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Repair Detection Procedure for Enumeration of Fecal Coliforms and Enterococci from Seafoods and Marine Environments†

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The repair detection procedure of Speck et al. (Appl. Microbiol. **29**:549-550, 1975) was adapted for the enumeration of coliforms, fecal coliforms, and enterococci in seafood and environmental samples. Samples were pour plated with Trypticase soy agar, followed by a 1- to 2-h incubation to effect repair; the plates were then overlaid with the selective medium and incubated. Violet red bile agar and an incubation temperature of 45°C were used as the selective conditions for fecal coliforms, and KF streptococcal agar was used for the enumeration of enterococci. The method was more efficient than the standard most-probable-number method for fecal coliform enumeration and also allowed enumeration of the injured cells, which might have remained undetected when selective medium in the most-probable-number method was used. The repair detection method effectively recovered the injured portion of the population of enterococci capable of growing on KF streptococcal agar. The repair enumeration method was not suitable for coliforms in marine samples because associative marine bacteria mimicked coliforms in violet red bile agar plates incubated at 35°C. The marine bacteria did not grow at 45°C and therefore did not interfere with fecal coliform enumeration.

Fecal coliforms, enterococci, and coliforms are used as index bacteria, and their presence in food and water indicates possible fecal contamination. These organisms can suffer nonlethal injury from stresses such as low heat (18), low temperature (13, 24), low pH (8, 22), low water activity (15, 28), irradiation (6), sanitizers (23), and starvation (4) and from various combinations of these stresses (12). Injured cells may lose their ability to grow in the presence of certain selective agents at elevated temperatures (14), yet methods for the enumeration of index bacteria by necessity often employ selective media and/or other selective environmental conditions that have been shown to exclude injured cells.

The most-probable-number (MPN) technique accepted by the United States Food and Drug Administration and the American Public Health Association for the enumeration of fecal coliforms requires a maximum of 48 h of incubation in lauryl sulfate tryptose broth or lactose broth at 35°C, followed by an additional 24 to 48 h of incubation in EC broth at either 44.5 or 45.5°C (1, 2, 7). Lauryl sulfate tryptose broth, which is selective, could interfere with the recovery of injured cells (19; B. Ray, J. Food Protect., in press). Additional drawbacks of the MPN method are that it is time consuming, expensive, and cumbersome. Also, since it is an indirect enumeration procedure, it inherently is less accurate than the direct plating method unless the population densities are very low (1). To overcome these difficulties, attempts have been made to use plating procedures for the enumeration of fecal coliforms (10, 11). These direct plating methods require a relatively short incubation period and allow the use of an air incubator, but problems have been reported. Varga et al. (26) found that some of these methods underestimated the fecal coliform population in clams by as much as 20%. The presence of injured cells and their inability to form colonies in the selective plating medium may explain this underestimation. Warseck et al. (27) have shown that violet red bile agar (VRBA) does not recover injured coliforms from food when incubated at 35°C, and the use of this medium with an incubation temperature of 45.5°C, as suggested by Klein et al. (11), would probably result in the exclusion of injured fecal coliforms.

The recommended method for enumerating enterococci from food is either a selective plating method or a selective MPN method (1, 7). The

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selective media employed have an inhibitory effect on cells which are injured by environmental stresses and thus may not be detected.

Plating techniques that enumerate both injured and uninjured coliforms in a food sample have been developed by Speck et al. (25) and others (9). This method was adopted for the enumeration of both injured and uninjured fecal coliforms and enterococci from seafoods and environmental samples. This method allows the injured cells to repair in a nonselective medium at room temperature before being exposed to the specific selective environment which promotes selective growth and colony formation of these index bacteria.

MATERIALS AND METHODS

Test organisms and preparation of samples. Three strains of *Escherichia coli* (all exhibiting a ++ −− IMViC pattern) were isolated for pure culture studies from clam and oyster samples. The isolates were maintained on Trypticase soy agar (TSA) slants and were subcultured daily for 1 week before experiments were performed.

For the freeze injury studies, isolates of *E. coli* were grown overnight in Trypticase soy broth, harvested by centrifugation, and resuspended in sterile distilled water. The cultures (at the desired population level) were transferred to either sterile distilled water or sterile oyster homogenate, divided into 20-ml portions in large tubes, and frozen to −20°C in an ethylene glycol bath maintained in a freezer cabinet. The sterile oyster homogenate was prepared by blending 100 g of oysters and liquor for 2 min and then autoclaving the homogenate for 20 min. The sterile homogenate was brought to its original volume by the addition of sterile distilled water. *E. coli* isolates were also added to nonsterile seafoods which were prepared by blending the seafood with an equal weight of sterile distilled water. The seafood homogenate was placed into large sterile tubes in 20-ml portions, and to each tube 1 ml of a dilute cell suspension was added to obtain the desired levels of *E. coli*. These were also frozen at −20°C in ethylene glycol.

Enumeration methods. The *E. coli* cells in the sterile water and oyster homogenate were enumerated before and after freezing by the following methods: (i) pour plating with TSA and incubating at 35°C; (ii) pour plating with VRBA and incubating duplicate plates at 35 and 45°C; (iii) using the standard MPN procedure (7); and (iv) using the repair enumeration technique (TSA/VRBA) (25). In the last method, the samples were first pour plated in duplicate sets with 5 ml of TSA, incubated for 1 h at room temperature for repair, and then overlaid with 12 to 15 ml of VRBA. One set of plates was incubated at 35°C, and the other set was air incubated at 45 ± 0.5°C. The colonies were enumerated after 18 to 24 h of incubation. *E. coli* cells inoculated into the nonsterile foods were similarly enumerated, except that TSA pour plates were not used.

Enumeration of coliforms and fecal coliforms from foods and environmental samples. The seafood samples were prepared for testing by blending 100 g of sample with 150 ml of 0.1% sterile peptone water. A 25-ml portion of this homogenate was added to 75 ml of peptone dilution water to obtain a 1:10 dilution. The zero dilution was obtained by plating 10 ml from the first dilution into three petri dishes. Water and sediment samples were diluted as needed without blending. Fecal coliforms and coliforms were enumerated by the standard MPN procedure (2) and by plating with VRBA and TSA/VRBA. Incubation was at 45°C for fecal coliforms and 35°C for coliforms for 18 to 24 h, and only characteristic colonies were counted. Representative colonies from the plates incubated at 45°C were confirmed by their ability to produce gas in EC medium within 24 h at 45.5°C. Coliform colonies were confirmed in brilliant green-lactose-bile broth (BGB). Small typical coliform colonies from the VRBA and TSA/VRBA plates which could not be confirmed in BGB were tested for their cytochrome oxidase activity (7) and for their ability to produce acid and gas in purple lactose broth (1).

Enumeration of enterococci. The seafood, water, and sediment samples were prepared as described above. The enterococci were enumerated by the recommended KF streptococcal (KF) agar pour plate method (7) and by the repair enumeration method. The latter technique consisted of pour plating a sample with 5 ml of TSA, incubating at room temperature for 2 h to effect repair, and then overlaying with 12 to 15 ml of KF agar. The plates were incubated at 35°C for 48 h, and the characteristic colonies were counted (1, 7). Representative colonies were confirmed biochemically by recommended procedures (1).

RESULTS

Relative recovery of frozen *E. coli* isolates. In studying recovery of three *E. coli* isolates frozen in water and oyster homogenate, it was found that the counts on VRBA (at 35 or 45°C) were lower than the TSA counts, indicating the presence of injured cells in the samples (Table 1). The MPN values were often nearly equal to and sometimes higher than the TSA counts, but fluctuated more than the plate counts. The repair detection method counts were similar to the TSA counts, indicating that injured cells were recovered by this method. The TSA/VRBA method recovered 18.8 to 69.4% more cells than VRBA pour plate method (Table 1). In general, incubation of VRBA plates at 45°C resulted in a decrease in counts; however, this decrease was not observed when the repair enumeration method was used.

Enumeration of *E. coli* inoculated into nonsterile food. Table 2 shows the recovery of *E. coli* (isolate 3) from four kinds of nonsterile seafoods which had been pretested by standard methods and found to be free of fecal coliforms. However, the oyster and shrimp contained 240 and 460 coliforms per g, respectively, before

TABLE 1. *Recovery of E. coli frozen in sterile water and oyster homogenate by the standard MPN method and by direct plating with VRBA and TSA/VRBA*[a]

Type of sample	Isolate no.	% Recovered in:					
		TSA[b]	VRBA at 35°C	TSA/VRBA at 35°C	VRBA at 45°C	TSA/VRBA at 45°C	Standard MPN method
Frozen in water (−20°C) for 24 h	1	100	56.6	102.7	41.7	111.1	68.8
	2	100	75	93.8	75.0	100.0	68.8
	3	100	80.3	100	80.8	100.0	106.9
Frozen in oyster homogenate (−20°C) for 7 days	1	100	56.3	112.5	51.5	106.3	71.9
	2	100	80.1	111.1	40.8	106.7	103.3
	3	100	51.4	94.4	49.4	93.8	60.5

[a] Results are the average of two trials. See text for other explanations.
[b] TSA was assumed to recover 100% of the viable cells.

TABLE 2. *Quantification of E. coli (isolate 3) in nonsterile seafood by different methods after freezing for 72 h at −20°C*[a]

Type of sample	pH of homogenate	Population (cells/g), determined by:								
		TSA/VRBA at 45°C before freezing	After freezing						% Survivors[c]	% Injured[c]
			MPN method (coliforms)	MPN method (fecal coliforms)	VRBA at 35°C[b]	TSA/VRBA at 35°C[b]	VRBA at 45°C[b]	TSA/VRBA at 45°C[b]		
Crabmeat	7.4	2,400	2,400	2,400	1,300	1,500	1,200	1,600	66.7	25.0
Shrimp[d]	8.0	2,300	1,500	1,500	1,900	2,300	1,600	2,200	95.7	27.3
Oyster[d]	5.8	2,300	460	93	410	520	100	250	10.9	60.0
Scallop	6.2	2,600	2,400	2,400	1,600	2,500	1,700	2,600	100.0	34.6

[a] It was assumed that TSA/VRBA detected all of the viable *E. coli* cells (at 45°C) and all of the coliforms (at 35°C) and that VRBA detected only the uninjured cells.
[b] At 35°C all coliforms and at 45°C only *E. coli* formed colonies on both types of plates.
[c] Percent survivors was calculated from the difference in TSA/VRBA counts before freezing (100%) and after freezing (45°C), and percent injured was calculated from the difference between the counts on TSA/VRBA and those on VRBA, both at 45°C. See also text and reference 21.
[d] The shrimp and oyster homogenates contained 460 and 240 nonfecal coliforms per g, respectively, before freezing.

freezing. It appears that the type of seafood can greatly influence the extent of death and injury in cells of *E. coli*. For example, if the TSA/VRBA method is assumed to recover all injured and uninjured *E. coli* cells, then in oyster samples 60% of the cells were injured, but in shrimp samples only 27% showed injury. Similar results were obtained with two other *E. coli* isolates in the same seafoods (data not shown). The pH of the different seafoods may have influenced injury caused by freezing. The pH of the oyster homogenate (pH 5.8) was lower than that of the shrimp homogenate (pH 8.0).

Enumeration of fecal coliforms from seafood and other samples. Fecal coliforms from naturally contaminated seafood, water, and sediment samples were enumerated by the standard MPN method, by VRBA (45°C), and by the TSA/VRBA method (45°C) (Table 3). Fecal coliforms were found in 30.7% of the 173 samples tested. Approximately 50% of the unshucked oyster and clam samples came from polluted beds, which might explain their relatively high average fecal coliform counts (1,073 cells per 100 g for oysters and 513 cells per 100 g for clams). Surprisingly, the shrimp samples which were obtained from retail markets also tended to have high fecal coliform counts (average, 1,580 coliforms per 100 g). In most samples, the MPN values and the TSA/VRBA counts were in close agreement; however, the VRBA pour plate method often underestimated the fecal coliform population. The percentage of injured cells was highest in the oyster and scallop samples (35%) and lowest (0%) in the clam samples. These results suggest that the number of injured cells varies with the type of seafood and the treatment it receives during processing (many of the scallop samples were frozen). The confirmation of the fecal coliforms from the TSA/VRBA

TABLE 3. *Mean values for fecal coliforms detected from seafood, water, and sediment samples by the standard MPN method and by plating with VRBA and TSA/VRBA*[a]

Type of sample	No. tested/no. positive	Mean fecal coliform MPN per 100 g	Mean of confirmed fecal coliform counts per 100 g on:		% Increase on TSA/VRBA over VRBA
			VRBA	TSA/VRBA	
Oyster (shucked)	15/4	813	832	1288	35.4
Oyster (unshucked)	35/12	1,073	1030	1575	34.6
Clam (unshucked)	33/18	513	622	622	0.0
Scallop	30/10	1,000	590	920	35.0
Shrimp	16/7	1,580	1420	1830	22.4
Crabmeat	16/2	360	400	400	0.0
Finfish	16/2	330	245	325	24.3
Water	6/6	275	370	513	27.7
Sediment	6/6	590	1290	1706	26.7

[a] Means were calculated only for the positive samples. For other explanations, see the text and Table 4. Mean values were calculated by using the logarithm of the numbers.

plates, for all samples, was over 97% (Table 4). Nonfecal coliforms occasionally formed small colonies on VRBA and TSA/VRBA plates incubated at 45°C, but these colonies were very small and never had the zone of bile precipitation; with a little practice the fecal coliforms were easily differentiated from the noncoliforms. The typical coliform colonies were usually at least 1 mm in diameter and were surrounded by a 2-mm or larger zone of bile precipitation.

Coliform analysis. Samples of seafood, water, and sediment which were tested for fecal coliforms (Table 3) were simultaneously tested for total coliforms by the standard MPN, VRBA pour plate, and TSA/VRBA procedures. Coliform counts by the MPN method and by the direct plating methods differed markedly, and colonies from these plates often could not be confirmed in BGB, especially in the summer months. The seasonal nature of the increased counts by the direct plating method suggested that marine microorganisms were possibly mimicking coliforms on VRBA. The colonies which could not be confirmed usually were red and 0.5 to 1 mm in diameter, but they did have a zone of bile precipitation. We selected 313 of these colonies (168 from VRBA plates and 145 from TSA/VRBA plates) from various seafoods and tested them for their production of gas in BGB and their production of cytochrome oxidase (Table 5). It was found that an average of 53% of the colonies from VRBA and 52.2% of the colonies from TSA/VRBA plates were cytochrome oxidase positive, indicating that these were not coliforms. Of the cytochrome oxidase-positive cultures, 50 were tested for their ability to ferment lactose; 72% of these produced acid from lactose, but no gas. The type of seafood influenced the number of false-positive colonies on VRBA and TSA/VRBA pour plates; finfish contained the highest percentage of false positives, and scallops contained the lowest.

TABLE 4. *Percentage of confirmation of fecal coliforms from different samples*[a]

Type of sample	Colonies tested from VRBA		Colonies tested from TSA/VRBA	
	No.	% Confirmed	No.	% Confirmed
Oyster (shucked)	16	100	20	95
Oyster (unshucked)	76	99	83	98
Clam (unshucked)	60	100	65	100
Scallop	25	100	34	100
Shrimp	27	93	32	97
Crabmeat	6	100	7	100
Finfish	5	100	4	100
Water	18	100	24	100
Sediment	25	100	28	100

[a] The numbers of colonies selected on the basis of their frequency of occurrence. For confirmation, each selected colony was tested for its ability to produce gas in EC medium at 44.5°C in 24 h. The data presented in Table 3 are based on the confirmation percentages presented here.

Enumeration of enterococci. The repair enumeration plating method was adapted for the enumeration of enterococci from seafood by using KF agar pour plates as the selective method and TSA/KF agar for the repair enumeration method; of 158 seafood samples tested by both procedures, 94 were found to contain enterococci (Table 6). Shucked oysters, scallops, shrimp, and finfish tended to have relatively

TABLE 5. *BGB and cytochrome oxidase reactions of selected colonies from VRBA and TSA/VRBA plates incubated at 35°C*[a]

Type of sample	Medium (-a)	No. of colonies tested	% BGB positive and cytochrome oxidase negative	% BGB negative and cytochrome oxidase positive[b]	% BGB negative and cytochrome oxidase negative
Oyster	VRBA	90	39	50	11
	TSA/VRBA	78	35	46	19
Clam	VRBA	15	47	40	13
	TSA/VRBA	16	25	62	13
Scallop	VRBA	15	53	40	17
	TSA/VRBA	13	38	38	24
Shrimp	VRBA	12	17	58	25
	TSA/VRBA	8	38	50	12
Crabmeat	VRBA	16	50	50	0
	TSA/VRBA	18	50	50	0
Finfish (whole)	VRBA	20	10	80	10
	TSA/VRBA	12	17	67	16

[a] Colonies selected were red, 0.5 to 1 mm in diameter, and did have zone of bile precipation. For further explanation, see text.

[b] Fifty of the cytochrome oxidase-positive cultures were tested for their ability to produce acid and gas in lactose broth, and 72% of the cultures were positive for acid production but not for gas production; 5% produced both acid and gas.

TABLE 6. *Mean values for enterococci from seafood, water, and sediment samples enumerated by the standard KF agar pour plate method and by the TSA/KF agar plating procedure*[a]

Type of sample	No. tested/ no. postive	Mean no. of enterococci per g by:		% Increase on TSA/KF agar over KF agar
		KF agar method	TSA/KF agar method	
Oyster (shucked)	18/15	88	171	48.2
Oyster (unshucked)	34/13	16	21	24.8
Clam	32/13	9	21	55.0
Scallop	26/17	124	273	54.5
Shrimp	17/16	750	5,300	85.8
Crabmeat	15/10	33	60	45.3
Finfish	16/10	160	205	22.4
Water	6/4	1	1	40.0
Sediment	6/6	11	24	54.6

[a] Means were calculated by using the positive samples only and by using the logarithms of the numbers.

high (>100/g) enterococcus counts. The TSA/KF agar repair enumeration procedure recovered 22.4 to 85% more cells than did the KF agar pour plating procedure, indicating the presence of injured enterococci. Representative colonies (50 on each medium) were picked from the KF agar and TSA/KF agar plates and tested biochemically (2). Approximately 66% of the colonies from both plating procedures gave positive confirmation for enterococci. The confirmation rate for colonies taken from fresh (unshucked) shellfish samples obtained from polluted water was much higher (about 90% by both methods).

DISCUSSION

Coliforms, fecal coliforms, and enterococci are used as index bacteria, and their populations in foods and environmental samples are used to measure the sanitary quality of those materials. The microorganisms are usually enumerated either by the MPN method or by plating methods which selectively allow their growth. The MPN method is an indirect statistical method and is particularly useful in measuring low populations. The MPN procedure is more costly and time consuming than plating procedures, and injured organisms may be missed when selective media such as lauryl sulfate tryptose broth or azide dextrose broth are used in the MPN procedure (13; Ray, in press). Therefore, a direct plating method, which can recover all of the survivors, including the injured cells, has the advantage of being less costly and time consuming, as well as being more accurate than the MPN procedure.

A direct plating method which can recover injured cells and still selectively enumerate only the desired group of microorganisms has been developed in this laboratory. This method is based on the concept that although injured cells lose their normal resistance to selective media, they can regain this resistance if first allowed to repair in a nutritionally rich nonselective environment (25). This relatively simple method,

which was found to be effective for the enumeration of injured and noninjured coliforms in dairy products (20), was adapted for the enumeration of coliforms, fecal coliforms, and enterococci in seafoods, water, and marine sediments. It appears that this method is quite effective for the enumeration of fecal coliforms and enterococci, but not for the enumeration of total coliforms in marine samples. Associative marine bacteria which are capable of mimicking coliforms on VRBA as well as on TSA/VRBA interfered with the effectiveness of this method. Previous work in this laboratory also indicated that direct plating with VRBA or TSA/VRBA is inefficient for the enumeration of coliforms from seafood because of the presence of noncoliform organisms (20). Our studies have demonstrated that the repair enumeration method can produce a selective environment as effective as that of the selective medium alone. VRBA has been proven to be effective for the selective enumeration of coliforms from dairy products. By incubating samples plated with this medium at 45°C, it also is very effective in the enumeration of fecal coliforms from seafood and other marine samples. The high rate of confirmation for the isolates of fecal coliforms indicates that the marine microorganisms do not grow at 45°C and thus do not interfere with the enumeration of fecal coliforms. Other workers have reported that cytochrome oxidase-positive marine bacteria of the genera *Vibrio* and *Aeromonas* do mimic coliforms on VRBA and Endo agar, respectively, at 35°C, but they do not grow at 45°C (3, 17, 21). In different types of samples, we observed that *Erwinia* sp., *Enterobacter agglomerans*, and *Enterobacter hafniae* can mimic coliforms on VRBA at 35°C (25).

Enterococci are used as index bacteria in water and as an indicator of unsanitary conditions in processed food (1, 7). KF agar or broth, which contains azide as the selective agent, is used for the enumeration of these organisms. We have found that the repair detection procedure can be adapted to recover injured cells of this group of organisms. The repair time for these organisms is longer than that of the fecal coliforms (about 1.5 to 2 h at 25°C). We tested 100 colonies of enterococci from the different seafoods and found that the confirmation rate (66%) was approximately the same for colonies from both the standard KF agar pour plates and the TSA/KF agar overlay procedure.

Other workers (5, 16) have reported similar enterococcus confirmation rates from KF agar. A possible explanation for the low rate of confirmation could be that certain enterococci of animal origin, such as *Streptococcus brevis* and *Streptococcus equinus*, which can form colonies on KF agar, are not included as fecal streptococci in the standard biochemical confirmation procedures (2).

In conclusion, the trend toward the use of fecal coliforms as a measure of sanitation or safety for seafoods and marine samples suggests that the repair enumeration method can be an advantageous monitoring method. In addition to not being restrictive to injured cells, it is more rapid and less cumbersome than the MPN procedure. An adaptation of this procedure is also more accurate for enumerating enterococci from such sources.

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