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Discrepancies in the Enumeration of *Escherichia coli*[1]

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Stationary-phase cells of *Escherichia coli* were enumerated by the pour plate method on Trypticase soy agar containing 0.3% yeast extract (TSYA), violet red-bile agar, and desoxycholate-lactose agar, and by the most-probable-number method in Brilliant Green-bile broth and lauryl sulfate broth. Maximum counts were assumed to be those on TSYA. In general, numbers detected were lower with the selective solid media and higher with the selective liquid media. Inhibitory effects, especially on selective solid media varied with the strains of *E. coli*. The lower detection on selective solid media was partly due to the stress induced in some cells by the temperature of the melted media used in the pour plate method. These cells apparently failed to repair and form colonies in the selective media. Improved detection on the selective solid media was achieved by using 1% nonfat milk solids, 1% peptone, or 1% $MgSO_4 \cdot 7H_2O$ in the dilution blanks. Higher detection on selective agar media was effected by surface plating or by surface-overlay plating of the cells. The surface-overlay method appeared to be superior for the direct enumeration of *E. coli* in foods.

Present recommended procedures for the enumeration of coliform bacteria in foods include the pour plate method with violet red-bile agar (VRBA) and desoxycholate-lactose agar (DLA) and the most-probable-number (MPN) method with Brilliant Green-bile broth (BGB) and lauryl sulfate broth (LSB) (1, 2, 5). Due to the greater degree of variability observed with the liquid media, in recent years many workers have preferred pour plating with the selective media for the enumeration of coliforms from foods (7-10). Recovery of *Escherichia coli* from inoculated foods by the pour plate method with VRBA has been reported to give variable results (4, 5). In our laboratory, while studying the enumeration of sublethally stressed *E. coli* cells, we observed that by the pour plate method unstressed cells showed restricted colony formation on selective solid media. In this work we report the results of our studies on causes of low recovery of *E. coli* cells by selective solid media. A preliminary report of these findings was presented previously (B. Ray, R. Swank, and M. L. Speck, Abst. Annu. Meeting Amer. Soc. Microbiol., 23-28 April 1972, p. 16).

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MATERIALS AND METHODS

Organisms. *Escherichia coli* strains NCSM, ML 30, B/r/1, D, 451B, and K-12 were obtained from the department stock collection. The cultures were transferred weekly and maintained in slants at 4 C on Trypticase soy agar containing 0.3% yeast extract (TSYA). *E. coli* from a slant culture was inoculated into Trypticase soy broth containing 0.3% yeast extract (TSYB) and grown for 14 to 18 h (stationary phase) at 35 C and used for enumeration.

Enumeration methods. Colony counts were made by using TSYA, VRBA, and DLA. BGB and LSB were used for the most-probable-number (MPN) estimations. All media were obtained from BBL. VRBA and DLA were prepared by steaming for 30 min, and TSYA, BGB, and LSB were autoclaved. For the pour plate method, after steaming the solid media were stored in 100-ml portions for no longer than 7 days. Plates for surface plating were poured with 12 to 15 ml of medium per plate and dried at room temperature for 48 h before use. These plates were stored at about 20 C and used within 4 days.

Cultures grown in TSYB were diluted serially in phosphate buffer (1), and 0.1 ml was plated in triplicate on each plating medium. The sample in the pour plate procedure was mixed with 10 ml of the tempered (45 ± 1 C) medium. In the surface plate method, the sample was measured onto the surface of a prepoured plate and distributed with a sterile glass hockey stick. A duplicate set of such plates was overlayed 10 min after surface plating with an

additional 4 ml of the same medium, and designated as "surface-overlay." The plates were incubated at 35 C for 24 h, and the colonies were counted.

A total of five tubes in each of three successive decimal dilutions were used for each medium in the MPN procedure. A 1-ml amount of the appropriate serially diluted sample was inoculated into a tube containing 9 ml of medium. The tubes were incubated at 35 C for 48 h. MPN was estimated based on the tubes showing gas production (2).

Enumeration of E. coli from food. Reconstituted nonfat dry milk (NFDM, 10% solids), meat broth (5% solids, prepared by boiling chopped veal and then filtering through cheese cloth), crab meat (1 part suspended in 9 parts of water and blended), and liquid whole egg were used. All other samples except liquid whole egg were autoclaved (121 C for 15 min). Liquid whole egg was taken aseptically from clean eggs and checked for bacteriological quality. Samples giving no more than 10 colonies/ml on TSYA and none on VRBA after 24 h at 35 C were used. *E. coli* K-12 cells from the stationary phase were added to these foods at a concentration of 1.5×10^3 to 2.0×10^3 cells/ml. A 0.1-ml portion of the food samples was used per plate for plate counts. For MPN, the respective food material was used to obtain the three requisite dilutions, and 1 ml was added to each tube containing 9 ml of the liquid media.

The effect of dilution of food materials on *E. coli* detection was studied by diluting food materials to several successive decimal dilutions with sterile water and inoculating these with about the same number of cells. A 0.1-ml amount from each sample was plated as above for enumeration with the solid media.

RESULTS

Enumeration of E. coli by solid and liquid media. Detection of six strains of *E. coli* on VRBA and DLA by the pour plate method and in BGB and LSB by the MPN method, and comparison with counts on TSYA are presented in Table 1. The VRBA and DLA yielded lower counts, whereas MPN estimates often were higher. Detection on DLA was lower than that on VRBA for all strains. However, there was considerable variation in resistance among strains to VRBA and DLA as determined from the differences in enumeration. Individual experiments gave wide variations in the estimates by the liquid media. For example, recovery ranges for strain NCSM were 77 to 101% on VRBA, 39 to 95% on DLA, 75 to 164% on BGB, and 26 to 270% on LSB (data not presented).

Effect of plating method on the detection of E. coli on solid media. The relatively sensitive strain K-12 was chosen for further study. Colony counts were made by using the three plating methods on TSYA, VRBA, and DLA. All platings were done at room temperature (25 C). The results (Table 2) indicated that the surface and surface-overlay methods detected much higher percentages of K-12 cells on VRBA and DLA. Detection on TSYA by the plating methods showed only slight differences. Studies with other sensitive strains gave similar results.

These data led us to suspect that manipulations in the pour plate method were contributing factors for the lower detection on VRBA and DLA. The temperature of the medium used for pour plating (45 ± 1 C) was one of the main differences in the three methods. Strain K-12 was tested by the three previously described plating methods. In the pour plate method, media tempered to 45 ± 1 C was used. Prepoured plates for use in the surface and surface-overlay methods were tempered at 25 C or at 45 C for 6 h before plating. The sample was added to the tempered plates while in the incubator, spread on the surface, removed to room temperature (25 C) within 1 to 2 min, and kept for 25 to 30 min before incubating at 35 C for 24 h. The medium for overlay in the surface-overlay method was added after the plates were at room temperature for about 20 min. The results (Table 3) showed that the

TABLE 1. *Enumeration of six strains of E. coli with solid and liquid media*

E. coli strain	Percent[a] cells detected by				
	Pour plate counts			MPN estimates	
	TSYA	VRBA	DLA	BGB	LSB
NCSM	100	94	72	98	125
B/r/1	100	71	51	135	121
ML 30	100	69	44	86	112
451B	100	44	6	107	104
D	100	28	26	83	190
K-12	100	33	4	110	54

[a] As percentage of counts on TSYA for each strain. Results are the average of three trials.

TABLE 2. *Enumeration of E. coli K-12 on selective and nonselective media by different methods of plating*

Plating method	Percent[a] cells detected on		
	TSYA	VRBA	DLA
Pour	100	38	15
Surface	103	70	59
Surface-overlay	98	70	43

[a] As percentage of counts on TSYA pour plates. Results are the average of four trials.

TABLE 3. *Effect of temperature of medium during plating on the detection of E. coli K-12*

Plating method	Plating temp (C)	Percent[a] cells detected on		
		TSYA	VRBA	DLA
Pour	45	100	27	4
Surface	25	107	55	46
	45	104	26	6
Surface-overlay	25	98	75	55
	45	104	34	11

[a] As percentage of counts on TSYA pour plates. Results are the average of four trials.

elevated temperature of media during plating lowered the detection of viable cells on VRBA and DLA. Detection by the surface and surface-overlay methods was lower at 45 C than at 25 C with VRBA AND DLA. Counts on TSYA were not affected by differences in temperature of the medium or plating method.

Exposure of the *E. coli* cells to the temperature of melted medium (45 C) was suspected of causing stress in the cells, such that colonies failed to form on VRBA and DLA, but not on TSYA. This was tested by using diluents at 25 and 45 C for serial dilution of the sample. Even 1 to 2 min of exposure at 45 C during dilution reduced the detection of strain K-12 from 36% to 8% on VRBA by surface plating at 25 C. Similar results were obtained on DLA but not on TSYA (data not presented).

Pour plate method on detection of E. coli. Several inherent variables of the pour plate method which might affect the efficiency of *E. coli* detection on VRBA and DLA were examined. The volume of medium used to mix with the sample was tested using volumes of 5 to 20 ml/plate. An additional 4 ml of the respective medium was overlaid in each plate. The results (Fig. 1) showed that, although the colony counts on TSYA remained essentially the same, counts on VRBA and DLA showed a reduction with the increase in medium volume. Similarly, an increase of about 3 C of the temperature of the medium during pouring (48 versus 45 C) reduced the recovery on VRBA from 40 to 18% (Table 4). When the sample volume was changed from 0.1 ml to 1.0 ml per plate (using 10 ml of medium/plate in all instances), higher detection was observed in plates with 1.0-ml samples with both selective media (data not presented). These variables probably produce differences in the time during which cells are exposed to sublethal temperature and thus cause differences in stress to the cells before they are permitted to form colonies on the particular plating medium.

Effect of the composition of diluents on the detection of E. coli. Sterile glass distilled water, and different concentrations of NFDM, peptone, $MgSO_4 \cdot 7H_2O$, and K_2HPO_4 were used as diluents for the enumeration of *E. coli* K-12 on TSYA, VRBA, and DLA by pour and surface-overlay plating methods (Table 5). The pH of the blanks was adjusted 7.0 to 7.3 (except water). Maximum detection on VRBA and DLA was obtained by using 1% peptone. NFDM and $MgSO_4 \cdot 7H_2O$ at 1% concentration were next best. All of the diluents at both concentrations had beneficial effects.

Effect of the food materials on the enumeration of E. coli. Food materials containing *E. coli* K-12 were plated on TSYA, VRBA, and DLA for enumeration by the pour and surface-overlay methods and inoculated into tubes containing BGB and LSB for MPN

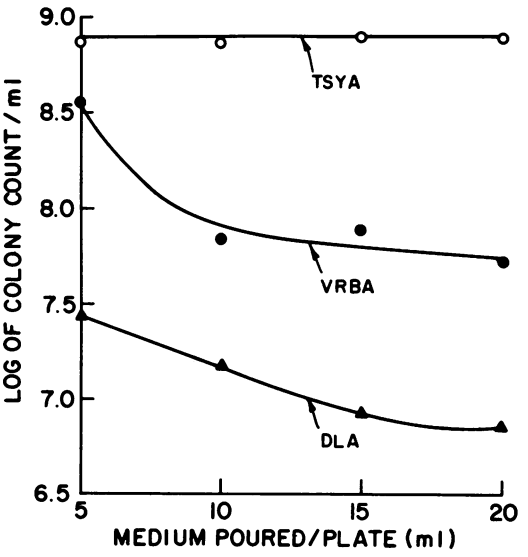

FIG. 1. *Effect of volume of medium per plate used to mix 0.1 ml of samples in pour plate method on the detection of E. coli K-12.*

TABLE 4. *Effect of temperature of plating media during pouring on the detection of E. coli K-12*

Temp of media (C)	Percent[a] cells detected on		
	TSYA	VRBA	DLA
45	100	40	5
48	98	18	3

[a] As percentage of counts on TSYA at 45 C. Results are the average of three trials.

estimates. The results (Table 6) showed that, in general, detection on solid media was lower but varied within a much narrower range between media than the estimates obtained by the MPN method. Surface-overlay did not show any advantage over the pour plate method. Similar observations were made also with surface plating.

Since the enumeration of higher populations of coliforms would result in the addition of smaller amounts of the food sample to the different media, the effects of such dilution were measured. Sterile reconstituted NFDM (10% solids) was diluted decimally four times with sterile water; each dilution was inoculated with approximately the same number of *E. coli* K-12 cells (about 10^3 cells/ml). A 0.1-ml portion of the sample was added to each plate and enumerated by pour and surface-overlay plate methods with TSYA, VRBA, and DLA. As the dilution factor increased, fewer cells were detected on VRBA and DLA by either method (Table 7). In any particular dilution more cells were detected by the surface-overlay method with VRBA and DLA.

DISCUSSION

The results of this study showed that *E. coli* cells may be stressed by numerous factors that complicate their accurate detection and enumeration with selective media. The selective agents, in concentrations present in VRBA and DLA, have inhibitory effects on the colony-forming ability of *E. coli*; the degree of inhibition varies with strains. The greater inhibition of DLA was more pronounced when the cells were enumerated by the pour plate rather than surface and surface-overlay methods. Availability of oxygen (6) was not the cause for the differences in detection on VRBA and DLA, as counts on TSYA remained essentially the same in all three plating methods. Also media freshly prepared and that which had been stored and reheated did not cause any difference in detection by the pour plate method.

Exposure to the customary temperature of tempered media used in the pour plate technique imposes sublethal and reversible stress to many cells in the population. In a nonselective solid medium, such as on TSYA, the stressed cells probably can repair and subsequently form colonies; in selective media, such as VRBA and DLA, repair is inhibited and such cells remain undetected. Inability of the freeze-

TABLE 5. *Effect of dilution blanks on the detection of E. coli K-12 by pour plate method.*

Dilution blanks	Percent[a] cells detected on		
	TSYA	VRBA	DLA
Water	100	11	0.7
NFDM, 1.0%	98	68	37
NFDM, 0.1%	100	35	14
Peptone, 1.0%	109	77	46
Peptone, 0.1%	106	53	5
K_2HPO_4, 1.0%	106	41	1.3
K_2HPO_4, 0.004%[b]	100	35	1.5
$MgSO_4 \cdot 7H_2O$, 1.0%	100	65	20
$MgSO_4 \cdot 7H_2O$, 0.1%	103	41	1.5

[a] As percentage of counts on TSYA with water as dilution blanks. Results are the average of three trials.

[b] Phosphate buffer used in reference 2.

TABLE 6. *Effect of the presence of food materials on the enumeration of E. coli K-12 by different procedures*

Detection method	Media	Percent[a] cells detected from			
		Milk (10% NFDM)	Meat broth (5% solids)	Liquid whole egg	Crab meat
Pour plating	TSYA	100	100	100	100
	VRBA	98	85	79	90
	DLA	95	76	75	76
Surface-overlay	TSYA	111	107	101	84
	VRBA	100	85	82	72
	DLA	86	72	79	71
5 Tubes MPN	BGB	168	83	67	341
	LSB	36	133	49	81

[a] As percentage of counts on TSYA pour plates for each food. Results are the average of four trials.

TABLE 7. *Effect of the concentration of milk solids on the detection of E. coli K-12 by two plating methods*

Plating method	Media	Concn of milk solids and no. of cells enumerated ($\times 10^3$)[a]				
		10.0	1.0	0.1	0.01	0.001
Pour	TSYA	87	87	86	95	85
	VRBA	93	57	17	11	14
	DLA	92	15	5	<1	<1
Surface-overlay	TSYA	89	83	92	93	87
	VRBA	94	79	65	56	48
	DLA	82	57	43	32	24

[a] Results are the average of two trials.

injured *E. coli* to form colonies on VRBA and DLA and in the medium containing desoxycholate have been observed (11). In surface plating the temperature stress is avoided. The 4 ml of overlay medium apparently does not cause stress. Other steps in the pour plate technique were found to contribute to stress by temperature and thus can possibly affect the accuracy of detection and enumeration of *E. coli* by selective media.

The decreasing amount of food materials which can be encountered on successive dilutions may also cause variation in the enumeration. This can be avoided by using suitable dilution blanks, e.g., 1% peptone or 1% NFDM. The food materials and the compounds used in the dilution blanks probably afford protection against the heat stress, rather than supplying necessary nutrients for the repair of the stress. Evidence of such protection of the gram-negative bacteria against heat damage has been reported (3).

This study suggests that the surface-overlay technique has inherent advantages that could be useful in the routine enumeration of coliform bacteria in foods. In addition to avoiding errors associated with temperature stresses, troublesome colonies which at times form between the plate and agar as well as inconsistencies associated with differences in the volume of media used in pour-plating can be avoided. Other recommended improvements are the use of dilution blanks of 1% peptone or NFDM. The effectiveness of these improved methods is currently being studied with naturally contaminated foods.

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