Journal of Food Protection, Vol. 62, No. 11, 1999, Pages 1346–1349

Research Note

Thin Agar Layer Method for Recovery of Heat-Injured *Listeria monocytogenes*

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MS 99-29: Received 12 February 1999/Accepted 24 May 1999

ABSTRACT

A thin agar layer (TAL) method was developed to recover heat-injured *Listeria monocytogenes.* Modified Oxford medium (MOX), a selective plating medium, inhibits heat-injured *L. monocytogenes* from growing, whereas tryptic soy agar (TSA), a nonselective medium, does not. In order to facilitate recovery of heat-injured *L. monocytogenes* cells while providing selectivity of isolation of *L. monocytogenes* from other bacteria in the sample, a unique TAL procedure was developed by overlaying 5 ml of nonselective medium (TSA) onto prepoured and solidified MOX medium in an 8.5-cm–diameter petri dish. The injured *L. monocytogenes* repaired and started to grow in the TSA during the first few hours after incubation of the plate. During the resuscitation of injured cells, the selective agents from MOX diffused to the TSA top layer to inhibit other microorganisms. *L. monocytogenes* showed a typical reaction (black colonies) on TAL after 24 h of incubation at 37°C. The recovery rate for heat-injured *L. monocytogenes* with the TAL method was compared with those rates associated with TSA, MOX, and the traditional overlay method (OV; pouring selective agar on top of resuscitated cells on TSA agar after 3 h incubation). Milk and 0.1% peptone water that were inoculated with *L. monocytogenes* (4 to 5 log CFU/ml) were heated for 15 min at 55°C. *L. monocytogenes* was enumerated on TSA, MOX, OV, and TAL media and procedures. No significant difference occurred among TSA, OV, and TAL ($P > 0.05$) in terms of enumeration of heat-injured *L. monocytogenes,* but these media recovered significantly higher numbers than did MOX agar ($P < 0.05$)—in both samples. The TAL method involves only one step, whereas OV is a more cumbersome two-step procedure.

Listeria monocytogenes is a foodborne pathogen that has been responsible for several outbreaks of human illness *(17).* Coleslaw *(20),* pasteurized milk *(8),* Mexican-style cheese *(12),* Vacherin Mont d'or cheese *(3),* and pickled pork tongue *(1)* have been implicated as vehicles of transmission. The organism has been isolated from commercial dairies and other food-processing plants and is ubiquitous in nature, being present on a wide range of unprocessed foods as well as in soil, sewage, silage, and river water *(21, 24).* Contamination of pasteurized commercial dairy products with *L. monocytogenes* has raised concern about the organism's thermotolerance. An additional health risk is posed by the ability of *L. monocytogenes* to grow at refrigeration temperature.

Numerous studies have been performed to develop isolation and identification methods and media for detecting *L. monocytogenes* in foods. These methods are based on traditional microbiological techniques consisting of enrichment, isolation, and confirmation. Enrichment techniques *(7, 10, 16, 17)* are time consuming, and several days to weeks are required before confirmation can be obtained. Direct plating to enumerate a target pathogen often proves challenging when food samples are heavily contaminated with other microorganisms. Chemicals with selective properties are often added to media to facilitate detection of specific microorganisms in foods. Some of these agents inhibit the repair of injured cells *(18). L. monocytogenes* is susceptible to injury on exposure to heat *(2, 9, 22).* Under favorable environmental conditions, such as in a nonselective medium, injured cells usually undergo repair and become functionally normal. Injured cells can fail to resuscitate when plated directly on media containing selective agents.

Special plating procedures (involving two steps) have been developed to allow for recovery from injury and subsequent enumeration *(11, 23).* First, injured cells are plated on a nonselective medium, such as tryptic soy agar (TSA), and are incubated for 2 to 4 h, and then a layer of selective agar is plated on top of the resuscitated cells. Thus, the recovered cells will not be affected by the selective agents, which will act on other bacteria in the sample. This is termed the overlay (OV) resuscitation method. Although the procedure is useful for enumeration of injured cells, it is cumbersome.

This paper reports the development and evaluation of a one-step thin agar layer (TAL) *(13)* method to recover heat-injured *L. monocytogenes* from 0.1% peptone water and heated milk. TAL method was compared with TSA (nonselective medium), modified Oxford medium (MOX; a

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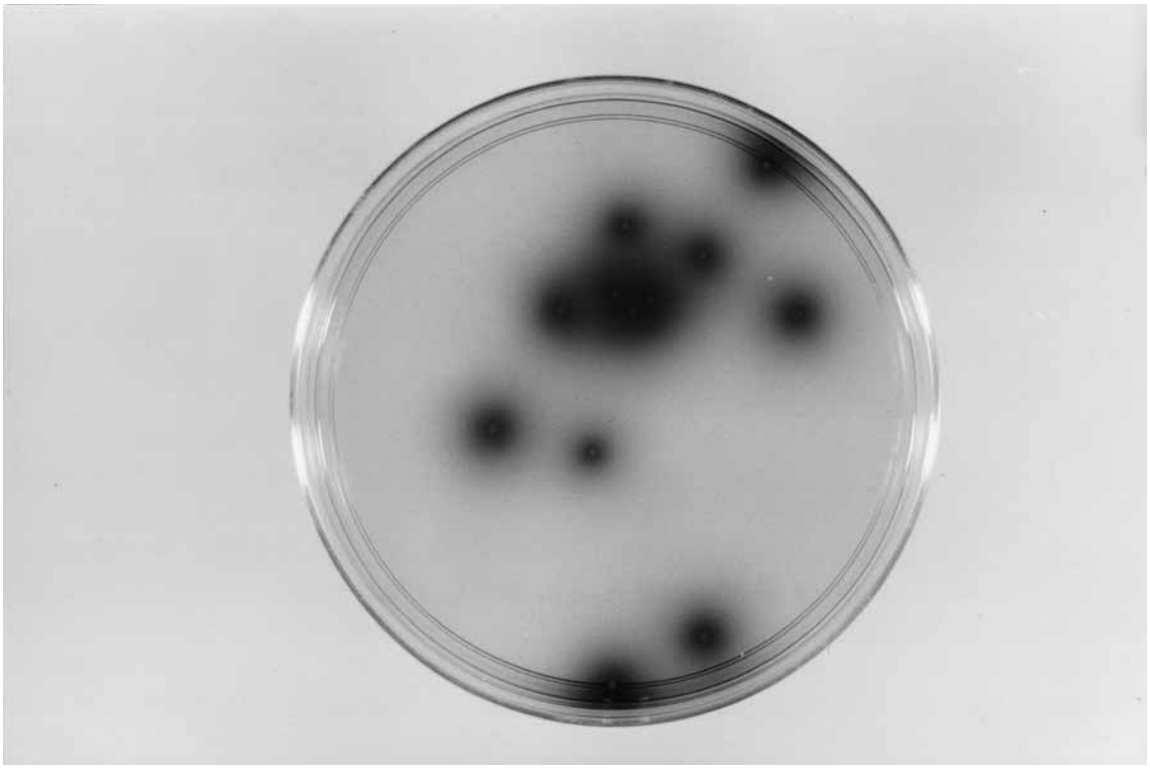

FIGURE 1. *Color development of TAL medium by* Listeria monocytogenes.

selective medium for *L. monocytogenes*), and OV methods and procedures for recovery of heat-injured *L. monocytogenes.*

MATERIALS AND METHODS

Media and development of the one-step TAL method. In order to enumerate both injured and uninjured *L. monocytogenes*, TSA (Difco, Detroit, Mich.) was used as a nonselective medium, and MOX (Oxoid, Hampshire, UK) was used as a selective medium. For the new TAL method, after solidification of sterilized MOX agar in a petri dish (8.5-cm diameter; 25 ml agar added to a height of 6 mm), 2.5 ml of melted TSA (45 to 48°C) was overlaid, and then another 2.5 ml TSA was overlaid. The top layer (2 to 3 mm) solidified in a few minutes. Heat-injured (55°C for 15 min) and uninjured *L. monocytogenes* cells were inoculated directly onto the TAL medium to allow nonselective repair and subsequent growth of recovered cells in the lower MOX-selective layer.

Two-step OV method. Sterilized TSA agar was poured into a petri dish and allowed to solidify. Then heat-injured (55°C for 15 min) *L. monocytogenes* cells were inoculated directly onto the TSA. After incubation at 37°C for 3 h (to allow for recovery of injured cells), 7 ml of MOX (45 to 48°C) was overlaid on the TSA. The plates were incubated for another 21 h, and then black colonies on the plates were counted. Uninjured cells were also plated separately as a control. This method was similar to that proposed by Hartman et al. *(11)* for evaluation of heat-injured *Escherichia coli* (using a two-step agar procedure with violet red bile).

Culture and cell suspension. *L. monocytogenes* (Scott A) obtained from the Food Microbiology Culture Collection at Kansas State University was transferred to 9.0 ml tryptic soy broth (Difco) and was incubated at 37°C for 24 h. After incubation, 1 ml of culture broth was transferred to sterilized 500-ml polycarbonate centrifuge bottles (Nalgene, Rochester, N.Y.) containing 100 ml of trypic soy broth, and the bottles were incubated at 37°C for 24 h. Cells were harvested by centrifugation (Model JA-22A, International Equipment Co., Needham Heights, Mass.) at 12,400 $\times$ *g* for 25 min at 4°C. After centrifugation, the pellet was resuspended in 0.1% peptone water. The resuspended cells were centrifuged and resuspended to be used as culture suspension. The numbers of *L. monocytogenes* were adjusted with diluent (0.1% sterile peptone water) to ca. 7.0 log CFU/ml.

Recovery of heat-injured *L. monocytogenes* from 0.1% peptone water and milk system. Fifty microliters of *L. monocytogenes* culture suspension (diluted to ca. 7.0 log CFU/ml) was added to each of three test tubes (5 ml of 0.1% peptone water/tube), which had been preheated and maintained at 55°C. After inoculation, the tube was sealed tightly, immersed completely in a shaking water bath, and heated at 55°C for 15 min. After heating, tubes were cooled immediately in slush ice, and after serial 10-fold dilutions, the contents were spiral-plated onto TSA, MOX, OV, and TAL. Plates were incubated at 37°C for 24 h. For the OV procedure, injured cells were spiral-plated on TSA and incubated at 37°C for 3 h; then 7 ml of MOX agar (45 to 48°C) was poured on top of the sample, and plates were incubated for 21 h more at 37°C. In order to study the recovery of heat-injured cells from a food system, *L. monocytogenes* was spiked into sterilized skim milk (to reach 4.0 to 5.0 log CFU/ml). The inoculated milk was heat-treated (55°C for 15 min) using the same procedures previously described. After cooling in slush ice, the milk sample was serially diluted with 0.1% peptone water and was spiral-plated on TSA, MOX, OV, and TAL media to evaluate the recovery rates with the four different media and procedures, as previously described. These experiments were performed three times.

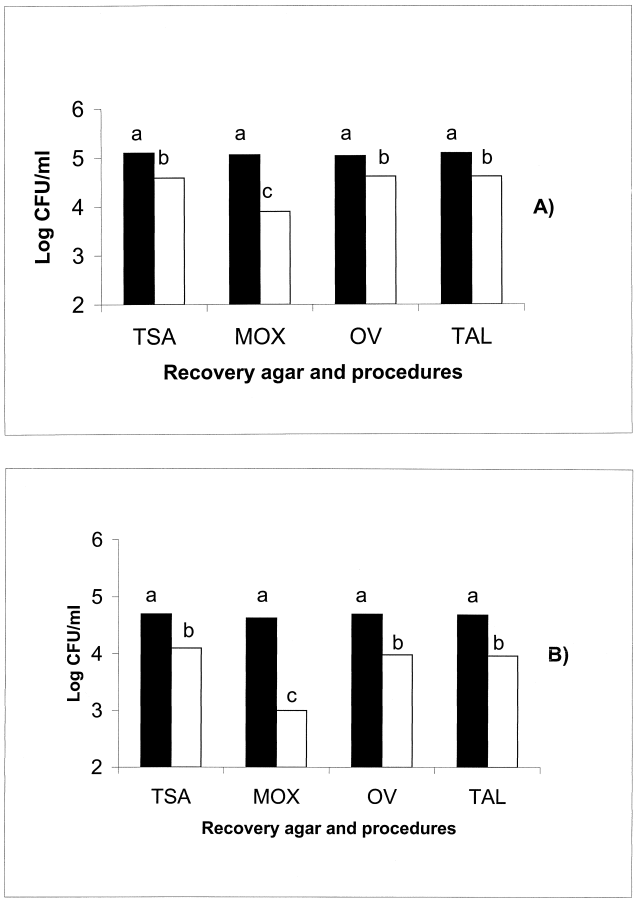

FIGURE 2. *Comparison of TSA, MOX, and OV MOX on TSA procedure (OV) and TAL medium for recovery of heat-injured (55°C for 15 min)* Listeria monocytogenes *in 0.1% peptone water (A) and in sterilized skim milk (B). Bars with different letters are different (*P *< 0.05).*

Evaluation of TAL with mixed cultures. *E. coli* (ATCC 11775), *E. coli* O157:H7 (Eh7-7), *Salmonella* Typhimurium (ATCC 23566), *Yersinia enterocolitica* (CDC 1806-96), and *Pseudomonas fluorescens* (ATCC 13525) were used to evaluate TAL selectivity. Each microorganism was incubated individually in brain heart infusion broth (BHI broth; Difco) at 37°C for 24 h. After incubation, 10 μl of each cultured broth was spotted on TAL. The spotted TAL medium was incubated at 37°C for 24 h. After incubation, the growth of each culture was monitored.

Statistical analysis. Data for each treatment and bacterial numbers were converted to $\log_{10}$ CFU/ml and analyzed statistically by analysis of variance using the SAS General Linear Models procedure *(19).* Means of three replicates were reported. Differences among treatments were examined for level of significance using Duncan's multiple range test.

RESULTS AND DISCUSSION

In the TAL plate method, the injured cells resuscitated and grew on the top agar layer of TSA and then formed black colonies, as shown in Figure 1. The diffusion of selective agents from MOX is important. During diffusion of selective agents from MOX to the top thin layer (TSA), the injured *L. monocytogenes* might be resuscitated. Once the selective agents diffused to the top agar layer, the resuscitated *L. monocytogenes* cells started to produce a typical reaction (black color), and other microorganisms were inhibited by the selective agents diffused from MOX. The selectivity of TAL was the same as that of the MOX medium. Preliminary experimentation showed that 5 ml of TSA (2- to 3-mm depth) on MOX was the optimum amount required to form a TAL to recover heat-injured *L. monocytogenes* without antimicrobial effects from MOX ingredients and to allow the injured cells to recover during diffusion. This TSA layer of 2 to 3 mm did not hinder black color production by *L. monocytogenes* on MOX. Figure 2A shows the enumeration of heat-injured *L. monocytogenes* in 0.1% peptone water using TSA, MOX, OV, and TAL media and procedures. No statistical difference occurred among TSA, OV, and TAL ($P > 0.05$), and they recovered higher numbers of heat-injured *L. monocytogenes* cells than did MOX ($P < 0.05$). This was because the sublethally injured microorganisms were sensitive to the selective agents when plated directly on MOX agar *(18).* These results indicated that the TAL medium provided a good combination to enumerate heat-injured *L. monocytogenes* with the same selectivity as afforded by the MOX medium used in the two-step OV procedure. Figure 2B shows the recovery rates of heat-injured *L. monocytogenes* from milk samples with TSA, MOX, OV, and TAL media and procedures. No statistical differences occurred among TSA, OV, and TAL ($P > 0.05$), and three recovery rates were higher than that of MOX ($P < 0.05$). Table 1 shows the growth of other microorganisms on TAL medium. All tested cultures were strongly inhibited on TAL, except *L. monocytogenes.*

TABLE 1. *Evaluated selectivity of thin agar layer (tryptic soy agar/modified Oxford medium) method*

Tested cultures	Reaction on thin agar layer	Reaction on modified Oxford medium
Escherichia coli (ATCC 11775)[a]	—[b]	—
E. coli O157:H7 (Eh7-7)	—	—
Salmonella Typhimurium (ATCC 23566)	—	—
Yersinia enterocolitica (CDC 1806-96)	—	—
Pseudomonas fluorescens (ATCC 13525)	—	—
Listeria monocytogenes (Scott A)	+++	+++

[a] ATCC, American Culture Collection; CDC, Centers for Disease Control and Prevention.
[b] —, no growth; +++, excellent growth (black color).

Cole et al. *(6)* reported that substantial repair of injured cells can occur in a nonselective medium, such as tryptic soy broth, within 1 h at 25°C. The disadvantage of this liquid method is that injured *L. monocytogenes* cells vary in terms of the time they require for repair, and, therefore, some cells may multiply before other cells recover. When accurate enumeration of cells is desired, this liquid repair method would not be appropriate. The TAL method can avoid this problem, because both injured and uninjured cells grow in TSA and then show a typical black color after incubation. Note that all four media and procedures provided similar counts ($P > 0.05$) when uninjured cells were plated (Fig. 2A).

Several studies have also been performed in order to improve techniques for recovering sublethally injured *L. monocytogenes* cells *(4, 5, 14–16).* Strict anaerobic conditions have been observed to promote recovery of heat-stressed *L. monocytogenes (14).* Busch and Donnelly *(5)* demonstrated that pyruvic acid enhanced the repair of heat-injured *L. monocytogenes,* whereas Yu and Fung *(25)* reported that several reducing agents greatly enhanced the recovery of *L. monocytogenes.* There are many reports suggesting that several agents can enhance the recovery of *L. monocytogenes.* The OV method *(11, 23)* was statistically similar to that of the nonselective medium (TSA). However, the OV method for recovery of heat-injured *L. monocytogenes* has the following limitations: (i) isolating pure colonies that grow under the selective medium is difficult; (ii) the selective medium must be kept in liquid form at 45 to 48°C until the overlay procedure; (iii) an analyst must remove plates from the incubator and pour the overlay several hours after the first plating; and (iv) the temperature of the melted selective agar (45 to 48°C) can further affect heat-injured target microorganisms that are being resuscitated on the nonselective agar. The new one-step TAL medium is convenient to prepare and use. Similar TAL systems are being tested to recover heat-injured *Salmonella,* with xylose lysone decarboxylase (XLD) as the selective agar layer, and *E. coli* O157:H7, using MacConkey Sorbital agar (SMAC) as the selective layer.

ACKNOWLEDGMENTS

This material is based upon work supported by the Cooperative State Research, Education, and Extension Service, United States Department of Agriculture, under Agreement no. 93-34211-8362. Contribution no. 98-375-J from the Kansas State Agricultural Experiment Station.

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