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Evaluation of Methods for Enumeration of *Vibrio parahaemolyticus* from Seafood

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The efficiency of several enrichment broths in recovering *Vibrio parahaemolyticus* inoculated into fish homogenates was studied. Recovery by the most probable number technique was very low in all the broths, while direct plating on thiosulfate citrate bile salt sucrose agar yielded better recovery. A decrease in the enrichment time to 8 from 18 h did not improve recovery. At concentrations exceeding 2.5 μg/ml, polymyxin was inhibitory to *V. parahaemolyticus*.

Enumeration of *Vibrio parahaemolyticus* from seafood has become important inasmuch as the International Commission on Microbiological Specifications for Foods (ICMSF) has recommended an acceptability limit of 10^2/g for this organism in some fishery products (4). The most probable number (MPN) technique generally has been employed for enumeration of *V. parahaemolyticus* from seafood, and several enrichment media have been used for this purpose (5, 6, 8–10).

The objective of the study was to evaluate the relative efficacy of various enrichment broths and to work out a suitable concentration of polymyxin to be included in enrichment broths.

GSTB was prepared as described in the *Bacteriological Analytical Manual* of the Food and Drug Administration (11). For use with iced fish and frozen or iced prawns, the modification suggested by Malin and Beuchat (7) for recovery of chill-stressed *V. parahaemolyticus* was incorporated. SPB contained 1% peptone, 0.3% yeast extract, 2% NaCl, and polymyxin B sulfate (Sigma Chemical Co., St. Louis, Mo.) at 250, 25, 2.5, or 0.25 μg/ml; the latter was added as a filter-sterilized solution after autoclaving. For comparative evaluation of broths, SPB with 2.5 μg of polymyxin per ml was used. SWYE was prepared as described by Kaneko and Colwell (6), and nutrient broth with eosin yellow (NBYE) was prepared as described by Hofer and Silva (3).

Samples of raw and processed shrimp were obtained from shrimp-processing factories around Mangalore. Samples of mullet (*Mugil cephalus*), oil sardine (*Sardinella longiceps*), white bait (*Anchoviella* sp.), and sole (*Cyanoglossus* sp.) were collected from the Mangalore fish market. Fresh droppings of birds also were collected from the market area.

The effect of polymyxin on growth of *V. parahaemolyticus* was determined as follows. A *V. parahaemolyticus* strain isolated from a seafood sample was grown in tryptic soy broth with 1% NaCl (TSBS) for 18 h, and 10-fold dilutions thereof were inoculated into SPB without polymyxin or with one of the following concentrations of polymyxin, which was added after autoclaving: 250, 25, 2.5, or 0.25 μg/ml. The inoculum contained 8.7×10^9 to 0.87×10^1 cells. The broths were incubated at 37°C for 24 h, and turbidity was noted.

Fish were washed and divided into three batches of 50 g each. Two batches were homogenized in 450 ml of 3% NaCl each. A total of 10 ml of an 18-h culture of *V. parahaemolyticus* containing 3.1×10^9 cells was inoculated into one homogenate, and 1 ml of the same culture was inoculated into the other. The third batch of fish was used to determine the level of the native population of *V. parahaemolyticus*. Enumeration of *V. parahaemolyticus* was done by direct plating on thiosulfate citrate bile salt sucrose (TCBS) agar and by the five-tube MPN technique with SPB without or with 0.25 and 2.5 μg of polymyxin B per ml. After incubation at 37°C for 18 h, the samples were subcultured onto TCBS agar. Two typical bluish green colonies were picked up from each subculture representing the original MPN tube. The colonies were purified and confirmed by the following biochemical reactions: oxidase test; triple sugar iron reaction; 0/129 sensitivity; ability to decarboxylate lysine, ornithine, and arginine; ability to grow in tryptic soy broth containing 0, 8, and 11% NaCl. At least 10 typical colonies were picked from a dilution showing countable colonies and subjected to biochemical tests, and the *V. parahaemolyticus* counts were estimated.

From Table 1, it can be noted that in the absence of polymyxin B, an inoculum of 8.7 *V. parahaemolyticus* cells per 10 ml of salt broth could initiate growth and cause turbidity. However when the polymyxin concentration was increased to 2.5 μg/ml, an additional 2 log units of cells was required to cause turbidity. At 25 μg of polymyxin per ml, on the other hand, 8.7×10^6 cells were required to initiate growth, and at 250 μg of polymyxin per ml, even 8.7×10^9 cells failed to multiply and cause turbidity. It should be

TABLE 1. Relation between number of *V. parahaemolyticus* cells and their ability to grow in salt broth containing different concentrations of polymyxin B

No. of cells inoculated	Growth at the following concn (μg/ml) of polymyxin B[a]:				
	0	0.25	2.5	25	250
8.7×10^9	+ +	+ +	+ +	+	−
8.7×10^8	+ +	+ +	+ +	+	−
8.7×10^7	+ +	+ +	+ +	+	−
8.7×10^6	+ +	+ +	+ +	+	−
8.7×10^5	+ +	+ +	+ +	−	−
8.7×10^4	+ +	+ +	+ +	−	−
8.7×10^3	+ +	+ +	+ +	−	−
8.7×10^2	+ +	+ +	+ +	−	−
8.7×10^1	+ +	+ +	−	−	−
8.7×10^0	+ +	+ +	−	−	−

[a] −, No growth; +, moderate growth; + +, good growth.

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TABLE 2. Recovery of *V. parahaemolyticus* from inoculated fish homogenates using various enrichment broths

Trial no.	Fish	No. of *V. parahaemolyticus* cells inoculated/ml	*V. parahaemolyticus* count/ml of homogenate recovered using:					
			Direct plating on TCBS	Salt broth	SPB (0.25/μg of polymyxin)	SPB (2.5 μg of polymyxin)	GSTB	SWYE
I	Silver bellies	5.2×10^6	ND[a]	11.0	6.0	5.4	2.0	2.0
		5.2×10^5	ND	4.0	19.0	9.0	4.0	2.0
II	Silver bellies	9.8×10^5	ND	8.0	110.0	13.0	1.8	15.0
		9.8×10^4	ND	9.0	13.0	17.0	1.8	12.0
III	Silver bellies	1.7×10^6	4.9×10^6	40.0	18.0	2.0	37.0	39.0
		1.7×10^5	1.85×10^4	60.0	2.0	2.0	2.0	45.0
IV	White bait	1.9×10^6	4.3×10^6	10.0	20.0	4.0	95.0	79.0
		1.9×10^5	4.3×10^4	3.6	11.0	2.0	11.0	210.0
VX	White bait homogenized and autoclaved	3.8×10^5	ND	2.4×10^3	2.4×10^3	3.5×10^2	2.0	2.4×10^3
		3.8×10^4	ND	2.4×10^3	2.4×10^3	5.4×10^2	2.0	2.4×10^3
VI	Muscle from live mullet removed aseptically	1.5×10^6	ND	2.4×10^3	2.4×10^3	3.5×10^2	2.0	2.4×10^3
		1.5×10^5	ND	2.4×10^3	2.4×10^3	0.23×10^2	2.0	2.4×10^3

[a] ND, Not done.

pointed out that such a high inoculum, which by itself caused slight turbidity on the day of inoculation, cleared out the next day, suggesting that the cells were killed due to exposure to polymyxin.

These results suggest that the concentration of polymyxin suggested by Sakazaki et al. (10) to be used in SPB, viz. 250 μg/ml, is toxic for *V. parahaemolyticus*. The success they obtained with their media perhaps could be due to loss of polymyxin during autoclaving, as has been shown to occur by Blanchfield et al. (1). Sakazaki et al. (10) autoclaved their medium of pH 8.6 to 9.0 containing polymyxin at 115°C for 10 min, and Blanchfield et al. (1) showed 97% loss of polymyxin when medium of pH 8.8 was autoclaved at 121°C for 15 min. Even Nakanishi and Murase (8), who reported success with SPB, autoclaved their medium containing polymyxin at 121°C for 15 min. Interestingly, they observed reduced positivity when the polymyxin concentration was raised to 500 and 1,000 U/ml. They observed best results with 250 U/ml, which is about 32.5 μg/ml, taking 1 mg as equivalent to 8,000 U, as with polymyxin B sulfate. Thus, our results indicate that there is a need to standardize the concentration of polymyxin to be used in broth when it is added as a filter-sterilized solution after the medium is autoclaved.

Because the purpose of adding polymyxin B to enrichment broth is to impart selectivity, it should be used at a concentration that supports growth of a small population of *V. parahaemolyticus*, which may be naturally associated with seafood, while it should inhibit the competing flora. Though results in Table 1 suggest that 0.25 μg of polymyxin per ml is not inhibitory to a small inoculum of *V. parahaemolyticus*, higher concentrations may be required in broth for inoculation with fish, in view of the demonstrated loss of polymyxin B by adsorption to fish (1). However, the unsuitability of SPB with 250 μg of polymyxin per ml was indicated by our studies on 27 samples of raw and processed shrimp, of which 10 samples showed the presence of *V. parahaemolyticus* when GSTB was the enrichment broth, whereas none were positive when SPB (250 μg of polymyxin per ml) was used. However, recovery from fish homogenates inoculated with a known number of *V. parahaemolyticus* cells was very poor in all five broths (Table 2). In two trials, in which direct plating was also done, recovery by this technique appeared to be good. This suggests that during the period of incubation of homogenates in broths, the *V. parahaemolyticus* isolates are perhaps overgrown by the other flora. This is further supported by the observation that from sterile fish homogenates inoculated with *V. parahaemolyticus* cells recovery reaches the upper limit of detection by the MPN technique in salt broth and SPB with 0.25 μg of polymyxin per ml and SWYE. However, even in the absence of competing flora, SPB with 2.5 μg of polymyxin per ml did not yield good recovery, suggesting the toxicity of polymyxin to *V. parahaemolyticus*. The least recovery was observed in GSTB (Table 2).

Dupray and Cormier (2) considered the growth of *V. parahaemolyticus* and other fecal and marine flora in enrichment broths and observed 8 h to be the optimal enrichment

TABLE 3. Recovery of inoculated *V. parahaemolyticus* from fish homogenates using enrichment for 8 and 18 h

Fish	No. of *V. parahaemolyticus* cells inoculated/ml of homogenate	*V. parahaemolyticus* count/ml by direct plating on TCBS	No. of *V. parahaemolyticus*/ml of homogenate recovered by MPN using broths on:							
			8-h culture				18-h culture			
			SPB (0.25 μg/ml)	SWYE	GSTB	NBEY	SPB (0.25 μg/ml)	SWYE	GSTB	NBYE
Sardine	5.5×10^6	1.2×10^5	160	1.2	0.2	0.74	35	0.92	0.37	2.5
Sole	5.5×10^6	8.4×10^4	43	0.37	2.8	7.2	1.7	0.36	0	1.1
Croaker	6.1×10^6	5.1×10^5	0	0.74	0.37	0.4	0.18	0.37	0.4	0.55
Pomfret	6.1×10^6	2.1×10^6	24	0	6.4	1.3	1.3	0.18	0.2	0.91

TABLE 4. *V. parahaemolyticus* counts using four different enrichment broths from different samples

Date	Fish or sample	*V. parahaemolyticus* count/g using:			
		GSTB	SPB (0.25/μg/ml)	SWYE	NBEY
10 October 1984	Mullet	2.3	0.45	3.9	6.4
	Oil sardine	0.2	2.20	1.7	6.9
	White bait	0.36	0.45	2.1	240.0
	Bird dropping	0.45	0.40	0.72	0.36
12 November 1984	Oil sardine	0	43.0	4.5	2.9
	Mullet	3.2	0.61	2.9	8.1
	White bait	0.18	3.9	1.4	2.0
	Sole	0	0.94	0.68	2.2
21 November 1984	Whole prawns (*Metapenaeus dobsoni*)	1.2	2.3	2.3	2.5
	Whole prawns (*Parapenaeopsis stylitera*)	3.9	1.9	2.6	4.5
	PUD[a] (*M. dobsoni*)	0.81	2.4	1.5	0.36
	PUD (*P. stylifera*)	0.94	240.0	2.1	1.1
5 December 1984	Whole prawns (*M. dobsoni*)	0.36	0.74	0.68	0.42
	Whole prawns (*P. stylifera*)	0.37	0.36	0	1.2
	PUD (*M. dobsoni*)	0.55	0.55	0.45	1.2
	PUD (*P. stylifera*)	2.6	0.82	0.68	2.9
12 December 1984	Oil sardine	0	1.4	0.68	1.3
	Mullet	1.7	0.54	2.1	3.9
	White bait	0.45	0	0	0
	Sole	1.3	0	0.45	0.7

[a] PUD, Peeled and undeveined.

time for the isolation of *V. parahaemolyticus* from seafood. To verify whether the low recovery obtained by us was due to longer enrichment, results of subcultures at 8 and 18 h were compared (Table 3). The results were analyzed statistically by the Kruskal-Walli nonparametric test of significance. The difference in results for subcultures at 8 and 18 h were not significant ($P = 0.05$). Direct plating on TCBS, however, yielded good recoveries.

V. parahaemolyticus counts obtained in various seafood samples with four enrichment broths are presented in Table 4. When the results obtained with 20 samples were analyzed statistically, no significant difference was observed ($P = 0.01$) among the different enrichment broths (F value, 1.20). Thus, the results of this study suggest that the effectiveness of these broths in enumerating *V. parahaemolyticus* from seafood is not significantly different. However, results in Table 2 suggest that direct plating on TCBS might be more useful in obtaining a good estimate of the *V. parahaemolyticus* counts in seafood. This technique is less laborious and the results are obtained a day earlier as compared with the MPN technique.

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