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Evaluation of the Ability of Primary Selective Enrichment To Resuscitate Heat-Injured and Freeze-Injured *Listeria monocytogenes* Cells

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Resuscitation rates of injured *Listeria monocytogenes* on conventional selective *Listeria* enrichment broth and nonselective Trypticase soy broth containing 0.6% yeast extract were compared. Cells were heated to 60°C for 5 min or frozen at −20°C for 7 days. Inoculation of Trypticase soy broth-yeast extract with the stressed cells resulted in growth that was superior to that in *Listeria* enrichment broth. Injured cells were fully recovered at 6 to 8 h.

Listeria monocytogenes is a Gram-positive facultative anaerobe. It is ubiquitous in nature and has been linked to food-borne illness outbreaks involving a wide range of foods (5, 8, 12).

Foods and food ingredients may be subjected to a variety of processes to preserve them against microbiological spoilage. The treatments may result in viable microorganisms that are physiologically deficient or injured because of the stress to which the cells have been subjected (9). Substances with selective properties are added to media for the selective detection of microorganisms from foods. Some of the agents are inhibitory to the process of repair, and others are toxic and cause death of the injured organisms (9).

Most of the methods and media used to detect microorganisms in food and environmental samples do not compensate for the injured state of the cells. As a result, the methods and media fail to detect them. In an ideal situation, the methods and media should detect both normal and physiologically injured cells. Under favorable environmental conditions, these cells may repair themselves and become functionally normal (3, 10).

There are two basic methods for detecting stressed microbial cells: liquid-repair and solid-repair methods. The liquid method allows stressed cells to repair in a nonselective broth before ingredients are added to facilitate selective culturing. In the solid method, a sample of the stressed cells is plated onto a nonselective agar. Agar with selective ingredients is then overlaid upon it. The selective ingredients diffuse through the basal nonselective agar.

The major obstacle associated with the liquid-repair method is that, because of variations in the time needed for repair, multiplication of certain cells may occur before other cells can be repaired (11). Since cells become immobilized in the solid-repair method, multiplication during the repair phase will not influence the final count.

Listeria spp., when present in a food sample, normally occur in very low numbers; levels of less than 100 CFU/g are common (6). Buchanan et al. (1) found *Listeria* levels of usually less than 2 CFU/g in retail-level meat, poultry, and seafood samples. Such low numbers of cells, similar to the numbers we have encountered in routine enumeration of *Listeria* spp. in seafood samples in our laboratory, make it impossible to detect *Listeria* spp. by direct plating.

Recent research has been conducted to compare media with selective ingredients (4, 6, 7). In this study we investigated the efficiency of the most commonly used primary selective enrichment procedure with *Listeria* enrichment broth (LEB) to recover heat-injured and freeze-injured cells of *L. monocytogenes*.

The three strains of *L. monocytogenes* (HPB 43, HPB 397, and HPB 563) used in this study were grown in Trypticase soy broth (TSB; Difco) for 24 h at 35°C before being used as inocula for the studies.

The cells were heated by adding 0.5 ml of an inoculum of each strain to 10 ml of TSB containing 0.6% yeast extract (TSBYE; Difco) in a test tube (15 by 150 mm) and placing the test tube in a water bath (Precision Scientific) at 60°C. The water level in the bath was adjusted so that the contents of the tubes were completely submerged throughout the heat treatment. After 5 min, the tubes were removed and immediately placed in an ice bath. One milliliter of each heat-treated strain was inoculated into 100 ml of TSBYE or LEB (prepared by adding *Listeria* selective supplement [Oxoid SR 141] to LEB base [Oxoid CM 862] in 250-ml Erlenmeyer flasks). Each flask was incubated at 30°C in a shaking water bath. After 24 h of incubation, samples were withdrawn, serially diluted in sterile 0.1% (wt/vol) peptone-water and immediately surface plated (0.1 ml) in triplicate on trypticase soy agar (TSA; Difco). Colonies were counted after 5 days of incubation at 35°C. The experiment was repeated three times. In another set of experiments, the same heating procedure was followed for strains HPB 397 and HPB 563, except that heating was done for 5 and 10 min, respectively. One milliliter of each strain was inoculated into TSBYE or LEB and incubated at 30°C in a shaking water bath. Every 2 h, a 1-ml sample from each flask was withdrawn under aseptic conditions and immediately surface plated as described above on TSA and TSA containing 6% NaCl. Colonies were counted after 5 days at 35°C. The experiment was repeated three times. In the freezing trials, an inoculum of each strain (0.5 ml) was added to a screw-cap test tube (15 by 150 mm) containing 10 ml of TSBYE and stored at −20°C. After 7 days, the culture was thawed under cool running tap water and 0.1-ml samples of each strain were inoculated into 100 ml TSBYE and 100 ml of LEB in 250-ml Erlenmeyer flasks. Flasks were incubated at 30°C in a shaking water

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TABLE 1. Recovery of heat-injured *L. monocytogenes* enriched in TSBYE or LEB at 30°C for 24 h[a]

Strain and trial no.	Log_{10} CFU/ml on:	
	TSBYE	LEB
HPB 43		
1	9.0	7.8
2	9.5	7.6
3	9.6	7.5
HPB 397		
1	9.5	6.3
2	9.0	6.0
3	9.6	6.5
HPB 563		
1	9.3	7.8
2	9.3	7.7
3	9.6	7.8

[a] Values shown are the means of triplicate plates of each trial on TSA as the plating medium. Recovery of all strains was significantly greater in TSBYE than in LEB ($P < 0.05$). Cells were heated at 60°C for 5 min in TSB, and 0.5-ml samples were inoculated into 100 ml of the indicated broth.

bath. After incubation for 24 h, samples were withdrawn, serially diluted in sterile 0.1% (wt/vol) peptone-water, and immediately surface plated (0.1 ml) in triplicate on TSA. Colonies were counted after 72 h at 35°C. The experiment was repeated three times.

Uninjured cells of all strains were used in a similar experiment with TSBYE or LEB as described above. Flasks were incubated at 30°C for 24 h and serially diluted in 0.1% (wt/vol) peptone-water 0.1-ml aliquots were surface plated in triplicate onto TSA. Colonies were counted after 72 h at 35°C. The experiment was repeated three times.

Two direct plating media most frequently used for isolating *Listeria* spp. were evaluated for their efficiency in the recovery of heat-injured cells of *L. monocytogenes*. The two media evaluated were lithium chloride phenylethanol-moxalactam agar (LPM), which consists of phenylethanol agar (Difco) with 10 g of glycine anhydride (Sigma) per liter and 5 g of lithium chloride (BDH) per liter, and Oxford agar (OXA), prepared by adding *Listeria* selective supplement (Oxoid SR140) to *Listeria* selective agar base (Oxoid CM 856). All strains of *L. monocytogenes* used were grown and heated as above, serially diluted in 0.1% (wt/vol) peptone-water, and surface plated (0.1 ml) in triplicate onto TSA, TSA containing 6% NaCl, LPM agar, and OXA agar. Colonies were counted after 5 days at 35°C. The experiment was repeated three times and also repeated with uninjured cells. Colonies were counted after 72 h of incubation at 35°C.

Results were evaluated by using Student's two-tailed *t* test and one-way analysis of variance by Tukey's honestly significant difference procedure. The level of significance was set at $P < 0.05$ for all comparisons.

The ability of heat-injured cells of *L. monocytogenes* to recover in TSBYE and LEB is shown in Table 1. All of the strains showed better growth in TSBYE than in LEB. Strain HPB 397 was more susceptible than strains HPB 563 and HPB 43 to inhibition of growth by LEB. Injured cells of strains HPB 397 and HPB 563 heated at 60°C for 5 and 10 min, respectively, were fully recovered at 6 to 8 h of incubation at 35°C in TSBYE but not LEB (Tables 2 and 3). This is shown by the similar numbers of CFU on TSA and on TSA containing 6% NaCl; TSA containing 6% NaCl prevented the growth of the injured cells.

TABLE 2. Recovery of *L. monocytogenes* HPB 397 after heat treatment at 60°C for 5 min[a]

Enrichment medium	Plating medium	Trial no.	Log_{10} CFU/ml at the following time (h)				
			0	2	4	6	8
TSBYE	TSA–6% NaCl	1	0.0	1.0	1.3	2.3	3.7
		2	0.0	1.1	1.3	2.1	3.5
		3	0.0	0.9	1.0	2.2	3.3
TSBYE	TSA	1	1.1	1.3	1.9	2.4	3.8
		2	1.2	1.6	1.8	2.5	3.5
		3	1.1	1.4	1.7	2.7	3.5
LEB	TSA–6% NaCl	1	0.0	0.0	0.6	1.7	2.3
		2	0.0	0.0	1.0	1.3	2.2
		3	0.0	0.0	1.0	1.6	2.0
LEB	TSA	1	1.1	1.0	1.5	2.0	2.8
		2	1.2	1.2	1.4	1.8	2.8
		3	1.0	1.1	1.4	2.0	2.8

[a] Values shown are the means of triplicate plates of each trial. Cells were serially diluted, surface plated onto TSA or TSA plus 6% NaCl, and incubated at 35°C for 5 days. Recovery with TSA was significantly greater than that with TSA plus 6% NaCl at 0, 2, and 4 h after enrichment in TSBYE ($P < 0.05$). Recovery with TSA was significantly greater than that with TSA plus 6% NaCl at all times except at 6 h when LEB was the enrichment medium.

The effect of growth of freeze-injured *L. monocytogenes* cells in TSBYE and LEB is shown in Table 4. The recovery in TSBYE as an enrichment medium was better than that in LEB for all strains. Strain HPB 563 recovered better in LEB than did strains HPB 43 and HPB 397. Normal cells grew better in TSBYE than in LEB (data not shown). The recovery of heat-injured cells was significantly greater on TSA than on OXA, LPM, or TSA with 6% NaCl. OXA was not significantly different from LPM for recovery of any of

TABLE 3. Recovery of *L. monocytogenes* HPB 563 after heat treatment at 60°C for 10 min[a]

Enrichment medium	Plating medium	Trial no.	Log_{10} CFU/ml at the following time (h):				
			0	2	4	6	8
TSBYE	TSA–6% NaCl	1	0.0	1.8	2.5	3.8	4.0
		2	0.0	1.8	2.6	3.6	4.0
		3	0.0	2.0	2.6	3.3	4.3
TSBYE	TSA	1	1.7	2.3	3.7	3.6	4.0
		2	1.3	2.2	3.5	3.8	4.3
		3	1.5	2.4	3.8	3.9	4.3
LEB	TSA–6% NaCl	1	0.0	1.5	2.0	2.6	3.8
		2	0.0	1.5	2.0	2.6	3.3
		3	0.0	1.0	1.8	2.6	3.6
LEB	TSA	1	1.7	2.0	2.4	2.8	3.9
		2	1.3	1.8	2.2	2.8	4.0
		3	1.5	1.7	2.2	2.8	4.0

[a] Values shown are means of triplicate plates of each trial. Cells were serially diluted, surface plated onto TSA or TSA plus 6% NaCl, and incubated at 35°C for 5 days. Recovery with TSA was significantly greater than that with TSA plus 6% NaCl for 0, 2, and 4 h after enrichment in TSBYE ($P < 0.05$). Recovery with TSA was significantly greater than with TSA plus 6% NaCl for all times when LEB was the enrichment medium.

TABLE 4. Recovery of freeze-injured cells of *L. monocytogenes* enriched in TSBYE or LEB for 24 h at 30°C[a]

Strain and trial no.	Log_{10} CFU/ml on:	
	TSBYE	LEB
HPB 43		
1	9.3	6.6
2	9.0	6.8
3	9.3	6.8
HPB 397		
1	9.0	6.4
2	8.9	6.4
3	8.9	6.3
HPB 563		
1	9.6	8.3
2	9.0	8.0
3	9.8	8.0

[a] Values shown are means of triplicate plates of each trial on TSA as the plating medium. Recovery of all strains was significantly greater in TSBYE than in LEB ($P < 0.05$). Cells were frozen at −20°C for 7 days.

the strains (data not shown). There was no significant difference between the growth rates on the different agars for the normal cells of all strains used (data not shown).

In this study, the primary selective enrichment procedure with LEB (the medium used most frequently for the recovery of *L. monocytogenes* from foods) was found not to encourage full resuscitation of all of the heat-injured and freeze-injured *L. monocytogenes* cells. Whereas LPM and OXA were not good for recovering sublethally injured *L. monocytogenes* cells, they were equivalent in their capacity to grow uninjured cells; this is in conformity with studies carried out by other workers. Buchanan et al. (1), who compared LPM and modified Vogel Johnson agars for the detection of *Listeria* species, found that these two media (in which the selective ingredients are different) gave roughly equivalent recoveries. It was also observed in this study that LEB with selective supplements suppressed the multiplication of viable cells. This makes LEB particularly inappropriate for isolating *Listeria* cells that have been sublethally injured.

It is difficult to monitor what usually goes on in a liquid repair process with respect to multiplication versus recovery of cells subjected to sublethal processes. For cultures involving sublethally injured cells, therefore, the solid repair method is more appropriate, since the cells are immobilized and multiplication does not interfere with recovery. There are two basic problems, however, with the use of solid repair. First, with very low numbers (less than 2 CFU/g [1]), it is possible but not likely that cells will be detected. Second, pouring an overlay with selective ingredients makes it inconvenient to pick colonies for further confirmation tests.

This study showed that, under favorable conditions (growth in TSBYE at 35°C), cells of *L. monocytogenes* sublethally injured by heating become fully repaired within 6 to 8 h (Tables 2 and 3). This is in conformity with work done by Busch and Donnelly (2), who incubated heat-injured *Listeria* cells in a formulated nonselective repair broth and observed complete repair in 5 h.

To allow the resuscitation of sublethally injured cells in the primary selective enrichment of *L. monocytogenes*, the sample should be incubated in a nonselective medium for about 6 to 8 h before selective ingredients are added to suppress the background microflora.

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