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Research Note

Comparison of Recovery of Airborne Microorganisms in a Dairy Cattle Facility Using Selective Agar and Thin Agar Layer Resuscitation Media†

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ABSTRACT

Thin agar layer (TAL) medium was developed at Kansas State University to improve the resuscitation of injured cells and has been shown to result in higher recovery than is obtained with selective media alone for cold-, heat-, salt-, and acid-injured cells. The experiment presented here was designed to determine the effectiveness of the TAL method for the recovery of possibly injured organisms from air. Eleven agar media were used for the experiment: tryptic soy agar (TSA), MacConkey sorbitol agar (MSA), TAL-MSA, Baird-Parker (BP) agar, TAL-BP agar, modified Oxford (MOX) agar, TAL-MOX agar, xylose lysine sodium desoxycholate (XLD) agar, TAL-XLD agar, *Yersinia*-selective (CIN) agar, and TAL-CIN agar. The TAL plates were prepared by pipetting 6 ml of selective agar into a BBL Rodac plate (65 by 15 mm). Selective agar was allowed to solidify, and then each plate was overlaid with 6 ml of TSA. Selective agar plates were prepared by pipetting 12 ml of agar into BBL Rodac plates and allowing the agar to solidify. Samples were taken at an indoor cattle facility at five separate locations with a BioScience SAS air-sampling instrument. For each plate, 60 liters of air was sampled. Three replications of the experiment were performed. The TAL method resulted in higher counts of microorganisms on all media tested. In addition, 175 isolates were selected randomly and identified in order to test the selectivity of TAL and the selective media for target organisms. The data obtained in this study show that the TAL resuscitation method is effective and necessary for the recovery of airborne organisms that may be injured.

Many different organisms are carried by dust (which can hold microorganisms and toxins *(2)*) and can be found in air. The type and amount of bacteria present in the air can be affected by environmental factors such as temperature, dust particle size, humidity, and air speed. Air sampled in close proximity to sewage treatment facilities or animal farms can support a reasonably diverse microbiota. The ability of organisms to survive drying stress is an important factor, especially for organisms such as gram-positive bacilli that are capable of producing spores, since they are more resistant to drying than are organisms without spores *(9, 16)*.

Many airborne microorganisms can cause infections and allergic reactions, depending on the type of bacteria and the susceptibility of an individual. Also, these bacteria can produce toxins that can cause toxicosis *(16)*. The numbers of organisms in the air has been studied because of increased concern about air quality. However, there are few data regarding the recovery of airborne organisms that may be injured.

When microorganisms are treated with acid, heat, cold, or chemicals, they may be killed (lethally injured), sublethally injured, or not injured. Noninjured and sublethally injured cells can grow in nonselective media (liquid or solid). However, sublethally injured cells may not be able to grow in selective agar as a result of additional stress caused by the selective agars. For this reason, the numbers of surviving microorganisms after treatment of foods may be underreported. It is possible for injured cells to recover when removed from the treatment environment. For these reasons, a resuscitation medium must be employed to recover sublethally injured cells *(1, 3, 4)*. One method that is commonly used is the overlay method *(8)*. This procedure involves plating the sample on a layer of nonselective medium such as tryptic soy agar (TSA), incubating it for about 3 h, and then overlaying it with a tempered (48°C) selective medium such as violet red bile agar. The plates are incubated at the appropriate temperature for 24 h, and then cells are counted and recorded *(14)*. This method has been effective but involves waiting to overlay the plates.

A recently developed method called the thin agar layer (TAL) method eliminates the overlay waiting period, allowing more efficient use of experimentation time. This method involves pouring a layer of selective medium, overlaying this layer with a nonselective medium, and then plating the sample on top of the nonselective layer and incubating it normally *(11)*. Injured cells then have a chance to resuscitate on the nonselective medium prior to the diffusion of selective agents into the nonselective agar.

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The objectives of this study were to compare the effectiveness of each of five different selective agars with that of the TAL version of that agar in recovering organisms from air and to identify the resulting isolates to determine the microflora of the air in a dairy cattle facility.

MATERIALS AND METHODS

In this experiment, TSA (Becton Dickinson, Sparks, Md.) was used for the recovery of the total count, MacConkey sorbitol agar (MSA; Becton Dickinson) and TAL-MSA were used for the recovery of *Escherichia coli* and coliforms, Baird-Parker (BP) agar (Becton Dickinson) and TAL-BP agar were used for the recovery of staphylococci, modified Oxford (MOX) agar (Becton Dickinson) and TAL-MOX agar were used for the recovery of *Listeria monocytogenes,* xylose lysine desoxycholate (XLD) agar (Becton Dickinson) and TAL-XLD agar were used for the recovery of gram-negative enteric bacilli, and *Yersinia*-selective (CIN) agar (Oxoid, Inc., Ontario, Canada) and TAL-CIN agar were used for the recovery of *Yersinia enterocolitica.*

Selective agar. Although the specific organisms listed are considered the ''target organisms,'' other bacteria may grow on the media if they can withstand the selective agents in the agar recipe. BP medium is prepared with egg yolk tellurite, contains lithium chloride, and is commonly used to isolate *Staphylococcus,* but other organisms, such as *Bacillus* and *Corynebacterium,* can reduce potassium tellurite and grow on the medium. The CIN agar is supplemented with cefsulodin, irgasan, and novobiocin and is very selective for *Yersinia.* However, it is possible that other genera, such as *Shigella,* may grow on the medium as well. MSA is commonly used to differentiate between enteropathogenic *E. coli* strains, but although most gram-positive organisms are inhibited by bile salts, other gram-negative organisms are able to grow. The MOX medium is supplemented with colistin sulfate and moxalactam and is used primarily for the isolation of *L. monocytogenes.* However, *Bacillus* and *Corynebacterium* are also capable of growing and hydrolyzing esculin to give the appearance of *Listeria.* The XLD agar is commonly used for the isolation of enteric organisms and can support the growth of *Enterococcus, Escherichia, Salmonella, Shigella,* and *Providencia* and other gram-negative organisms *(5).*

Plate preparation. The agar sampling plates used for the experiment were prepared with BBL Rodac plates (65 by 15 mm; Becton Dickinson) and the following media: TSA, MSA, BP agar, MOX agar, XLD agar, and CIN agar. Rodac plates were used because theirs is the plate size most commonly used in air-sampling procedures. Selective agar plates were prepared by pipetting 12 ml of sterilized tempered agar into each plate and allowing the agar to solidify. For TAL plates of each selective agar, 6 ml of a selective medium was pipetted into each Rodac plate and allowed to solidify. Each plate was then overlaid with 6 ml of TSA, which was allowed to solidify. Research by Hajmeer et al. *(7)* showed that the preparation of TAL plates 0, 1, and 7 days in advance of use did not statistically significantly affect injured cell resuscitation for *E. coli* O157:H7. For this experiment, all plates were used within 24 h of preparation for practical reasons.

Microbial counts. Samples were taken on three different days at an indoor cattle facility. The relative humidity, temperature, wind speed, and wind direction based on information from the state climatologist (Weather Data Library, Kansas State Research and Extension, Kansas State University, Manhattan, Kans.) were recorded for each day of sampling. This information is provided as a supplement for greater insight into the climactic con-

TABLE 1. *Average temperature, relative humidity, wind speed, wind direction, and plate counts for each day of sample collection*

Date	Temp (°C)	Relative humidity (%)	Wind speed (kph[a])	Wind direction[b]	Plate count (CFU/m³) for medium										
					TSA	TAL-BP	BP	TAL-CIN	CIN	TAL-MSA	MSA	TAL-MOX	MOX	TAL-XLD	XLD
12 January 2001	10.5	56	5.6	NE	530	937	573	77	0	3	10	630	153	7	0
16 January 2001	−1.7	75	18.5	NE	963	837	630	147	13	187	43	817	337	90	0.4
19 January 2001	−7.8	57	20.5	N	1,213	893	557	120	20	63	20	853	400	73	0.6
SD	9.3	10.7	8.1		345.6	50.1	38.4	35.3	10.1	93.8	16.9	119.7	128.3	43.8	0.3

[a] kph, kilometers per hour.
[b] NE, northeast; N, north.

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TABLE 2. *Identification of bacteria on TAL media*

	% of samples positive for bacteria in medium[a]				
	TAL-BP	TAL-CIN	TAL-MSA	TAL-MOX	TAL-XLD
Aerococcus	18.9 (7/37)	5.6 (1/18)	5.9 (1/17)	10 (3/10)	8.3 (1/12)
Acinetobacter	—	5.6 (1/18)	11.7 (2/17)	—	8.3 (1/12)
Alloiococcus	—	—	—	3.3 (1/30)	—
Bacillus	2.7 (1/37)	—	—	36.7 (11/30)	—
Chryseomonas	—	—	—	—	8.3 (1/12)
Corynebacterium	13.5 (5/37)	5.6 (1/18)	5.9 (1/17)	20 (6/30)	25 (3/12)
Enterobacter	2.7 (1/37)	22.2 (4/18)	5.9 (1/17)	—	8.3 (1/12)
Gardenella	2.7 (1/37)	—	—	—	—
Klebsiella	—	33.3 (6/18)	5.9 (1/17)	—	16.7 (2/16)
Leuconostoc	2.7 (1/37)	5.6 (1/18)	—	—	—
Micrococcus	8.1 (3/37)	5.6 (1/18)	11.7 (2/17)	10 (3/30)	—
Nonculturable	—	—	—	3.3 (1/30)	8.3 (1/12)
Oerskovia	—	—	—	3.3 (1/30)	—
Pseudomonas	—	—	5.9 (1/17)	—	—
Shigella	—	16.7 (3/18)	29.4 (5/17)	—	16.7 (2/16)
Staphylococcus	45.9 (17/37)	—	—	—	—
Stomatococcus	—	—	5.9 (1/17)	—	—
Streptococcus	2.7 (1/37)	—	11.7 (2/17)	10 (3/30)	—

[a] Number of positive samples/total number of samples shown in parentheses.

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ditions at the time of the experiment. The air samples were taken about 1.07 m from the floor with a SAS-Super100 air-sampling instrument (BioScience International, Rockville, Md.). All plates were exposed to 60 liters of air deposited in 20 s. After sampling, all plates were incubated at 37°C for 24 h. After 24 h, the TSA plates were removed, and all other media were allowed an additional 24 h of incubation for cell growth and resuscitation. For each location and sampling time, counts were recorded. In addition to a total count for each agar plate, a "visually typical" count was carried out to record the appearance of organisms that looked like the target organism for each agar as described by the manufacturer of the agar. The visually typical colony appearance for each medium is as follows: BP agar and TAL-BP agar, black or very dark with halos; CIN agar and TAL-CIN agar, small and red; MSA and TAL-MSA, pink-red or clear; MOX agar and TAL-MOX agar, dark with black halos; XLD agar and TAL-XLD agar, black. Within the facility, five sampling locations were chosen. All 11 media were tested at each location and were used in random order.

Isolate identification. A total of 112 microbial isolates were selected from TAL plates and identified. All isolates were Gram stained with the 3-Step Gram Staining method (Becton Dickinson). Gram-negative colonies were identified with API 20E (bioMerieux, Inc., Hazelwood, Mo.) and BBL Enterotube (Becton Dickinson) test kits. API 20E is a rapid microbiological detection method that can show results of 23 biochemical reactions for the identification of *Enterobacteriaceae* and other nonfastidious gram-negative rods. Gram-positive colonies were identified with BBL Crystal Gram-Positive test kits. BBL Crystal Gram-Positive tests use 29 biochemical and enzymatic reactions for the identification of aerobic gram-positive bacteria.

Statistical analysis. Statistical analysis of the data was performed with the PROC general linear model and nonparametric sign tests. The PROC general linear model was implemented with the SAS system *(15)*. The significance level used was $P \leq 0.05$. The experiment was repeated three times in its entirety.

TABLE 3. *Identification of bacteria on selective media*

	% of samples positive for bacteria in medium[a]				
	BP	CIN	MSA	MOX	XLD
Aerococcus	5.6 (1/18)	—	—	—	—
Alloiococcus	—	—	—	14.3 (4/28)	—
Bacillus	—	—	—	39.3 (11/28)	—
Citrobacter	—	—	—	—	50 (1/2)
Corynebacterium	33.3 (6/18)	—	18.2 (2/11)	28.6 (8/28)	—
Enterobacter	—	50 (1/2)	—	—	—
Klebsiella	—	50 (1/2)	—	—	—
Lactococcus	16.7 (3/18)	—	—	—	—
Micrococcus	—	—	9.1 (2/11)	3.6 (1/28)	—
Nonculturable	—	—	—	3.6 (1/28)	—
Oerskovia	—	—	—	3.6 (1/28)	—
Pseudomonas	—	—	18.2 (2/11)	—	50 (1/2)
Rhodococcus	—	—	27.3 (3/11)	—	—
Shigella	—	—	27.3 (3/11)	—	—
Staphylococcus	33.3 (6/18)	—	—	—	—
Streptococcus	11.1 (2/18)	—	—	7.1 (2/28)	—

[a]Number of positive samples/total number of samples shown in parentheses.

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RESULTS

The temperature, relative humidity, wind speed, and wind direction, in addition to average plate counts for each medium, were recorded for each day of sampling. The indoor facility at which the samples were collected runs east and west, with an open garage door facing east. In general, TSA recovered more organisms; however, with the other media, the counts varied, with a clear trend of TAL plates recovering higher counts than the selective media (Table 1).

TAL-BP agar. TAL-BP agar recovered higher total counts ($P \leq 0.0024$) than did BP agar. TAL-BP agar also recovered higher counts ($P \leq 0.0007$) of visually typical colonies than did BP agar alone. A total of 37 isolates were randomly selected from the TAL-BP plates for identification (Table 2). For comparison with the selective agar, 18 isolates were randomly selected from BP plates and identified (Table 3).

TAL-CIN agar. TAL-CIN agar recovered higher counts ($P \leq 0.0027$) than did CIN agar alone. The TAL-CIN plates also recovered higher counts ($P \leq 0.0050$) of visually typical colonies. A total of 18 isolates were randomly selected from the TAL-CIN plates for identification (Table 2). For comparison with the selective agar, 2 isolates were randomly selected from CIN plates and identified (Table 3).

TAL-MSA. TAL-MSA recovered higher counts ($P \leq 0.0433$) than did MSA agar alone. The TAL-MSA plates also recovered higher counts of visually typical colonies ($P \leq 0.0112$). A total of 17 isolates were randomly selected from the TAL-MSA plates for identification (Table 2). For comparison with the selective agar, 11 isolates were randomly selected from MSA plates and identified (Table 3).

TAL-MOX agar. TAL-MOX agar recovered higher counts ($P < 0.0001$) than did MOX agar alone. The TAL-MOX plates also recovered higher counts ($P \leq 0.0080$) of visually typical colonies. A total of 30 isolates were randomly selected from the TAL-MOX plates for identification (Table 2). For comparison with the selective agar, 28 isolates were randomly selected from MOX plates and identified (Table 3).

TAL-XLD agar. TAL-XLD agar recovered higher counts ($P \leq 0.0128$) than did XLD agar alone. No visually

typical black colonies were observed on either medium. A total of 12 isolates were randomly selected from the TAL-XLD plates for identification (Table 2). For comparison with the selective agar, 2 isolates were randomly selected from XLD plates and identified (Table 3).

DISCUSSION

For all comparisons the TAL media recovered much higher counts than did selective media used alone. Although organisms other than the target organism were identified on TAL, it must be noted that the selective agar used alone also recovered nontarget organisms. Previous work has been carried out by Kang and Fung *(11, 12)* to evaluate the effectiveness of TAL for the recovery of heat-injured cells (55°C for 15 min). These researchers found that the TAL method resulted in significantly higher counts of heat-injured *L. monocytogenes* and *Salmonella* Typhimurium than did the selective media used alone (MOX agar and XLD agar, respectively).

In another study, the effectiveness of the TAL method for the recovery of salt (NaCl)–injured bacteria was compared with that of selective media used alone, and the results showed that the TAL method recovers larger numbers of pathogens than do selective media used alone *(6).* The results from these studies also support previous work in reporting that the TAL method recovered significantly higher counts than did selective media used alone.

Furthermore, the bacteria identified from the agar plates are consistent with organisms found in milk and beef. Common bacterial contaminants of cow's milk that were identified in this experiment are *Enterococcus, Lactococcus, Streptococcus, Lactobacillus, Oerskovia, Micrococcus,* and *Bacillus.* Many of the bacteria isolated, such as *Micrococcus, Enterococcus,* and *Staphylococcus,* are also common meat contaminants resulting from meat fabrication *(13).* In addition, soil and water are primary sources of *Acinetobacter, Aeromonas, Corynebacterium,* and *Pseudomonas,* making them good candidates for airborne contaminants. *Bacillus, Corynebacterium,* and *Micrococcus* are considered common organisms found in air and dust *(10).*

On the basis of our findings and previous work, the results from this experiment are significant, and they show that the TAL method of resuscitation should be used for the recovery of airborne organisms that may be injured. There are many organisms in air, and the use of selective media alone may result in underestimation and inaccurate counts of airborne bacteria.

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