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Detection of Low Levels of *Listeria monocytogenes* Cells by Using a Fiber-Optic Immunosensor

Tao Geng,[1] Mark T. Morgan,[2] and Arun K. Bhunia[1]*

Molecular Food Microbiology Laboratory[1] and Sensors and Controls Laboratory,[2] Department of Food Science, Purdue University, West Lafayette, Indiana

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Biosensor technology has a great potential to meet the need for sensitive and nearly real-time microbial detection from foods. An antibody-based fiber-optic biosensor to detect low levels of *Listeria monocytogenes* cells following an enrichment step was developed. The principle of the sensor is a sandwich immunoassay where a rabbit polyclonal antibody was first immobilized on polystyrene fiber waveguides through a biotin-streptavidin reaction to capture *Listeria* cells on the fiber. Capture of cells on the fibers was confirmed by scanning electron microscopy. A cyanine 5-labeled murine monoclonal antibody, C11E9, was used to generate a specific fluorescent signal, which was acquired by launching a 635-nm laser light from an Analyte 2000 and collected by a photodetector at 670 to 710 nm. This immunosensor was specific for *L. monocytogenes* and showed a significantly higher signal strength than for other *Listeria* species or other microorganisms, including *Escherichia coli*, *Enterococcus faecalis*, *Salmonella enterica*, *Lactobacillus plantarum*, *Carnobacterium gallinarum*, *Hafnia alvei*, *Corynebacterium glutamicum*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, and *Serratia marcescens*, in pure or in mixed-culture setup. Fiber-optic results could be obtained within 2.5 h of sampling. The sensitivity threshold was about 4.3×10^3 CFU/ml for a pure culture of *L. monocytogenes* grown at 37°C. When *L. monocytogenes* was mixed with lactic acid bacteria or grown at 10°C with 3.5% NaCl, the detection threshold was 4.1×10^4 or 2.8×10^7 CFU/ml, respectively. In less than 24 h, this method could detect *L. monocytogenes* in hot dog or bologna naturally contaminated or artificially inoculated with 10 to 1,000 CFU/g after enrichment in buffered *Listeria* enrichment broth.

Listeria monocytogenes is a gram-positive, rod-shaped foodborne pathogen that causes listeriosis particularly in immunocompromised populations and abortion in pregnant women (9, 27, 32). The recent well-publicized outbreaks and food recalls due to *L. monocytogenes* (9, 23, 31, 39) have increased the need for more rapid, sensitive, and specific methods for detection of this bacterium in foods.

Conventional methods for the detection and identification of bacteria in food are greatly restricted by prolonged assay times (up to 7 days), requiring initial enrichment for detection of pathogens that are initially present in low numbers. Immunological assays with antibodies provide specific, reproducible, and reliable detection of bacteria, viruses, or toxins. Even though antibody-based detection may greatly reduce the assay time compared to traditional culture techniques, it still lacks the ability to detect biomolecules in real time. Biosensors use a combination of biological receptors and physical or chemical transducers, which represent a new and unique technology with great potential to meet the need for the rapid detection of low levels of biomolecules (5, 14). Fiber-optic biosensors exploit the measurement of fluorescent light excited by an evanescent wave generated by a laser to quantitatively detect biomolecules immobilized on the fiber surface (1, 24, 26). A portable sensor (Analyte 2000; Research International, Woodinville, Wash.) has been developed by using the above principle. The assay principle is based on a sandwich immunoassay, using a capture antibody, immobilized onto the optical fibers, and a cyanine 5 (Cy5)-labeled antibody for detection (24). The Analyte 2000 uses a 635-nm laser diode as an excitation light that is launched into the proximal end of an optical fiber. The Cy5 fluorescent molecules within several hundred nanometers of the fiber are excited by an evanescent field, and a portion of their emission energy reemits into the fiber. A photodiode allows for quantization of the collected emission light at wavelengths of 670 to 710 nm (1). This assay has been used to detect 2,4,6,-trinitrotoluene and hexahydro-1,3,5-trinitro-1,3,5-triazine (2), staphylococcal enterotoxin B, (36), *Clostridium botulinum* toxin (29), polymyxin B (15), *Salmonella enterica* serovar Typhimurium (40), *Escherichia coli* O157:H7 (7, 8), and PCR products of *Listeria* spp. (35). Recently, Tims et al. (37) used this assay to detect pure cultures of *L. monocytogenes*, reported a detection limit of 4.1×10^8 CFU/ml, and concluded that the quality of antibodies is key in improving sensitivity. The feasibility of their assay to differentially detect *L. monocytogenes* in the presence of other *Listeria* spp. or other common food-contaminating microorganisms was not tested. Also, no food samples were tested in their study (37).

Sensitivity and specificity of antibodies are critical for immunodetection of biomolecules in antibody-based biosensors. In our project, we attempted to increase the sensitivity and specificity of the fiber-optic biosensor by using two different antibodies. A polyclonal antibody (PAb) developed against whole cells of *L. monocytogenes* in the rabbit was used as a capture antibody, while a Cy5-labeled murine monoclonal antibody (MAb), C11E9 (4), which reacts with *L. monocytogenes* and some selected strains of *Listeria innocua* (4, 20), was used for detection. Sensitivity and specificity of the sensor were

* Corresponding author. Mailing address: Molecular Food Microbiology Laboratory, Department of Food Science, Purdue University, 745 Agriculture Mall Dr., West Lafayette, IN 47906-2009. Phone: (765) 494-5443. Fax: (765) 494-7953. E-mail: bhunia@purdue.edu.

further evaluated by testing with other microorganisms in a mixed-culture setup. Finally, the ability of this sensor to detect *L. monocytogenes* cells from artificially inoculated (10 to 1,000 CFU/g) and naturally contaminated ready-to-eat (RTE) hot dog or bologna samples following enrichment was tested.

MATERIALS AND METHODS

Bacteria. *Listeria monocytogenes* strain V7, a milk isolate (4), *Listeria ivanovii* ATCC 19119, *Listeria grayi* ATCC 19120, *Listeria seeligeri* SE 31, *Listeria welshimeri* ATCC 35897, *L. innocua* strains F4248 and LA-1, *Citrobacter freundii* ATCC 3624, *E. coli* ATCC 52739, *Enterococcus faecalis* ATCC 344, *Salmonella enterica* serovar Typhimurium, *Salmonella enterica* serovar Enteritidis, *Bacillus cereus* 4AC, *Lactobacillus plantarum* NCDO 955, *Hafnia alvei*, *Corynebacterium glutamicum* ATCC 31834, *Enterobacter aerogenes*, *Pseudomonas aeruginosa* ATCC 10145, *Carnobacterium gallinarum* ATCC 49517, and *Serratia marcescens* ATCC 14756 from our collection were maintained on brain heart infusion (BHI) agar (1.5%) slants (Difco Laboratories) at 25°C for the duration of this study. All fresh cultures for experiments were obtained by inoculating a loop of slant cultures into BHI broth and incubating them at 37°C for 16 h. In some cases, bacteria were adjusted to approximately the same concentration by using a spectrophotometer (Beckman-Coulter, Fullerton, Calif.) (12).

Preparation of polyclonal (capture) and monoclonal (detection) antibodies. Anti-*Listeria* PAb was used as a capture antibody and was prepared according to the method described by Harlow and Lane (13). Briefly, freshly cultured *L. monocytogenes* cells (final concentration, approximately 1×10^9 CFU/ml) were inactivated at 70°C for 15 min and were subcutaneously injected at 2-week intervals for 3 months into a New Zealand White rabbit (Harlan Sprague-Dawley, Indianapolis, Ind.). The rabbit was maintained in the Purdue Biology Animal Facility (West Lafayette, Ind.). The serum from the third bleeding, showing very high antibody titer (1:10,000), was partially purified by ammonium sulfate precipitation (13). A Protein A column (ActaPrime; Pharmacia-Amersham, Uppsala, Sweden) was used for affinity purification of antibodies, and the final concentration was adjusted to 2.0 mg/ml. The reaction characteristics of this antibody to different bacterial cultures were determined by an indirect enzyme-linked immunosorbent assay (ELISA) (12, 20).

MAb C11E9 was used as a detection antibody. Frozen, stored hybridoma cell line C11E9 (4) was grown in cell culture medium in a cultivation chamber of CELLine 1000 (Integra Biosciences, East Dundee, Ill.) as previously described (12). The antibody from the supernatant was partially purified by ammonium sulfate precipitation (13), followed by the use of a Protein G column (Acta Prime) for affinity purification, and the final concentration was adjusted to 1.4 mg/ml.

Antibody labeling. An antibody labeling kit (Cy5-Ab labeling kit; Amersham Biosciences, Piscataway, N.J.) was used for labeling MAb C11E9 according to the manufacturer's instructions and the suggestions of Kada et al. (18). Briefly, purified antibody was first ion exchanged from 0.1 M glycine-HCl (pH 2.7) to 0.1 M carbonate-bicarbonate buffer (pH 9.3) by 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. Then, free dye was removed by a gel filtration column provided by the labeling kit. The final concentration of labeled antibody, measured by a DU-640 spectrophotometer (Beckman-Coulter), was 0.7 mg/ml, and the final molecular ratio of dye to antibody was estimated to be 2:1.

A long-chain biotin (EZ-Link NHS-LC-Biotin; Pierce, Rockford, Ill.) was used for biotinylation of the polyclonal capture antibody according to the manufacturer's instructions. One milligram of biotin was dissolved in 1 ml of dimethyl sulfoxide, and 75 μl of this solution was added to 1 mg of the antibody in 1 ml of carbonate-bicarbonate solution (5.7 g of $NaHCO_3$, 3.4 g of Na_2CO_3 in 1 liter of water, pH 9.3). The solution was then incubated in an ice bucket for 2 h. Free biotin was removed by column chromatography (PD-10; Amersham Biosciences), and the final biotinylated antibody was estimated to be 0.7 mg/ml. 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.

Western blotting. *L. monocytogenes* cells grown in BHI broth at 37°C for 16 to 18 h were harvested by centrifugation (6,000 × *g*, 10 min) and washed three times with 20 mM PBS, pH 7.4, and cell concentration was adjusted to an A_{595} of 0.93. The washed cell pellets were resuspended with sample solvent buffer containing sodium dodecyl sulfate buffer (5%), β-mercaptoethanol (0.5%), and Tris (1.5%), pH 6.8, and incubated at 37°C for 60 min (4). After centrifugation (10,000 × *g*, 10 min), the cellular protein extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5% polyacrylamide), transferred to Immobilon-P membranes (Millipore, Bedford, Mass.) and immunoprobed with MAb C11E9 (1:400) or anti-*Listeria* PAb (1:1,000). Membranes were developed with horseradish peroxidase-conjugated goat anti-mouse (for MAb C11E9) or anti-rabbit (for PAb) antibody (1:20,000) utilizing diamino benzidine tetrahydrochloride (Sigma) containing H_2O_2 as a substrate (6).

Determination of optimal concentration of detection antibody required for detection of *L. monocytogenes*. *L. monocytogenes* V7 cells, with serial dilutions from 10^9 CFU/ml to 10^2 CFU/ml in 1 ml of PBS, were centrifuged, and the supernatants were discarded. The pellets of each concentration were incubated with 200 μl of 500-, 200-, 100-, or 50-μg/ml Cy5-labeled C11E9 for 1 h at 37°C. Then the cells were centrifuged, washed with PBS three times, and resuspended in 200 μl of PBS. Finally, cell suspensions were added into a 96-well black microtiter plate (Costar, Corning, N.Y.) and measured by a spectrofluorometer (Molecular Devices, Sunnyvale, Calif.) at excitation and emission wavelengths of 650 and 667 nm, respectively.

Fiber preparation. The fibers were prepared according to the method described by Tims et al. (37) with modifications. The polystyrene fibers (4 cm in length and 0.78 mm in diameter) (Research International) were precleaned with 50% isopropanol and air-dried under a biosafety cabinet with laminar flow of air. Fibers were inserted into glass capillary tubes (VWR, Willard, Ohio) to form a reaction chamber and were incubated overnight at 4°C with 80 μl of 0.1-mg/ml streptavidin (Promega, Madison, Wis.). The fibers were rinsed with PBS containing 0.05% Triton X-100 (PBS-Triton) and then incubated with 80 μl of biotinylated capture antibody (0.1 mg/ml) at room temperature for 1 h in capillary tubes.

Blocking and background reading. Fibers were first rinsed with PBS-Triton and then incubated with 80 μl of 1-mg/ml biotinylated bovine serum albumin (Pierce) in capillary tubes at room temperature for 1 h to block nonspecific binding sites. PBS-Triton was used to rinse fibers before inserting them into the waveguide holder that was prefabricated with proximal and distal outlets. The waveguide holder was injected with 200 μl of PBS and then connected to the Analyte 2000 equipped with a 635-nm laser light source (Research International) for a final reading taken at a wavelength of 670 to 710 nm. This reading value, recorded in picoamperes (pA), was considered to be the background for each fiber.

Fiber-optic assays. Eighty microliters of bacteria in PBS was injected into capillary tubes and incubated with fibers at room temperature for 1 h. After rinses with PBS-Triton, fibers with captured bacteria were placed in a waveguide holder. Briefly, 200 μl of Cy5-labeled C11E9 (100 μg/ml) was injected into the holder through the proximal outlet, with the distal outlet blocked by a clip. Then, the fiber was inserted into the holder and incubated with the detection antibody for 252 s (on-site signals were read every 28 s for 10 readings). Consequently, 1 ml of PBS-Triton was injected into the holder by a syringe through the proximal outlet to wash out unbound antibody. Again, readings were taken every 28 s, and the values at the end of 252 s (10 readings) were considered final. The changes in signal due to the binding of bacteria were calculated as the final value minus the background reading. For each concentration of *L. monocytogenes* or each of the bacterial species, three to four fibers were used to generate average values and standard deviations.

Sensitivity and specificity of the biosensor. To determine the sensitivity (detection limit) of this sensor, fresh culture of *L. monocytogenes* was washed and serially diluted (from 4.3×10^9 to 4.3×10^2 CFU/ml) in sterile PBS, pH 7.4. Eighty microliters of each dilution was incubated with fibers separately, and signals were acquired as described above. The minimum concentration with a signal significantly ($P < 0.05$) greater than the signal for a negative control (0 CFU/ml) was considered to be the limit of detection. The ability of the biosensor to detect stressed *L. monocytogenes* cells (2.8×10^9 CFU/ml) grown at low temperature (10°C) in the presence of NaCl (3.5%) for 48 h was also determined. The cells were serially diluted (from 2.8×10^7 to 2.8×10^5 CFU/ml) in PBS, and signals were acquired as described above.

The specificity of the biosensor to discriminate *L. monocytogenes* from closely related *Listeria* species (*L. ivanovii*, *L. grayi*, *L. seeligeri*, *L. welshimeri*, and *L. innocua*) or competitive microflora (*Lactobacillus plantarum*, *Carnobacterium gallinarum*, *Serratia marcescens*, *H. alvei*, *Enterobacter aerogenes*, *P. aeruginosa*, and *Corynebacterium glutamicum*) and other bacterial cultures (*E. coli*, *Enterococcus faecalis*, and *S. enterica* serovar Typhimurium) was tested. Cultures were grown in BHI separately, washed with PBS, and adjusted to uniform concentrations (approximately 4×10^9 CFU/ml), and 80 μl of each culture was tested separately.

To determine the fiber-optic signal response for *L. monocytogenes* cells in the presence of other bacteria, two mixed-culture conditions were used. In the first one, *L. monocytogenes* (1.0×10^9 CFU/ml) was mixed in equal proportions with

E. coli, *Enterococcus faecalis*, and *S. enterica* serovar Typhimurium. For the control without *L. monocytogenes*, an equivalent volume of PBS was added. In the second one, *L. monocytogenes* (about 7.8×10^8 CFU/ml) was mixed with equal concentrations of other *Listeria* spp. listed above. In the control, *L. monocytogenes* or *L. innocua* was omitted.

The performance of the fiber-optic immunosensor in the presence of competitive microflora was evaluated. *L. monocytogenes* cells at 4.1×10^5, 4.1×10^4, and 4.1×10^3 CFU/ml were mixed separately with a constant number of *Lactobacillus plantarum* cells (2.6×10^7 CFU/ml) and analyzed. In addition, the same culture mixture was added to hot dog samples, enriched in buffered *Listeria* enrichment broth (BLEB) for 20 h, and analyzed. The fibers with PBS or *L. monocytogenes* cells only (4.1×10^9 CFU/ml) were used as controls.

Scanning electron microscopy. Binding patterns of *L. monocytogenes* to fibers coated with capture antibody (PAb) were analyzed by scanning electron microscopy (SEM). No detection antibody was used in this experiment. The fibers were prepared and blocked as described above and incubated with *L. monocytogenes* for 1 h. The control fibers were devoid of the capture antibody. The fibers were then washed in three changes of buffered 2% gluteraldehyde (0.025 M KH_2PO_4, 0.025 M Na_2HPO_4, pH 7.2) for 15 min per change, rinsed quickly with buffer, immersed in buffered osmium tetrachloride (1%) for 30 min, rinsed briefly with distilled water, dehydrated in a graded series of ethanol exchanges until reaching 100%, dried in a critical-point dryer (Ladd Research Industries, Burlington, Vt.), mounted on metal stubs with silver paste, and coated with a layer of gold (Hummer I Sputter Coater). Finally, three portions, the top, the middle, and the bottom, of the fibers were examined under an SEM (JSM 840; Jeol USA Inc., Peabody, Mass.) at 5 kV.

The number of cells captured on the surface of cylindrical fibers was calculated with two formulae. First, the surface area was calculated as $A_t = \pi \times d \times l$, where A_t is the surface area, d is the diameter, and l is the length. Second, the cells in SEM images (N_i) were counted, and image area (A_i) was determined. Finally, the total number of cells (N_t) captured by the fibers was estimated by $N_t = N_i \times A_t/A_i$. Two fibers were used to generate average values. These calculated values were confirmed by the plate-counting method on BHI agar plates, where the captured cells were released by vigorous vortexing for 10 min in Eppendorf tubes.

Selection of suitable selective enrichment broth for testing meat samples with fiber-optic sensor. Several selective enrichment broths commonly used for *Listeria* detection were examined for their suitability for use with the fiber-optic immunosensor application. This selection was done by assaying reaction patterns of viable *L. monocytogenes* cells grown in different selective enrichment broths for 20 h at 37°C with the capture antibody (anti-*L. monocytogenes* rabbit PAb) and the detection antibody (murine MAb C11E9) separately in an ELISA format as described previously (12, 28). The following selective enrichment broths were purchased from Becton Dickinson (Sparks, Md.) or Oxoid (Ogdensburg, N.Y.): University of Vermont medium (UVM), Fraser broth (FB), *Listeria* repair broth (LRB), and BLEB. All media were prepared according to the manufacturer's directions or as described previously (33). In the immunoassay, *L. monocytogenes* cells grown in BLEB gave overall the highest reactions with both antibodies; therefore, BLEB was a logical choice for food sample analyses using the fiber-optic sensor.

Detection of *L. monocytogenes* from artificially inoculated or naturally contaminated hot dog and bologna. Packages of hot dog and bologna were purchased from area grocery stores, and 25 g of each was aseptically transferred into a stomacher bag with a filter lining (Seward, Cincinnati, Ohio). *L. monocytogenes* cells were artificially inoculated into the selected meat samples, with final concentrations of 10^1, 10^2, and 10^3 cells/g, and allowed to stand for 10 min. Meats with or without inoculation were suspended in 100 ml of BLEB (Oxoid) in stomacher bags and were massaged by hand for 2 min and incubated at 37°C for 20 h. One-milliliter aliquots of the samples were withdrawn, the cells were harvested by centrifugation ($6{,}000 \times g$ for 5 min) and washing (three times), and the cells were finally resuspended in 1 ml of PBS. Eighty microliters of each sample was used for fiber-optic detection. Three fibers were used to generate average values and standard deviations. After fiber-optic analysis, fibers with adsorbed cells were cut, and the thin tips were transferred to Eppendorf tubes with 1 ml of PBS. The cells captured on the fibers were released into PBS by vigorous vortexing for 10 min and plated on Oxford agar plates. Bacterial counts in enriched samples were determined by serial dilution and plating on Oxford and BHI agar plates. In parallel, samples were analyzed by the U.S. Department of Agriculture Food Safety and Inspection Service (USDA FSIS) method (25). Colonies on Oxford plates recovered from the enrichment broth or the fibers were confirmed by CAMP test and ribotyping (16).

Statistical analysis. Data were presented as means ± standard deviations. Statistical analysis of the results was done using a Duncan multiple comparison test or a Tukey's *t* test. Differences were significant if P was <0.05.

RESULTS

Reaction characteristics of anti-*Listeria* PAb. A limited number of cultures were tested by ELISA (A_{490}). As expected, the anti-*Listeria* PAb showed strong reactions with all species of *Listeria* tested, including *L. monocytogenes* strains V7 (2.95) and Scott A (1.66), *L. innocua* strains LA-1 (2.74) and F4248 (1.49), *L. welshimeri* (0.98), *L. ivanovii* (2.20), *L. seeligeri* (1.71), and *L. grayi* (2.79). Among the non-*Listeria* cultures, antibody positively reacted with *Citrobacter freundii* (1.99) but showed weak or no reactions (<0.5) with *S. enterica* serovar Enteritidis, *S. enterica* serovar Typhimurium, *E. coli*, and *B. cereus*.

Determination of optimal concentration of detection antibody. Concentrations of cells or detection antibodies (Cy5-conjugated C11E9) needed to show optimum reaction were determined in a spectrofluorometer, and the strongest signal was seen when 10^9 cells/ml was mixed with 100 μg of Cy5-conjugated C11E9/ml. When the cell concentrations decreased, the signal strengths decreased logarithmically (Fig. 1). When antibody concentrations increased, the signal strengths also decreased because of fluorescent quenching (21). Based on this experiment, Cy5-conjugated C11E9 was used at 100 μg/ml.

Western blotting reaction patterns of two antibodies. Western blotting showed that the major proteins recognized by anti-*Listeria* PAb were about 68, 62, 58, 50, 43, and 30 kDa, while the expected bands for MAb C11E9 were 76, 66, and 52 kDa (4). Molecular weights of the bands for each antibody were apparently different from each other; therefore, it is anticipated that there will be less competition for epitopes between capture and detection antibodies on the waveguide (Fig. 2).

Sensitivity and specificity of the biosensor. An *L. monocytogenes* concentration of 4.3×10^9 CFU/ml gave the strongest signal (1,700 pA), while the signal strength decreased logarithmically when the cell concentration decreased (Table 1). The lowest cell concentration that gave a positive signal compared to a background control (no bacteria) was 4.3×10^3 CFU/ml and was considered to be the detection limit for this sensor. The strength of the signal obtained for this cell concentration was 230 pA, which was significantly higher than that of the signal emitted by a control (89 pA) or by a cell concentration of 4.3×10^2 CFU/ml (93 pA).

When cells were subjected to a stress environment of cold (10°C) and salt (3.5% NaCl) concurrently, the signal output was severely diminished. Signals emitted by cells at bacterial concentrations of 2.8×10^5 CFU/ml (299 pA) and 2.8×10^6 CFU/ml (348 pA) were not significantly stronger than that of the control (332 pA). A positive signal was obtained only from cell concentrations of 2.8×10^7 CFU/ml (738 pA), thus indicating that bacterial cells subjected to stress can severely affect the result (Table 1). When the sensor was evaluated for its reaction with other *Listeria* spp. at approximate concentrations of 4×10^9 CFU/ml each, *L. monocytogenes* showed a significantly ($P < 0.05$) stronger signal (1,728 pA) than *L. ivanovii*

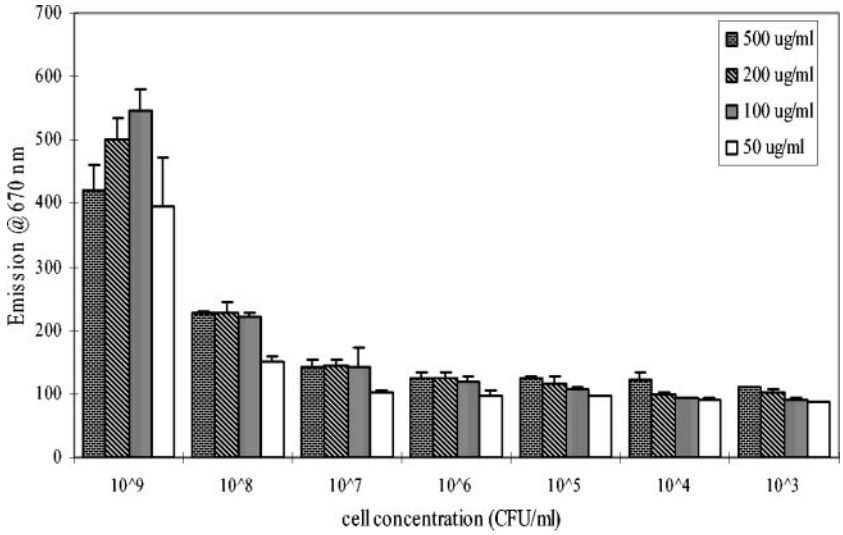

FIG. 1. Determination of optimal concentration of detection antibody, Cy5-conjugated C11E9, and *L. monocytogenes* cells required in a 96-well microtiter plate using a spectrofluorometer. Measurement was done at excitation and emission wavelengths of 650 and 670 nm, respectively.

(346 pA), *L. grayi* (204 pA), *L. seeligeri* (381 pA), *L. welshimeri* (533 pA), or *L. innocua* (720 pA) (Table 2).

Evaluation of the sensor with other common food contaminants indicated that *L. monocytogenes* (~10^9 CFU/ml) generated a signal of 1,636 pA, which was significantly ($P < 0.05$) stronger than that of the equivalent concentrations of *S. enterica* serovar Typhimurium (375 pA), *E. coli* (110 pA), *Enterococcus faecalis* (553 pA), *Lactobacillus plantarum* (310 pA), *Serratia marcescens* (147 pA), *H. alvei* (73 pA), *Enterobacter aerogenes* (141 pA), *P. aeruginosa* (327 pA), *Carnobacterium gallinarum* (342 pA), and *Corynebacterium glutamicum* (281 pA) (Table 2).

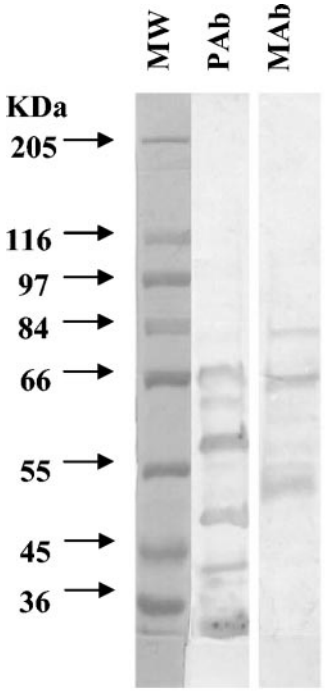

FIG. 2. Reaction patterns of antibodies with surface protein extract of *L. monocytogenes* with Western blotting. Lane 1, molecular weight standard (MW); lane 2, *L. monocytogenes* protein extracts reacted with anti-*Listeria* rabbit PAb; and lane 3, mouse MAb C11E9.

In a mixed-culture setup, the signal for *L. monocytogenes* with other *Listeria* spp. was 475.5 pA, while that for *L. monocytogenes* alone was 456 pA (Table 3). Mixed cultures without *L. monocytogenes* or *L. monocytogenes* and *L. innocua* had a significantly ($P < 0.05$) lower-strength signal than *L. monocytogenes*-containing mixed culture. The bacterial concentrations for each were about 7.8×10^8 CFU/ml, and the signal for *L. monocytogenes* alone was slightly lower in strength than the expected value for this concentration observed in Table 1. When *L. monocytogenes* was mixed with a high concentration of *Lactobacillus plantarum*, the signal for *L. monocytogenes* at 4.1×10^3 CFU/ml was 316 pA, equivalent to that of a negative control (PBS). Signals from *L. monocytogenes* at 4.1×10^4 CFU/ml (883 pA) and 4.1×10^5 CFU/ml (1,041 pA) were significantly stronger than that of the control (Table 3), which suggests that the presence of competitive microflora could decrease the detection limit by 1 log. The signal strength from the mixed cultures containing *L. monocytogenes*, *E. coli*, *Enterococcus faecalis*, and *S. enterica* serovar Typhimurium was 1,009 pA, while that from cultures containing *L. monocytogenes* alone was 995 pA (Table 3). When *L. monocytogenes* was absent from the mixture, the signal strength was significantly decreased and was similar to that of the negative control (PBS only). These studies suggest that a specific signal for *L. monocytogenes* could be acquired even in the presence of other *Listeria* spp. or common food contaminants.

SEM analysis of fibers. SEM images taken from three sections, i.e., the top, the middle, and the bottom of the fibers, showed that the number of cells captured by the fibers decreased from the top to the bottom (Fig. 3A). Cell distributions on fibers appeared to be in clusters. The average number of cells captured by fibers was estimated to be 7.6×10^6 cells/fiber, and the recovery rate was about 10% from 8×10^7

TABLE 1. Sensitivity of the immunosensor using a serial dilution of *L. monocytogenes* suspended in PBS (pH 7.2) grown in optimal (37°C, 0.5% NaCl) or stressful (10°C, 3.5% NaCl) environments

Species or control	Density (CFU/ml)	Preculture	Mean signal strength (pA)[a] ± SD	Results[b]
L. monocytogenes V7	4.3×10^9	37°C, 0.5% NaCl	1,649 ± 218	Positive
	4.3×10^8		649 ± 114	Positive
	4.3×10^7		446 ± 112	Positive
	4.3×10^6		419 ± 108	Positive
	4.3×10^5		381 ± 28	Positive
	4.3×10^4		309 ± 21	Positive
	4.3×10^3		230 ± 10	Positive
	4.3×10^2		93 ± 24	Negative
Control (PBS)	0		89 ± 14	Negative
L. monocytogenes V7	2.8×10^7	10°C, 3.5% NaCl	748 ± 140	Positive
	2.8×10^6		348 ± 35	Negative
	2.8×10^5		229 ± 135	Negative
Control (PBS)	0		332 ± 52	Negative

[a] Signal strengths are based on the average of results from three independent measurements (fibers) from a representative experiment.
[b] Results were positive when the values were significantly greater than that of the control without bacteria ($P < 0.05$).

bacteria present in 80 μl of solution. The number of cells bound to fibers without capture antibody was calculated to be about 4.6×10^5 CFU/fiber, and the recovery rate was about 0.6%. When cells were released from fibers by vortexing, the average count from two fibers was 8.6×10^6 CFU/fiber, with a 10% recovery rate, and the count from control fibers was 2.8×10^5 CFU/fiber, with about 0.4% recovery. These values were comparable to the estimated values.

The numbers of cells captured on the surface of the fibers decreased from the top to the bottom. This result could be due to the concentration gradient when cells were incubated with fibers in a vertical tube without agitation for a long time (1 h).

Close inspection of the fibers also revealed dents, scratches, and manufacturing defects with attached bacterial cells (Fig. 3B). These defects are thought to mask the acquisition of signals from bacteria and may contribute to fiber-to-fiber variation in signal output.

BLEB as a suitable enrichment broth for fiber-optic assay. ELISA data (A_{490}) showed that the reaction of anti-*Listeria* PAb to *L. monocytogenes* cells (2.30) grown in BLEB was significantly ($P < 0.05$) higher than that of cells grown in UVM (1.5), LRB (0.62), or FB (0.05) (Fig. 4). The reaction of MAb C11E9 to *L. monocytogenes* cells grown in BLEB (1.0) was similar to the reaction to cells grown in LRB (1.10) but significantly ($P < 0.05$) higher than that to cells grown in UVM (0.64) or FB (0.3). BHI broth was used as a nonselective medium control, and the reaction to cells grown in it was lower than that to cells grown in BLEB for both antibodies.

Testing of food samples. Artificially contaminated hot dogs with initial inocula of *L. monocytogenes* at concentrations of

TABLE 2. Specificity of the immunosensor for *L. monocytogenes* when compared with *Listeria* spp. and other microorganisms[a]

Species or control	Density (CFU/ml)	Mean signal strength (pA) ± SD[b]
L. monocytogenes V7	4.0×10^9	1,728 ± 325 A
L. ivanovii ATCC 19119	4.0×10^9	346 ± 85 D
L. grayi ATCC 19120	4.0×10^9	203 ± 9 D
L. seeligeri SE 31	4.0×10^9	381 ± 186 BCD
L. welshimeri ATCC 35897	4.0×10^9	533 ± 57 BC
L. innocua F4248	4.0×10^9	720 ± 11 B
Control (PBS)	0	114 ± 87 D
L. monocytogenes V7	$\sim 10^9$	2,179 ± 366 A
S. enterica serovar Typhimurium	$\sim 10^9$	553 ± 77 B
E. coli ATCC 52739	$\sim 10^9$	374 ± 39 BC
Enterococcus faecalis ATCC 344	$\sim 10^9$	110 ± 41 CD
Lactobacillus plantarum NCDO 955	$\sim 10^9$	310 ± 82 BCD
Serratia marcescens ATCC 14756	$\sim 10^9$	147 ± 82 CD
H. alvei	$\sim 10^9$	73 ± 51 D
Enterobacter aerogenes	$\sim 10^9$	141 ± 28 CD
P. aeruginosa ATCC 10145	$\sim 10^9$	327 ± 44 BCD
Corynebacterium glutamicum ATCC 31834	$\sim 10^9$	281 ± 58 BCD
Carnobacterium gallinarum ATCC 49517	$\sim 10^9$	342 ± 78 BCD
Control (PBS)	0	347 ± 12 BCD

[a] Bacterial cultures were precultured at 37°C and with 0.5% NaCl.
[b] Mean signal strengths were based on the average of results from three independent measurements (fibers) from a representative experiment. Results with different letters (A, B, C, and D) are significantly ($P < 0.05$) different from each other.

TABLE 3. Fiber-optic signal acquisition for *L. monocytogenes* in mixture in PBS, pH 7.2[a]

L. monocytogenes density (CFU/ml)	Added organism, density (CFU/ml)	Mean signal strength (pA)[b] ± SD	Results[c]
7.8×10^8		476 ± 204	Positive
7.8×10^8	All other *Listeria* spp., $\sim 3 \times 10^9$	456 ± 30	Positive
0 (control)		104 ± 6	Negative
0	All other *Listeria* spp., $\sim 3 \times 10^9$	165 ± 45	Negative
0	All other *Listeria* spp., except *L. innocua*, $\sim 3 \times 10^9$	198 ± 37	Negative
1.0×10^9		995 ± 77	Positive
1.0×10^9	*E. coli*, *Enterococcus faecalis*, and serovar Typhimurium, $\sim 3 \times 10^9$	1,009 ± 142	Positive
0		304 ± 33	Negative
0	*E. coli*, *Enterococcus faecalis*, and serovar Typhimurium, 1.0×10^9	298 ± 68	Negative
4.1×10^9		1,844 ± 123	Positive
4.1×10^3	*L. plantarum*, 2.6×10^7	316 ± 55	Negative
4.1×10^4	*L. plantarum*, 2.6×10^7	883 ± 205	Positive
4.1×10^5	*L. plantarum*, 2.6×10^7	1,041 ± 187	Positive
0		294 ± 111	Negative

[a] Cells were precultured at 37°C and with 0.5% NaCl.
[b] Signal strengths are based on the average of results from three independent measurements (fibers) from a representative experiment.
[c] Results were positive when the values were significantly greater than that of the control without bacteria ($P < 0.05$) for a given experiment.

approximately 10^1, 10^2, and 10^3 CFU/g followed by a 20-h enrichment gave fiber-optic signals of 1,064, 1,540, and 1,652 pA, respectively. The final cell concentrations after enrichment were enumerated to be 3.8×10^8, 2.3×10^9, and 4.2×10^9 CFU/ml, respectively (Fig. 5). These values were significantly ($P < 0.05$) greater than those from samples without inoculation. Likewise, spiked bolognas with initial inocula of 10^1, 10^2, and 10^3 CFU/ml gave fiber-optic signals of 764, 790, and 1,452 pA, respectively, and the final cell concentrations after enrichment were estimated to be 2.3×10^8, 6.8×10^8, and 3.9×10^9 CFU/ml (Fig. 5). No background *Listeria* spp. were found in either hot dog or bologna samples. The cells captured on the fibers were randomly picked from Oxford plates and were positive by CAMP test, identifying them as *L. monocytogenes*.

Analyses of 11 hot dog or bologna samples purchased from area grocery stores (six stores) with the fiber-optic sensor revealed only one hot dog sample to be positive for *L. monocytogenes*, with an average signal of 1,400 pA. The isolate obtained from this positive fiber formed a black colony on an Oxford agar plate, gave a positive CAMP reaction, and was identified as *L. monocytogenes* with a ribotype pattern of DUP 1093. The presence of *L. monocytogenes* in this sample was further confirmed by the USDA FSIS method. The average signals for negative meat samples ranged from 338 to 549 pA, and none of the samples contained any *L. monocytogenes* or other *Listeria* species, as analyzed by the USDA FSIS method and CAMP testing.

DISCUSSION

L. monocytogenes accounted for the greatest number of food product recalls (9, 31, 39) and several recent outbreaks (10, 11, 23, 31). Therefore, rapid and sensitive methods are required to detect this pathogen in food. Conventional methods are time-consuming. Several rapid methods, such as PCR (17) and biosensors based on their transducer properties (21), are sensitive and specific but often expensive, sophisticated, and nonportable, and thus with limited usefulness. Furthermore, the efficacy of those biosensors to detect pathogens from real-world food samples has not been thoroughly investigated (5, 14).

Antibody-based fiber-optic biosensors could be one of the best methods to meet the need for a rapid, sensitive, and portable microbial detection system. A previous report showed that *E. coli* O157:H7 could be detected at levels as low as 30 CFU/ml with a fiber-optic sensor (8). Recently, Liu et al. (22) developed a chemiluminescent fiber-optic biosensor that could detect about 100 CFU of *E. coli* O157:H7/ml from ground beef, chicken carcass, and lettuce samples within 1.5 h, but preseparation with immunomagnetic beads was required. Zhou et al. (40) used a compact fiber-optic evanescent system that could detect 10^4 CFU of *S. enterica* serovar Typhimurium/ml. Using a fiber-optic system, Tims et al. (37) reported the detection limit for *L. monocytogenes* to be $\sim 10^8$ CFU/ml; however, this study did not evaluate the performance of the sensor with other *Listeria* spp. or other microorganisms or with food samples.

In this study, our goal was to develop a fiber-optic immunosensor and optimize conditions that would be able to detect viable *L. monocytogenes* cells from ready-to-eat meat samples in less than 24 h from the point of food sampling. To develop a sensitive immunosensor, the quality and specificity of the antibodies are important. We developed a PAb in rabbit against whole cells of *L. monocytogenes*, which was used as a capture antibody on the fiber waveguide. Subsequent detection was accomplished by using MAb C11E9 (4, 20). This combination improved the sensitivity of the fiber-optic biosensor to 4.3×10^3 CFU of *L. monocytogenes*/ml. In contrast, when MAb C11E9 was used for both capture and detection, the sensitivity was very poor and results were extremely variable (data not shown). Overall, the strength of the fiber-optic signals decreased logarithmically when *L. monocytogenes* cells were serially diluted. A similar reaction pattern was observed when antibody binding (detection antibody; MAb C11E9) was examined by immunofluorescence assay in a 96-well microtiter plate, and the detection limit appeared to be 10^7 to 10^8 CFU/ml (Fig. 1). The detection limit for *L. monocytogenes* by sandwich or indirect ELISA methods could vary from 10^6 to 10^8 CFU/ml (3, 30, 34, 38). A similar detection limit was reported for the fiber-optic sensor (37) or surface plasmon res-

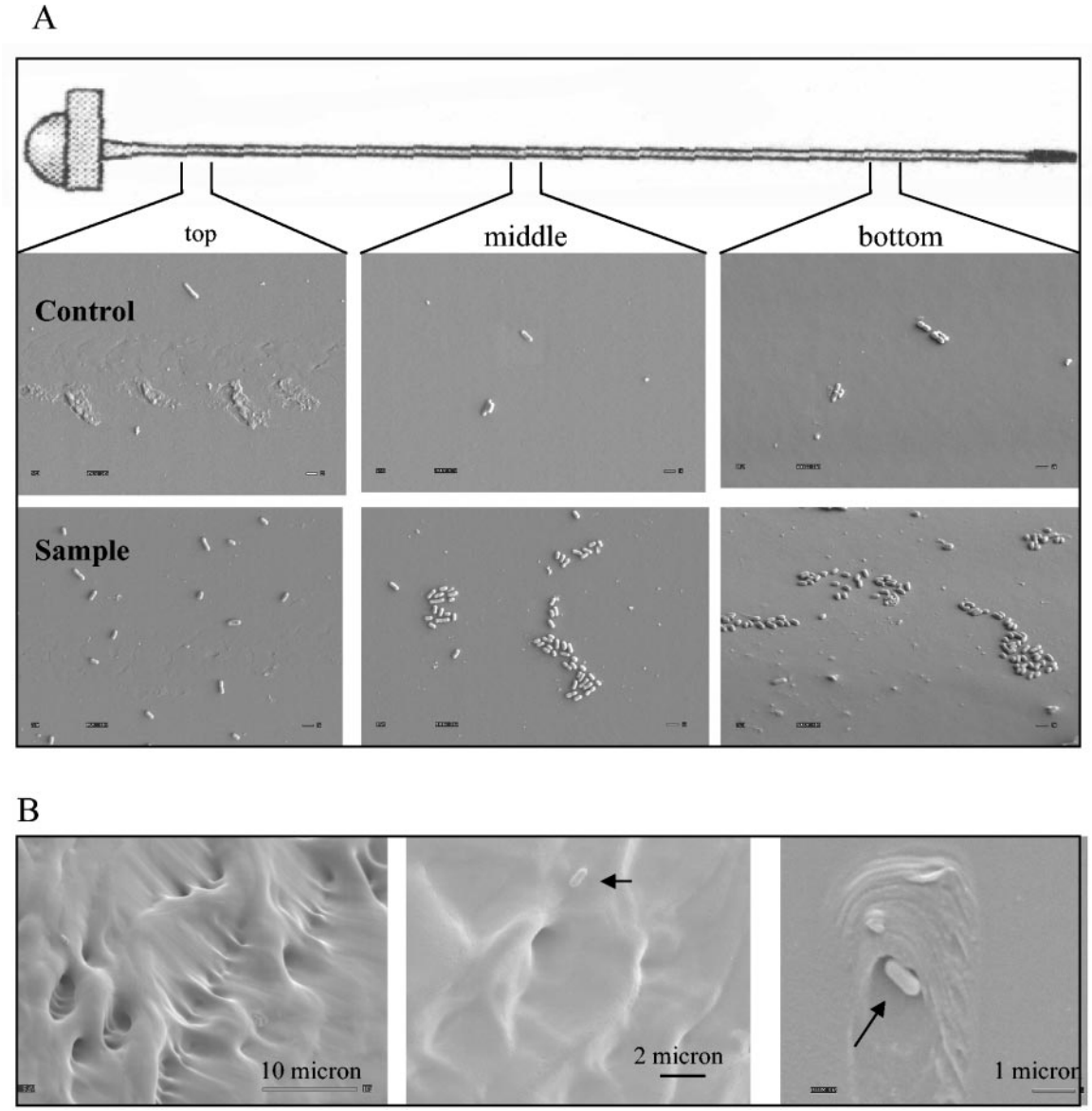

FIG. 3. SEM analyses of fibers with captured *L. monocytogenes* cells. (A) *L. monocytogenes* cell binding was examined in three arbitrary sections (top, middle, and bottom) of the fiber. The top row (control) is without capture antibody, and the bottom row (sample) is with rabbit polyclonal capture antibody. Magnification, ×4,000. Bar, 1.0 μm. (B) Fibers showing scratches and manufacturing defects. Magnifications for each panel are presented in the figure.

onance sensor (19, 20) for *L. monocytogenes*. In comparison, the sensitivity of the present fiber-optic sensor is severalfold higher.

When a low concentration of *L. monocytogenes* cells was mixed with a high concentration of competitive microflora, the sensitivity of the sensor decreased and the detection limit increased by 1 log. This result implied that the presence of microflora might interfere with the binding of antibodies to *L. monocytogenes*, thus affecting the detection sensitivity. When cells were grown in a stress environment with a combination of cold and salt, the sensitivity of the sensor decreased by 4 log. This result was expected, since cold (4°C) or salt (1.5 to 10.5% NaCl) treatments displayed diminished reactions with MAb C11E9 (12). The introduction of an enrichment step prior to detection by immunosensor would eliminate such problems.

The specificity of this fiber-optic biosensor was tested in the presence of *E. coli*, *Enterococcus faecalis*, *S. enterica* serovar Typhimurium, different *Listeria* species, and common bacterial contaminants, which may be present in a food product. Taken together, these data indicated that the fiber-optic sensor was specific for *L. monocytogenes*. However, the detection antibody (C11E9) used here is known to show cross-reactions with certain strains (23%) of *L. innocua* (20). It is possible that the fiber-optic sensor in its present configuration may pick up a signal from certain *L. innocua* strains. Therefore, a more specific *L. monocytogenes* antibody would be able to eliminate this problem.

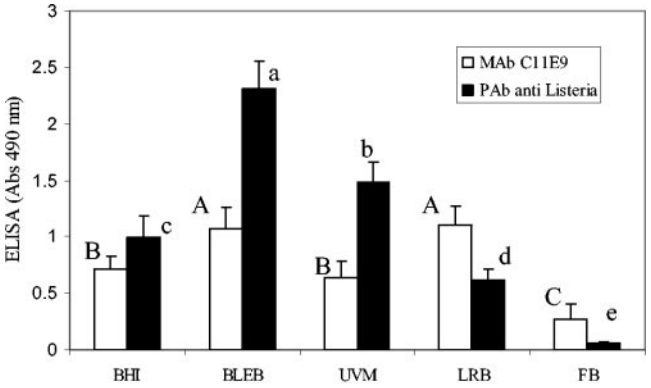

FIG. 4. Comparison of reactions of MAb C11E9 and anti-*Listeria* PAb to *L. monocytogenes* grown in BHI broth, BLEB, UVM, LRB, and FB in an ELISA. Cultures were obtained after 20 h of growth and centrifuged to remove media, and cell concentrations were adjusted to an A_{595} of 0.37 with a spectrophotometer. Bars marked with different capital letters (A, B, or C) for MAb C11E9 and different lowercase letters (a, b, c, d, or e) for anti-*Listeria* PAb are significantly different, with P values of <0.05.

The fiber-optic sensor sometimes showed fiber-to-fiber variation in signal strength, which was counted as background and subtracted from the final reading. This background reading varied from 300 pA to 500 pA (data not shown). SEM analyses of surface topography of some of the fibers revealed scratches, dents, and manufacturing defects that possibly contributed to fiber-to-fiber variation in signal acquisition. Besides, the ratios of biotin or Cy5 to antibodies were changed batch-to-batch when antibodies were labeled (data not shown). The in-batch variation could also contribute to signal variation. The standardization of this procedure needs to be improved in future studies.

In this study, we had set as our goal that fiber-optic sensor-based detection be completed in less than 24 h from the point of food sampling. To achieve that goal, we needed a suitable enrichment broth that would support sufficient antigen expression for capture and detection antibodies to generate strong signals in the fiber-optic sensor. Several enrichment broths (UVM, FB, LRB, and BLEB) were evaluated. Certain selective enrichment broths were reported previously to affect antibody-based detection of *L. monocytogenes*. Nannapaneni et al. (28) indicated that *Listeria* enrichment broth supported antigen expression for MAb EM-7G1 (6), while UVM and FB suppressed the expression. In this study, we also observed a similar trend, where cells cultured in BLEB had the overall highest level of reaction, followed by those cultured in UVM, LRB, and FB. Cells from FB showed the lowest level of reaction, and incidentally, this broth is used in the USDA FSIS method (25) for detection of *L. monocytogenes*. Based on this data, we used BLEB as an enrichment broth for the fiber-optic sensor application.

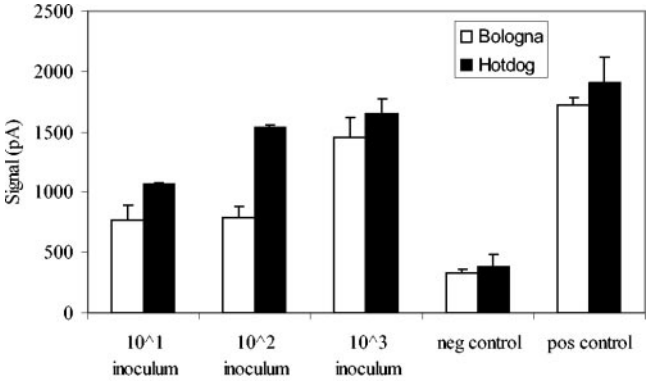

FIG. 5. Fiber-optic signal acquisition for *L. monocytogenes* artificially inoculated into hot dog or bologna at 10^1, 10^2, or 10^3 CFU/ml. The meat samples were enriched for 20 h in BLEB before fiber-optic analysis. The negative (neg) control contained only PBS, and the positive (pos) control contained pure culture of *L. monocytogenes.*

Since *Listeria* is problematic in ready-to-eat meats, artificially inoculated hot dogs and bolognas were tested after 20 h of enrichment in BLEB. Results showed that *L. monocytogenes* cells could be detected directly from the enrichment broth even with low levels of inoculations (10 to 1,000 cells/g). The enriched samples were centrifuged and washed to remove food particles, since excessive food particles gave increased background noise (data not shown).

In an attempt to validate the performance of the fiber-optic sensor to detect *L. monocytogenes* from food samples, we tested 11 RTE meat samples and found only one to be positive. The sell-by date for this particular positive hot dog sample had expired. Although a limited number of samples were tested, this study indicates that the fiber-optic sensor can detect *L. monocytogenes* from enriched RTE meat samples with initial low levels of inoculation (contamination) in under 24 h. Our future goal is to determine the earliest time, preferably in a single working day (8 h), in which the assay could be completed with an initial contamination level of 1 to 100 CFU/g of RTE products. This achievement would require optimization of both enrichment conditions and the fiber-optic assay method.

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