

Survey of *Listeria monocytogenes* in Ready-to-Eat Foods

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MATERIALS AND METHODS

Product categories

The products sampled were ready-to-eat (RTE) and not likely to be further treated in such a way that would decrease the levels of *L. monocytogenes* after purchase. The categories included sliced luncheon meats (i.e., ham, bologna and poultry) and prepared “deli” salads (i.e., potato, tuna, pasta and coleslaw), fresh soft “Hispanic-style” cheese; bagged, precut leafy vegetable salad; blue-veined or soft mold-ripened cheeses; seafood salads (except tuna); and smoked seafood. Products included those packaged by the manufacturer and those packaged in the store.

Number of samples

Because the level of contamination was expected to be low (<5%) for most products, and because the cost of enumerating *L. monocytogenes* is high, we attempted to estimate the total sample size based on the following equation:

$$n = \frac{z^2 P(1-P)}{d^2}$$

where n is the number of positive samples, P is the actual value of the proportion or percentage positive for the population, d is the desired upper bound on the absolute error (i.e., margin of error), and $z=1.96$, corresponding to a 95% confidence level for the probability that the estimate is within $\pm d$ of the population value (Garthright et al. 2000). For a fixed value of d , the sample size is at its maximum when $P = 0.5$ (50%). To be conservative, and since we did not know the values of the percentages to be estimated, we assumed $P=50\%$. Using this approach, sample sizes (n values) of 125, 250 and 500 correspond to upper bounds on absolute error of 8.8%, 6.2%, and 4.4%, respectively. Assuming that 5% of the total samples tested would be positive for *L. monocytogenes*, the corresponding total sample sizes required would be 2,500, 5,000 and 10,000, respectively. Based on this analysis, we decided to collect 2,500 samples of each of two types of products, luncheon meats and deli salads, at each of two FoodNet sites (see sampling site selection, below), for a total of 5,000 samples per food type, or 10,000 samples in total. In the course of the study, we found that prevalence of *L. monocytogenes* in luncheon meats and deli salads was considerably lower than had been expected, and thus we decided to double the total number of luncheon meats and deli salads samples collected. For the other product categories, we targeted a total of 2500-3000 samples per food type.

Within the category of luncheon meats or deli salads, we weighted the number of samples by consumption of product. For example, with luncheon meats, the proportion of ham, bologna and chicken/turkey samples was based on the frequency of consumption of these meats in the geographical area according to the Continuing Survey of Food Intakes by Individuals 1994-1996 (CSFII): West for California and South for Maryland. Thus, for Maryland, we targeted half the luncheon meat samples to be ham, 30% bologna, and 20% turkey/chicken; for California, we targeted 43% of the samples to be ham, 30% bologna, and 27% turkey/chicken. Due to the

diverse nature of the additional product categories, there was no weighting within those categories.

Sampling site selection

The CDC conducts active surveillance for listeriosis at nine FoodNet sites (FoodNet website, 2003), which provides the most accurate estimate of listeriosis possible. In addition, during the period of this study, the CDC performed case-control studies for listeriosis in these sites, which provided a potential opportunity to obtain *L. monocytogenes* isolates from listeriosis patients and compare them with isolates from foods in the same geographic area. Thus, in order to relate exposure data and food isolates to illness, food samples were collected at the Northern California (CA) and the Maryland (MD) FoodNet sites. The rationale for selecting these sites was as follows. Although FoodNet data indicated that the incidence of listeriosis was similar for all sites (approximately 0.5 cases per 100,000 people), the 1997 FoodNet final report indicated a slightly higher rate at the Northern California site (0.7 cases per 100,000 people). The potential for this site to have a higher number of cases of listeriosis, and therefore more isolates for comparison to food isolates, was one factor in selecting this as one of our sampling sites. In addition, the limited geographical area (Alameda and San Francisco counties) simplified the sampling procedure (compared to sampling across an entire state such as Minnesota or Georgia). We selected Maryland because the FoodNet site was relatively small (comprising 5 counties plus Baltimore City) and it was geographically far removed from the other site chosen. However, in Maryland, listeriosis data for FoodNet are collected statewide, so we elected to sample all counties containing more than 2% of the population (10 counties) plus Baltimore City, covering 87.5% of the population. Counties were omitted from the sampling list where products were not available (e.g., fresh soft Hispanic-style cheeses).

Selection of sampling locations within the FoodNet sites

Sampling within the sites was weighted by the population in the counties involved (1 July 1998 estimate from www.census.gov). For example, since it was determined that approximately 65% of the population resided in Alameda County and 35% in San Francisco County, the study was designed to collect 65% of the samples in Alameda County and 35% in San Francisco County. The sampling strategy for fresh soft Hispanic-style cheeses was based on the Hispanic population in the sampling area. In order to simplify sample collection and minimize travel costs, samples were generally collected in only one county on each sampling day. The order in which the counties were sampled within a site was determined using a random number table (ICMSF 1978).

Selection of collection sites within counties

Given the ubiquitous nature of the organism, foods may be contaminated with *L. monocytogenes* at foodservice establishments or in the home, but we focused on evaluating levels of *L. monocytogenes* in foods purchased at retail. Purchasing products at retail stores allowed us to sample a variety of products (brand name product and unbranded products) representative of what the consumer would purchase and consume in the areas of the study. Logistically it was easier to sample at retail than at foodservice establishments or to obtain foods from consumers' homes, and samples were in packages/containers more readily shipped to the laboratories than, for example, foodservice meals. Further, collecting retail samples avoided the potential for cross-contamination by the consumers' handling.

Lists of large and small retail markets were created from current telephone directories accessed at the Library of Congress. For each county, the list of stores was divided into List A (major supermarkets) and List B (other grocers). It was assumed that List A stores would carry

luncheon meats and deli salads. All List B stores were contacted by telephone to determine if they carried the specific product and to verify the address; stores were deleted from the list if they did not respond to three phone calls during business hours, if their phone number was incorrect, or if they did not carry the product to be sampled. For the additional product categories, List A-stores were also contacted to verify product availability. The stores on the lists were numbered, and the random number table (ICMSF 1978) was used to select stores for each collection week (5 major supermarkets and 10 other grocers). It was assumed (based on the experiences of the authors and others in the retail industry) that 75% of shopping is done at major supermarket chains and 25% at other grocers, and the number of samples from Lists A and B were weighted accordingly. “Supplementary lists” of stores reported to have specific products (smoked seafood, seafood salads, soft cheeses, and bagged salads) were provided to use as needed to meet the selected number of samples for these products.

Collection of samples

The NFPA Research Foundation (RF) contracted with independent third parties to collect samples at retail markets of all products.

Luncheon Meats and Deli Salads. For luncheon meats and deli salads, each week, in alternating weeks, 120 samples were collected in both California and Maryland for approximately 90 weeks over 23 months. The NFPA RF provided specific instructions for collectors and store lists for each collection week. The information specified the product category, specific types of products included in the category, the number of samples of each type of product to be obtained, the size of the sample to be purchased, and how to collect the sample. For example, for the sampling of luncheon meats in California, collectors were instructed to purchase 39 samples of ham, 27 samples of bologna, and 24 samples of poultry from stores on

List A and 13 samples of ham, 9 samples of bologna, and 8 samples of poultry from stores on List B. Ham could include products made from pork or poultry (such as turkey), and could include regular, low salt/sodium, low-fat, extra lean, and fat-free varieties. Bologna could include products made from pork, beef, turkey or mixtures of these, and could include regular, low salt/sodium, low-fat, and fat-free varieties. Turkey or chicken could be smoked or not smoked. All products were to be sliced (either pre-sliced by manufacturers or sliced by the retail stores). Samples were to be obtained first from the delicatessen where a delicatessen was present (¼-½ lb samples of each different type of ham, bologna, and chicken or turkey luncheon meat) and then from the refrigerated cases. No attempt was made to pre-specify the percentage collected from the delicatessen vs. the refrigerated case. For each type of manufacturer-packaged product, the top unit of each “facing” (hook or stack) of 4 to 16-oz packages (except variety packs) was to be selected. If this did not produce the specified number of samples, the back or bottom unit of every facing was to be selected, starting again at the top row. A similar approach was used for deli salads.

Sample collectors were instructed to make purchases from at least two stores on List A and two stores on List B, until the specified number of samples had been collected. If the required number of samples could not be purchased in two stores, shoppers were to go to additional stores on the list until the specified number was obtained. To ensure that at least two stores of each type (major supermarket and other grocers) were visited, shoppers were to collect no more than 2/3 of a category goal in the first store. In order to minimize shopping time and travel costs, shoppers were instructed to not go to more than five List B stores; if the required number of samples was not obtained in the 5 List B stores, they were to select a store from List A that had not been sampled, if possible, to collect the remaining samples needed. Also, if

shoppers were not able to obtain adequate numbers of samples of a particular type of product they could select the needed number of samples from other products in the category (e.g., if there were insufficient sliced poultry samples, they could be replaced by ham or bologna; or tuna salad, pasta salad or coleslaw could be substituted for potato salad to obtain adequate numbers).

Smoked seafood, seafood salad, blue-veined or soft mold-ripened cheeses, bagged salads, and fresh soft cheeses. For approximately 50 weeks over 14 months, 28 samples of each of the five additional product types were collected in each of the two locations. Four product types (smoked seafood, seafood salad [except tuna], blue-veined or soft mold-ripened cheeses, and bagged salads) were collected from the same locations from which sampling luncheon meats or deli salads had been obtained the previous day. Collectors were instructed to make purchases for each product type from at least two List A/major supermarkets and two List B/other grocers until the specified number of samples was collected and to not collect more than 2/3 of a category goal at the first store. However, if shoppers were unable to find a sufficient amount of the product of the desired type in the stores used the previous day, they were to select stores from the supplementary lists for the area and product type, with a maximum of eight stores (to minimize costs), until the specified number of samples had been obtained.

Twenty-eight samples of fresh soft Hispanic-style cheese were collected in a similar manner from northern California and Maryland each week for approximately 50 weeks. The NFPA RF randomized the order of the counties for shopping and store lists (major supermarkets and other grocers) for each county where the type of product was available (Alameda and San Francisco counties in California; Montgomery County, Prince George's County, and Baltimore City and County in Maryland). Shoppers were to select any of the stores from the lists but to ensure that all the stores were visited during the study. Shoppers were instructed to make

purchases from at least two major supermarkets and two other grocers (except in one county, where none of the major supermarkets reported carrying the product) until the specified number of samples was collected. To ensure that at least two stores in a category were visited, shoppers were to collect no more than 2/3 of the samples in any one store. If the required number of samples was not obtained in the four stores, shoppers were instructed to select any of the stores on either list to collect the remaining samples needed and to not go to more than eight stores altogether.

Shoppers were provided with information on what was included in or excluded from each group and the sizes of the samples (8 - 16 oz, or two packages of the same code if only a <8-oz size was available). They were instructed to obtain samples from both the delicatessen (if there was one) and the refrigerated case, where applicable. They were to select one package of each type of product from within the specific category from the deli and to select the top container and the bottom container of each stack or facing (hook) when purchasing manufacturer-packaged products.

Handling of samples after selection

Samples were placed in insulated shipping coolers containing frozen gel packs, which were placed on the sides, middle, and the tops of the product. A data logger (model SL100 or SX100, Dickson, Addison, IL) was placed between two products in the middle of the cooler to monitor temperature during product transportation to verify that the temperature remained within desirable limits. When the cooler was opened at the laboratory, a thermometer or other temperature-sensing device was inserted between packages, and the temperature was recorded. The temperature profile recorded by the logger was evaluated together with the temperature

recorded at the laboratory, and samples would be rejected if both the data logger temperature and the lab-measured temperature were above 10 °C. No such occasions occurred.

Selection of testing laboratories

Two testing laboratories were selected on the basis of 1) familiarity with procedures for detecting *L. monocytogenes* in foods, 2) accuracy in detecting and quantifying *L. monocytogenes* in several NFPA-prepared test samples, and 3) an on-site audit of the laboratory's standard operating procedures. The laboratories ultimately selected to test the product samples were in Green Bay, WI and in Modesto, CA. Samples collected in Maryland were shipped to the Green Bay laboratory overnight. Samples collected in California were sent to the Modesto laboratory by ground transportation on the day of collection or the next morning, and the cooler was held refrigerated overnight prior to further handling.

Handling of samples upon receipt

The laboratories were instructed to discard any sample with package damage such that the microbiological integrity of the sample was compromised and any sample that was collected but did not meet the description for the product category. After culling unacceptable samples, for luncheon meats and deli salads, 100 samples were selected for testing. For the other product categories, 50 samples were selected for the first several weeks of sampling, and 25 samples were selected each week thereafter for testing. In a week when less than 100 or 25 samples were purchased (due to product shortage in the stores), all acceptable samples were tested.

The laboratories were instructed to aseptically transfer samples into individual sterile plastic bags and discard the original retail packages. For MD samples, the selection of 100 or 25 samples to be tested occurred after all samples had been transferred into plastic bags. For CA

samples, the selection occurred prior to the transfer of the samples. The selected samples were stored at 2 ± 2 °C until use. Sample testing was initiated within 24 h after receipt.

Testing procedures

The general scheme for sample testing is shown in Figure 1. Four combinations of testing procedures were used to screen the samples for *L. monocytogenes*. The choice of screening procedure was based on product type and which laboratory performed the testing (see below). Samples were screened by recognized methods typically used by the laboratory for *L. monocytogenes*. The Gene-Trak[®] assay (Neogen, Lansing, MI) and the BAX[®] assay (DuPont Qualicon, Wilmington, DE) were used to screen samples collected in MD and CA, respectively. In a pre-study evaluation of the laboratories, both DNA-based assays generated comparable results for the detection of *L. monocytogenes* in samples provided by NFPA (data not shown). USDA or FDA testing procedures for *L. monocytogenes* were modified for use in enumeration and isolation. Methods described in chapter 8 of the revised *Microbiological Laboratory Guide* (USDA 1998) were adapted for meat products, and those in the *Bacteriological Analytical Manual* (BAM; FDA 1998) were adapted for non-meat products.

Sample screening

Each sample was divided into two portions for screening and enumeration, respectively. For screening, approximately half (up to 100 g) of a sample was aseptically transferred to a sterile stomacher bag and blended with an equal amount of enrichment broth. For MD samples, University of Vermont broth 2 (UVM-2 broth) was used as the enrichment broth for luncheon meats, smoked seafood, and seafood salads; phosphate-buffered *Listeria* enrichment broth was used for other product types. For CA samples, demi-Fraser broth was used as the enrichment broth for all products. After blending, 50 g of the homogenate was added to 200 ml of

enrichment broth and stomached for one minute. This procedure resulted in detection sensitivity equivalent to that of the current regulatory methods (i.e., 1 colony-forming-unit [cfu] in 25 g). The initial sample-blending step was performed to account for the potential heterogeneous distribution of *L. monocytogenes* in the sample. The 250-ml enrichment was incubated at 35 °C for 24±2 h.

After incubation, individual MD sample enrichments were swabbed onto two plates of modified lithium chloride-ceftazidime agar (mLCA), which were incubated at 35 °C for 24±2 h. Cells grown were collected from one of the plates with a cotton swab for composite screening with Gene-Trak[®], and the other plate was held for individual screening if needed. For each CA sample, 0.1 ml of the demi-Fraser enrichment culture was transferred to 10 ml of morpholinepropanesulfonic acid (MOPS)-*Listeria* enrichment broth and incubated at 35 °C for 20-24 h. After the secondary enrichment, 1 ml of the broth culture was used for screening with the BAX[®] assay as described below.

Up to five like-product samples were composited and screened. For MD samples, cells from five cotton swabs (representing 5 samples) were suspended in 5 ml of phosphate-buffered saline (PBS, pH 7.5). The suspension was centrifuged at 3000xg for 10 min; the cell pellet was resuspended in 1 ml of PBS and tested by the Gene-Trak[®] assay, according to the manufacturer's procedures. For CA samples, five 1-ml samples of secondary enrichment broths were composited, and the 5-ml composite was tested by the BAX[®] assay, according to the manufacturer's procedures.

If a composite tested negative, all samples in the composite were considered negative (in 25 g) and were not tested further. If a composite was positive, samples in the composite were tested individually by the Gene-Trak[®] assay with the PBS from the second mLCA plate or by the

BAX[®] assay with the secondary enrichment broths. Individual samples that tested negative were not further tested. If an individual sample was found positive, the retained portion was subjected to an enumeration assay. The PBS suspension or the secondary enrichment broth was also streaked onto an agar plate (modified Oxford agar [MOX] for luncheon meats and Oxford agar [OXA] for the other product types), which was incubated at 35 °C for 24-48 h and held for confirmation if needed.

Enumeration

Individual positive samples were enumerated by both the most probable number (MPN) method (FDA 1998, USDA 1998) and direct plate count. Up to 100 g of the retained portion of a sample was blended using the same procedure as in the sample preparation for screening (see above). The media used in sample preparation and MPN enumeration for samples from both MD and CA were UVM-1 for luncheon meats and phosphate-buffered *Listeria* enrichment broth for other product types. A 250-ml sample homogenate in an appropriate medium, representing 25 g of the original sample, was prepared.

MPN method. A 9-tube MPN method was used. The nine tubes were divided into three sets of three tubes. The second and third sets of tubes contained 10 ml of the appropriate broth medium. Three aliquots of the sample homogenate, in volumes of 10, 1, and 0.1 ml, were dispensed into the first, second and third set, representing 1.0, 0.1, and 0.01 g of the original sample, respectively. For a non-luncheon meat sample, the tubes were incubated at 30 ± 2 °C for 48 h and subjected to confirmation. For a luncheon meat sample, the tubes were incubated at 30 ± 2 °C for 22 ± 2 h, and 0.1 ml from each tube was transferred to a new tube containing 10 ml of Fraser broth. The tubes were incubated at 35 ± 2 °C for 26 ± 2 h. Darkened Fraser tubes (an indication of esculin hydrolysis and potential presence of *L. monocytogenes*) were subjected to

confirmation. If a Fraser broth tube did not darken, it was examined again after an additional 26 ± 2 h of incubation.

For MD samples, the MPN pattern was determined by the Gene-Trak[®] assay. Darkened Fraser tubes for a luncheon meat sample, and all nine tubes for a sample of the other product types, were individually streaked onto mLCA plates, which were incubated at 35 ± 2 °C for 24±2 h. Individual plates were swabbed and cells were suspended in 1 ml of PBS and tested by the Gene-Trak[®] assay. The MPN was determined based on the number of positive tube(s) in each of the three sets and an MPN table (FDA 1998, USDA 1998). Each sample for which at least one MPN tube was found positive was tested further by biochemical assays and to obtain an *L. monocytogenes* isolate. If only one tube tested positive, the corresponding PBS suspension was subcultured onto an appropriate agar plate (MOX for luncheon meat samples and OXA for other samples) and incubated at 35 ± 2 °C for 24 - 48 h. If tubes from more than one set tested positive, the PBS suspension from the highest MPN dilution was subcultured. The isolation of *L. monocytogenes* was carried out by the method described in the confirmation section, below.

For CA samples, the MPN pattern was determined by cultural methods and biochemical assays. Darkened Fraser tubes for a luncheon meat sample and all nine tubes for a sample of the other product types, were individually subcultured onto an appropriate agar plate (MOX for luncheon meat and OXA for other product types) and incubated at 35 ± 2 °C for 24 - 48 h. If suspected colonies on the plate were confirmed to be *L. monocytogenes* (see below), the corresponding MPN tube was deemed positive. The MPN pattern for the sample was determined from the number of positive tubes yielding confirmed *L. monocytogenes* colonies.

The MPN method resulted in direct estimates of *L. monocytogenes* levels in the range of 0.3-110 MPN/g. For some samples, a value of <0.3 was also obtained through further

calculation, where a 10-tube MPN pattern (including the screening step as one tube with 25-g test portion) was used in MPN determination (Chen et al. 2003).

Direct plating. All positive samples from both MD and CA were tested by direct plating as follows. The 250-ml sample homogenate that was used for MPN enumeration was also used for direct plating. A volume of 0.2-ml homogenate was evenly spread onto each of five agar plates (MOX for luncheon meat samples and OXA for other samples). The total number of colonies on the five plates represented a 0.1-g test portion. The homogenate was also diluted 10-fold, and 0.1 ml of the dilution was plated in duplicate. The average of the counts on these two plates represented a 0.001-g test portion. The plates were incubated at 35 ± 2 °C for up to 48 h and examined for suspected *L. monocytogenes* colonies.

Up to 20 different individual colonies were subjected to confirmation analysis. Ten suspected colonies from each of the 0.001-g plates, or a total of twenty colonies from the five 0.1-g plates were individually picked and point transferred onto a section of a horse blood agar plate. After incubation at 35 ± 2 °C for 19 ± 3 h, the blood plates were examined for the presence of β -hemolytic colonies. Up to three clearly isolated β -hemolytic colonies were subjected to confirmation analysis. If a colony was confirmed as *L. monocytogenes*, all the β -hemolytic colonies were considered *L. monocytogenes*. The ratio of β -hemolytic colonies to the total number of colonies picked (up to 20) was calculated, and used to determine the count (cfu/g) for the plate. The concentration of *L. monocytogenes* in a sample was determined by multiplying the ratio by the total count. In a few of the positive samples, the MPN and direct count methods provided different results. In those cases, the larger number was used in further calculations.

Confirmation of *L. monocytogenes*

Agar plates obtained at the direct-plating, MPN, or screening step, were examined at 24 h and then at 48 h for suspected *L. monocytogenes* colonies (on a MOX agar plate, distinctive 1-2 mm round colonies surrounded by darkened zones of esculin hydrolysis; on an OXA plate, distinctive 1-2 mm round colonies surrounded by a black halo). If suspected colonies were present on a plate obtained from the MPN step or screening step, up to 20 colonies were picked (by running a loop through them), and streaked onto a horse blood agar plate. Suspected colonies on a plate from the direct plating step were individually picked and point transferred onto a horse blood plate. The plate was incubated at 35 ± 2 °C for 19 ± 3 h and examined for the presence of translucent colonies surrounded by a small zone of β -hemolysis. When necessary, colonies from the horse blood plate were re-streaked onto a second horse blood plate to obtain isolated colonies. A clearly isolated β -hemolytic colony, if present, was subjected to further biochemical confirmation. If it was confirmed as *L. monocytogenes*, all the β -hemolytic colonies were considered *L. monocytogenes*. If it was confirmed not to be *L. monocytogenes*, up to two more β -hemolytic colonies were subjected to biochemical confirmation. If all three colonies were confirmed as not to be *L. monocytogenes*, none of the β -hemolytic colonies was considered to be *L. monocytogenes*.

Biochemical confirmation was carried out with the use of the API *Listeria* ID strip (bioMérieux, Inc., Hazelwood, MO) or the Micro ID *Listeria* kit (Organon Teknica Corp., Durham, NC), according to the manufacturer's procedures. CA samples collected prior to October 2000 were confirmed by the Micro ID method, and all other samples were confirmed by the API method.

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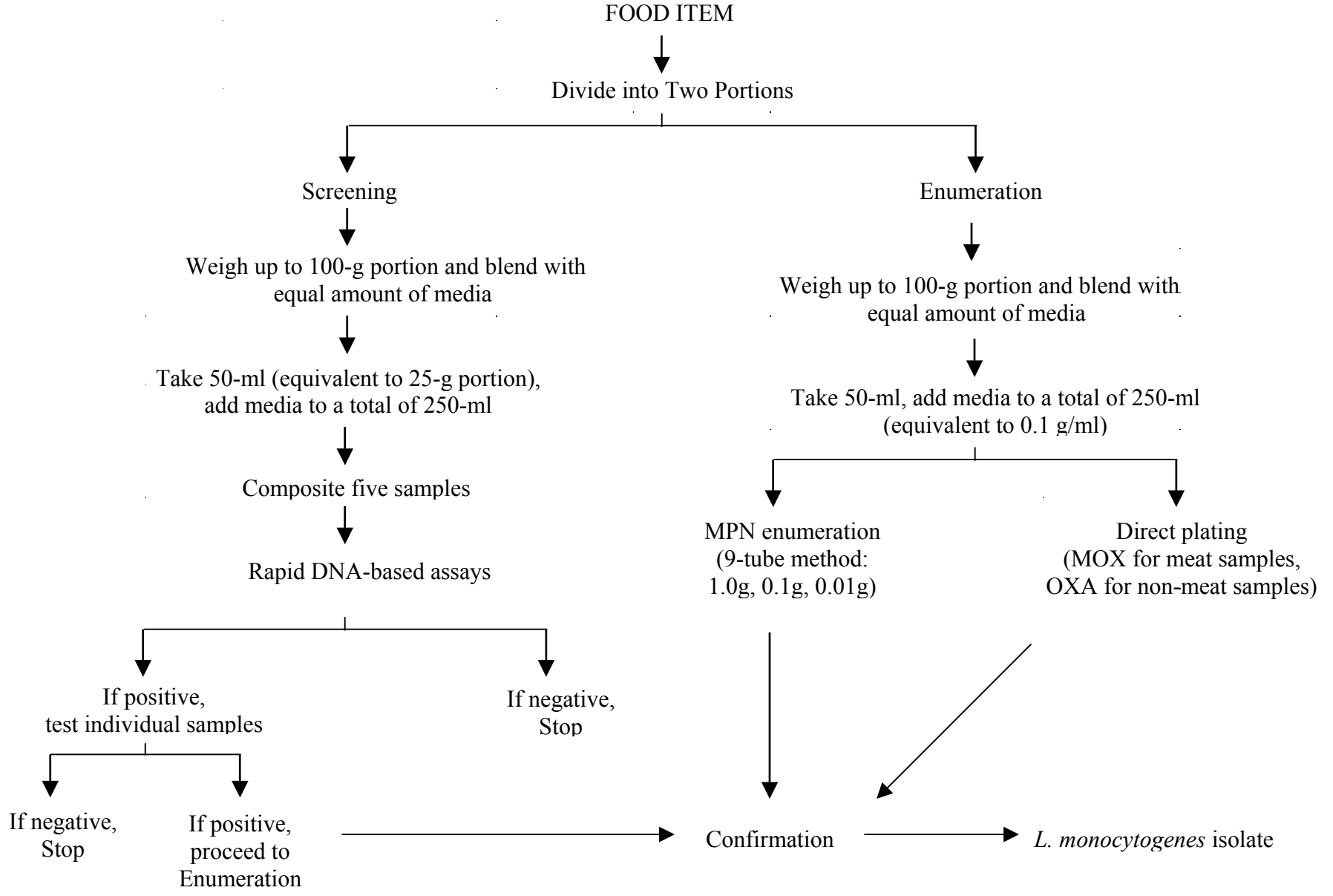


Figure 1 Detection and enumeration of *L. monocytogenes* in ready-to-eat food samples