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An Automated Fiber-optic Biosensor Based Binding Inhibition Assay for the Detection of *Listeria Monocytogenes*

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Abstract Conventional methods for pathogen detection and identification are labor-intensive and take days to complete. Biosensors have shown great potential for the rapid detection of foodborne pathogens. Fiber-optic biosensors have been used to rapidly detect pathogens because they can be very sensitive and are simple to operate. However, many fiber-optic biosensors rely on manual sensor handling and the sandwich assay, which require more effort and are less sensitive. To increase the simplicity of operation and detection sensitivity, a binding inhibition assay method for detecting *Listeria monocytogenes* in food samples was developed using an automated, fiber-optic-based immunosensor: RAPTOR (Research International, Monroe, WA, USA). For the assay, fiber-optic biosensors were developed by the immobilization of *Listeria* antibodies on polystyrene fiber waveguides through a biotin-avidin reaction. Developed fiber-optic biosensors were incorporated into the RAPTOR to evaluate the detection of *L. monocytogenes* in frankfurter samples. The binding inhibition method combined with RAPTOR was sensitive enough to detect *L. monocytogenes* (5.4×10^7 CFU/mL) in a frankfurter sample.

Keywords: *Listeria monocytogenes*, biosensor, fiber-optic, fluorescence, immunosensor

Introduction

Listeria monocytogenes is one of the major foodborne pathogens and current food safety regulatory policy maintains a 'zero tolerance' in ready-to-eat (RTE) foods. It is a Gram-positive, rod-shaped intracellular pathogen that causes listeriosis in the elderly, those with weakened immune systems, and pregnant women (1). Recent *L. monocytogenes*-related outbreaks from various food sources have increased public awareness of this pathogen. The greatest threat of listeriosis is from RTE products that do not require further cooking at home. A recent risk assessment estimated the risks of serious illness and death associated with consumption of RTE foods possibly contaminated with *L. monocytogenes* (2, 3). The results included a list of 23 food categories of seafood, produce, dairy, and meat which were classified as very high risk (>100 cases per year), high risk, moderate risk, and low risk (<1 case per year). The very high and high risk categories included: deli meats, pasteurized fluid milk, high fat and other dairy products, and frankfurters that are not reheated. The Healthy People 2010 goals for national health promotion and disease prevention called on federal food safety agencies to reduce foodborne listeriosis by 50% by the end of the year 2005.

Conventional methods for *Listeria* detection and identification involve prolonged, multiple enrichment steps (4). Even though some rapid immunological assays are available, these assays still require enrichment steps and give results in 18-48 hr. Biosensors have shown great potential for the rapid detection of foodborne pathogens. Biosensors use a combination of biological recognition elements and electrical or chemical transducers to produce great specificity and sensitivity in the rapid detection of low levels of biomolecules. Among the biosensors, fiber-optic biosensors provide specific, reproducible, and reliable detection of various biomolecules. Fiber-optic biosensors use light transmittable tapered fibers to send excitation laser light and receive emitted fluorescence, usually from a fluorophore-labeled antibody. The fluorescent light excited by an evanescent wave generated by the laser is quantitatively related to the number of labeled biomolecules in close proximity to the fiber surface (5). These biosensors have been used to rapidly detect various microorganisms including: *Vaccinia* virus (6), *Escherichia coli* O157:H7 (7), *Bacillus globigii* (8), *Salmonella enteritidis* (9), and *L. monocytogenes* (10, 11). Inconvenience due to the manual handling of these biosensors necessitates improvements in the portability and automation of the fiber-optic biosensor. The recently commercialized automated fiber-optic biosensor system (RAPTOR™, Research International, Monroe, WA, USA) has increased the usefulness of this detection device.

The automated RAPTOR system can perform four assays on the same sample allowing replicate measurements of the same analyte or simultaneous detection of four different targets. The RAPTOR uses four 635 nm diodes to excite each of four, 4.5 cm long fiber-optic probes. The fibers are assembled in a coupon which has fluidic channels for automated operation. Fluorescent molecules bound on the surface of the sensing region are excited by an evanescent wave generated by the laser. Photodiodes collect emitted light at wavelengths over 670 nm. The emission signal is recorded in picoamperes (pA) and is related to the concentration of analyte. The automated RAPTOR system has been used to detect *Salmonella*

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typhimurium (12) and *Giardia lamblia* (13). However, few such studies have been conducted to detect *L. monocytogenes* in food samples. Furthermore, many fiber-optic biosensors rely on the sandwich assay, which uses secondary antibodies and may be less sensitive.

In this study, a binding inhibition assay for detecting *L. monocytogenes* using the RAPTOR system was devised to increase the simplicity and sensitivity of detection. To evaluate the assay method, fiber-optic biosensors were developed by the immobilization of *Listeria* antibodies on polystyrene fiber waveguides through a biotin-avidin reaction. Developed fiber-optic biosensors were incorporated into the RAPTOR to detect the presence of *L. monocytogenes* in frankfurter samples.

Materials and Methods

Bacteria and media *L. monocytogenes* strain V7, a milk isolate from our collection, was used for all experiments. The bacteria were maintained on brain heart infusion (BHI) agar (1.5%) slants (Difco Laboratories, Detroit, MI, USA) at 25°C for the duration of this study. Fresh cultures of *L. monocytogenes* were prepared by incubating the slant cultures in BHI broth at 37°C for 16 hr. In some cases, cultures were adjusted to approximately the same concentration using a spectrophotometer (Beckman-Coulter, Fullerton, CA, USA).

Buffered *Listeria* enrichment broth (BLEB) and Oxford *Listeria* agar base were purchased from Acumedia (Baltimore, MD, USA). Modified oxford antimicrobic supplement was purchased from Becton, Dickinson (Sparks, MD, USA). Packaged frankfurters were purchased from a local grocery store.

Frankfurter sample preparation *L. monocytogenes* cells were inoculated in 5 mL of BHI broth and incubated at 37°C with shaking (150 rpm). After 16 hr of culture, which was the time necessary for cell numbers to reach approximately $1\text{-}2\times10^9$ CFU/mL, the cells were diluted to the desired concentration with 20 mM phosphate-buffered saline (PBS, pH 7.2), and 10 g of each frankfurter sample was spiked by dropping 100 mL of the cell suspension onto the sample surface. To ensure absorption of the fluid, the spiked frankfurter samples were air-dried for 20 min at room temperature and then placed in a sterilized enrichment container filled with 30 mL of buffered *Listeria* enrichment broth (BLEB) medium for enrichment. The containers with frankfurter samples were further incubated at 37°C for 20 hr with shaking (150 rpm). As a negative control, containers with frankfurter samples were incubated without prior inoculation of *L. monocytogenes*. The enriched cell suspensions were collected after filtration to remove any remaining food particles and used for the immunoassay. Enumeration of the enriched *L. monocytogenes* population was performed using the modified oxford (MOX) plate method (14).

After 20 hr of incubation, the numbers of enriched *L. monocytogenes* cells were counted using a MOX plate. There was a one-log difference between cultures inoculated with 10 and 100 CFU/g after 20 hr. The cell concentrations were 5.4×10^7 (pH 6.85) and 6.2×10^8 CFU/mL (pH 6.78) for the cultures inoculated with 10 and 100 CFU/g, respectively.

Regeants and antibodies Purified anti-*Listeria* monoclonal antibody (C11E9) (15) was provided by Dr. Arun Bhunia (Purdue University, IN, USA). This antibody was prepared according to the method described by Geng *et al.* (11). Biotinylated anti-mouse IgG for the binding inhibition assay was purchased from AnaSpec (San Jose, CA, USA). Casein, bovine serum albumin (BSA), and phosphate buffer were purchased from Sigma (St. Louis, MO, USA). SuperBlock was purchased from Pierce (Rockford, IL, USA).

Antibody labeling An antibody labeling kit (Cy5-Ab labeling kit; Amersham Biosciences, Piscataway, NJ, USA) was used for labeling MAb C11E9 according to the manufacturer's instructions. Briefly, purified antibody was first subjected to ion exchange ranging from 0.1 M glycine-HCl (pH 2.7) to 0.1 M carbonate-bicarbonate buffer (pH 9.3) using a desalting column (Amersham Biosciences). Second, 2 mL of antibody (1 mg/mL) was added to a dye vial wrapped with aluminum foil and incubated at room temperature for 30 min with mixing approximately every 10 min. The free dye was then removed on a gel filtration column provided by the labeling kit.

Cy5-and biotin-labeled antibodies were stored in phosphate-buffered saline (PBS) containing bovine serum albumin (1 mg/mL; Sigma) at 4°C until used.

Fiber preparation and instrument setup Fibers were prepared to have active binding sites for Cy5-labeled MAb detection as shown in Fig. 1. A fiber was inserted into a 100 μL pipette tip with the dispensing end sealed with a sealant and incubated overnight (18-22 hr) at 4°C with 90 μL of 100 μg/mL streptavidin. Fibers were rinsed with PBS-Triton (0.02 M PBS containing 0.05% Triton X-100) and incubated with 90 μL of 100 μg/mL biotinylated capture antibody (anti-mouse IgG) in PBS at room temperature for 1 hr in a pipette tip. Finally, the fibers were rinsed with PBS-Triton again and incubated with 90 μL of SuperBlock followed by 90 μL of 1 mg/mL biotinylated bovine serum albumin (bBSA) at room temperature for 1 hr each to block non-specific binding sites.

After the immobilization of capture antibody onto the

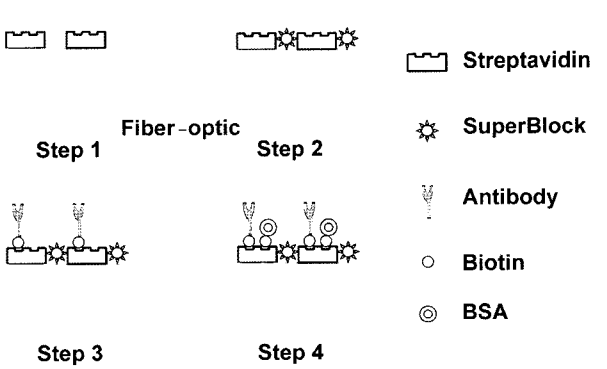

Fig. 1. Immobilization procedure for the fiber-optic biosensor.

fiber, the fibers were mounted into a disposable coupon according to the manufacturer's procedures (RAPTOR™). The fiber was inserted through the mounting hole in the edge of the coupon and optically cured adhesive was applied at the mounting hole edge. The coupon was then put under an ultraviolet (UV) light for 40 min to cure the glue. Once the fibers had been glued into the coupon, the coupon cover tape was applied to form the last surface of the fluidics channels.

An assembled coupon was inserted into the RAPTOR and measurements were done automatically by running a preprogrammed baseline recipe for each baseline reading and an assay recipe for each sample reading. The baseline recipe involved first incubating the fibers with internal washing buffer (PBS-Triton) for 5 min. The emission signal was then recorded for 6 sec. The assay recipe consisted of incubating the fibers with a 0.9 mL sample for 8 min, and rinsing three times with PBS-Triton. The excitation laser was then turned on, and the emission signal recorded for 10 sec.

Blocking of non-specific binding Three different blocking methods were evaluated for reducing the non-specific binding of fluorophor-labeled detection antibody to the fiber/capture-antibody-surface. The first blocking method involved incubation with biotinylated bBSA only (Pierce, Rockford, IL, USA) immediately after immobilizing the capture antibody to block any unoccupied streptavidin not linked to biotinylated capture antibody. The other two blocking methods included additional incubation with superblock or BSA followed by an additional incubation with bBSA. All blocking reagent incubations were done at room temperature for 1 hr with 90 μL of 1 mg/mL blocking reagents.

To further reduce non-specific binding of fluorophor-labeled detection antibody to the fiber/capture-antibody-surface, a modified sample buffer was evaluated. The sample buffer modification included the addition of BSA and casein to the sample buffer prior to injection into the coupon. The modified sample buffer contained 2 mg/mL blocker BSA and 2 mg/mL casein. For the sample buffer, BSA and casein were added to 0.02 M PBS (pH 7.4) or directly to the frankfurter sample extract. All modified buffer tests were performed with fibers blocked using SuperBlock followed by bBSA.

The amount of non-specific binding with each method was determined by the iterative measurement of samples that did not contain bacteria. When non-specific binding of the detection antibody occurs, the fluorescence signal continues to increase for each subsequent assay with the same fiber.

Binding inhibition assay For the binding inhibition assay, capture antibodies specific for the *Listeria* detection antibodies were immobilized on the fiber. As a capture antibody, commercial anti-mouse IgG antibodies were immobilized onto the fibers to capture *Listeria* MAb C11E9.

The binding inhibition assay included incubating samples with 10 μg/mL of dye conjugated detection antibody Mab C11E9 for 30 min at room temperature. After the incubation, the sample was centrifuged and the supernatant containing the remaining C11E9-Cy5 was injected into the coupon.

Signal resulting from anti-*Listeria* detection antibody binding to capture antibody on the fiber surface was measured by running two measurement-recipes sequentially. A background signal was measured first using the baseline measurement recipe which uses PBS-Triton as a sample. After the baseline measurement, the assay recipe was loaded and signals were measured for each test sample.

With the same coupon, consecutive measurements were performed using serially diluted bacteria samples (10^9-10^2 CFU/mL) in PBS or prepared food samples.

Figure 2 shows the binding inhibition assay procedure. Optimum detection of antibody concentration for the assay

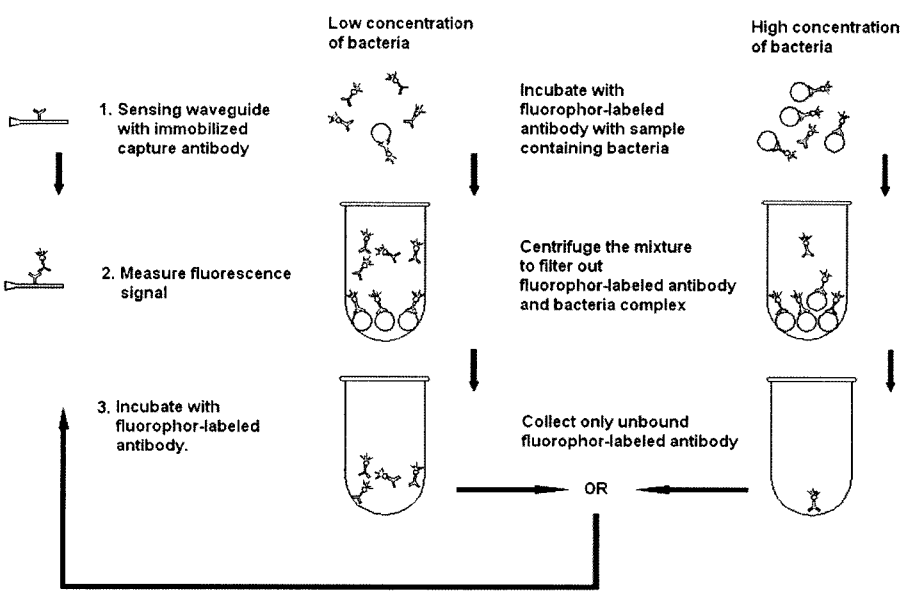

Fig. 2. Binding inhibition assay procedure.

was determined by serially diluting the detection antibody.

Data analysis For the binding inhibition assay, the signal difference between two consecutive assay signals was used to represent the data. The detection limit of the binding inhibition assay was calculated by using 4 last control signals taken immediately after the sample measurement. The limit of detection was designated as three times the standard deviation of the three control signals minus the background signal. A result was considered positive if the signal difference was higher than the limit of detection.

For each experiment, the standard deviation of the mean (SEM) signals from 4 fibers within a coupon was calculated. The error bars on each graph designate ± SEM.

Results and Discussion

Blocking non-specific binding Among the three immobilization methods used, SuperBlock with bBSA showed the least non-specific binding (Fig. 3). Even though the SuperBlock with bBSA decreased non-specific binding, it did not effectively prevent non-specific binding enough to acquire meaningful results from the binding of bacteria.

The use of modified buffers with SuperBlock immobilization drastically reduced non-specific binding. The addition of blocking agents to the sample buffer stabilized the background signal. Bars in the figures show the differences between two consecutive assay signals (Fig. 4). Other researchers (12) have also made several background signal measurements to compensate for continuous signal increase resulting from non-specific binding.

Binding inhibition assay By analyzing data acquired from serially diluted C11E9-Cy5, a concentration range between 1 and 10 μg/mL produced the highest signal change (Fig. 5). Therefore, for the binding inhibition assay 10 μg/mL of C11E9-Cy5 was incubated with each sample.

The responses of the biosensor to increasing concentrations of *L. monocytogenes* in PBS are shown in Fig. 6. Higher concentrations of *L. monocytogenes* in PBS produced higher signal differences between two consecutive

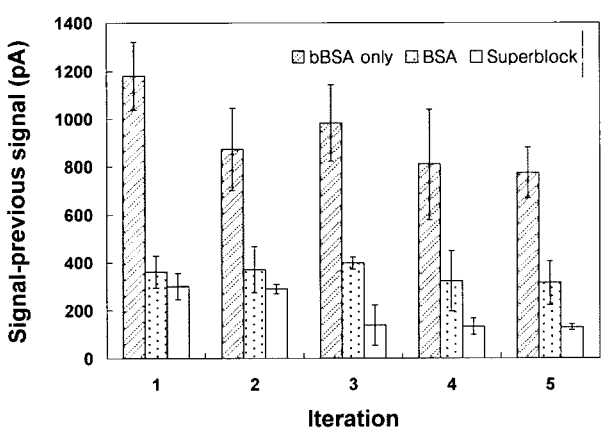

Fig. 3. Non-specific binding of fluorophor-labeled antibodies on the surface of the biosensor for each immobilization method.

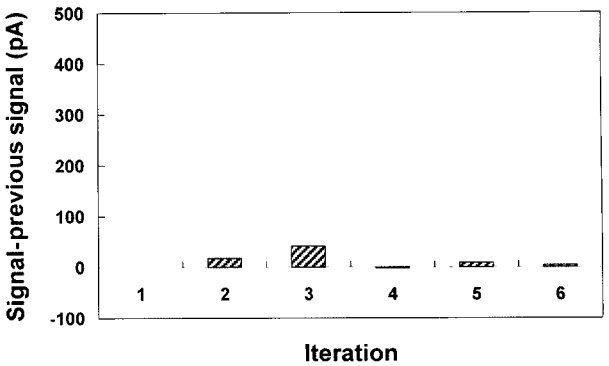

Fig. 4. Non-specific binding of modified detection antibody buffer and sample buffer containing 2 mg/mL blocker BSA and 2 mg/mL casein.

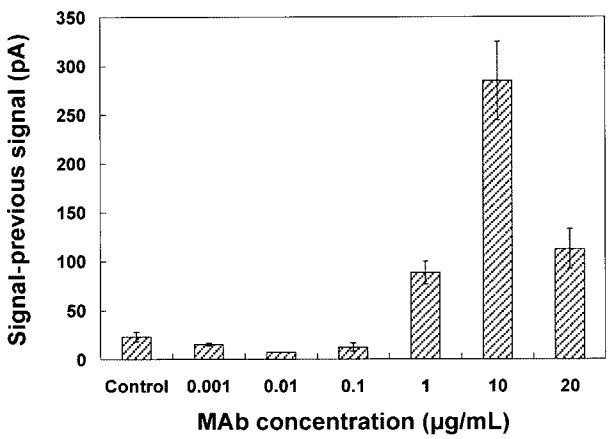

Fig. 5. Concentration response curve for detection antibody C11E9-Cy5. Serially diluted C11E9-Cy5 was introduced into the coupon to determine the optimum concentration of the detection antibody for the binding inhibition method.

assay signals. This method showed the detection of 1×10^7 CFU/mL of *L. monocytogenes* in PBS samples.

In our previous research (unpublished), a sandwich assay in which the bacteria bind to a primary antibody immobilized on the surface of a biosensor produced better detection results (1×10^4 CFU/mL) for samples in PBS. However, the sandwich assay produced false negative detection of *L. monocytogenes* in real food samples.

A possible reason of this false negative detection of *L. monocytogenes* comes from the interference of antigen-antibody binding due to the complex nature of food (16). Other competitive microorganisms or the food ingredients themselves could interfere with the signal measurements. Furthermore, detection could be affected by the stressful environment within the food which might alter the physiology and metabolism of microorganisms thus interfering with antibody binding (11). This complexity requires a more sensitive and robust method to detect *L. monocytogenes* in food samples.

In order to test the detection of *L. monocytogenes* in food, frankfurter samples were prepared as described above. Frankfurter samples containing serially diluted C11E9-Cy5 excluded from antigen and antibody detection

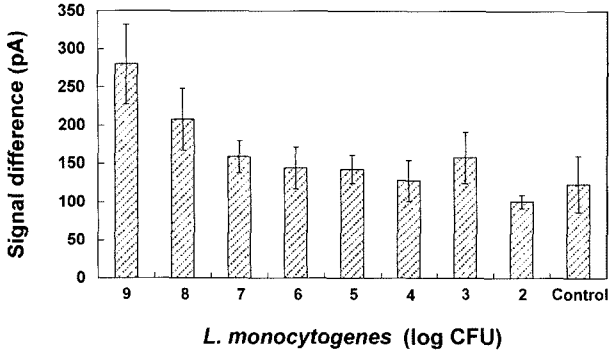

Fig. 6. Biosensor response to different concentrations of *L. monocytogenes*.

reactions were introduced into the coupon.

The binding inhibition assay was successful at detecting both high (6.2×10^8 CFU/mL) and low (5.4×10^7 CFU/mL) concentrations of *L. monocytogenes* in frankfurter samples (Fig. 7).

This increased sensitivity seen with prior incubation of C11E9-Cy5 with antigen can be explained partly by the principle of evanescent wave biosensors. Theoretically, the evanescent wave that excites the dye to produce fluorescent light penetrates into the lower refractive index sample medium with an exponential decay. The depth at which the intensity of the wave drops to 1/e of its original value is known as the depth of penetration. The depth of penetration depends on the incident angle, the refractive indices of the fiber and sample medium, and the wavelength of the excitation light. Usually, the penetration depth reaches less than 300 nm (17). Because the penetration depth of the evanescent wave covers a small fraction of the cells, with an average size of 1-2 μm, only the C11E9-Cy5 within this depth can be excited to produce the signal. Incubating the C11E9-Cy5 with antigen prior to the assay and introducing only the remaining detection antibodies might increase the sensitivity of the biosensor. Since the typical size of antibodies is less than 100 nm, detection antibodies bound to the capture antibodies immobilized on the fiber should be fully excited by the evanescent wave.

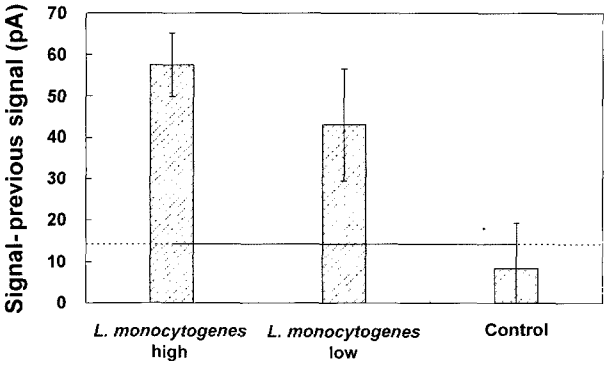

Fig. 7. Detection of *L. monocytogenes* in frankfurter matrix with the binding inhibition method. The line indicates the detection limit (14.3 pA). Cell numbers determined by MOX were 6.2×10^8 and 5.4×10^7 CFU/mL for the high and low concentrations, respectively.

Tims *et al.* (10), who also used a fiber-optic biosensor to detect pure cultures of *L. monocytogenes*, reported a detection limit of 4.1×10^8 CFU/mL. They used the sandwich assay method and the detection limit of their biosensor was lower than the biosensor used in this study. The binding inhibition assay method used with the automated fiber-optic biosensor lowered the detection limit, however enrichment steps are still needed to reach the detection limit. To reduce the enrichment times, the sensitivity of the biosensor should be improved by means of enhancing the detection methods along with improving the quality of the antibodies.

In this study, a method for detecting *L. monocytogenes* in food samples was developed using an automated fiber-optic-based immunosensor: RAPTOR. A binding inhibition method was devised and evaluated to detect *L. monocytogenes* in PBS and frankfurter samples. The binding inhibition method, which was devised to overcome the low penetration depth of evanescent waves, could detect 10^7 CFU/mL of *L. monocytogenes* in PBS. Also the binding inhibition assay method could detect 5.4×10^7 CFU/mL of *L. monocytogenes* in frankfurter samples.

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