Research Note

Combined Secondary Enrichment of Primary Enrichment Broths Increases *Listeria* Detection

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ABSTRACT

The efficacy of combining dual primary enrichment cultures into a single secondary broth was evaluated for detecting *Listeria* in naturally contaminated meats and environmental samples obtained from dairy processing plants. A total of 336 samples were tested using University of Vermont modified *Listeria* enrichment broth (UVM) and *Listeria* repair broth containing selective agents (LRBS) as primary enrichment media. Eighty samples (23.8%) yielded *Listeria* by at least one method. Neither primary enrichment broth was significantly better (*P* > 0.05) than the other in identifying *Listeria*-positive samples. UVM media, when used as a primary enrichment broth, identified 66 *Listeria*-positive samples, while the use of LRBS as a primary enrichment broth identified 65 *Listeria*-positive samples. *Listeria* detection improved significantly (*P* < 0.01) when two primary enrichment media were used for sample analysis. It is not clear whether this improvement was due to simply replicating the primary enrichment or to the particular pair of primary enrichment media used. The use of a dual secondary enrichment procedure was better (*P* < 0.05) than the use of either individual primary enrichment medium alone. The overall rate of recovery increased from 81.3 to 82.5% for single secondary enrichment to 93.8% using a dual secondary enrichment technique. Analysis of results obtained when combining two independent isolation methods versus combining two primary enrichment media into one single secondary enrichment broth indicated that there was no significant difference (*P* > 0.05) in either procedure. Inoculum size (0.1 ml versus 0.2 ml) did not have an effect on the overall rate of recovery. The procedure developed increased the sensitivity of testing while decreasing the potential workload associated with an increase in enrichment procedures.

*Listeria monocytogenes* is a ubiquitous gram-positive-shaped bacterium capable of causing the disease listeriosis, which ranges from flu-like illness, spontaneous abortion, meningitis, to even death. It has been estimated that there are, on average, 1,092 cases of listeriosis that result in 248 deaths every year in the United States (15). Because of the potentially devastating illness *L. monocytogenes* can cause, a zero tolerance has been established for this organism in ready-to-eat products (14).

The need to evaluate perishable foods with limited shelf-life has resulted in procedures that can identify *Listeria*-negative samples within 48 h. Current regulatory procedures consist of those developed by the U.S. Department of Agriculture–Food Safety and Inspection Services (USDA-FSIS) for meat and poultry products and the Food and Drug Administration (FDA) for dairy products and vegetables. While the presently used methods are relatively rapid, they do not reflect the true number of *Listeria*-positive samples (6). Several studies (4, 10, 17) have demonstrated that results obtained are at least partially dependent on the type and number of primary enrichment media used. Others have shown (8, 13, 15) that the primary enrichment medium used may in fact influence which particular strains are identified within a sample.

This study was undertaken to evaluate an isolation scheme that would increase the sensitivity of current *Listeria* testing procedures while decreasing overall labor demands.

MATERIALS AND METHODS

Sample collection. Sponge samples (*n* = 324) were obtained from dairy processing plant environments, refrigerated (4°C), and evaluated within 24 h of receipt. Retail meat samples (ground turkey, ground beef, hot dogs) were obtained from local supermarkets, stored at refrigeration temperature (4°C), and evaluated within 24 h of receipt.

Isolation procedure. Neutralizing buffer (30 ml) was added to each sponge sample, which was then stomached for 2 min. The resulting fluid was diluted 1:10 in 99 ml of University of Vermont modified *Listeria* enrichment broth (UVM) (Difco Laboratories, Detroit, Mich.) (3) and 99 ml of *Listeria* repair broth (2) as modified by Flanders et al. (LRBS) (4). Meat samples were stomached for 2 min and then enriched 1:10 in 225 ml of UVM medium and 225 ml of LRBS.

Primary enrichment cultures were incubated at 30°C for 24 h. A 0.1-ml portion of UVM primary enrichment culture was used to inoculate 10 ml of Fraser broth (5) (secondary enrichment). A second tube of Fraser broth was inoculated with 0.1 ml of LRBS primary enrichment culture, and a third Fraser broth tube was inoculated with 0.1 ml of each primary enrichment broth (total of 0.2 ml of inoculum). Fraser broth tubes were incubated at 35°C.
for 26 ± 2 h. All Fraser broth tubes were streaked with modified Oxford medium (MOX) (11), regardless of blackening due to esculin hydrolysis, and then incubated 24 to 48 h at 35°C. Two black colonies showing typical convex centers were isolated on tryptone soy agar with 0.6% yeast extract and purified for further analysis.

**Confirmation of isolates.** Presumptive isolates were identified based on Gram reaction, catalase activity, umbrella motility, carbohydrate utilization (rhamnose, mannitol, xylose), and CAMP reaction (18).

**Evaluation of inoculum size.** Environmental sponge samples (n = 69) were obtained from processing plants and analyzed as outlined above. Following incubation, a total of five Fraser broth tubes were inoculated from the two primary enrichment broths (Figure 1). Fraser broth cultures were streaked on MOX plates, incubated, and evaluated. Two colonies showing typical blackening with dimpled centers were evaluated via Gram reaction, umbrella motility, catalase reaction, and the API Listeria (BioMerieux Vitek, Hazelwood, Mo.).

**Statistical analysis of results.** Data were evaluated using the Student-Newman-Keuls multiple comparison test to determine significance (P < 0.05) both for individual broths and combined broths.

### RESULTS

The combined use of all detection methods identified 80 Listeria-positive samples (Table 1). Both primary enrichment media identified 53 common Listeria-positive samples. UVM medium identified 66 positive samples (82.5%), 13 of which could not be identified as Listeria-positive by LRBS. Similarly, LRBS identified 65 positive samples (81.3%), 12 of which could not be identified using UVM media. The combined results of both primary enrichment procedures identified 78 of the 80 Listeria-positive samples. Statistical analysis of the two individual media indicated that neither primary enrichment broth was significantly better than the other in identifying Listeria-positive samples. The use of two isolation procedures significantly increased (P < 0.01) the probability of detecting Listeria-positive samples. The combined results (i.e., UVM and LRBS results) indicated that the difference was significant (P < 0.05) when comparing the combined data to either individual primary enrichment procedure.

The use of a dual secondary enrichment step significantly (P < 0.05) increased the probability of identifying Listeria-positive samples over the use of either individual primary enrichment media. The results of a dual secondary enrichment (75/80) versus two primary enrichment media (78/80) did not show a significant difference (P > 0.05) in the level of detection of Listeria-positive samples. Both the dual secondary enrichment procedure and the combined results of the two primary enrichment media identified a common 73 Listeria-positive samples. Two additional Listeria-positive samples were identified by the use of a dual secondary enrichment.

Environmental sponge samples (n = 69) were evaluated to determine if the volume of Fraser broth inoculum effected the results obtained. Thirty-eight samples (55%) were positive for Listeria. The use of 0.1 ml of UVM as an inoculum for secondary enrichment identified 36 of the 38 sites. Identical results were obtained when 0.2 ml of UVM was used as an inoculum for the secondary enrichment. The use of LRBS as the inoculum for the secondary enrichment broth gave similar results to those seen with UVM. Both media identified a common 34 sites and each identified 1 site that the other did not identify. The use of a dual secondary enrichment broth identified all 38 positive sites.

### DISCUSSION

The present study reconfirms that the true incidence of Listeria in samples may not be accurately reflected by established protocols. Our results indicate the use of a single isolation procedure may result in a 17 to 18% underreporting of actual Listeria-positive samples. In our study both UVM and LRBS performed similarly. UVM media identified 13 unique Listeria-positive samples, whereas LRBS identified another 12 unique Listeria-positive samples. Similar results have been obtained by previous investigators. Flanders et al. (4) evaluated samples from dairy processing plants and determined that while no single isolation procedure could identify all positive samples, the use of two cultural methods did increase the number of samples iden-
tified as *Listeria* positive. Similarly, Lund et al. (10) evaluated raw milk samples for the presence of *Listeria* using three primary enrichment media and determined that the use of three primary enrichment broths increased the overall incidence of *Listeria* by almost 41%. Warburton et al. (17) evaluated food and environmental samples for the presence of *L. monocytogenes* using both the FDA and the USDA-FSIS procedures. The combined results for both media increased the *Listeria*-positive results 30% over the use of single isolation procedures.

Noah et al. (12) evaluated naturally contaminated seafood and seafood products using five different procedures. The *Bacteriological Analytical Manual* (BAM) (9) procedure was used as a control against which to evaluate the efficacy of the other procedures. The BAM procedure failed to identify 7 samples that were found to harbor *Listeria* via other procedures. The overall incidence of *Listeria* was increased 11.7% by the use of more than one testing procedure.

In two separate studies Hayes et al. (6, 7) evaluated foods by the use of various enrichment techniques. In one study, food samples from the refrigerators of *Listeria*-positive patients were analyzed using three isolation procedures (7). Although no one procedure was significantly better than the others, a combination of any two methods significantly (*P < 0.02*) increased the rate of detection by 17 to 21%.

In a second refrigerator study, Hayes et al. (6) compared the use of the USDA-FSIS procedure with the use of cold enrichment (CE) as means of identifying *L. monocytogenes* in suspect foods. The USDA-FSIS procedure identified 21 unique samples, while the CE procedure identified an additional 2 *Listeria*-positive samples.

In all cases, the use of more than one testing procedure significantly increased the sensitivity of identifying a *Listeria*-positive sample. The use of two or more procedures also increased the amount of work required to perform the testing. By performing two primary enrichments and combining them into a dual secondary enrichment broth, it is possible to both increase sensitivity of the testing and decrease the overall workload required to obtain accurate results. The results of the dual secondary enrichment were significantly better (*P < 0.05*) than any one test procedure, and yet it was as sensitive as carrying out both procedures individually. It is not clear whether this effect was due to simply replicating the primary enrichment or to the particular pair of primary enrichment media used.

The use of dual secondary enrichment procedure identified two unique samples. Although the dual secondary enrichment procedure failed to identify five *Listeria*-positive samples, all five false-negative samples did give characteristic Fraser broth blackening and black colonies on MOX media. Ryser et al. (13) have suggested that 10 isolates should be evaluated to ensure that all potential biotypes of *Listeria* are identified. In our study we examined two colonies per plate showing typical *Listeria* characteristics. The failure of the dual secondary enrichment sample to identify *Listeria* in these five samples may be because of the evaluation of too few suspect colonies.

We attempted to determine if the inoculum size (0.1 ml versus 0.2 ml) was responsible for the increased rate of identification of *Listeria* in the dual secondary enrichment broth. The results indicated that the volume of the inoculum did not effect the outcome of the test. It should also be noted that some procedures suggest the use of 1.0 ml of primary enrichment to inoculate the Fraser broth (1).

Given the zero tolerance for *Listeria* in ready-to-eat foods, our present surveillance systems are inadequate to maintain this level of confidence in our food supply. Currently used methods for evaluating *Listeria* in food and environmental samples do not accurately identify all positive samples. We have developed a combined protocol that appears to significantly increase the sensitivity of testing and decrease the workload required to obtain the results.

REFERENCES