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Rapid Enrichment Strategy for Isolation of *Listeria* from Bovine Hide, Carcass, and Meat Samples†

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ABSTRACT

Since the outbreak of foodborne illness linked to *Escherichia coli* O157:H7 bacteria in ground beef in the early 1980s, the beef processing industry has focused on increasing the safety of beef products by implementing procedures for surveying live cattle, carcasses, and beef products for bacterial pathogens. Effective methods are in place for screening cattle and beef products for the presence of *E. coli* O157:H7 contamination, and recent work has established the acceptability of these methods for surveillance of *Salmonella.* In keeping with the need to continually improve the food safety of beef products, new work investigating pathogen prevalence now includes surveillance for *Listeria monocytogenes.* Tryptic soy broth (TSB) has been documented as a robust nonselective medium for the enrichment of both *E. coli* and *Salmonella* from bovine hide, carcass, and meat samples. The University of Vermont modification medium is most often used as the primary enrichment medium for surveillance of *Listeria* spp. In this study, samples from bovine hides ($n = 50$), preevisceration carcasses ($n = 50$), and beef trim ($n = 193$) were used to evaluate TSB as a primary enrichment medium for the isolation of *Listeria* spp., including *L. monocytogenes.* No significant difference ($P > 0.05$) between TSB and the University of Vermont modification medium was observed when all three sample types underwent primary enrichment for the isolation of *Listeria* spp. Furthermore, the standard secondary enrichment ratio for Fraser broth used for *Listeria* recovery can be modified to accommodate a high-throughput method for processing multiple samples.

The intracellular pathogen *Listeria monocytogenes* is the causative agent of epidemic and sporadic listeriosis *(9, 13, 21).* Although outbreaks are limited, the consequences of contracting listeriosis can be particularly severe in pregnant women, newborns (younger than 1 year of age), elderly people (older than 65 years), and immunocompromised individuals, with mortality rates approaching 30% in individuals who become infected *(9).* This pathogen is present throughout the environment and is routinely isolated from numerous animal sources, including cattle *(18).* A clear relationship between food source and disease was established in 1981, when an outbreak of listeriosis was linked to contaminated coleslaw *(9, 22).*

The presence of *L. monocytogenes* is generally regarded as a food safety issue in ready-to-eat foods; its presence in fresh beef that is to be cooked has been considered inconsequential. Because of this point of view, information on the prevalence of *Listeria* in beef processing plants in the United States is minimal *(18).* The lack of data on *L. monocytogenes* is compounded by the additional work and media required to process samples for *Listeria* isolation. Conventional U.S. Department of Agriculture methods used for the detection and isolation of *L. monocytogenes* in foods consist of a primary enrichment (University of Vermont modification medium [UVM]), then a secondary enrichment (Fraser broth), followed by isolation, which then leads into biochemical identification and serotyping *(23).* These methods are time-consuming and, in general, require 7 to 8 days to determine the species *(9, 23).* Trypticase soy broth (TSB) has been documented *(1, 2)* as a robust medium for the enrichment of both *Escherichia coli* and *Salmonella* from bovine hide, carcass, and meat samples. The use of a universal nonselective medium for the isolation of multiple pathogens is advantageous because it allows researchers and regulators to more comprehensively assess the microbial quality of food products delivered to the consumer. Our objectives for this work included evaluating the use of TSB as a primary enrichment medium for the recovery of *Listeria* and *L. monocytogenes* and assessing a modified method for enhanced sample throughput during secondary enrichment.

MATERIALS AND METHODS

Hide and carcass sample processing protocol. Hide (1,000 cm^2) and carcass sponge samples (2,000 cm^2) were collected at beef processing plants ($n = 50$ of each). Each prehydrated sponge (9 by 4 cm) sample (20 ml of buffered peptone water from Difco [Becton Dickinson, Sparks, Md.]) was squeezed by hand five or more times to thoroughly mix contents before dividing into 5-ml aliquots for a comparison of primary enrichment strategies using either TSB (Becton Dickinson) or UVM (Neogen, Lansing, Mich.). All samples were incubated at the prescribed temperatures in a Precision Scientific (Thermo Electron Corp., Milford, Mass.) model 818 incubator. For the TSB enrichment, 5 ml from the sponge sample was incubated in 20 ml of TSB. This dilution was chosen because it represents the currently used ratio of sample to

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† Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

TABLE 1. *Isolation of* Listeria *spp. from hide and from carcass sponge sample bags*

Sample type	No. of samples	No. of samples positive for *Listeria* spp.[a]					
		UVM+[b]	TSB+[b]	UVM+/TSB+	UVM+/TSB−	UVM−/TSB+	UVM−/TSB−
Hide	50	50	50	50	0	0	0
Preevisceration carcass	50	13	11	5	8	6	31

[a] *Listeria* spp. represent all *Listeria* spp. except *L. monocytogenes.* No *L. monocytogenes* was found in any of the hide or preevisceration carcass sponge samples.

[b] A chi-square statistical analysis found no significant difference between enrichment media ($P > 0.05$).

TSB in the U.S. Meat Animal Research Center *E. coli*/*Salmonella* assay *(1, 2).* TSB enrichment was incubated at 25°C for 2 h, then at 42°C for 6 h, followed by a hold at 4°C for ≥4 h prior to the secondary enrichment. Samples are ready for processing following the 42°C incubation, but for convenience during these experiments, the samples were held for 4 h at 4°C. For the UVM enrichment, a 5-ml sample of the buffered peptone water from the sponge was used to prepare a 1:10 dilution in 45 ml of UVM. UVM enrichments were incubated at 30°C for ~20 h. Following each primary enrichment strategy, a 100-μl aliquot from each sample was incubated in Fraser broth (Becton Dickinson) at 35°C for 40 h. Samples were then plated onto one of two media, either *Listeria* CHROMagar (DRG International, Mountainside, N.J.) or Oxford agar (Becton Dickinson). *Listeria* CHROMagar was incubated at 37°C for 24 h and Oxford agar was incubated at 35°C for up to 48 h. *Listeria* CHROMagar became the agar of choice because it easily allowed for preliminary identification of samples containing *L. monocytogenes* within 24 h. Subsequent analysis of each sample was by PCR, as described below.

Beef trim and ground beef sample processing protocol. Both TSB and UVM primary enrichments were compared by using beef trim and ground beef. For experiments with beef trim ($n = 193$), 25-g samples of trim were placed in Whirl-Pak bags (Nasco, Fort Atkinson, Wis.) with 225 ml of UVM or TSB medium and stomached on high speed for 30 s in a Lab Blender 400C (Seward Co., Essex, UK). TSB and UVM primary enrichments and Fraser broth secondary enrichments were incubated and then plated onto chromogenic agar medium as just described. Subsequent analysis of each sample by PCR was as described below.

Molecular identification of presumptive *Listeria.* Three presumptive colonies were confirmed for *Listeria* spp. (*L. monocytogenes, L. grayi, L. innocua, L. seeligeri, L. welshimeri,* and *L. ivanovii*), and *L. monocytogenes*–specific serovars by multiplex PCR *(4, 7).*

Evaluation of a high-throughput method for secondary enrichment sample processing. A secondary enrichment of each sample in Fraser broth from Difco (Becton Dickinson) is part of the normal procedure for isolating *Listeria (8).* The secondary enrichment incubation procedure was evaluated by using two different dilutions of the primary enrichment in TSB. In this experiment, 100 μl of the primary enrichment was placed into either a 3- or 10-ml volume of Fraser broth. The 10-ml Fraser incubation was performed with sterilized glass borosilicate tubes (150 by 16-mm; Kimble Glass Inc., Vineland, N.J.), and the 3-ml incubation was performed in a 48 deep-well gamma-irradiated block (Axygen, Union City, Calif.). A 35°C incubation for 40 h was used for both volumes.

Statistical analysis. Pair-wise comparisons between the two methods were made by using PROC FREQ and Mantel-Haenszel chi-square analysis procedures of SAS (SAS Institute, Inc., Cary, N.C.).

RESULTS AND DISCUSSION

Comparison of primary enrichment media. Current culture conditions designed for the isolation of *Listeria* require a significant number of manipulations *(23).* Samples are first incubated in UVM for ~24 h; then an aliquot is transferred to Fraser broth for 48 h and is subsequently plated on a chromogenic medium requiring 24 to 48 h of incubation prior to confirmatory tests. Once colonies are available, they may be screened by PCR or biochemical assays to determine the species and, in the case of *L. monocytogenes,* the serovar designation. Although speciation and serotyping are not commonly used in environmental sampling programs, the determination of species and serovar is beneficial to the beef industry for enabling quality-assurance personnel to track the source of contamination, thereby increasing the safety of beef products. The most frequently used primary enrichment medium is UVM, a selective medium developed for the enrichment of *Listeria* spp. *(19, 23).* Using artificially inoculated samples, Petran and Swanson *(17)* demonstrated that TSB with 0.6% yeast extract (TSBYE) could be used for the growth of *L. innocua* and *L. monocytogenes* and that performance was comparable to growth in UVM. Our laboratory has previously shown that nonselective enrichment in TSB is useful for the isolation of *E. coli* and *Salmonella* spp. *(1, 2)*; therefore, TSB was evaluated as a primary enrichment medium for the isolation of *Listeria* spp.

The U.S. Meat Animal Research Center nonselective enrichment strategy *(1, 2)* was evaluated for the recovery of *Listeria* from hide, carcass, and beef trim samples (Table 1). When the recovery and isolation of *Listeria* spp. with UVM or TSB for primary enrichment (Table 1) of hide sponge samples were compared, the two enrichments were not different (chi-square, $P > 0.05$). Primary enrichment of preevisceration carcass samples with UVM and TSB showed no statistically significant difference between the distribution of positive samples. Of the 19 positive *Listeria* samples, five were positive from both primary enrichments (UVM+/TSB+), eight were identified as positive only from a primary UVM enrichment (UVM+/TSB−), and six were identified as positive solely from the TSB primary enrichment (UVM−/TSB+) (Table 1). These data demonstrate that neither medium outperformed the other; however, as was previously documented by Ryser et al. *(19),* UVM alone is

TABLE 2. *Isolation of* Listeria *spp. and* L. monocytogenes *from beef trim samples*

	No. of samples[a]	UVM$^+$	TSB$^+$	UVM$^+$/TSB$^+$	UVM$^+$/TSB$^-$	UVM$^-$/TSB$^+$	UVM$^-$/TSB$^-$
Listeria spp.[b,c]	193	7	7	3	4	4	182
L. monocytogenes[c,d]	193	18	18	10	8	8	167

[a] *Listeria* spp. and *L. monocytogenes* were recovered from the same 193 beef trim samples.
[b] *Listeria* spp. represent all *Listeria* spp. except *L. monocytogenes*.
[c] A chi-square statistical analysis found no significant difference between enrichment media ($P > 0.05$).
[d] These values represent only samples containing *L. monocytogenes*.

not capable of identifying all samples which are contaminated.

Data collected from 193 beef trim and ground beef samples with both UVM and TSB as the primary enrichment media demonstrate that the two are comparable in performance. In total, 37 of the 193 samples were positive for *Listeria* spp. In these samples, the total number of recovered *Listeria* and *L. monocytogenes* in both UVM and TSB were identical (Table 2). A further analysis, shown in Table 2, demonstrates that of the 11 *Listeria* recovered, only three came from both the UVM and TSB (UVM$^+$/TSB$^+$) enrichment of the same sample. UVM and TSB primary enrichments each detected four additional *Listeria*-positive samples. More important is the comparison of *L. monocytogenes* recovered by both methods. Because *L. monocytogenes* is considered the causative agent of foodborne listeriosis *(9, 13, 21, 22),* any newly developed technique must exhibit high sensitivity in detecting this important pathogen. As shown in Table 2, of the 26 total *L. monocytogenes* isolates recovered, only 10 were recovered by both methods from the same samples. An additional eight isolates were recovered from unique samples with either TSB or UVM. These data further demonstrate that neither medium outperformed the other in the isolation of *L. monocytogenes.*

A further analysis of the 26 samples demonstrates that both media provided an environment capable of supporting growth for the four major *L. monocytogenes* serovars (1/2a, 1/2b, 1/2c, and 4B) attributed to foodborne illness outbreaks (Table 3). Using ribotype analysis to compare the recovery of *Listeria* spp. from UVM and *Listeria* repair broth, Ryser et al. *(19)* found major differences in the number and distribution of ribotypes recovered. In the data presented in Table 3, all serovars are equally distributed among the two media, but ~50% of the time, a serovar recovered from one primary enrichment method was not identified by the other method. In sample 9, a unique *L. monocytogenes* serovar was recovered by both primary enrichment methods. In sample 20, both serovars 1/2b and 1/2c were recovered but only from the TSB primary enrichment. Individual meat samples were split; therefore, it is possible that the starting material that contained the bacteria was segregated into the processing bag for a particular method, thus creating this discrepancy in *L. monocytogenes* recovery. More than likely, as supported by the work of Ryser et al. *(19),* the use of multiple primary enrichment media on a single sample would enable the researcher to better identify *Listeria* spp. in each sample.

Numerous publications have documented the use of multiple broths, including *Listeria* repair broth *(19),* UVM *(19),* TSBYE *(17),* and *Listeria* enrichment broth *(10)* for *Listeria* enrichment procedures. Bruhn et al. *(3)* demonstrated that UVM can exhibit a bias in the enrichment of certain *Listeria* lineages. The data presented in this report demonstrate that the frequency of recovery of *Listeria* and *L. monocytogenes* from naturally contaminated samples with TSB is statistically the same as with UVM. The benefit of using TSB over UVM is threefold. First, at least three other foodborne pathogens, including *E. coli* O157:H7, *Salmonella* spp., and non-O157 *E. coli,* can be enriched from sponge samples with TSB *(1, 2).* Second, TSB is less expensive than UVM. Third, the shorter incubation time in TSB, which requires only 2 h at 25°C and 6 h at 42°C, can provide an initial time savings ≥8 h. The use of a nonselective enrichment medium such as TSB also has the advantage (over using a selective medium) in that it may not prohibit the recovery of damaged bacteria. This work did not directly address whether injured *Listeria* had a better chance of survival in TSB, but other research has demonstrated that nonselective media provide a growth environment that is more hospitable to injured *Listeria* spp. *(5, 11, 12).* TSB has a pH of 7.2, and it is likely that other bacteria will grow in this enrichment, but this procedure uses a selective temperature incubation (25 and 42°C) as well as a secondary enrichment to selectively enrich for *Listeria* spp. Additional considerations include the ease of preparation of TSB and the wide availability of this medium in microbiology laboratories.

Comparison of high-throughput method of secondary enrichment sample processing. After validating this TSB protocol for *Listeria* recovery, we investigated ways in which the secondary enrichments could be modified for higher throughput. The secondary enrichment medium most commonly used for *Listeria* isolation is Fraser broth. Normally, a 1:100 dilution of the primary enrichment is incubated for up to 48 h.

In a recent report by our laboratory, the validity of using a sequential immunomagnetic separation for the recovery of *E. coli* O157:H7 and *Salmonella* spp. from enrichments was investigated *(14).* In this format, enriched samples are placed in a 96-well block so that an eight-channel immunomagnetic separation device can be used for

TABLE 3. *Distribution of* Listeria monocytogenes *serovars recovered from beef trim and ground beef with TSB and UVM*

Sample no.[a]	*L. monocytogenes* serovar[b]: 1/2a	1/2b	1/2c	4b
1	+/+			
2		+/+		
3	−/+			
4				+/−
5	+/+			
6				−/+
7		+/+		
8	+/−			
9	+/−	−/+		
10	+/+			
11	+/−			
12	+/+			
13		−/+		
14	−/+			
15			−/+	
16			+/−	
17		+/+		
18				−/+
19		−/+		
20[c]		+/−	+/−	
21				+/−
22				+/−
23			+/−	
24			+/+	
25			−/+	
26				+/+
Total[d]	7/6	4/6	4/3	4/3

[a] Samples were renumbered 1 through 26 for simplicity in reporting this information in this table.

[b] The four *L. monocytogenes* serovars attributed to foodborne illness are presented here. +/+ represents a sample that had the *L. monocytogenes* serovar recovered from both TSB and UVM. +/− represents a sample that was found to have the *L. monocytogenes* serovar recovered from TSB only. −/+ represents a sample that was found to have the *L. monocytogenes* serovar recovered from UVM only.

[c] In sample 20, both a 1/2b and a 1/2c *L. monocytogenes* serovar were identified from the same TSB enrichment.

[d] The total represents the sum of *L. monocytogenes* serovars recovered from each primary enrichment (TSB/UVM).

immunomagnetic recovery of the pathogen. Because samples are in an eight-well format, it is logical to use a multichannel pipette to set up the secondary enrichment. The customary dilution of primary enrichment into the secondary enrichment is 1:100 *(23),* but for adapting to a more convenient high-throughput method, we tested a 1:30 dilution. Data collected from 96 hide and 48 preevisceration carcass samples with both tubes and blocks for the secondary enrichment demonstrate that each system performs similarly (Table 4). Overall, 95 of 96 natural hide samples yielded *Listeria* spp. with a 1:100 ratio (tubes), whereas all 96 samples diluted 1:30 were positive. Although not statistically significant, *L. monocytogenes* was recovered from only 25 of the samples diluted 1:100, whereas 35 of the 1:30 diluted samples were positive. One concern was that

TABLE 4. *Comparison of secondary enrichment dilutions for the isolation of* Listeria *spp.*

Sample type	No. of samples	Organism	Secondary enrichment dilution[a]: 1:100	1:30
Hide	96	*Listeria* spp.	95	96
		L. monocytogenes	25	35
Preevisceration carcass	48	*Listeria* spp.	4	9
		L. monocytogenes	1	2

[a] The 1:100 dilution was accomplished by pipetting 100 μl of primary enrichment into 10 ml of Fraser broth. The 1:30 dilution was performed by pipetting 100 μl of the primary enrichment into 3 ml of Fraser broth. A chi-square statistical analysis found no significant difference between dilutions ($P > 0.05$).

with the lower dilution, background organisms might overtake *Listeria* in the Fraser broth or on the subsequent agar plates, but this did not appear to be a problem. For 48 preevisceration carcass sponge samples, the 1:30 dilution in Fraser broth yielded a slightly higher prevalence level, although this finding was not statistically significant (Table 4).

The significance of this work is that TSB can be used as a primary enrichment medium for the isolation of *Listeria.* With this medium, there is a time savings and a cost savings, and the number of manipulations required for each sample is decreased. Other methods exist for the rapid identification of *Listeria* in food samples *(11, 16),* but these methods do not result in the isolation of a bacterial colony. The importance of obtaining a colony is that it provides researchers with an opportunity to determine the antimicrobial sensitivity *(6, 20)* and diversity *(15, 24).* Additionally, this work provides a method development that may enable the end user to screen food samples for *Listeria* spp. by using a high-throughput method.

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