Research Note

Evaluation of Thin Agar Layer Method for Recovery of Acid-Injured Foodborne Pathogens†


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ABSTRACT

The thin agar layer (TAL) method of Kang and Fung was used to enumerate acid-injured foodborne pathogens. This method involves overlaying 14 ml of nonselective medium (tryptic soy agar [TSA]) onto a pre-poured and solidified pathogen-specific, selective medium in a petri dish. After surface plating, injured cells resuscitated and grew on TSA during the first few hours of incubation; then, the selective agents from the selective medium diffused to the top layer, interacted with the recovered microorganisms, and started to produce typical reactions. Foodborne pathogens were exposed to 2% acetic acid for 1, 2, or 4 min, and the recovery rate with the TAL method was compared with the rate of TSA and pathogen-specific, selective media. No significant difference occurred between TSA and TAL (P > 0.05) for enumeration of acid-injured Escherichia coli O157:H7, Salmonella Typhimurium, Staphylococcus aureus, and Yersinia enterocolitica, and both recovered significantly higher numbers than the selective medium for each respective pathogen (P < 0.05). For recovery of acid-injured Listeria monocytogenes, no difference (P > 0.05) occurred among TSA, TAL, and selective media. However, fewer cells were recovered in the selective media. The TAL method is a one-step, convenient procedure for recovery of acid-injured cells.

Foodborne infections and intoxications are increasing in both industrial and developing countries today. In the United States, 6 to 81 million people experience foodborne infections each year; however, only a fraction of cases are recognized or reported (1, 5).

Many studies have been done to develop isolation and identification methods and media for detecting foodborne pathogens. These methods are based on traditional microbiological techniques, which include enrichment, isolation, and confirmation. Enrichment techniques are time-consuming and require several days before confirmation can be made (7). Many selective media have been developed to isolate target microorganisms, as detailed in such references as the DIFCO, OXOID, and REMEL manuals. These selective media contain agents designed to select for healthy target organisms. Commonly used selective agents include organic dyes, antibiotics, bile salts, and surfactants. However, these agents may inhibit the repair of injured cells of the target organisms. After food processing (heating, freezing, drying, freeze-drying, irradiation, fermentation, or additions of antimicrobials and chemicals), one population will be killed (lethally injured), another population will survive (noninjured cells), and a third population will be injured sublethally. Nonselective agars will allow the growth of both noninjured and sublethally injured cells but cannot differentiate target pathogens from a mixed population. Selective agars, which can differentiate the target pathogens, may not allow the growth of sublethally injured cells. For this reason, many studies have attempted to improve techniques for recovery of sublethally injured cells (8).

Hartman et al. (6) and Speck et al. (12) developed a two-step overlay method to allow for the recovery of injured cells and provide subsequent differentiated enumeration. Although it is useful for enumeration of injured cells, it is quite cumbersome because of the complicated manipulations (7). Kang and Fung (7) developed the thin agar layer (TAL) method to recover heated-injured Listeria monocytogenes, Salmonella Typhimurium, and Escherichia coli O157:H7, and obtained good selectivity and recovery for all of them. However, further research is needed to evaluate TAL for more foodborne pathogens and other modes of injury induction.

The objectives of this study were (i) to apply the TAL method to recovery of acid-injured cells of five foodborne pathogens (E. coli O157:H7, L. monocytogenes, Salmonella Typhimurium, Staphylococcus aureus, and Yersinia enterocolitica), and (ii) to compare the selectivity and efficiency of the TAL method against those of commercial selective media and nonselective media.

MATERIALS AND METHODS

Culture preparation. Five foodborne pathogen species (two strains of each) were obtained from the American Type Culture Collection (ATCC; Manassas, Va.) and the Food Microbiology Culture Collection at Kansas State University: E. coli O157:H7 (ATCC 43894, ATCC 43895); L. monocytogenes (ATCC 43256, ATCC 43257); Salmonella Typhimurium (ATCC 6994, ATCC

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FIGURE 1. Comparison of TSA, 202 agar, and TAL media for recovery of acid-injured (1% acetic acid/2 min) E. coli O157: H7 ATCC 43894 in 0.1% peptone water. Bars with different letters are different (P < 0.05).

FIGURE 2. Comparison of TSA, MOX agar, and TAL media for recovery of acid-injured (2% acetic acid/4 min) L. monocytogenes ATCC 43256 in 0.1% peptone water. Bars with different letters are different (P < 0.05).

7823); S. aureus (ATCC 4012, ATCC 6341); and Y. enterocolitica (virulent serotypes O:3, and O:9). Each culture was grown in 10.0 ml tryptic soy broth (TSB) and incubated at 37°C for 24 h. All cultures were checked for purity and authenticity by Gram-stain reaction, cell morphology, and growth on selective media. Cultures were kept under refrigeration (4°C) as stock cultures and transferred weekly to maintain viability. Cell numbers were adjusted with diluent (0.1% w/v sterile peptone water; Difco Laboratories, Detroit, Mich.) to approximately 10^7 CFU/ml for all subsequent experiments (7, 8).

Media used. Tryptic soy agar (TSA; Difco) was used for the nonselective medium. The selective medium 202 agar (4) was used for E. coli O157:H7 (green colonies). Modified Oxford (MOX; Difco) agar medium was used for L. monocytogenes (black colonies). Xylose lysine desoxycholate (XLD; Difco) agar medium was used for Salmonella Typhimurium (black colonies with clear and opaque zone). Baird-Parker agar medium (BP; Difco) was used for S. aureus (black colonies). Cefsulodin-Irgasan-Novobiocin (CIN; Oxoid Limited, Hampshire, UK) was used for Y. enterocolitica (red colonies). Each culture was incubated aerobically at 37°C for 24 h.

One-step TAL method. To enumerate both injured and uninjured pathogens, TSA was used as a nonselective medium, and 202, MOX, XLD, BP, and CIN agars were used as selective media for different pathogens.

After solidification of the sterilized selective agar in a petri dish, 7 ml of melted TSA (48°C) was overlaid. After this first layer solidified for 1 min, another 7 ml of TSA was overlaid. The top layer solidified in a few minutes. Then, cells of foodborne pathogens treated with acid (to be described) were applied directly onto the TAL medium, using the spread plate method (8). Similar treated populations were applied to nonselective agar (TSA) alone and to the selective agars alone. Untreated populations were applied to all three agars as positive controls.

Acid-injury foodborne pathogens in acetic acid solution. Three 50-µl samples of cell suspension (diluted to about 7.0-log
CFU/ml) of each foodborne pathogen were added separately into three screw-cap test tubes with 5 ml of acetic acid per tube at room temperature (about 25°C). E. coli O157:H7 was opposed to 1% (v/v) acetic acid for 2 min. L. monocytogenes was opposed to 2% acetic acid for 4 min. Salmonella Typhimurium and Y. enterocolitica were opposed to 1% acetic acid for 1 min. S. aureus was opposed to 2% acetic acid for 2 min. The concentration of acetic acid and time of injury for each pathogen were determined by preliminary experiments (2, 3, 9, 10). After the acid treatment, 1-ml samples from tubes were serially diluted 10-fold with 0.1% sterile peptone water, and suspensions (0.1-ml aliquots) were spread onto nonselective medium (TSA), selective media, and TAL media as described above. Plates were incubated at 37°C for 24 h. The recovery rates for the foodborne pathogens (log CFU/ml) with the three different media and methods were evaluated.

Statistical analysis. The experiments were repeated three times (the data of each time were averaged from three tubes, resulting in a total of nine data points). Bacterial numbers were converted to log_{10} CFU/ml. Analysis of variance was performed on cell counts using the SAS General Linear Models procedure (11). Means of three replicates were plotted in graphs, and the significance of differences was determined at the 95% confidence limit. Differences among treatments were examined for level of significance by the Least Square Difference and Bonferroni (Dunn) tests.

RESULTS AND DISCUSSION

In the TAL method, the injured cells resuscitated and grew on the top layer (nonselective agar, TSA), then formed typical reaction colonies when the selective agents from the bottom layer (selective agar) diffused to the top thin layer of TSA. The injured microorganisms resuscitated during diffusion of selective agents from selective agar to top thin layer (TSA). The selective agents would inhibit other microorganisms. Kang and Fung (8) showed that 14 ml of nonselective agar (TSA, 3- to 4-mm depth) on XLD was the optimum amount to form a thin layer for recovery of
injured microorganisms (in this case, *Salmonella Typhimurium*) without antimicrobial effects from the ingredients of the selective agar and to allow the recovered cells to produce a typical reaction. This 3- to 4-mm layer of TSA did not influence the typical colors of colonies produced by target microorganisms (7).

Preliminary data on acid injury indicated that various pathogens were affected differently by the concentration of acetic acid and time of exposure. The concentrations and exposure times used produced different results among the three plating combinations.

**E. coli O157:H7.** Figure 1 shows the viable counts of acid-injured *E. coli* O157:H7 ATCC 43894 in 0.1% peptone water using TSA, 202, and TAL media. No difference (*P* > 0.05) occurred between TSA and TAL, and both recovered higher numbers of acid-injured cells than 202 (*P* < 0.05). Similar results were obtained for acid-injured *E. coli* O157:H7 ATCC 43895 (data not shown). The TAL method provided a good combination to enumerate acid-injured *E. coli* O157:H7 with selectivity similar to the 202 medium.

**L. monocytogenes.** Figure 2 shows the enumeration of acid-injured *L. monocytogenes* ATCC 43256 in 0.1% peptone water using TSA, MOX, and TAL media. No differences (*P* > 0.05) occurred among these three media and methods. Similar results were obtained for acid-injured *L. monocytogenes* ATCC 43257 (data not shown). However, the number of acid-injured cells recovered from TAL was numerically higher than these obtained from MOX. The lack of significant differences among the TSA, TAL, and MOX methods could indicate that there were not too many injured cells. The cells were alive and capable of growth on MOX, or they were dead (acid treatment was too mild or severe). A more suitable acid concentration or treatment time may be used to show better recovery with the TAL method versus direct plating.

**Salmonella Typhimurium.** Figure 3 shows the enumeration of acid-injured *Salmonella Typhimurium* ATCC 6994 in 0.1% peptone water using TSA, XLD, and TAL. No difference (*P* > 0.05) occurred between TSA and TAL, and both recovered much higher numbers of acid-injured cells than XLD (*P* < 0.05). Similar results were obtained for *Salmonella Typhimurium* ATCC 7823 (data not shown). Sublethally acid-injured *Salmonella Typhimurium* cells were very sensitive to the selective agents in XLD agar. The TAL method provided a good combination to enumerate acid-injured *Salmonella Typhimurium* with the similar selectivity XLD medium.

**S. aureus.** Figure 4 shows the viable counts of acid-injured *S. aureus* ATCC 4012 in 0.1% peptone water using TSA, BP, and TAL media. No difference (*P* < 0.05) occurred between TSA and TAL, and both recovered higher numbers of acid-injured cells than BP. Similar results were obtained for *S. aureus* ATCC 6341 (data not shown). BP agar has been considered a satisfactory selective medium for enumerating injured cells compared with other staphylococcal selective media. However, the TAL method provided a better combination to enumerate acid-injured *S. aureus* with the same selectivity as the BP medium.

**Y. enterocolitica.** Figure 5 shows the enumeration of acid-injured *Y. enterocolitica* virulent serotype O:3 in 0.1% peptone water using TSA, CIN, and TAL. No difference (*P* > 0.05) occurred between TSA and TAL, and both recovered much higher numbers of acid-injured cells than CIN (*P* < 0.05). Similar results were obtained for *Y. enterocolitica* virulent serotype O:9 (data not shown). These results indicated that the sublethally acid-injured *Y. enterocolitica* cells were sensitive to selective agents when plated directly on CIN agar. They confirm that TAL is a good method to enumerate acid-injured *Y. enterocolitica*.

The variable degrees of injury for different pathogens exposed to acetic acid are quite different from behaviors of pathogens injured by heat or cold. It suggests that acid in-
jury occurs by different mechanisms in different pathogens compared with physical treatments of heat or cold. Nevertheless, the TAL method still recorded better recovery of acid-injured pathogens compared to selective media. Application of the TAL method on mixed culture and natural flora in food systems will be performed in further studies to ascertain the usefulness of the system in the food industry.

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REFERENCES