Validation of the USDA/ARS Package Rinse Method for Recovery of Listeria monocytogenes from Naturally Contaminated, Commercially Prepared Frankfurters†

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ABSTRACT

The utility of the U.S. Department of Agriculture/Agricultural Research Service (USDA/ARS) package rinse method for recovering Listeria monocytogenes from the surface of contaminated foods was validated in comparison to the standard USDA/Food Safety and Inspection Service (FSIS) product composite enrichment method and two other methods using frankfurters from a lot with a known package prevalence rate of approximately 16% for this pathogen. One hundred packages from this batch of naturally contaminated, commercially prepared frankfurters were examined as follows: (i) the package exudative fluid was removed and tested using the standard USDA/FSIS product composite enrichment method; (ii) approximately 5 to 7 portions of frankfurters were removed to obtain a 25-g composite of meat that was then processed using the standard USDA/FSIS product composite enrichment method; (iii) 50 ml of 0.1% peptone water was added to each package, and the USDA/ARS package rinse method was performed on the remaining contents; and (iv) after removing the rinse fluid, the solids remaining in each package were directly enriched using the USDA/FSIS product composite enrichment method. These four methods identified that 7, 6, 15, and 9 of the 100 packages tested positive for the pathogen, respectively. Although no single approach yielded a positive result for every package that tested positive for L. monocytogenes by any one of the four sampling strategies, the USDA/ARS package rinse method was appreciably (P < 0.05) better than either the package exudate enrichment method or the standard USDA/FSIS product composite enrichment method at recovering the bacterium. These findings validate the sensitivity and ease of use of the USDA/ARS package rinse method using naturally contaminated frankfurters and argue strongly for its adoption for routine screening of ready-to-eat products that are prone to surface contamination with undesirable microbes such as L. monocytogenes.

The recovery rate of Listeria monocytogenes in ready-to-eat (RTE) foods, including frankfurters, reportedly ranges from 1.6 to 7.6% (5, 6, 8, 12, 13). If present, the bacterium is usually found at <10 CFU/g, but levels of the bacterium in RTE foods can range from as low as 0.3 most probable number (MPN) per gram up to 10⁷ CFU/g (3, 6, 7, 12). In addition, L. monocytogenes is widespread in the environment and capable of growth or survival at much lower temperatures than other common foodborne pathogens (3, 11). Although listeriosis accounts for only a small fraction of the total illnesses associated with food, because of the severity of the ensuing infection, L. monocytogenes accounts for about one in every four deaths from foodborne illnesses in the United States (10). To monitor the safety of RTE meats, the U.S. Department of Agriculture/Food Safety and Inspection Service (USDA/FSIS) routinely samples products produced in plants under Federal inspection. In 1999, the most recent year for which USDA/FSIS data has been published, 38 of 2,162 (1.76%) small-diameter sausage samples, which includes frankfurters, tested positive for this foodborne pathogen. For comparison, between 2000 and 2002, Wallace et al. (12) sampled about 2,700 pounds/packages from a single production run from each of 12 commercial manufacturers: L. monocytogenes was recovered from 532 of 32,800 (1.6%) packages tested. The latter study relied on the recently developed USDA/Agricultural Research Service (ARS) package rinse method (9). This method is approximately six times more effective and significantly less labor intensive than the standard USDA/FSIS product composite enrichment method (2) for recovery of L. monocytogenes from packages of frankfurters inoculated with relatively low levels (~20 CFU per package) of the pathogen (9).

In validation or challenge studies, frankfurters are typically inoculated with stationary-phase cells of a liquid culture containing either a single strain or a mixture of strains of L. monocytogenes. In a processing facility, frankfurters are more likely contaminated via product contact surfaces, aerosols, or both (11). As such, there is some concern that different routes of natural “inoculation,” versus artificial contamination, might lead to differences in the tenacity with which the bacterium can adhere to the product or in

† Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.
the niche of the bacterium within the package and that this, in turn, could affect the efficiency of the method(s) used to recover the bacterium. For these reasons, the present study was designed to compare the efficacy of the USDA/ARS package rinse method with the USDA/FSIS product composite enrichment method using the same packages of vacuum-sealed and commercially prepared frankfurters. A second objective was to determine whether the bacterium was more likely to be found in the exudative fluid, on the frankfurter, or on the packaging film from the same lot of frankfurters previously confirmed as being naturally contaminated with \textit{L. monocytogenes} at a rate of 16\% (12).

**MATERIALS AND METHODS**

**Source of frankfurters.** In a previous study (12), about 2,700 one-pound packages of freshly processed and peeled frankfurters were obtained from one production run from each of 12 commercial processors. From a statistical perspective, product from three of the plants tested positive, including 437 of the 2,800 (16\%) packages tested from plant 133. One hundred additional packages from plant 133 were used in the present study, and each package was stored at 4°C for 150 days prior to testing herein.

**Comparison of sampling methods to determine the location of \textit{L. monocytogenes} in naturally contaminated packages of frankfurters.** After 150 days of refrigerated storage, the 100 vacuum-sealed packages of frankfurters from plant 133 were sampled for \textit{L. monocytogenes}. The packages were disinfected by wiping with 70\% ethanol, and a sterile, single-use scalpel was used to make a 1- to 2-cm incision along one of the corners of the package. A 5-ml pipette was used to remove as much of the exudative fluid as possible from within the package; the volume recovered ranged from 6 to 13 ml per package. About 70\% of the exudative fluid recovered was diluted 1:10 into University of Vermont (UVM) broth (Twirl 'Em Sample Bags, Lab Plas, Quebec, Canada). The remaining 30\% of the exudative fluid was transferred into a sterile, conical-bottom centrifuge tube and stored at −20°C for subsequent enumeration. The remaining contents of each package were transferred to a sterile work surface, and a 25-g composite was prepared by taking approximately five to seven sagittal sections from different locations of several links with a sterile, single-use scalpel. The 25-g composite was added to a sterile nylon-polyethylene bag containing 225 ml of UVM enrichment broth (Oxoid Ltd., Ogdensburg, N.Y.). The contents were stomached for 1 min, then subsequently incubated at 30°C for 22 ± 2 h and processed according to the standard USDA/FSIS product composite enrichment method (1). The remaining product was transferred to a sterile nylon-polyethylene bag (8 by 12 in., 3-mil thick, standard barrier package; Koch Industries, Kansas City, Mo.).

Concomitantly, a 50-ml volume of 0.1\% peptone water was used to wash and rinse the original package, and the resulting rinse fluid was transferred to the nylon-polyethylene bag containing the corresponding product. After each bag of product was massaged by hand for 2 min, the rinse fluid was transferred to two sterile, 50-ml conical centrifuge tubes. One 25-ml portion of the rinse fluid was transferred to a sterile nylon-polyethylene bag containing 225 ml of UVM broth and incubated at 30°C for 22 ± 2 h before being processed according to the USDA/ARS package rinse method (9). The remaining portion of the rinse fluid was stored at −20°C for subsequent enumeration. The remaining product (∼425 to 430 g) from each original package (i.e., without the exudative fluid, minus a 25-g composite sample, and after rinsing) was added to a nylon bag containing 225 ml of UVM broth for processing according to the USDA/FSIS product composite enrichment method. As described previously (12), both positive and negative controls were performed each day for these experiments. Presumptive \textit{Listeria} isolates were biochemically confirmed as \textit{L. monocytogenes} and characterized using the Riboprinter (Qualicon/ DuPont, Wilmington, Del.) as described previously (12).

**Enumeration of \textit{L. monocytogenes} from frozen samples of exudative fluid and package rinse fluids.** Exudative fluid and package rinse fluid samples were held at −20°C for between 4 and 7 days pending confirmation that \textit{L. monocytogenes} was recovered from the corresponding package by at least one of the four sampling strategies being evaluated. Select frozen samples were then thawed in a water bath maintained at 37°C. Three milliliters of each thawed sample of package rinse fluid was assayed, with and without prior dilution, by spread plating 333-μl portions onto modified Oxford (MOX) agar plates (2). The remainder of the thawed rinse fluid was appropriately discarded. All of the thawed exudative fluid (1.8 to 3.9 ml) was assayed, with and without prior dilution, by spread plating 333-μl portions onto MOX agar plates. Plates were incubated at 37°C for 48 h, and the resulting colonies were counted manually. On the basis of the total volume of rinse fluid or exudate plated, counts were normalized to estimate the total number of \textit{L. monocytogenes} per package. Up to five colonies were authenticated, as described previously (12), using USDA/FSIS procedures (2).

**Statistical analyses.** Data were analyzed using version 8.0 of the SAS statistical program (SAS Institute, Inc., Cary, N.C.). Frequency analysis was performed using McNemar’s test (4) to determine whether there was a difference among methods. A simple kappa statistic was calculated to determine how well the methods were in agreement in classifying the samples as positive or negative for \textit{L. monocytogenes} (4). For the comparison of all four sampling methods, Fischer’s test was performed on the resulting six “two by two” pairs, and a kappa statistic for each pair was also generated (4).

**RESULTS AND DISCUSSION**

In this study, 100 packages of product from plant 133, previously shown as being naturally contaminated with \textit{L. monocytogenes} at a package prevalence of 16\% (9), were analyzed to determine the whereabouts of the pathogen in the package. Analyses of the data (Table 1) using McNemar’s test indicated that the USDA/ARS package rinse method was significantly more effective (\(P < 0.05\)) for recovery of \textit{L. monocytogenes} than the USDA/FSIS product composite enrichment method or than the enrichment of exudative fluid from packages. However, from a statistical perspective, it was not significantly better than direct enrichment of the remaining approximately 425 g of product from a given package after the exudate was removed, after a 25-g composite of product was obtained, and after the package and product were rinsed. Pairwise comparison of results among the four sampling regimens generated a kappa value of 0.3534 to 0.5926, indicating there was only poor to fair agreement among the sampling methods in the identification of a given package as either positive or negative for the pathogen (Table 1). Although no single method detected the pathogen in all of the packages that tested positive by at least one other method, the USDA/ARS package rinse method detected \textit{L. monocytogenes} in 15 of 18 (83\%)
packages that tested positive by at least one of the four sampling regimens. The second most effective sampling regimen was direct enrichment of the remaining 425 g of frankfurters from a given package. By this approach, nine packages tested positive for *L. monocytogenes* and six of these nine packages also tested positive by the USDA/ARS package rinse method. Of the remaining two sampling regimens, the enrichment of the exudative fluid generated seven positive packages, all but one of which also tested positive by the USDA/ARS package rinse method. Only six packages tested positive by the USDA/FSIS product composite enrichment method, and all of these packages also tested positive using the USDA/ARS package rinse method. These findings substantiate the utility of the USDA/ARS package rinse method.

A few points deserve mention relative to the usefulness of some of the sampling regimens tested. Although the direct enrichment of the remaining 425-g portion of frankfurters was the second most effective sampling regimen, problems were encountered with the recovery and subsequent confirmation of the pathogen because of an outgrowth of indigenous flora. Perhaps if a more typical ratio of 9 ml of enrichment broth to 1 ml of sample were used, a better result would be obtained. However, this method would require an enrichment volume of >4 liters, which would be cumbersome as part of a regulatory sampling program. Such problems could hinder the routine use of this method. In addition, removal of the exudative fluid might preclude recovery of the pathogen by any other methods that follow. Regardless, testing exudative fluid could prove to be an easy and effective method for routine sampling of frankfurter packages. Finally, the standard USDA/FSIS product composite enrichment method identified six packages as positive, and all six of these packages also tested positive by the USDA/ARS package rinse method. Because only 6 of the 15 packages testing positive by the USDA/ARS package rinse method also tested positive by the USDA/FSIS product composite enrichment method, more than twice as many packages would need to be tested by the latter method to approach the effectiveness of the former for recovery of low levels of the pathogen in packages.

As another component of this study, enumeration from the frozen samples of package exudative fluid and the frozen package rinse fluid showed large variations in the levels of the pathogen that were present. Some packages did not yield any colonies by direct plating, whereas other packages harbored high levels of the pathogen. From the frozen portions of the 16 samples from which *L. monocytogenes* were detected by the exudative fluid enrichment method, the USDA/ARS package rinse method, or both, direct plating revealed that nine packages did not yield any viable colonies of the pathogen and that two packages had $10^1$ to $10^2$, two packages had $10^2$ to $10^3$, and three packages had $10^4$ to $10^5$ CFU per package. It should be noted that the minimum detectable number of *L. monocytogenes* was estimated as 6 and 120 CFU per package in exudative and rinse fluid, respectively, on the basis of the limited volume of each sample plated and on an estimated 50 to 80% loss in viability following storage at −20°C for 7 or fewer days (12).

Molecular subtyping was conducted on at least one isolate for each method that identified a given package as testing positive for *L. monocytogenes*; 37 total isolates were characterized. More specifically, if a given package tested positive by three of the four methods, then at least three isolates were ribotyped from that package. Molecular characterization revealed that all 37 isolates displayed the same ribotype, previously designated as “profile A” (12). In fact, profile A (serotype 1/2a) was displayed by 90% of the 887 isolates typed from the 437 packages that tested positive from plant 133 (12). As such, it is not surprising that all of the isolates from the present study recovered from frankfurter packages produced by plant 133 displayed profile A. It is possible that certain strains, such as those displaying profile A, are better able to survive within the environment of a vacuum-sealed package of frankfurters or within a processing facility.

*L. monocytogenes* continues to pose challenges to the food producers, who need to control contamination of foods by this pathogen; to government agencies, which need to identify and regulate problems that occur in foods; and to consumers, who need to better understand how to maintain the safety of foods in the home. Certain RTE foods, such as frankfurters, could pose a greater public health risk than other RTE foods as reported in the *L. monocytogenes* risk assessment for selected RTE foods (5). This finding is even
more significant given that about 20 billion frankfurters are consumed each year in the U.S. alone (1). Better methods of screening for \textit{L. monocytogenes} in RTE foods will decrease the risks associated with this pathogen. Nonetheless, it must be noted that even the most carefully crafted sampling program will miss some lots of product that are contaminated at a low package prevalence. The widely varying levels of the pathogen recovered from positive packages in the present study, and the fact that it may not be possible to recover the pathogen from all packages, even from a heavily contaminated batch of frankfurters, highlights the need for faster, cheaper, easier, and more sensitive sampling regimens and detection methods. The USDA/ARS package rinse method is a significant improvement over the current regulatory-sanctioned approach as demonstrated in our previous study using artificially inoculated packages of commercially prepared frankfurters (9) and in this study using naturally contaminated packages. These findings suggest that the USDA/ARS package rinse method should be instituted for routine testing of RTE foods suspected of surface contamination by foodborne pathogens such as \textit{L. monocytogenes}.

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