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Research Note

Spray Method for Recovery of Heat-Injured *Salmonella* Typhimurium and *Listeria monocytogenes*

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ABSTRACT

Selective agar is inadequate for supporting recovery of injured cells. During risk assessment of certain foods, both injured and noninjured cells must be enumerated. In this study, a new method (agar spray method) for recovering sublethally heat-injured microorganisms was developed and used for recovery of heat-injured *Salmonella* Typhimurium and *Listeria monocytogenes*. Molten selective agar was applied as an overlay to presolidified nonselective tryptic soy agar (TSA) by spray application. Heat-injured cells (55°C for 10 min in 0.1% peptone water or 55°C for 15 min in sterilized skim milk) were inoculated directly onto solidified TSA. After a 2-h incubation period for cell repair, selective agar was applied to the TSA surface with a sprayer, and the plates were incubated. The recovery rate for heat-injured *Salmonella* Typhimurium and *L. monocytogenes* with the spray method was compared with the corresponding rates associated with TSA alone, selective media alone, and the conventional overlay method (selective agar poured on top of resuscitated cells grown on TSA and incubated for 2 h). No significant differences ($P > 0.05$) were found in pathogen recovery obtained with TSA, the overlay method, and the spray method. However, a lower recovery rate ($P < 0.05$) was obtained for isolation of injured cells on selective media. Overall, these results indicate that the agar spray method is an acceptable alternative to the conventional overlay method and is a simpler and more convenient approach to recovery and detection of injured cells.

Salmonella Typhimurium and *Listeria monocytogenes* are recognized as important foodborne pathogens and have caused numerous outbreaks of human illness *(3, 10, 23, 25, 31, 35)*. These microorganisms will not grow in harsh environments. Methods such as drying, heating, freezing, irradiation, high hydrostatic pressure, freeze-drying, fermentation, or the addition of antimicrobials and chemicals are commonly used to control bacterial contamination and pathogens in foods *(15, 36, 37)*. Severe conditions can cause cell injury and cell death. However, any injured bacterial population (stressed or sublethally or reversibly injured) in a food matrix may be able to resuscitate. After the antimicrobial treatments, one subpopulation of microorganisms may be killed (lethally injured), another subpopulation may survive (noninjured), and a third subpopulation may be injured sublethally *(26, 28, 32, 39)*. Factors affecting the recovery of microorganisms include availability of essential nutrients, optimum pH and temperature, and incubation period *(27)*. The ability to detect the presence of injured microorganisms in food and their recovery during culturing procedures is critical, because these injured cells can repair themselves, grow, and regain their pathogenicity under suitable conditions *(13, 29, 36)*. Therefore, injured microorganisms should be accounted for in many contexts, such as the preservation and spoilage of foods, consumer protection, the manufacture of safe foods, and evaluation of the effectiveness of processing interventions for reduction of microorganisms.

Injured *Salmonella* Typhimurium and *L. monocytogenes* cannot undergo repair and form colonies on their respective selective media (xylose lysine decarboxylase agar [XLD] and Oxford agar base [OAB]) because these media contain various agents designed to select for healthy target microorganisms. Commonly used selective agents include organic dyes, antibiotics, bile salts, and surfactants, which may impair the ability of microorganisms to repair themselves. These effects on microorganisms can be considered growth retardation *(11, 24, 29)*. Significant differences between selective and nonselective media for recovery of injured microorganisms have been observed *(17, 18, 38–40)*. The difference in bacterial counts obtained on selective and nonselective media can be used to determine the degree to which a microbial population is sublethally injured *(2, 24, 37)*.

Several identification methods and media for detecting sublethally injured foodborne pathogens in foods have been described *(6, 7, 9, 17, 18)*. Among these methods, the agar pour overlay (OV) method is widely used to recover injured microorganisms because it ensures both good selectivity and

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high recovery. However, one limitation of this method is the difficulty of enumerating injured cells that grow between the layers of nonselective and selective media *(20)*. Overlaying with molten agar may detach cells from the bottom agar layer, which can lead to erroneous results.

We describe herein the new agar spray overlay (SP) method, which includes use of a hand-operated trigger sprayer to apply an agar overlay layer as a fine mist, for recovery of heat-injured *Salmonella* Typhimurium and *L. monocytogenes* in peptone water and milk. Milk was chosen as a test vehicle because it is an important vehicle of transmission of these pathogens *(14, 21, 30)*. The SP method may reduce the number of difficult-to-isolate bacterial colonies. The aim of this study was to evaluate the efficacy of the SP method by comparing recovery of heat-injured microorganisms obtained using this method, nonselective medium (tryptic soy agar [TSA]), selective media for *Salmonella* Typhimurium and *L. monocytogenes* (XLD and OAB, respectively), and the pour OV method.

MATERIALS AND METHODS

Bacterial strains. *Salmonella* Typhimurium (ATCC 19585, ATCC 43971, and ATCC 700408) and *L. monocytogenes* (ATCC 15315, ATCC 19114, and ATCC 19115) strains were obtained from the Bacterial Culture Collection at Seoul National University (Seoul, Korea). All strains were stored at −80°C in 0.7 ml of tryptic soy broth (TSB; Difco, BD, Sparks, MD) and 0.3 ml of 50% glycerol (vol/vol). Working cultures were maintained on TSA (Difco, BD) slants at 4°C and subcultured monthly.

Bacterial cultures and cell suspension preparation. Each strain of *Salmonella* Typhimurium and *L. monocytogenes* was cultured in 5 ml of TSB for 24 h at 37°C, harvested by centrifugation at 4,000 × *g* for 20 min at 4°C, and washed three times with buffered peptone water (BPW; Difco, BD). The final pellets were resuspended in BPW, corresponding to approximately 10^9 CFU/ml.

OV method. Sterilized TSA was poured into petri dishes and allowed to solidify, and heat-treated cells were spread plated directly onto the solidified TSA. Untreated cells also were plated separately as a control. After incubation at 37°C for 2 h to allow recovery of injured cells, 7 ml of each selective medium, XLD (Difco, BD) or OAB with the antimicrobial supplement Bacto (Difco, BD), was poured over the inoculated plates. The plates were then incubated for another 22 h (*Salmonella* Typhimurium) or 46 h (*L. monocytogenes*), and colonies were counted. This method was similar to that proposed by Hartman et al. *(12)* for evaluation of heat-injured *Escherichia coli* using a two-step agar procedure with violet red bile agar.

SP method. A hand-operated trigger sprayer (650 ml; A5, Apollo, Siheung-si, Korea) was purchased at a local hardware store. The ejecting nozzle was adjustable and could be selectively adjusted to produce a conical pattern of fine spray. The nozzle discharge rate was 1 ml per squeeze of the sprayer trigger. To apply the selective agar, the distance between the petri dish and the sprayer was approximately 45 cm. Sterilized TSA was prepared and inoculated as described above. After incubation at 37°C for 2 h to allow recovery of injured cells, approximately 7 ml of selective medium (XLD or OAB) was sprayed onto the TSA (Fig. 1). Plates were sprayed inside a clean bench, and the hand sprayer was filled with approximately 300 ml of each selective medium at 50°C. Plates were incubated for another 22 h (*Salmonella* Typhimurium) or 46 h (*L. monocytogenes*), and colonies were counted.

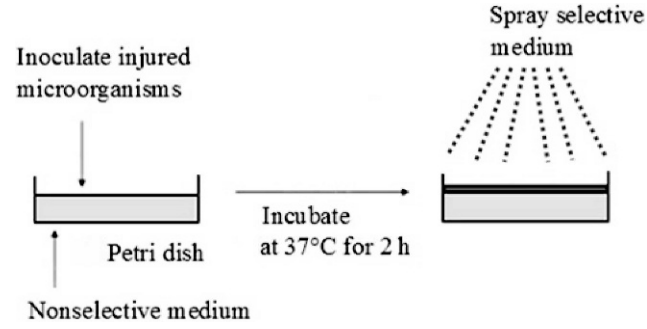

FIGURE 1. *Spray method for recovery and enumeration of injured microorganisms.*

Recovery of heat-injured foodborne pathogens from 0.1% peptone water. Culture suspension (100 μl) of *Salmonella* Typhimurium and *L. monocytogenes* (diluted to ca. 9.0 log CFU/ml) was added separately to screw-cap test tubes containing 10 ml of 0.1% peptone water that had been preheated and maintained at 55°C. After inoculation, the tube caps were tightly closed, and the portion of the tube containing liquid was immersed completely in a shaking 55°C water bath and heated for 10 min *(1, 16, 20)*. Tubes were then removed from the water bath and cooled immediately in slush ice to room temperature (about 25°C, 10 min). Ten-fold serial dilutions were made with 0.1% sterile peptone water, and 0.1-ml aliquots were spread onto TSA (nonselective medium) and XLD or OAB (selective media). The inoculated plates were incubated at 37°C for 24 h (*Salmonella* Typhimurium) or 48 h (*L. monocytogenes*). The OV and SP methods were then performed as described above. These experiments were conducted three times.

Recovery of heat-injured foodborne pathogens in sterilized skim milk. Culture suspension (100 μl) of *Salmonella* Typhimurium and *L. monocytogenes* (diluted to ca. 9.0 log CFU/ml) were added separately to screw-cap test tubes containing 10 ml of sterilized skim milk that had been preheated and maintained at 55°C. After inoculation, the tube caps were tightly closed, and the portion of the tube containing liquid was immersed completely in a shaking 55°C water bath and heated for 15 min. After heating, the same cooling, plating, and incubating protocols were followed as used with the nonselective and selective media and the OV and SP methods. These experiments were conducted three times.

Statistical analysis. All experiments were conducted three times with duplicate samples, and means of duplicate plate counts from the three replications were converted to units of log CFU per milliliter. Data were analyzed with the analysis of variance procedure of SAS (version 8.1, SAS Institute, Cary, NC) for a completely randomized design. When the main effect was significantly different ($P < 0.05$), means were separated using Duncan's multiple range test.

RESULTS

Injured cells recovered and grew under conditions of both the SP and OV methods, forming typical colonies in the overlaid XLD (black colonies) or OAB (black colonies) (Figs. 2 and 3).

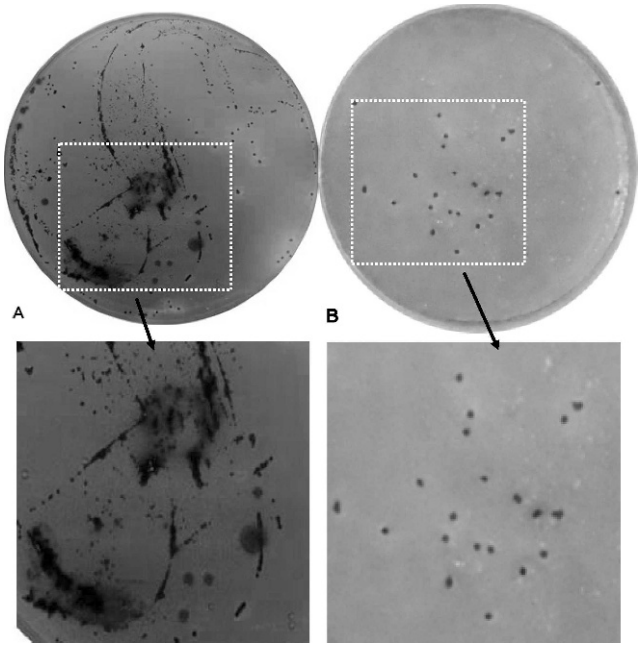

FIGURE 2. *Detection of heat-injured* Salmonella *Typhimurium with the OV method (A) and SP method (B).*

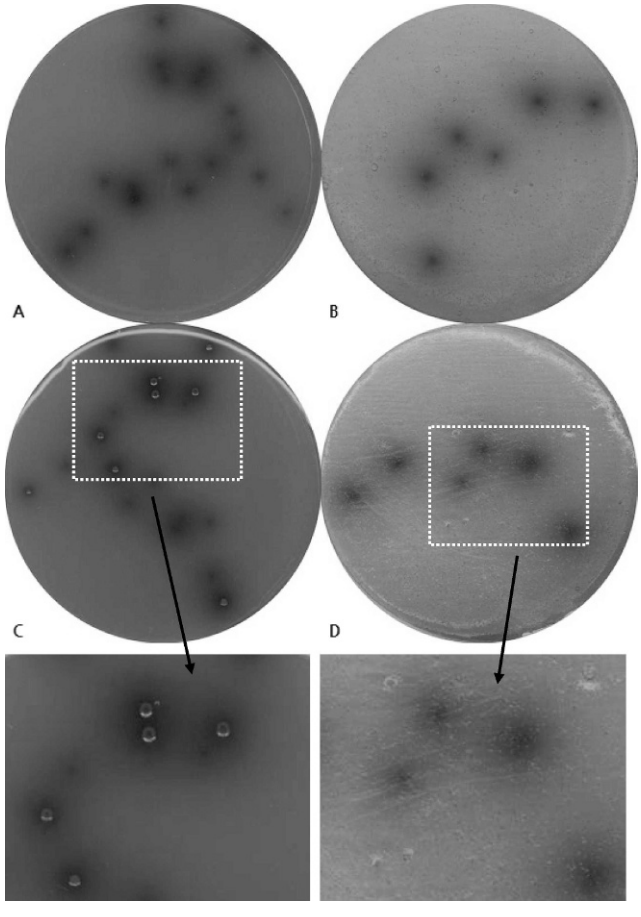

FIGURE 3. *Detection of heat-injured* L. monocytogenes *with the OV and SP methods. (A) Lower surface of an OV plate; (B) lower surface of an SP plate; (C) upper surface of an OV plate; (D) upper surface of an SP plate.*

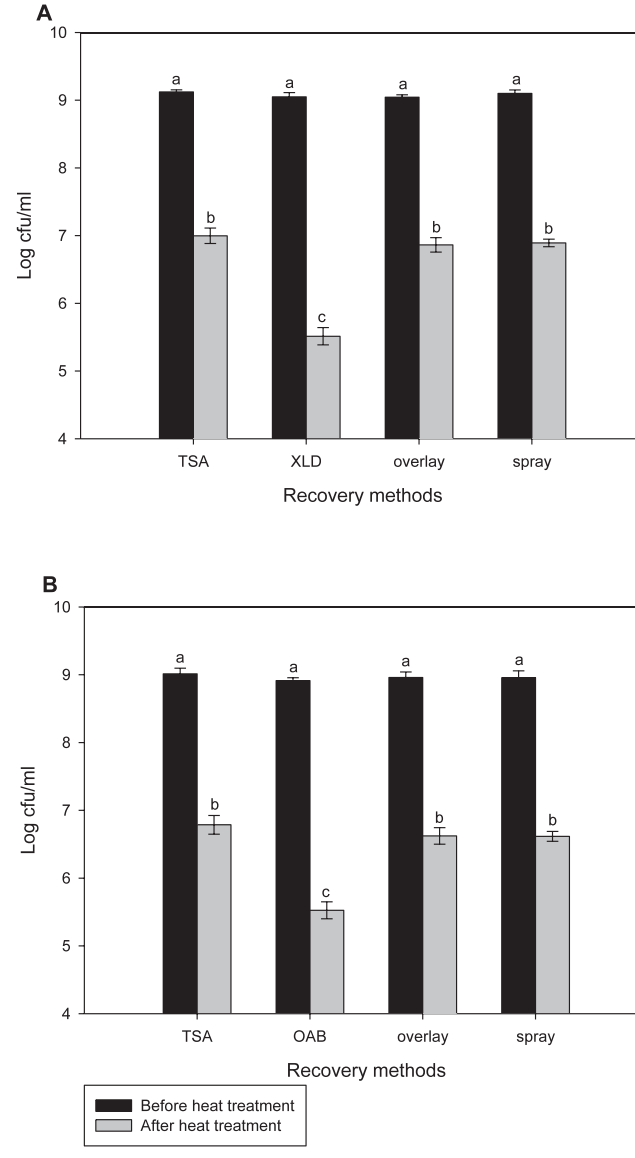

FIGURE 4. *Comparison of nonselective tryptic soy agar (TSA), selective agars (XLD or OAB), selective agar poured over TSA, and selective agar sprayed over TSA for recovery of heat-injured (55°C for 10 min)* Salmonella *Typhimurium (A) and* L. monocytogenes *(B) in 0.1% peptone water. Bars with different letters are significantly different (*$P < 0.05$*).*

Recovery of heat-injured *Salmonella* Typhimurium or *L. monocytogenes* from 0.1% peptone water. Growth of untreated *Salmonella* Typhimurium cells plated according to the SP method was not significantly different ($P > 0.05$) from that of cells plated on nonselective medium (TSA), selective medium (XLD) or cells plated using the OV method. However, significantly higher numbers of heat-injured *Salmonella* Typhimurium cells ($P < 0.05$) were recovered on the nonselective medium and when using the SP and OV methods than on the selective medium (Fig. 4A) because sublethally injured *Salmonella* Typhimurium cells were sensitive to the selection agents when plated directly onto XLD.

For untreated *L. monocytogenes* cells, no significant differences in recovery ($P > 0.05$) were observed between nonselective medium (TSA), selective medium (OAB), the

SP method, and the OV method. However, a lower recovery rate ($P < 0.05$) was observed for injured *L. monocytogenes* cells on the selective medium (OAB) compared with the nonselective medium and the SP and OV methods (Fig. 4B).

Recovery of heat-injured *Salmonella* Typhimurium or *L. monocytogenes* from sterilized skim milk. *Salmonella* Typhimurium and *L. monocytogenes* inoculated into sterilized skim milk and subsequently heat injured were enumerated by plating on nonselective agar (TSA), selective agar (XLD or OAB), and the OV and SP methods. Before heat treatment, the growth of *Salmonella* Typhimurium and *L. monocytogenes* after direct plating on selective media (XLD or OAB), nonselective medium (TSA), the OV method, and the SP method was not significantly different ($P > 0.05$) (Fig. 5). After heat treatment at 55°C for 15 min, the recovery of *Salmonella* Typhimurium and *L. monocytogenes* by the SP method was not significantly different from recovery on nonselective medium and by the OV method (Fig. 5). However, significantly lower level of recovery ($P < 0.05$) was obtained after direct plating on the selective media compared with plating on TSA, the OV method, and the SP method.

DISCUSSION

Effective methods for recovery of all types of microorganisms from various foods are necessary to ensure the detection of microorganisms that may repair themselves and proliferate after they have been injured *(4, 5, 26)*. The need to quantify these organisms has led several researchers to develop novel techniques for the recovery of injured microorganisms *(9, 17–19, 22)*. Several injury repair protocols have been published *(7, 8, 12, 24, 33, 34, 41)*. Most include combinations of selective and nonselective media to enumerate injured cells.

For cell suspensions in both water and sterilized skim milk in the present study, nonselective and selective media produced the highest and lowest levels of recovery, respectively, for both *Salmonella* Typhimurium and *L. monocytogenes*. Only healthy cells were assumed to grow on XLD and OAB because of the harsh conditions of these media for injured cells. Conversely, the nonselective medium provided an environment in which both noninjured and sublethally injured cells could grow, but the target pathogens could not be differentiated.

Injury repair protocols are generally based on the principle that sublethally injured bacteria are more sensitive to selective agents *(27, 29)* and require a resuscitation step or growth period under nonselective conditions. Cole et al. *(8)* reported that substantial repair of *L. monocytogenes* can occur in a nonselective medium such as tryptic soy broth within 1 h at 25°C. The disadvantage of this liquid recovery step is that injured cells vary in the time required for repair and thus uninjured and nontarget cells can multiply before the population of interest recovers *(34)*. In an effort to avoid this problem and allow the enumeration of both injured and uninjured cells, numerous researchers have used the OV method developed by Speck et al. *(34)* and Hartman et al.

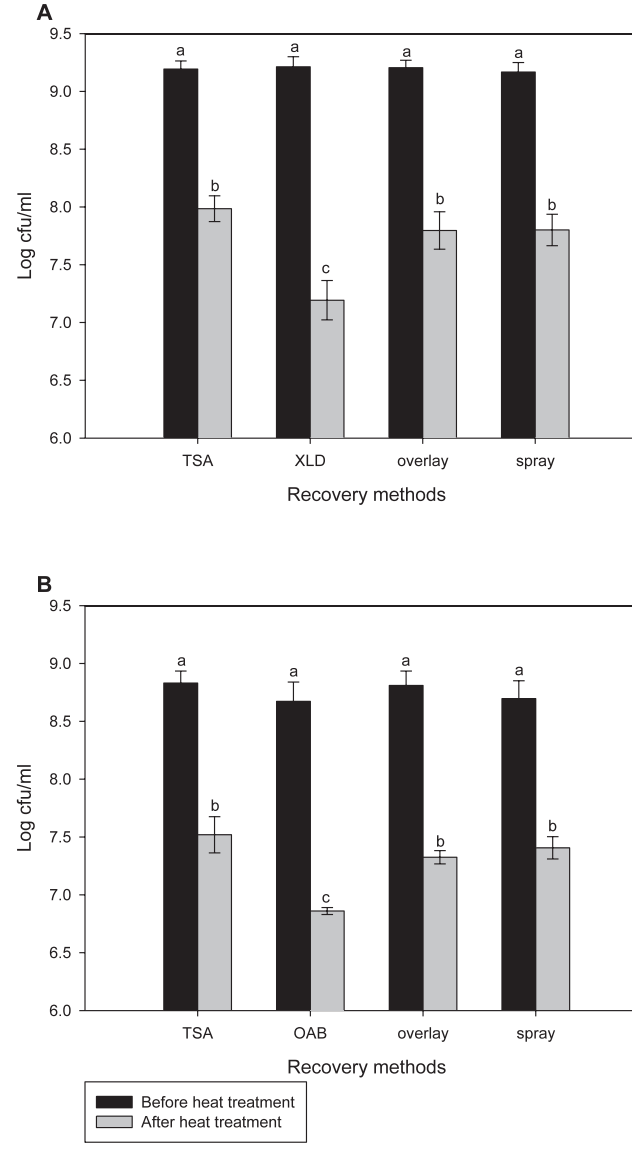

FIGURE 5. *Comparison of nonselective tryptic soy agar (TSA), selective agars (XLD or OAB), selective agar poured over TSA, and selective agar sprayed over TSA for recovery of heat-injured (55°C for 15 min)* Salmonella *Typhimurium (A) and* L. monocytogenes *(B) in sterilized skim milk. Bars with different letters are significantly different (*$P < 0.05$*).*

(12). In the current study, the recovery rates for the OV method were statistically similar to that for nonselective medium (TSA). However, the OV method for recovery of heat-injured cells has the following limitations: enumeration of *Salmonella* Typhimurium cells that grow between the two agar layers is difficult *(19, 37)*, and pouring the liquid medium onto the existing cell culture can result in clumping and undesirable spreading throughout the plate (Fig. 2A), making the isolation and enumeration of individual colonies difficult. Some colonies of *Salmonella* Typhimurium or *L. monocytogenes* also moved upward onto the top of the freshly poured overlay. The addition of the molten medium can dislodge cells from the nonselective layer, and colonies growing on or in the bottom and top layers may overlap each other (Fig. 3C). The colonies on the top layer were larger than those grown on the bottom layer, possibly

because of frictional force due to the viscosity of the molten medium. Conversely, with the SP method the selective agar is applied as a fine mist, which did not dislodge cells or disrupt the bottom nonselective agar layer (Figs. 2B and 3D). Thus, the SP method can compensate for the limitations of the OV method, and the colonies obtained are easier to enumerate than those obtained with the OV method.

In conclusion, the data presented in this study indicate that the SP method is an efficient approach to recovery and subsequent selective culture of sublethally heat-injured microorganisms and offers several advantages compared with the conventional OV method. The recovery of bacteria with the SP method was statistically similar to recovery on the nonselective medium TSA, but TSA has the disadvantage of not allowing differentiation of target microorganisms from background flora. The SP method incorporates a selective overlayer applied as a fine mist, which can isolate the target microorganisms. This new spray method allows injured cells to repair themselves and grow, is convenient, and makes it easier to enumerate colonies. The SP method allows both enumeration and differentiation of heat-injured pathogens. Limitations of the SP method include nonuniformity of the sprayed medium and lack of control of sprayed volume. A new spray apparatus is being designed to address these limitations.

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