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Comparative Study of Procedures for Quantification of *Vibrio parahaemolyticus* in Seafoods

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ABSTRACT

A collaborative study was made comparing the efficacy of combinations of two broths and two agar media for recovery and quantification of *Vibrio parahaemolyticus* and *Vibrio alginolyticus*. Broths used in the study were Glucose Salt Teepol broth (GSTB) and Horie broth (HB); agars were Thiosulfate-Citrate-Bile Salts-Sucrose agar (TCBS) and water blue-alizarin-yellow agar (WB). Frozen shrimp samples seeded with high, medium and low concentrations of *V. parahaemolyticus* and medium concentrations of *V. alginolyticus* were used in this study. Results showed that in general all media using HB gave MPN values 10 times greater than combinations employing GSTB. The HB-WB combination gave *V. parahaemolyticus* recoveries 10-fold higher than the other media combinations tested.

Since the initial isolation of *Vibrio parahaemolyticus* in 1950 from Japanese victims of seafood poisoning (*8*), the world wide distribution of these organisms in sea water and sea fish has been accepted (*2*). Because many formulae are purported to be effective for isolating and identifying *V. parahaemolyticus* (*12*), laboratories must select an appropriate procedure. The Food and Drug Administration (FDA) *Bacteriological Analytical Manual* (BAM) (*7*) specifies, as pre-enrichment, Glucose Salt Teepol Broth (GSTB) to be subsequently streaked on Thiosulfate-Citrate-Bile Salts-Sucrose agar (TCBS) in a protocol designed to isolate the organism from contaminated seafoods. Both GSTB and TCBS have been used extensively since first described in 1963 (*1,11*). Another teepol-containing medium, described in 1963 by Sakazaki (*14*), is water blue-alizarin-yellow agar (WB). Horie et al. (*10*) devised a broth used successfully for isolation of *V. parahaemolyticus* from plankton and fish. Studies by FDA (Division of Microbiology, unpublished) and Beuchat (*3,4*) indicate that Horie broth (HB) and WB may be superior to GSTB and TCBS, particularly in recovery of cold-stressed organisms. The main difference in the composition of these media is the inhibitors employed. HB is a non-selective broth and GSTB contains teepol. Teepol is also used in WB agar. The selectivity of TCBS agar depends on oxgall and sodium cholate.

This report describes a study constrasting the efficacy of GSTB with HB and TCBS with WB. Nineteen analysts in three university, three food industry, and eleven FDA laboratories participated. Shrimp were inoculated with mixtures of *V. parahaemolyticus* and *Vibrio alginolyticus*. These organisms are often isolated from common environments and food samples; however, their close association, similar habitat, morphology and growth requirements can be confusing to the analyst.

MATERIALS AND METHODS

The organisms used in this study, supplied by Robert M. Twedt, FDA, Cincinnati, OH, were *V. alginolyticus* No. Z106 and *V. parahaemolyticus* No. 553, K-type 4, Kanagawa positive. Cultures for sample seeding were grown in Trypticase Soy Broth (BBL) with 3% (w/v) NaCl (TSBS) for 24 h at 35 C. Viable counts were determined by the plate count technique, using Trypticase Soy agar (BBL) with 3% (w/v) NaCl (TSAS). The final viable count in the sample was made after frozen storage for 5 days. The initial inoculum levels were adjusted to provide a loss of one log due to freezing and storage.

Frozen, raw, peeled shrimp, demonstrated to be free of indigenous *Vibrio* bacteria, were chopped into small pieces and placed in sterile Oster blenders. The chopped shrimp were seeded with *V. alginolyticus* at a level that would provide a final viable count of 4000/g. The shrimp were then divided into three groups and further seeded with *V. parahaemolyticus* at levels that would provide final viable counts of approximately 70,000, 7000, and 700/g, respectively. The samples were diluted 1:10 with 3% saline-10% glycerol solution, blended to a homogeneous slurry, weighed into sample jars and frozen at 30 C. Each microbiologist received six samples that were shipped frozen in dry ice; two from each of the 70,000, 7000, and 700/g *V. parahaemolyticus* inoculum levels.

Each medium, ingredient and chemical employed was from a single manufacturer's batch, lot or control number. All participating analysts were provided with the same materials and formulae.

A common analytical procedural outline was followed by each microbiologist. The scheme is shown in Fig. 1. GSTB and HB were inoculated in parallel with the serially diluted sample, into three tubes at each of six dilutions, to determine the most probable number (MPN). One-gram portions were examined by inoculating double-strength GSTB and HB with 10 ml of the sample as received. After broths were incubated for 20 h at 35 C, all tubes were streaked on TCBS and WB plates with a 3.0-mm loop. After incubating the plates for 20 h at 30 C, one characteristic *V. parahaemolyticus* colony/plate was selected for the biochemical confirmation tests shown in Table 1.

RESULTS AND DISCUSSION

After frozen storage, expected counts for the three sample sets designed to contain high, medium and low *V. parahaemolyticus* concentrations were 70,000, 7000 and 700/g, respectively. Geometric means of analysts' data and estimates of replicate error are given in Table 2. For purposes of statistical analysis, all MPN values reported as > 110,000/g were given the value of 240,000/g. More than half of the high concentration sample values were reported as > 110,000/g when media combinations of HB-TCBS and HB-WB were used. In general, all media

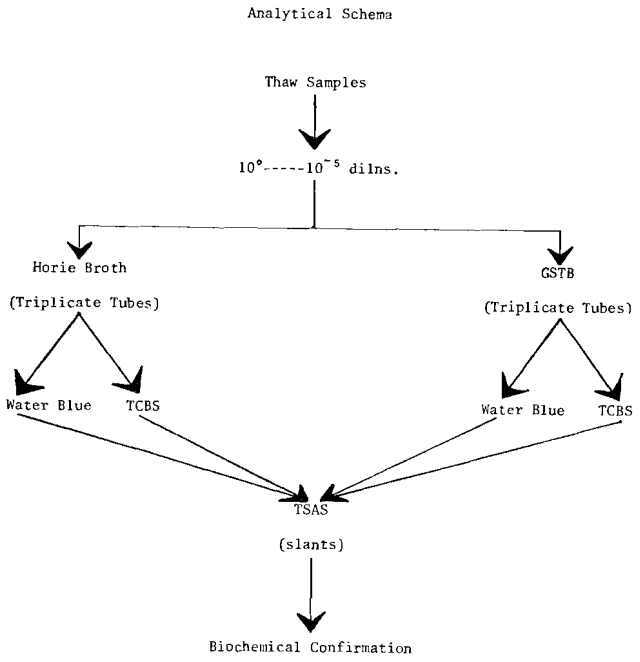

Figure 1. *Analytical schema for* V. parahaemolyticus.

TABLE 1. *Confirmation tests for* V. parahaemolyticus.

Test	Reaction
Cytochrome oxidase	+
Glucose aerobic	+
Glucose anaerobic	+
Sucrose fermentation	−
Lysine decarboxylation	+
Growth at 42 C	+
Halophilism, 8% NaCl	+
Halophilism, 10% NaCl	±
Acetoin (V.P.)	−
Triple Sugar Iron Agar Slants	alkaline slant, acid butt, no H_2S
Morphology (gram stain)	rods (negative)

using HB gave MPN values 10 times greater than those combinations employing GSTB. A non-parametric (*6*) test of significance (Kruskal-Wallis H test) was performed. Data were ranked for each concentration to determine if one condition had a significantly different average rank. All three tests (H = 17.7, 125.2, 10.9) were significant at the $a = 0.05$ level. The HB combinations seemed to be the best choice.

Components of variance (*15*) were computed for the medium and low concentrations in the HB-WB media combination. The high concentration contained too many > 110,000 values to be included in the computation. Estimates of experimental error (σ^2A) and interactions between analysts and samples (σ^2AB) were 0.11302, 0.36833 and 0.10039, respectively. The sum of these components is 0.76272. Variance values of the magnitude this study yielded are unacceptable for a regulatory procedure. However, the HB-WB combination gave *V.parahaemolyticus* recoveries 10-fold higher than other media in the study.

A previous study (*5*) demonstrated that HB was superior to GSTB in the recovery of *V. parahaemolyticus* from chilled and frozen crab meat. The work of Ray et al. (*13*) demonstrates the need to consider providing a non-selective medium for injury-repair before more selective media and may be the clue to the superiority of HB in this study. Additionally, the study contrasted WB agar with TCBS agar. Most participants in this study found it difficult to differentiate *V. alginolyticus* from *V. parahaemolyticus* colonies on WB agar. Although not indicated in the procedure, it was found that identifiable colonies would develop on WB agar with an additional 20 h of aging at room temperature. Our data and all recent publications (*5,9,13*) support the necessity for continued research in the methodology of *V. parahaemolyticus* detection.

ACKNOWLEDGMENTS

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TABLE 2. *Geometric Means and Replicate Variances for Four Sets of* V. parahaemolyticus *Broth and Solid Media.*

		Broth			
		GSTB		HORIE	
		Solid media			
Concentration		TCBS	WB	TCBS	WB
High	Geometric Mean	23,000 (18)[a]	21,000 (17)	130,000	130,000
	S^2	0.37477	0.47746	b	b
Medium	Geometric Mean	2,500 (17)	2,700 (16)	25,000 (18)	35,000
	S^2	0.40040	0.14888	0.17810	0.15917
Low	Geometric Mean	250 (17)	240 (16)	1,300 (16)	1,800 (11)
	S^2	0.13533	0.16818	0.43372	0.06419

[a]Degrees of Freedom.
[b]More than 10% of values > 110,000 (set equal to 240,000).

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Harmon, *con't. from p. 917*

biology. He is a systematic, conscientious teacher, one who is attentive to students' perception of subject matter. His research has continued throughout his academic career, and the list of his publications is impressive. At the 1966 ADSA Annual Meeting, he received the Pfizer Award. Dr. Harmon has been a longtime member of the Editorial Board of the *Journal of Food Protection.*

The Award of Honor is presented in recognition of unusually outstanding contributions to the welfare of the ADSA. Dr. Harmon served the ADSA in 1966 as its representative at the World Dairy Congress in Munich, Germany. He has accepted numerous committee assignments, was a member of the Board of Directors from 1968 to 1971, was Vice-President in 1974-75 and President in 1975-1976. He then served in the positions of treasurer and Chairman of the General Arrangements Committee, which organized the combined meeting of the American Dairy Science Association and the American Society of Animal Science. at Michigan State University.

Friend, *con't from p. 917*

After receiving her M.S. degree, Friend worked as a research technician in the Biochemistry Department of the University of Nebraska Medical School, Omaha, for two years. She then moved with her husband, who is in the U.S. Air Force, to Texas and then Illinois. In June 1978, after her husband was sent to Korea, she returned to the University of Nebraska to continue work toward the Ph.D. degree.

She has been involved in a number of research areas, but has especially excelled in enzyme biochemistry, particularly the understanding of lactase immobilization and its significance to the dairy industry. Her Ph.D. work has further improved the process by which lactase is made more stable and immobilized. She also developed ways by which lactase can be regenerated once inactivated.

Economical enzymatic hydrolysis of lactose in dairy products can be a significant development for the dairy industry for several reasons: production of low lactose dairy products suitable for consumption by lactose intolerant individuals is made possible; approximately one-half billion pounds of lactose per year which is currently being wasted can be made usable; cost savings for waste treatment of cheese whey can be realized; the fermentation in producing industrial alcohol from whey for the manufacture of gasohol can be accelerated.

Sandine, *con't from p. 917*

tured products. His research has considered the importance of bacterial physiology and genetics in understanding control of fermentative processes.

Dr. Sandine holds membership in Phi Kappa Phi, Sigma Xi, Gamma Sigma Delta, American Society for Microbiology, IAMFES, and ADSA. He has served on award selection committees for ADSA and on the editorial boards of the *Journal of Dairy Science* and *Journal of Food Protection.* He has appeared as an invited speaker at numerous conferences and seminars.