 - 100 colonies/plate, +++ = >100 colonies/plate, - = negative for *Listeria*, OGr = Overgrown.

TABLE 2. *Estimate*[a] *of* L. monocytogenes *growth after 7 d at 4°C from various enrichment combinations.*

Inoculated strain	Pre-enrichment broth	Dilution ratio	Selective-enrichment/Plating medium: Direct AN	Direct MLA	Direct TA	Thio-Nal AN	Thio-Nal MLA	Thio-Nal TA	Thio-Nal-Acri AN	Thio-Nal-Acri MLA	Thio-Nal-Acri TA
SA	NB2	1:10	OGr[b]	+	OGr	+ +	+ +	+	+ +	+ +	+
		1:5	OGr	+	OGr	+ +	+	+	+ +	+ +	+ +
	TB	1:10	OGr	+	OGr	+ + +	+ +	+ +	+ + +	+ +	+ +
		1:5	OGr	-	OGr	+ +	+	+	+ +	+	+
V7	NB2	1:10	OGr	+ +	OGr	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
		1:5	OGr	+	OGr	+ +	+ +	+ +	+ +	+ +	+ +
	TB	1:10	OGr	+	OGr	+ + +	+ +	+ +	+ +	+ +	+ +
		1:5	OGr	-	OGr	+ + +	+ +	+ +	+ + +	+ +	+ + +

[a]Average of three trials, each with duplicate plating.
[b]The density of growth was estimated by scoring the number of well-separated colonies observed:
+ = <10 colonies/plate, + + = 10 - 100 colonies/plate, + + + = >100 colonies/plate, - = negative for *Listeria,* OGr = Overgrown.

TABLE 3. *Estimate*[a] *of* L. monocytogenes *growth after 14 d at 4°C from various enrichment combinations.*

Inoculated strain	Pre-enrichment broth	Dilution ratio	Selective-enrichment/Plating medium: Direct AN	Direct MLA	Direct TA	Thio-Nal AN	Thio-Nal MLA	Thio-Nal TA	Thio-Nal-Acri AN	Thio-Nal-Acri MLA	Thio-Nal-Acri TA
SA	NB2	1:10	OGr[b]	+	OGr	+	+	+ +	+	-	+
		1:5	OGr	+	OGr	+	+	+	+ +	+	+
	TB	1:10	OGr	+ +	OGr	+ +	+	+ +	+ +	+ +	+ +
		1:5	OGr	+ +	OGr	+ +	+	+ +	+ +	+	+
V7	NB2	1:10	OGr	+ +	OGr	+ + +	+ +	+ +	+ +	+ +	+ +
		1:5	OGr	+	OGr	+	+	+	+ +	+	+
	TB	1:10	OGr	+	OGr	+	+	+	+	+	+
		1:5	OGr	+ +	OGr	+ +	+ +	+ +	+ +	+	+ +

[a]Average of three trials, each with duplicate plating.
[b]The density of growth was estimated by scoring the number of well-separated colonies observed:
+ = <10 colonies/plate, + + = 10 - 100 colonies/plate, + + + = >100 colonies/plate, - = negative for *Listeria* , OGr = Overgrown.

TABLE 4. *Estimate*[a] *of* L. monocytogenes *growth after 21 d at 4°C from various enrichment combinations.*

Inoculated strain	Pre-enrichment broth	Dilution ratio	Selective-enrichment/Plating medium: Direct AN	Direct MLA	Direct TA	Thio-Nal AN	Thio-Nal MLA	Thio-Nal TA	Thio-Nal-Acri AN	Thio-Nal-Acri MLA	Thio-Nal-Acri TA
SA	NB2	1:10	OGr[b]	+	OGr	+	+	+	-	+	-
		1:5	OGr	+	OGr	+	+	+	-	-	-
	TB	1:10	OGr	+	OGr	+	+ +	+	+ +	+ +	+
		1:5	OGr	-	OGr	+	+	+	+	-	+
V7	NB2	1:10	OGr	+	OGr	+	+	+	+ +	+ +	+
		1:5	OGr	+	OGr	+	+	+	+	+	+
	TB	1:10	OGr	-	OGr	+	+	+	+	+	+
		1:5	OGr	+	OGr	+ +	+ +	+	+ +	+ +	+

[a]Average of three trials, each with duplicate plating.
[b]The density of growth was estimated by scoring the number of well-separated colonies observed:
+ = <10 colonies/plate, + + = 10 - 100 colonies/plate, + + + = >100 colonies/plate, - = negative for *Listeria,* OGr = Overgrown.

TABLE 5. *Estimate*[a] *of* L. monocytogenes *growth after 28 d at 4°C from various enrichment combinations.*

Inoculated strain	Pre-enrichment broth	Dilution ratio	Selective-enrichment/Plating medium								
			Direct			Thio-Nal			Thio-Nal-Acri		
			AN	MLA	TA	AN	MLA	TA	AN	MLA	TA
SA	NB2	1:10	+[b]	+	OGr	+	+	+	+	+	+
		1:5	+	+	OGr	+	+	+	-	-	-
	TB	1:10	+ +	+ +	OGr	+	+	+	+	+	+
		1:5	+	-	OGr	+	+	+	+	+	+
V7	NB2	1:10	+	+ +	OGr	+	+	+	+	+	+
		1:5	+	+	OGr	+	+	+	+	+	+
	TB	1:10	-	-	OGr	+	+	+	+	+	+
		1:5	+ +	-	OGr	+	+	+	+	+	+

[a]Average of three trials, each with duplicate plating.
[b]The density of growth was estimated by scoring the number of well-separated colonies observed:
+ = <10 colonies/plate, + + = 10 - 100 colonies/plate, + + + = >100 colonies/plate, - = negative for *Listeria,* OGr = Overgrown.

by enrichment in Thio-Nal-Acri broth yielded best recoveries of test organisms. AN and TA yielded more isolates after selective enrichment. Limited effect of the dilution ratio of milk to pre-enrichment broth was observed; dilution 1:10 in either broth yielded slightly more listeriae. The selective enrichment combinations allowed better recoveries than direct plating after 7 d at 4°C (Table 2). The Thio-Nal-Acri broth provided excellent growth on all three agar media. Again, there was a small difference between dilution 1:10 and 1:5. Following Thio-Nal enrichment, slightly more growth was obtained from the 1:10 dilutions. The density of *L. monocytogenes* growth was similar from TB and NB2. After 14 d cold enrichment (Table 3), little difference was observed between the two pre-enrichment broths at either dilution ratio. The AN and TA media yielded better recoveries than MLA from both selective enrichment broths. The growth of interfering colonies was more pronounced on TA streaked from Thio-Nal broth. After 21 d at 4°C (Table 4), the populations generally declined. Enrichment in Thio-Nal broth allowed recovery of *Listeria* strains in some instances where Thio-Nal-Acri did not. Some effect of the dilution ratios were observed. In two instances, the 1:10 dilution was better for recovery of the test organisms when enrichment was in Thio-Nal-Acri broth. The plating media were equally effective. Data in Table 5 indicate that at the completion of 28-d cold enrichment, the Thio-Nal broth produced marginally better recoveries, but populations have declined considerably from their 7-14 d highs. Differences between dilutions or plating media did not affect recoveries.

Uninoculated control samples were negative for *L. monocytogenes* from every combination at every period (data not tabulated).

Naturally contaminated milks

The combined naturally contaminated milks had an average APC of 2.3×10^3 CFU/ml.

L. monocytogenes was not detected from any enrichment combination at zero time (0 d) or after 21 d and 28 d at 4°C. After 7 d and 14 d at 4°C, *L. monocytogenes* were detected (Table 6). Isolations were readily demonstrated after 7 d, but not so well after 14 d. After 7 d, slightly better isolations were obtained using a combination of milk pre-enriched in NB2 at either dilution with subsequent selective enrichment in Thio-Nal-Acri followed by plating onto any of the three agars.

DISCUSSION

Tryptose broth (TB) is the traditional medium for cold enrichment of samples containing *Listeria*. TB is preferred over NB2 since improved isolations were obtained with the former. The main difference between the two is that TB contains glucose in its formulation.

More importantly, dilution of the samples 1:10 yielded results superior to dilution 1:5. Hayes et al. (*6*) found that 1:5 dilution appears to enhance isolation of listeriae, but it is not clear which factors cause the enhancement. Our results indicate that 1:10 dilution improves the situation even more. Perhaps increased dilution has the effect of further reducing the concentrations of antagonistic microflora such that *L. monocytogenes* is able to compete more successfully during the course of cold enrichment.

McBride *Listeria* agar (MLA) was the only medium tested useful for direct plating of pre-enrichment broth. AN and TA allowed overgrowth of competing flora. This was expected from the latter, but AN medium has been shown to give good yields when a loopful of pre-enrichment broth is streaked (*8*), unlike our spreading of 0.1 ml. In some instances, direct plating on MLA yielded positives when some selective enrichment combinations did not.

The use of Thio-Nal broth has been shown to allow adequate selection of *L. monocytogenes* following several weeks' cold enrichment. (*6,11,12*). Watkins (*11*) found that the method allows *L. monocytogenes* to recover and grow rapidly, thus aiding its recognition on TA by the

TABLE 6. *Growth[a] of wild* L. monocytogenes *in naturally contaminated milk after 7 d and 14 d at 4°C from various enrichment combinations.*

Time at 4°C	Pre-enrichment broth	Dilution ratio	Selective-enrichment/Plating medium: Direct AN	Direct MLA	Direct TA	Thio-Nal AN	Thio-Nal MLA	Thio-Nal TA	Thio-Nal-Acri AN	Thio-Nal-Acri MLA	Thio-Nal-Acri TA
7d	NB2	1:10	OGr[b]	-	OGr	+ + +	+	+	+ + +	+ + +	+ +
		1:5	OGr	+	OGr	+ +	+ +	+ +	+ + +	+ + +	+ + +
	TB	1:10	OGr	-	OGr	+ +	+ +	+ +	+ +	+	+ +
		1:5	OGr	-	OGr	+	-	+	+	-	+
14 d	NB2	1:10	OGr	-	OGr	+ +	-	+	-	-	-
		1:5	OGr	-	OGr	+	-	-	-	-	-
	TB	1:10	OGr	-	OGr	+	-	-	-	-	-
		1:5	OGr	-	OGr	-	-	-	-	-	-

[a]Average of duplicate trials.

[b]The density of growth was estimated by scoring the number of well-separated colonies observed: + = <10 colonies/plate, + + = 10 - 100 colonies/plate, + + + = >100 colonies/plate, - = negative for *Listeria*, OGr = Overgrown.

presence of large numbers of colonies. Furthermore, he found that pure cultures are often obtained from heavily contaminated environmental samples, thus allowing rapid identification within 14-21 d. We found that AN medium was better than TA at providing isolated colonies and may be useful should prolonged cold enrichment be necessary.

Our results indicate that addition of acriflavine to Thio-Nal broth provides much earlier, improved isolations of *L. monocytogenes*. Like Fenlon (*4*), we obtained good recoveries within 7-14 d. This supports Hayes et al. (*6*) who intimated that two-stage enrichment shortens the time required for isolation. The addition of acriflavine to enrichment and plating media is thought to aid in suppressing the growth of enterococci (*4*). Generally, the plating media, AN, MLA and TA gave similar results from each selective combination. Following enrichment in Thio-Nal-Acri broth, use of AN medium probably does not provide further selectivity, so its use may be redundant.

The isolation of *L. monocytogenes* from heavily contaminated environmental samples has been facilitated by the use of a two-stage enrichment technique, as found previously (*12*), and suggested for dairy products (*3,6*). In addition to the recovery of strains inoculated into raw milk, the isolation of strains indigenous to milk has been demonstrated.

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