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Procedure for Isolation and Enumeration of *Vibrio parahaemolyticus*[1, 2]

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An evaluation of criteria used in the identification of *Vibrio parahaemolyticus* showed that cultural responses varied with respect to growth in broth with 10% NaCl, type of hemolysis, reactions in triple sugar-iron-agar, and serological reactions. With few or no exceptions, cultures were positive for cytochrome oxidase, utilized glucose fermentatively, were sensitive to pteridine (O/129) and novobiocin, and failed to grow in Trypticase soy broth (TSB) without NaCl. A procedure employing a direct plating technique, with or without prior enrichment, was designed for the isolation and enumeration of *V. parahaemolyticus*. The plating medium consisted of 2.0% peptone, 0.2% yeast extract, 1.0% corn starch, 7% NaCl, and 1.5% agar, with the *p*H adjusted to 8.0. The enrichment broth was TSB with 7% NaCl. Dilutions of food homogenates were either spread directly on the plates or inoculated into enrichment broth. TSB enrichments were incubated at 42 C for 18 hr. A loopful of the TSB tubes then was streaked onto the direct plating medium. Incubation of plates was at 42 C for 24 to 48 hr. Smooth, white to creamy, circular, amylase-positive colonies were then picked as suspect *V. parahaemolyticus*. Confirmation of gram-negative, fermentative, oxidase-positive, pleomorphic rods sensitive to pteridine O/129 was made by a fluorescent-antibody technique. With this procedure, a satisfactory quantitative recovery of known *V. parahaemolyticus* from inoculated seafoods was made possible. *V. parahaemolyticus* was not isolated from other salted foods.

Information on the isolation, identification, and public health significance of *Vibrio parahaemolyticus* is presented in a recent review (R. Nickelson and C. Vanderzant, J. Milk Food Technol., *in press*). Much of the published information concerning this organism originates from Japan where *V. parahaemolyticus* is responsible for the majority of foodborne gastroenteritis. Although several isolations have been reported from marine environments and seafoods in the United States, little is known about its presence in other salted foods and about its significance in foodborne illnesses in this country. Present isolation and identification procedures are lengthy and require large quantities of various media. Confirmation of suspect *V. parahaemolyticus* involves numerous biochemical tests and serological typing. The objectives of this investigation were (i) to evaluate criteria now used for presumptive identification of *V. parahaemolyticus* and to determine the fewest and most reliable characteristics for identification and (ii) to develop a rapid and reliable procedure for the isolation and enumeration of *V. parahaemolyticus*.

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[2] A preliminary account of this work was presented at the 71st Annual Meeting of the American Society for Microbiology at Minneapolis, Minn., 2-7 May 1971.

MATERIALS AND METHODS

Cultures. The sources of the *V. parahaemolyticus* cultures and other *Vibrio* species are shown in Table 1. Other organisms mentioned in this study were purchased from the American Type Culture Collection or were isolated from seafoods. All cultures were maintained at 25 C on Trypticase soy agar (TSA; BBL) with 3% NaCl.

Morphological and biochemical characteristics. Gram stains of 24-hr agar slant cultures were made by Hucker's modification (13). For flagella stains, cultures were incubated at 25 C for 18 to 24 hr in a flagella broth (*p*H 7.0) consisting of 1% Tryptose (Difco), 0.1% K_2HPO_4, and 3.0% NaCl. After centrifugation (3,500 × *g* for 10 min), packed cells were resuspended in distilled water and poured over a process clean slide (Corning no. 2948). Air-dried

TABLE 1. *Sources of Vibrio cultures*

Organism	Strain designation	Obtained from[a]	Source
Group I	17802, 17803	ATCC	Food poisoning, Japan
V. parahaemolyticus	SAK 8, SAK 15	FDA, Dallas	Food poisoning, Japan
Group II	O	C. Vanderzant and R. Nickelson	Gulf Coast shrimp
V. parahaemolyticus	WL1, WL2, WL3, WL4	W. L. Landry, FDA, Dallas	Gulf Coast oysters
Group III	5DBW, 12A, 12B, 5B, 7BW, 8C	R. R. Colwell	Chesapeake Bay
V. parahaemolyticus	5DBW SPR, 7D, 12B SPR, 5A 4A-A, 5DA, 4A-B, 5DBS		
Group IV	A4281	R. Weaver, CDC	Rat
V. parahaemolyticus	A4280		Bovine
	A6540		Fish
	A7606		Human wrist
	B1650		Human foot
Group V	1BH4422, 1BOH422, 1BH4423, 28PO31	J. Baross	Washington State water, clam, oyster
V. parahaemolyticus			
Group VI	14181S, 19264	ATCC	Unknown, lesion of cod
V. anguillarum	SOY2A, SOY1	J. Baross	Oysters
Group VII			
V. alginolyticus	17749	ATCC	Japan
Vibrio sp. "01"	11171	ATCC	Sewage
V. haloplanktis	14393	ATCC	
V. marinofulvus	14395	ATCC	
V. marinus	15381	ATCC	Seawater
V. alcaligenes	14736	ATCC	Endocarditis
V. metschnikovii	7708	ATCC	
V. marinopraesens	19648	ATCC	Marine mud
V. marinagilis	14398	ATCC	

[a] Abbreviations: ATCC, American Type Culture Collection; FDA, Food and Drug Administration; CDC, Center for Disease Control.

slides were stained for 6 to 18 min with flagella stain (Difco). Motility was determined by microscopic examination of the broth by the hanging-drop method (13). Growth from TSA slants was used to determine catalase production in 3% H_2O_2 and cytochrome oxidase on filter paper moistened with 1% aqueous tetramethyl-*p*-phenylenediamine. Utilization of glucose was tested in Hugh-Leifson medium at 25, 35, and 42 C (5). Sensitivity to pteridine O/129 and novobiocin was determined on TSA plates by the paper disc method. Reactions on triple sugar-iron-agar (TSI; Difco) were recorded in 24 hr. Salt tolerance was determined in Trypticase soy broth (TSB) with 0, 3, 7, and 10% NaCl. Hemolysis was determined on sheep, human, and rabbit blood with 0.5, 5.0, and 7.0% NaCl. Hemolysis was also tested on the medium proposed by Wagatsuma (7) for the "Kanagawa phenomenon." Starch hydrolysis was determined on a corn starch medium (R. M. Twedt et al., Bacteriol. Proc., p. 6, 1970). Unless stated otherwise, 3% NaCl was used in all media.

Serological identification. Serological identification was accomplished by slide agglutination with K antisera (Nichimen Co., New York, N.Y.). K pool and corresponding monovalent reactions were determined by the scheme of Sakazaki et al. (10). A suspension of a 24-hr TSA culture and diluent (3% NaCl) was mixed with an equal volume of polyvalent or monovalent antiserum. After mixing for 30 to 60 sec, agglutination was rated (1+ to 4+) by visual observation. Cultures were considered nontypable with any of the following: no reaction with K pool, reaction with more than one K pool, reaction with K pool and absence of any monovalent reaction, and reaction with K pool plus reaction with more than one monovalent antiserum.

Isolation of antigenic substance from V. parahaemolyticus. The procedure was a modification of that reported by Miwatani et al. (6) for the isolation of A substance. Thirty grams of wet-packed cells of *V. parahaemolyticus* (ATCC 17802) was used. The procedure was only carried out to the elution of the partially purified A (PPA) substance, and diethylaminoethyl (DEAE) Sephadex A-50 was used in place of DEAE cellulose.

Preparation of antiserum for PPA. A 40-ml sample of fraction PPA containing 3.44 mg of protein was reduced to 2.5 ml with Lyphogel (Gelman Instrument Co.). This was mixed with 2.5 ml of Freund's complete adjuvant (Difco). The mixture was injected subcutaneously into five sites (1 ml per site) of a rabbit's back. Titer was determined by slide agglutination with live cells of culture 17802. After 17 days the rabbit was exsanguinated, yielding 120 ml of whole blood. After clotting, the serum (49 ml) was removed and the globulin fraction was separated by dialysis (overnight at 4 C) against 50% saturated $(NH_4)_2SO_4$. The precipitate was removed by

centrifugation (16,000 × *g* for 20 min) and was washed three to four times with 50% saturated $(NH_4)_2SO_4$ until most of the red pigment was removed. The precipitate was suspended in 0.85% NaCl and desalted on a G-25 Sephadex column.

Conjugation of rabbit anti-PPA globulin with fluorescein isothiocyanate. Labeling procedures were those described by Goldman (4). The globulin solution was reduced to 16 ml with Lyphogel. Protein concentration by the method of Lowry et al. was 17.5 mg/ml. To this solution in an ice bath was added 10% (v/v) 0.5 M carbonate buffer (*p*H 9.5) in saline. Fluorescein isothiocyanate (FITC) was added at the rate of 15 μg of dye per mg of protein. The labeling reaction was allowed to proceed overnight at 4 C with gentle stirring. Unreacted dye was removed by passing the solution through a Sephadex G-25 column. The column was eluted with 0.0175 M phosphate buffer at *p*H 6.3. Highest working dilution of conjugate was found to be 1:8; hence a 1:4 dilution with FA buffer (Difco) was made yielding a total of 84 ml.

Fluorescent-antibody examination of V. parahaemolyticus and other organisms. Cultures of *Proteus*, *Salmonella*, *Aeromonas*, *Escherichia coli*, *Bacillus*, *Flavobacterium*, Providence group, *Staphylococcus*, *Alcaligenes*, *V. parahaemolyticus*, and other *Vibrio* species were streaked on TSA and incubated at 35 C for 24 hr. A loopful of each culture was spread in 3% NaCl on a FA slide (Clay Adams, Parsippany, N.J.) and allowed to dry in the air. They were then lightly heat-fixed before staining. A few drops of diluted labeled antibody were placed on each smear and incubated in a humidifier at 25 C for 30 min. Excess conjugate was removed by rinsing with FA buffer (Difco). Slides were then soaked for 10 min in the same buffer with at least one change of buffer in that time period. After air-drying, the slides were either mounted by using FA mounting fluid (Difco) and a cover slip or were examined directly. Controls were run by exposing antigen to normal (no antibody to antigen) conjugate and exposing antigen to unlabeled and then to labeled antibody. Indirect stains were made in a similar manner with minor exceptions. Smears were first reacted with unlabeled anti-PPA serum for 30 min and then were stained with fluorescein-conjugated goat anti-rabbit globulin (Nutritional Biochemicals, Cleveland, Ohio). Intensity of fluorescence was rated from 0 to 4+. Reactions of greater than 2+ were considered positive.

RESULTS

Morphological, biochemical, and serological characteristics. All *V. parahaemolyticus* cells were short, gram-negative rods exhibiting pleomorphism. Curved, straight, coccoid, and swollen forms were observed. There was also a strong tendency towards bipolar staining. All cultures were motile, had a single polar flagellum, and hydrolyzed starch. With few or no exceptions, cultures of *V. parahaemolyticus* (i) showed a weak catalase reaction; (ii) were positive for cytochrome oxidase; (iii) utilized glucose fermentatively at at least two of the three incubation temperatures; (iv) were sensitive to pteridine (O/129) and novobiocin; (v) did not grow in TSB without NaCl; (vi) grew well in TSB with 3 or 7% NaCl; and (vii) produced an alkaline slant, acid butt, and no H_2S or gas in TSI. Growth in 10% NaCl varied. Other *Vibrio* species were sensitive to pteridine O/129 and novobiocin. Hemolytic activity of the test strains varied greatly, depending on the type of blood and concentration of NaCl (Table 2). Table 3 shows the serological reactions observed in this laboratory and those reported by others. Only eight cultures were typable. The other 20 cultures were nontypable for the following reasons: 9 cultures failed to react with any K pool, 3 reacted with more than one K pool, 7 reacted with a K pool and failed to react with the monovalent antisera in that pool, and 1 reacted with more than one monovalent antiserum. Reactions on 13 cultures had been reported previously. Only 7 of the 13 cultures showed similar reactions in the present study. *V. anguillarum* (14181S) showed a 2+ reaction with the group III K pool and a slight agglutination with monovalent antisera 4 and 29. *V. alginolyticus* (17749) showed no reaction.

Isolation and identification procedure. The various plating media tested were as follows: Staphylococcus 110 medium with 5 IU of penicillin per ml; Twedt medium (Bacteriol. Proc., p. 6, 1970); Brain Heart Infusion agar with 5% NaCl; Brain Heart Infusion agar with tetramethyl-*p*-phenylenediamine (0.1%); bismuth-sulfite-agar; Brilliant Green-sulfadiazine-agar; tellurite-polymyxin-egg yolk-agar; tellurite-polymyxin-egg yolk-agar without polymyxin; Baross and Liston medium (1); Twedt medium with 5% NaCl and 0.2% bile salts; Twedt medium with crystal violet (0.0001%); Twedt medium with 1% corn starch, 7% NaCl, and 10 IU of penicillin per ml; and Twedt medium with 1% corn starch, 7% NaCl, and no penicillin. The enrichment broths were as follows: Trypticase soy broth with 5% NaCl at *p*H values from 8 to 11, Trypticase soy broth with 7% NaCl and 10 IU penicillin per ml, Trypticase soy broth with 7% NaCl. Plating media were inoculated by spreading 0.1 ml of appropriate dilutions of TSB cultures on the surface of the plates. In addition, seafoods with a natural microbial flora were inoculated with *V. parahaemolyticus* cultures and plated in a similar manner. All plating and enrichment media were incubated at 35 and 42 C. With few exceptions, a modification of a medium

TABLE 2. *Hemolysis of Vibrio parahaemolyticus with different types of blood and with various concentrations of NaCl*

Culture	Sheep			Rabbit			Human			Wagatsuma's "Kanagawa"
	0.5% NaCl	5.0% NaCl	7.0% NaCl	0.5% NaCl	5.0% NaCl	7.0% NaCl	0.5% NaCl	5.0% NaCl	7.0% NaCl	
17802	—[a]	—	—	β	β	β	—	—	β	α
17803	—	—	—	β	β	β	—	—	β	α
SAK 8	β	β	—	β	β	β	—	β	β	β
SAK 15	—	—	—	β	β	β	—	—	β	β
O	—	—	—	β	β	β	—	—	β	α
WL1	β	β	—	β	β	β	—	—	β	α
WL2	β	β	—	β	β	β	—	—	β	α
WL3	—	—	—	β	β	β	—	—	β	α
WL4	—	—	—	α	α	α	—	—	β	α
5DBW	—	β	—	β	β	β	—	—	β	α
12A	—	—	—	β	β	β	—	—	β	α
12B	α	α	—	β	β	β	—	β	β	α
5B	β	β	—	β	β	β	β	β	β	—
7BW	α	α	α	α	α	α	—	—	β	β
8C	—	—	—	β	β	β	—	—	β	β
5DBW SPR	—	β	β	β	α	β	—	—	β	α
12B SPR	β	β	—	β	α	β	—	β	β	α
5A	—	—	—	β	α	β	—	—	β	α
4A-A	—	—	—	β	β	β	—	β	β	—
5DA	—	β	—	β	α	β	—	—	β	α
7D	—	—	—	—	α	β	—	—	β	β
4A-B	β	—	—	β	α	β	—	—	β	—
5DBS	—	—	—	β	α	β	—	—	β	α
A4281	β	—	—	β	β	β	β	β	β	β
A4280	β	β	β	β	β	β	β	β	β	β
A6540	β	β	β	β	β	—	—	β	β	β
A7606	β	—	—	β	α	β	—	α	β	β
B1650	β	α	α	β	α	α	β	β	β	—

[a] Indicates nonhemolytic.

recently proposed for *V. parahaemolyticus* by Twedt et al. (Bacteriol. Proc., p. 6, 1970) was best suited for recovery and selective isolation of *V. parahaemolyticus*. This medium (MT) contained 2.0% peptone, 0.2% yeast extract, 1% corn starch, 7% NaCl, and 1.5% agar (*p*H 8.0). Enrichment broths were compared by subsequent plating on MT medium and on TSA with 3% NaCl. Enrichment was best in TSB with 7% NaCl adjusted to *p*H 7.3. Cultures of *V. parahaemolyticus* on MT medium were white to creamy, circular, smooth, and amylase-positive. The isolation procedure selected is as follows. Food (50 g) to be examined for *V. parahaemolyticus* was blended for 2 min with 450 ml of 7% NaCl. For direct plating, serial dilutions were made in sterile 7% NaCl. Quantities (0.1 ml) of appropriate dilutions were spread over the surface of MT agar plates. Enrichment consisted of placing 10-, 1-, and 0.1-ml quantities of homogenate into TSB with 7% NaCl. After 18 hr at 42 C, the tubes were streaked onto MT agar plates with a wire loop. All MT agar plates were incubated aerobically at 42 C for 24 to 48 hr. White to creamy, circular, smooth, amylase-positive colonies were picked as suspect *V. parahaemolyticus*. These isolates were then tested for Gram reaction, morphology, glucose utilization, presence of cytochrome oxidase, and sensitivity to pteridine O/129. Confirmation of gram-negative, fermentative, oxidase-positive, pleomorphic rods sensitive to pteridine O/129 was made by fluorescent-antibody technique.

Recovery efficiency of plating and enrichment media. Seafoods (50 g) were blended with 450 ml of sterile 7% NaCl and inoculated with *V. parahaemolyticus*. The population level of the inoculum (TSB culture) was determined by plating on MT medium and on TSA with 3% NaCl. The number of cells recovered was determined by direct plating on MT medium. Recovery of *V. parahaemolyticus* from seafoods inoculated with various isolates was generally acceptable (Table 4). The average recovery of *V. parahaemolyticus* from all seafoods was 85%. All enrichment broths which contained seafoods inoculated with *V. parahae-*

TABLE 3. *Serology of Vibrio parahaemolyticus and other cultures*

Culture	Reaction[a]	Reactions reported by others
17802	I++++, V++, 1++++	
17803	I++++, 1++++	
SAK 8	V++, 8++	8 (FDA, Dallas)[b]
SAK 15	II++++, 15++++	15 (FDA, Dallas)
O	IV++++, NT	III, 30 (R. R. Colwell)
WL1	NT	NT (FDA, Dallas)
WL2	IV+++, NT	25 (FDA, Dallas)
WL3	VII+++, 50++	NT (FDA, Dallas)
WL4	NT	25 (FDA, Dallas)
5DBW	IV+++, NT	
12A	V++++, NT	
12B	V++++, II++, 28+++	
5B	NT	
7BW	II++++, 17++++	
8C	NT	
5DBW SPR	IV+++, NT	
12B SPR	II++++, 17++++	
5A	II+++, NT	
4A-A	I+, II++, V++, VI+	
5DA	IV++, 37++++, 31+++, 33+, 43+	
7D	NT	
4A-B	V+++, 34++	NT (R. R. Colwell)
5DBS	NT	NT (R. R. Colwell)
A4281	NT	NT (14)
A4280	NT	41+++, 45++++ (14)
A6540	NT	NT (14)
A7606	II+++, 17+++	17 + + + (14)
CO (Texas A&M isolate)	VI++++, NT	
V. anguillarum	III++, 4+, 29+	
V. alginolyticus	NT	

[a] Roman numerals refer to polyvalent antisera, and arabic ones refer to monovalent antisera; NT = did not react with polyvalent and/or monovalent antisera; + to ++++ refers to degree of reaction.
[b] FDA, Food and Drug Administration.

TABLE 4. *Recovery of Vibrio parahaemolyticus from various seafoods*[a]

Culture	Shrimp		Oysters		Crab meat		Swordfish steaks	
O	900/1,800[b]	24,000/20,000	3,300/2,400	21,000/26,000	1,400/2,400	10,000/26,000	400/900	8,000/9,400
WL1	2,200/1,600	25,000/19,000	2,500/2,800	8,000/31,000	1,500/2,800	25,000/31,000	1,200/1,000	12,000/12,000
5A	3,000/1,900	22,000/19,000	500/700	9,000/7,700	700/700	12,000/7,700	900/1,400	9,000/9,000
12A	700/1,000	12,000/11,000						
17802	1,000/1,300	7,000/14,000						

[a] All enrichments from low and high cell populations were positive for *V. parahaemolyticus* when streaked on MT medium.
[b] Expressed as number per gram recovered/inoculated at low and high cell density.

molyticus yielded *V. parahaemolyticus* when subsequently streaked on MT medium. When food homogenates (two samples) were inoculated with very low cell concentrations (calculated 1 to 10 per g), *V. parahaemolyticus* was not recovered by direct plating on MT medium. However, enrichment in TSB and subsequent plating on MT medium yielded *V. parahaemolyticus* from these samples.

Immunofluorescent reaction. When tested with the conjugated globulin, all *V. parahaemolyticus* cultures (except 7BW, 8C, A4280, A6540, and A7606) were positive. These five cultures differed in one or more characteristics

from the majority of *V. parahaemolyticus* cultures. Some enteric pathogens such as Providence group, *Proteus*, and *Salmonella* showed positive reactions. Of the *V. anguillarum* cultures, only one (14181S) exhibited a positive reaction. Other species of *Vibrio* (including *V. alginolyticus*) exhibited slight or no fluorescence. The indirect staining procedure increased the intensity of fluorescence in only one case (Providence group). Fluorescence remained the same or diminished with the other test organisms.

Comparison of isolation procedures. Eighteen commercial seafoods were examined for *V. parahaemolyticus* by the present procedure and that described in the *Bacteriological Analytical Manual* (15). Seafoods included were oysters, clams, shrimp, squid, and crab meat. These samples were either fresh from the Gulf of Mexico or were purchased in local restaurants. Both procedures recovered *V. parahaemolyticus* from Galveston Bay clams and oysters from the Gulf of Mexico. With the new procedure, *V. parahaemolyticus* was recovered from two additional samples, oysters and crab from Galveston Bay. *V. parahaemolyticus* cells detected with both procedures were in low numbers and were recovered from 1:10 and 1:100 dilutions placed in enrichment broths. Organisms which mimicked *V. parahaemolyticus* on MT medium (gram-negative, oxidase-positive, and amylase-positive rods) were usually *V. alginolyticus*. Organisms picked as suspect *V. parahaemolyticus* from thiosulfate citrate bile salts sucrose agar (TCBS) were screened by the oxidase reaction and reactions in TSI. Many of these produced H_2S, gas, or were oxidase-negative. The number of colonies picked as suspect *V. parahaemolyticus* from the MT medium for further confirmatory tests was lower than that from TCBS medium.

Incidence of V. parahaemolyticus in other salted food products. Sixteen commercial foods including ham, salt pork, corned beef, stuffed crab, olives, pickles, and some canned seafoods (oysters, clams, sardines, tuna) were checked for the presence of *V. parahaemolyticus*. No *V. parahaemolyticus* was isolated from the salted foods. Amylase-positive colonies were noted on plates which contained corned beef. These were gram-negative rods which failed to utilize glucose fermentatively. With the enrichment procedure, amylase-positive colonies were recovered from three samples, but none could be confirmed as *V. parahaemolyticus*. The predominant organisms on MT plates usually were gram-positive cocci.

DISCUSSION

Numerous morphological and biochemical characteristics have been reported for *V. parahaemolyticus*. One of the objectives of this study was to determine a set of characteristics which would best identify *V. parahaemolyticus*. Strains of *V. parahaemolyticus* used in this study were morphologically similar to those reported by Sakazaki et al. (9) and Twedt et al. (14). They were short, gram-negative pleomorphic rods, with a single polar flagellum.

Sakazaki (8) recommended growth in 10% NaCl as one of the methods of separating *V. alginolyticus* (+) from *V. parahaemolyticus* (−). Results of the present study and those reported by Twedt et al. (14) showed that growth of *V. parahaemolyticus* in media with 10% NaCl was variable and that this characteristic should not be used as a key test in identification. *Aeromonas* can be separated from *Vibrio* because of its resistance to pteridine O/129. This substance is a known vibriostatic compound as described by Collier et al. (2), Shewan et al. (12), and Sakazaki et al. (9). Reactions in TSI agar as a preliminary screening test (*Bacteriological Analytical Manual*) are not considered useful, since many other organisms, for example anaerogenic *Aeromonas* and some *Enterobacteriaceae*, produce reactions similar to those of *V. parahaemolyticus*. Hemolysis has been related to pathogenicity by Miyamoto et al. (7) and Sakazaki et al. (11). It is also used as a preliminary screening criterion by Baross and Liston (1). Fishbein et al. (3) reported that only 50% of *V. parahaemolyticus* strains isolated from crab meat were hemolytic. Twedt et al. (14) were unable to separate pathogenic strains of *V. parahaemolyticus* from nonpathogens on sheep blood. The results of the present study indicate that hemolysis is dependent on type of blood and sodium chloride concentration and is neither a measure of pathogenicity (*unpublished data*) nor an adequate screening test for suspect *V. parahaemolyticus*. No differences could be established between human isolates and marine isolates on any of the blood media tested.

Serological identification of *V. parahaemolyticus* seemed to be of little value at the present time. With the commercial antisera obtained from Japan, only 8 of 28 known strains were typable. Of these eight, three were Japanese isolates, indicating perhaps that United States isolates may differ in serotypes from those found in Japan. With antisera of Japanese origin, Fishbein et al. (3) were able to type only

22 of 56 isolates from crab meat. All strains in their study were biochemically identical to Japanese cultures. In this study, only 7 of 13 cultures gave results comparable to those previously reported. Serological identification of *V. parahaemolyticus* is in such a state of uncertainty as to preclude its role as a diagnostic tool at the present time.

Another objective of this study was to develop an isolation procedure that would require less time than those currently employed. Baross and Liston (1) used anaerobic hydrolysis of starch and hemolysis of human blood in 1% NaCl as criteria for selecting suspect colonies for further identification of *V. parahaemolyticus*. The lack of dependability of hemolysis as a criterion for identification has been discussed. Although all strains of *V. parahaemolyticus* tested in this study hydrolyzed starch, results on salt water-starch-agar (Baross-Liston) were not clear. Growth of *V. parahaemolyticus* was not good, and in mixed cultures amylase-positive colonies were difficult to recognize. Twedt et al. (Bacteriol. Proc., p. 6, 1970) eliminated the tedious process of anaerobic incubation; however, many other marine organisms are capable of growth on their starch medium. Starch hydrolysis was used as a means of picking suspect *V. parahaemolyticus*. The procedure described in the *Bacteriological Analytical Manual* (15) is a combination of Baross and Liston's and Japanese methods. The procedure employs two plating and two enrichment media. Suspect colonies are screened on TSI reaction with 34 additional biochemical tests that could require up to 9 days for the final identification of *V. parahaemolyticus*. The procedure reported in this study requires 3 to 4 days for the identification of *V. parahaemolyticus*. Gram-positive bacilli and cocci are frequently encountered but present no problem because of distinct morphological differences of colonies and cells. Confirmation of suspect colonies is based on the previously mentioned morphological and biochemical characteristics and fluorescent-antibody reactions. Although *V. alginolyticus* mimicked *V. parahaemolyticus* on MT plates, separation was easy on the basis of morphology and fluorescent-antibody reaction. Recovery of *V. parahaemolyticus* from inoculated seafoods by this new procedure was good. The method is comparable or more effective than those currently used for the isolation of *V. parahaemolyticus* from fresh seafoods. Although *V. parahaemolyticus* was not isolated in this study from other salted foods, the possible presence of this halophilic organism in other salted foods should not be discounted.

Although serological identification of *V. parahaemolyticus* is at the present time questionable, the A substance described by Miwatani et al. (6) seems a very specific antigen. The isolation of this substance is quite complex and requires large numbers of wet-packed cells. With the modifications in procedure recommended in this study, the isolation of fraction PPA (assumed to be partially purified A substance) is not difficult. Fluorescein conjugated anti-PPA rabbit globulin was specific for *V. parahaemolyticus* when tested with other species of the genus *Vibrio*. Some cross-reactions were observed with enteric pathogens (*Enterobacteriaceae*), but it is doubtful that these organisms would be encountered on MT medium containing 7% NaCl.

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