Evaluation of the Thin Agar Layer Oxyrase® Method for Recovery of Heat-Injured Foodborne Pathogens in Liquid Media and Food Systems

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INTRODUCTION

Recovery of injured microorganisms from processed food and environmental samples has been a major concern of applied bacteriologists [1]. Injured cells are cells that survive a stress but lose some of their distinctive qualities [2]. As opposed to a dead cell, an injured cell can repair the cellular damage (resuscitation) when supplementing the restricted medium with specific nutrients and then regain its ability to form a colony in the presence of the selective agent; however, the dead cell cannot form a colony under any conditions [3]. Pathogens and spoilage organisms in foods can become injured within food products after processing and handling procedures. Injury to pathogens eliminates their ability to cause disease, but once the cells are repaired pathogenicity is totally restored [4]. Therefore, determining the presence of impaired microorganisms is important in all aspects of food processing and preservation [2].

The utility of a sterile suspension of bacterial membrane fragments and their associated enzymes as reagents for elimination of dissolved oxygen was first reported by Adler and Crow [5]. After the addition of membrane fractions, the redox potential in a medium is reduced to -200 to -300 mV becoming completely anaerobic within about a minute. The partially purified membrane fragments from Escherichia coli O157:H7, Listeria monocytogenes, Salmonella Typhimurium, and Yersinia enterocolitica individually in liquid medium. We evaluated the TALO method for recovery of the above four heat-injured (35°C, 10 min) pathogens in a mixture environment, as well as in food systems (ground beef and milk). The TALO method recovered greater numbers of pathogens (P>0.05) than selective media for four pathogens from all conditions, thus providing more sensitive detection levels for practical applications.
Oxrase® could be considered as a supplement to the resuscitation Thin Agar Layer system (non-selective agar overlaid on selective agar) [20-22] to increase the capability of recovery for injured pathogens. We have developed the Thin Agar Layer Oxrase Method (Tryptic Soy Agar with 1:30 dilution of "Oxrase® for Agar" overlaid onto a prepoured and solidified pathogen-specific, selective medium in a petri dish). This method improved the capability for recovery of heat-injured *Versinia enterococitica*, *Salmonella Typhimurium*, *L. monocytogenes*, and *E. coli* O157:H7 individually in pure culture liquid medium. The objective of this study was to evaluate and apply the Thin Agar Layer Oxrase Method (TALO) for the detection of injured foodborne pathogens from a mixture of *Y. enterococitica*, *Salmonella Typhimurium*, *L. monocytogenes*, and *E. coli* O157:H7 in liquid medium, as well as solid food using ground beef (22% fat) and liquid foods using milk samples (skim milk).

**MATERIALS AND METHODS**

**Culture preparation**

Four foodborne pathogens were used: *E. coli* O157:H7 (American Type Culture Collection, ATCC 35150), *L. monocytogenes* (ATCC 49594), *S. Typhimurium* (United States Department of Agriculture, USDA), and *Y. enterococitica* virulent serotypes O:8 (National Animal Disease Center, NADC). All cultures were checked for purity and authenticity by commercial diagnostic kits (API 20E, latex agglutination test and Crystal ID systems). Each culture was grown in 9.0 ml Tryptic Soy Broth and incubated at 37°C for 24 hrs. After incubation, 1 ml of each culture was checked for purity and authenticity by commercial systems. Each culture was grown in 9.0 ml Tryptic Soy Broth and incubated at 37°C for 24 hrs. After incubation, 1 ml of each culture was checked for purity and authenticity by commercial systems. Each culture was grown in 9.0 ml Tryptic Soy Broth and incubated at 37°C for 24 hrs. After incubation, 1 ml of each culture was checked for purity and authenticity by commercial systems. Each culture was grown in 9.0 ml Tryptic Soy Broth and incubated at 37°C for 24 hrs. After incubation, 1 ml of each culture was checked for purity and authenticity by commercial systems. Each culture was grown in 9.0 ml Tryptic Soy Broth and incubated at 37°C for 24 hrs. After incubation, 1 ml of each culture was checked for purity and authenticity by commercial systems. Each culture was grown in 9.0 ml Tryptic Soy Broth and incubated at 37°C for 24 hrs. After incubation, 1 ml of each culture was checked for purity and authenticity by commercial systems. Each culture was grown in 9.0 ml Tryptic Soy Broth and incubated at 37°C for 24 hrs. After incubation, 1 ml of each culture was checked for purity and authenticity by commercial systems. Each culture was grown in 9.0 ml Tryptic Soy Broth and incubated at 37°C for 24 hrs. After incubation, 1 ml of each culture was checked for purity and authenticity by commercial systems. Each culture was grown in 9.0 ml Tryptic Soy Broth and incubated at 37°C for 24 hrs. After incubation, 1 ml of each culture was checked for purity and authenticity by commercial systems.

**Media and recovery methods**

To enumerate both injured and uninjured pathogens, Tryptic Soy Agar (TSA, Difco, Detroit, MI) was used as a basic nonselective medium. Selective media were MacConkey Sorbitol Agar (MSA, Difco) for *E. coli* O157:H7, Modified Oxford (MOX, Difco) for *L. monocytogenes*, Xylose Lysine Deoxycholate (XLD, Difco) for *S. Typhimurium*, and Cefsulodin-Irgasan-Novobiocin (CIN, Oxoid, England) for *Y. enterococitica*. Single TAL plates were made by overlaying a total of 14 ml of nonselective medium (TSA) on a prepoured and solidified pathogen-specific, selective medium in a petri dish. One ml of the pathogen cocktail (diluted to about 7.0 log CFU/ml) was added to a screw-cap test tube containing 9 ml of 0.1% peptone water. The tubes and peptone water had been preheated and maintained at 55°C. After heating, the tubes with the pathogen suspension were removed from the water bath and cooled immediately in slush ice to room temperature (25°C, 10 min). The cap of the tube was tightly screwed, and the portion of the tube containing liquid was completely immersed in a 55°C water bath shaker and heated for 10 min [24,26,27]. A thermometer was used to measure the temperature of water in an unincubated tube to ensure accuracy of heating at 55°C. After heating, the tubes with the pathogen suspension were removed from the water bath and cooled immediately in slush ice to room temperature (25°C, 10 min). Unheated samples were used as the control. After serial 10-fold dilutions using 0.1% sterile peptone water, individual pathogen suspensions were spiral plated onto duplicate TSA, pathogen selective agar, TAL, TALO and 4-TALO media with a Spiral Plater (Spiral Biotech, Inc., Bethesda MD). Plates were incubated at 37°C for 24 hrs.

**Recovery of Heat-injured Foodborne Pathogens from a Mixture in 0.1% Peptone Water**

One ml of the pathogen cocktail (diluted to about 7.0 log CFU/ml) was added to a screw-cap test tube containing 9 ml of 0.1% peptone water. The tubes and peptone water had been preheated and maintained at 55°C. The cap of the tube was tightly screwed, and the portion of the tube containing liquid was completely immersed in a 55°C water bath shaker and heated for 10 min [24,26,27]. A thermometer was used to measure the temperature of water in an unincubated tube to ensure accuracy of heating at 55°C. After heating, the tubes with the pathogen suspension were removed from the water bath and cooled immediately in slush ice to room temperature (25°C, 10 min). Unheated samples were used as the control. After serial 10-fold dilutions using 0.1% sterile peptone water, individual pathogen suspensions were spiral plated onto duplicate TSA, pathogen selective agar, TAL, TALO and 4-TALO media with a Spiral Plater (Spiral Biotech, Inc., Bethesda MD). Plates were incubated at 37°C for 24 hrs.

**Recovery of Heat-injured Pathogens from Ground Beef**

A pathogen cocktail was made by combining four individual cultures in a sterile tube (2.5 ml of each culture). After vortexing the mixtures well, 90 g of fresh ground beef purchased from a local grocery store (22% fat) were inoculated with 10 ml of a pathogen cocktail (approximately 8 log CFU/ml) to achieve initial inoculum level of approximately 7 log CFU/g and thoroughly mixed for 2 minutes by manual massaging. The beef sample was set at room temperature for 1 hr to assist absorption of inocula. Three 25-g portions of sample were added to a sterile filter stomacher bag (Spiral Biotech, Inc., Bethesda MD) and immersed in a shaking water bath. Each 25-g sample was spread evenly in the bag to facilitate uniform heat transfer during heat treatment. Parameters for injury were set at 55°C for 10 minutes.
A thermometer was used to measure the temperature of meat (25 g) in an uninoculated stomacher bag to ensure accuracy of heating at 55°C. After heating, the samples were cooled in an ice bath until the beef had reached room temperature (ca 25°C, ca 10 min). Unheated samples served as controls. Two hundred and twenty five ml of 0.1% peptone was added to each filter stomacher bag and the samples were stomached for 2 minutes with the Stomacher (Seward Medical London, UK) [28]. After serial 10-fold dilutions using 0.1% sterile peptone water, suspensions were spiral plated onto duplicate nonselective (TSA) agar, pathogen selective agar, TAL, TALO and 4-TALO media with a Spiral Plater (Spiral Biotech, Inc., Bethesda, MD). Plates were incubated at 37°C for 24 hrs.

Recovery of Heat-injured Pathogens from Milk

Four individual cultures (250 µl of each culture) were combined into a sterile tube and vortexed. One ml of pathogen cocktail approximately 8 log CFU/ml was spiked into 99 ml of skim milk (approximately 6 log CFU/ml). The milk sample was set at room temperature for 1 hr to facilitate absorption of inocula. Three 10-ml portions of sample were heated and treated and monitored at 55°C for 10 min [23] as previously described. After cooling in slush ice, milk samples were diluted serially with 0.1% peptone water and spiral plated onto duplicate nonselective (TSA) agar, pathogen selective agar, TAL, TALO and 4-TALO media with a Spiral Plater (Spiral Biotech, Inc., Bethesda, MD). Plates were incubated at 37°C for 24 hrs. Unheated samples served as controls were spiral plated onto the same medium method as heated samples after serial dilutions.

Data analysis

The recovery capacity was compared among pathogen selective agar, TAL, TALO, and 4-TALO except TSA in liquid medium and food samples. TSA could not differentiate target pathogen from mixed flora and only provided total counts of the mixtures in the mixed culture study. The experiments were repeated three times. The percentage of increased recovery was calculated by the formula: Percent increased recovery = [(counts on recovery medium - counts on selective medium)/counts on selective medium] x 100 using actual bacterial numbers counted from the media [29-31]. Bacterial numbers were also converted to log10 CFU/ml for statistical analysis. The experiment design was a Randomized Complete Block (RCB). Analysis of variance (ANOVA) was performed on cell counts using the SAS General Linear Models (GLM) procedure with SAS software version 6.12 (SAS Institute, Cary, NC). Means of three replicates were plotted in graphs, and significant differences determined at the 95% confidence limit. Least Square Difference (LSD) test was employed to quantify significant differences among means of bacterial numbers that were transformed to log10 CFU/ml prior to analysis. Fisher’s confidence interval was calculated as:

\[ (\bar{y}_r - \bar{y}_c) \pm t \cdot s_e \]

where \( \bar{y}_r \) and \( \bar{y}_c \) are two means for pairwise comparison [32].

RESULTS AND DISCUSSION

Mixed cultures in liquid medium

According to our previous studies, the Thin Agar Layer Oxyrase® Method was an improvement over the existing Thin Agar Layer Method when evaluated for individual cultures in a liquid medium (0.1% peptone water). The TALO method recovered greater numbers of pathogens than selective media as well as the TAL method did. In addition, the TALO method had better recovery than the existing TAL method when compared with pathogen-specific selective media. In this study, *E. coli O157:H7, L. monocytogenes, S. Typhimurium, and Y. enterocolitica* were studied as a mixture and recovery capacity was compared among pathogen selective agar; TAL, TALO, and 4-TALO for detecting target pathogen form the mixed cultures.

This mixed culture study also showed that both TALO and TAL methods recovered injured cells, provided nutrients for injured organisms to grow on the top layer (nonselective agar, TSAO in the TALO method, TSA in the TAL method). Then the selective agents from the bottom layer (selective agar) diffused into TSA to form typical reaction colonies with the target microorganisms. Neither the TSAO nor the TSA layer influenced the typical colors of colonies produced by target microorganisms in the mixtures. Both TALO and TAL methods recovered greater numbers of pathogens than selective media. Moreover, the TALO method showed increased recovery than the existing TAL method when compared with pathogen-specific selective media. The TALO method was also applied to a 4-compartment petri dish (4-TALO). It showed greater recovery capability than pathogen-specific selective media and higher percent increase of recovery than the TAL method. The 4-TALO system was able to recover and distinguish, on the appropriate compartment, each individual pathogen from the 4-pathogen mixture. Moreover, it would allow simultaneous selective recovery of all four pathogens studied, improving the overall efficiency of the single-TALO and TAL systems.

For heat-injured (55°C/10 min) *E. coli O157:H7* from a mixture (*E. coli O157:H7, L. monocytogenes, S. Typhimurium, and Y. enterocolitica*) TALO (TSAO/MSA), 4-TALO (4-TSAO/MSA) and TAL (TSA/MSA) showed similar increases in recovery. The recovery capacity of MSA was increased by TALO by 365%; by 4-TALO with 346%; and by TAL with 332%. When recovered cells were analyzed in the form of logarithm (Figure 1a), TALO, 4TALO, and TAL recovered more (approximately 1 log) (P<0.05) heat-injured *E. coli O157:H7* than MSA. The 95% confidence interval was from –0.09 to 0.15 log for the difference between TALO and TAL (TALO-TAL). The TALO method did not improve the existing TAL method greatly for heat-injured *E. coli O157:H7* from the mixed cultures, indicating either method could be used as the recovery method and replace the commercial selective medium, MSA. However, considering both injured and non-injured cells, Oxyrase® in TALO would facilitate the enumeration of organisms in terms of actual cell numbers. TALO, 4-TALO and TAL distinguished *E. coli O157:H7* as clear colorless colonies.

Heat-injured *L. monocytogenes* was recovered well by TALO (TSAO/MOX) with the highest percent increase of recovery, 821% when compared with the selective medium, MOX. The second highest percent increase of recovery was 4-TALO (4-TSAO/MOX) with 786%, followed by TAL (TSA/MOX) with a 531% increase. Figure 2a shows that TALO, 4-TALO, and TAL had greater recovery of heat-injured *L. monocytogenes*, (approximately 1 log) than MOX (P<0.05). The 95% confidence interval for the
Figure 1 Comparison of TSA, selective media, TAL, TALO and 4-TALO for recovery of heat-injured (55 °C/10 min) E. coli O157:H7 from the mixed culture in a) 0.1% peptone water, b) ground beef, and c) in milk.
difference between the TALO and TAL methods was -0.18 to 0.47 log. The TAL and TALO methods differentiated *L. monocytogenes* as black colonies.

Compared with XLD, greater recovery of heat-injured *S. Typhimurium* was observed for 4-TALO (4-TSAO/XLD, 2842%), TALO (TSAO/XLD, 2636%) and then TAL (TSA/XLD, 1767%). The TALO method (TALO or 4-TALO) gave approximately 1.6 times higher recovery than the TAL method. In the analysis of log numbers (Figure 2b), all 4-TALO, TALO and TAL showed greater recovery (approximately 1-1.5 log) of heat-injured *S. Typhimurium* than XLD (P<0.05). The 95% confidence interval for the difference between TALO and TAL was -0.45 to 0.76 log. TALO, 4-TALO and TAL distinguished *S. Typhimurium* as black colonies.

The percent increase of recovery for heat-injured *Y. enterocolitica* as compared with CIN was 12218%, 5285% and 2733% for 4-TALO, TALO and TAL, respectively. The 4-TALO (4-TSAO/CIN) and TALO (TSAO/CIN) showed approximately 2 to 4.5 times greater percent increase of recovery than TAL. This indicated an improvement over the TAL method by the TALO method for heat-injured *Y. enterocolitica*. Comparison in log numbers showed a significantly greater recovery of Y. *enterocolitica* for 4-TALO, TALO and TAL (approximately 1-1.5 log) over CIN (P<0.05) (Figure 2a). The 95% confidence interval was -0.52 to 1.14 log for the difference between TALO and TAL (TALO-TAL) and TAOLO, 4-TALO, and TAL differentiated Y. *enterocolitica* as pink colonies.

Considering enumeration of both non-injured and injured pathogens, the TALO method showed increased recovery over the existing TAL method, thus providing more sensitive detection levels than the TAL method.

**Recovery of heat-injured pathogens from ground beef**

All four injured pathogens were recovered well by the TALO and TAL methods, indicating greater recovery of sublethally injured organisms than selective media. The 4-TAL system was able to recover and distinguish each individual pathogen on the appropriate compartment from the 4-pathogen mixture in ground beef.

Heat-injured (55°C/10 min) *E. coli* 0157:H7 was recovered with highest percent increase of recovery by TALO (TSAO/MSA, 290%), followed by 4-TALO (4-TSAO/MSA, 213%), and then TAL (TSA/MSA, 178%) when compared with MSA. The TALO method showed a slight improvement over the TAL method for recovery of heat-injured *E. coli* 0157:H7 cells in ground beef samples. The analysis of log numbers in (Figure 1b) indicated TALO, 4TALO, and TALO recovered better than MSA (P<0.05). The 95% confidence interval for the difference between TALO and TAL was -0.06 to 0.31 log. Compared with the selective medium MOX, heat-injured *L. monocytogenes* was recovered well by 4-TALO (TSAO/MOX) with the highest percent increase of recovery, 5424%. The second highest percent increase of recovery was TALO (TSAO/MOX) with a 4599% increase, followed by TAL (TSA/MOX) with a 4434% increase. All these methods, 4-TALO, TALO and TAL, recovered more heat-injured *L. monocytogenes* (approximately 1.3-1.8 log) from the pathogen mixture in ground beef than MOX (P<0.05) (Figure 2b). The 95% confidence interval for the difference between the TALO method and the TAL method was -0.30 to 1.71 log.

Greater percent increase of recovery of heat-injured *S. Typhimurium* as compared with XLD was observed for 4-TALO (4-TSAO/XLD) with 58233%, TALO (TSAO/XLD) had a 3993% increase and TAL (TSA/XLD) had a 3063% increase. In the analysis of log numbers (Figure 3b), 4-TALO, TALO and TAL showed greater recovery (approximately 1-1.6 log) of heat-injured *S. Typhimurium* than XLD (P<0.05) from the mixed cultures in the ground beef samples. The 95% confidence interval for the difference between the TALO method and the TAL method was -0.22 to 1.24 log.

Both 4-TALO (4-TSAO/CIN) and TALO (TSAO/CIN) showed greater recovery than TAL for heat-injured *Y. enterocolitica* when compared with the selective medium, CIN. The percent increase of recovery as compared with CIN was 1419%, 1207% and 447% for 4-TALO, TALO and TAL, respectively. This indicated that the existing TAL method was greatly improved by the addition of Oxyrase® (TALO method). Figure 2b indicated that greater (P<0.05) recovery of *Y. enterocolitica* was observed for 4-TALO, TALO and TALO over CIN (P<0.05) and both TALO methods were also statistically better than TAL.

**Recovery of heat-injured pathogens from milk**

The TALO method showed 1.1 to 5.7 times improvement over the TAL method. Moreover, when the TALO method was applied on the four-compartment petri dishes, the system was able to recover and distinguish each individual pathogen on the appropriate compartment from the 4-pathogen mixture in milk samples.

The recovery capacity of MSA was increased by TALO with 264%, followed by 4-TALO with 238% and then TAL with 224% recovery of heat-injured (55°C/10 min) *E. coli* 0157:H7 from a mixture in milk samples. In the analysis of logarithm-recovered cells (Figure 1c), TALO and 4-TALO recovered injured *E. coli* 0157:H7 better than MSA (P<0.05), while TAL was not different from MSA (P>0.05). This supports the assertion that an improvement over TAL has been made by the TALO method.

Heat-injured *L. monocytogenes* was recovered well by TALO (TSAO/MOX) with the highest percent increase (587%) of recovery over MOX. The second highest was TAL (TSA/MOX) with a 427% increase, followed by 4-TAL (4-TSAO/MOX) with a 346% increase. Figure 2c shows that TALO, 4-TALO, and TAL had greater recovery of heat-injured *L. monocytogenes* than MOX (P<0.05).

Compared with XLD, greater percent increase of recovery of heat-injured *S. Typhimurium* was observed for 4-TALO (4-TSAO/XLD, 813%), TALO (TSAO/XLD, 557%) and then TAL (TSA/XLD, 516%) (Figure 3c). In the analysis of log numbers all 4-TALO, TALO and TAL showed greater recovery (approximately 1 log) of heat-injured *S. Typhimurium* than XLD (P<0.05). The 95% confidence interval for the difference between TALO and TAL was -0.25 to 0.62 log.

Greater percent increase of recovery for heat-injured *Y. enterocolitica* as compared with CIN was obtained from TALO.
Figure 2 Comparison of TSA, selective media, TAL, TALO and 4-TALO for recovery of heat-injured (55°C/10 min) *L. monocytogenes* from the mixed culture in a) 0.1% peptone water, b) ground beef, and c) in milk.
Figure 3 Comparison of TSA, selective media, TAL, TALO and 4-TALO for recovery of heat-injured (55°C/10 min) S. Typhimurium from the mixed culture in a) 0.1% peptone water, b) ground beef, and c) in milk.
Figure 4 Comparison of TSA, selective media, TAL, TALO and 4-TALO for recovery of heat-injured (55 °C/10 min) Y. enterocolitica from the mixed culture in a) 0.1% peptone water, b) ground beef, and c) in milk.
TSAO/CIN) and 4-TALO (4-TSAO/CIN) with 3617% and 2428%, respectively (Figure 4c). The TALO method recovered more than the TAL (TSA/CIN, 639%) method. Both TALO and 4-TALO showed greater recovery of Y. enterocolitica than CIN (P<0.05) while there was no significant difference between TAL and CIN (P>0.05). The 95% confidence interval was –0.06 to 1.23 for the difference between TALO and TAL.

In this study, a Thin Agar Layer Oxyrase® Method was used to recover heat-injured foodborne pathogens. This method uses Oxyrase®, which reduces oxygen in the environment during the resuscitation of injured-cells. Oxygen may become toxic for facultative anaerobic pathogens when cells are injured. When detecting pathogens in food samples, Oxyrase® worked with the nonselective medium (TSA) to enumerate injured and non-injured anaerobes as well as facultative anaerobes, then the selective agents from the bottom layer (selective medium) diffused into TSAO to inhibit background flora but allowed the target recovered cells to produce colonies with typical reaction color. The whole system combined both sensitive enumeration and good differentiation for detecting pathogens in the food samples. Moreover, applying the TALO method in a four-compartment petri dish is at least four times more efficient than the single plate system because it enumerated four pathogens at the same time [25].

CONCLUSIONS

The Thin Agar Layer Oxyrase® Method was developed as a modification of the TAL method and has shown better percent increase of recovery than the existing TAL method, thus providing more sensitive detection than the TAL method for practical applications in mixed cultures and food samples as shown in the present study. The procedure of the TALO method is also less cumbersome and complicated compared to the traditional two-step overlay method, which requires a long wait between each manipulation. The improvement for the TALO methods has been demonstrated to be more effective for the detection of both non-injured and injured pathogens and is more rapid and reliable than the traditional methods. Moreover, applying the TALO method with a four-compartment plate allows simultaneous selective recovery of all four pathogens studied and is more efficient than the single plate system, considering the efficiency of operation and savings in material, space and labor.

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