Prevalence of Pandemic Thermostable Direct Hemolysin-Producing *Vibrio parahaemolyticus* O3:K6 in Seafood and the Coastal Environment in Japan

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Although thermostable direct hemolysin (TDH)-producing *Vibrio parahaemolyticus* has caused many infections in Asian countries, the United States, and other countries, it has been difficult to detect the same pathogen in seafoods and other environmental samples. In this study, we detected and enumerated *tdh* gene-positive *V. parahaemolyticus* in Japanese seafoods with a *tdh*-specific PCR method, a chromogenic agar medium, and a most-probable-number method. The *tdh* gene was detected in 33 of 329 seafood samples (10.0%). The number of *tdh*-positive *V. parahaemolyticus* ranged from <3 to 93/10 g. The incidence of *tdh*-positive *V. parahaemolyticus* tended to be high in samples contaminated with relatively high levels of total *V. parahaemolyticus*. TDH-producing strains of *V. parahaemolyticus* were isolated from 11 of 33 *tdh*-positive samples (short-necked clam, hen clam, and rock oyster). TDH-producing strains of *V. parahaemolyticus* were also isolated from the sediments of rivers near the coast in Japan. Representative strains of the seafood and sediment isolates were examined for the O:K serovar and by the PCR method specific to the pandemic clone and arbitrarily primed PCR and pulsed-field gel electrophoresis techniques. The results indicated that most O3:K6 *tdh*-positive strains belonged to the pandemic O3:K6 clone and suggested that serovariation took place in the Japanese environment.

Vibrio parahaemolyticus inhabits the marine environment and is a major food-borne pathogen. Strains carrying the *tdh* gene, encoding the thermostable direct hemolysin (TDH), or the *trh* gene, encoding the TDH-related hemolysin (TRH), or both genes are considered virulent strains (18).

In recent years, outbreaks of *V. parahaemolyticus* infection have increased in Japan and Taiwan with the increase in the incidence of the O3:K6 serovar (8, 14). Outbreaks of O3:K6 infection also occurred in the United States, linked to ingestion of raw seafoods (6, 7). The O3:K6 strains were demonstrated to carry the *tdh* gene but not the *trh* gene and to be a new clone by an arbitrarily primed PCR (AP-PCR) technique (17, 20). AP-PCR and group-specific PCR (GS-PCR), a PCR method targeting the clone-specific bases in the *toxRS* operon (17), revealed that the new clone was responsible for pandemic spread to Taiwan, Laos, Japan, Thailand, Korea, and the United States since 1996 (4, 17, 24, 25). Furthermore, sero-

variants of the pandemic clone belonging to O4:K68, O1:K untypeable, O1:K25, O1:K41, and O4:12 were found (5, 17; M. Nishibuchi, unpublished data).

The new O3:K6 clone, in addition to other virulent strains of *V. parahaemolyticus*, has rarely been isolated from food and other environmental samples, although many infections with the O3:K6 clone have been epidemiologically linked to consumption of seafoods after 1997 in Japan (14). Virulent strains of *V. parahaemolyticus* are usually found together with much larger populations of avirulent strains in the environment. The relatively low population of virulent strains in environmental samples (3, 10, 21) and the similarity in growth kinetics of the virulent and avirulent strains have made it difficult to selectively detect and enumerate virulent strains in environmental samples. Therefore, undifferentiated total *V. parahaemolyticus* instead of virulent *V. parahaemolyticus* has long been used as an indicator for control of food contamination toward prevention of infection, but the appropriateness of this approach remains uncertain because of the lack of adequate information on the incidence of virulent strains in environmental samples.

To gain insights into the incidence and population of TDH-producing O3:K6 strains in environmental samples, we tried to isolate and enumerate these strains in seafoods marketed in

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Enrichment
Sample 25 g+225 ml APW

Qualitative analysis   **Quantitative analysis**
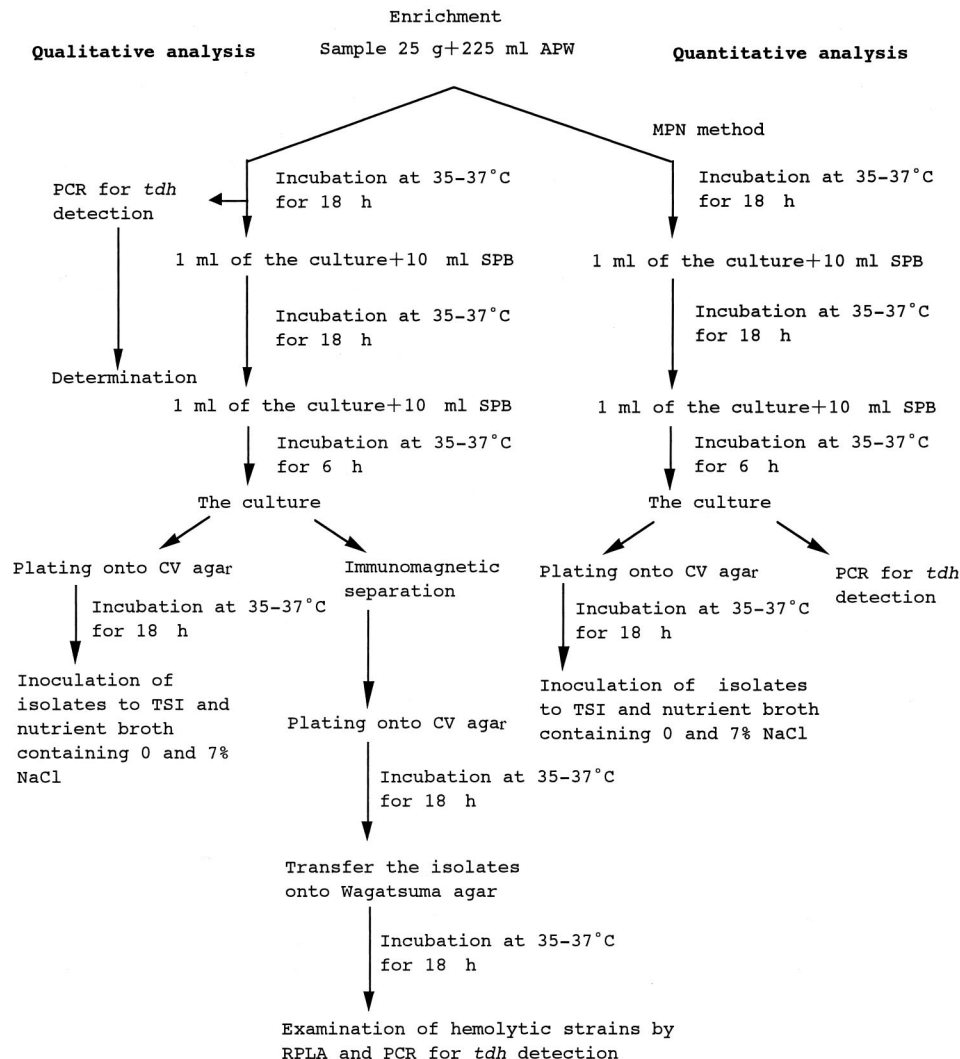
MPN method

FIG. 1. Schematic representation of qualitative and quantitative analyses of total and *tdh*-positive *V. parahaemolyticus* in seafood. APW, alkaline peptone water; SPB, salt polymyxin broth; CV, CHROMagar Vibrio; RPLA, reversed passive latex agglutination test; TSI, triple sugar iron.

Japan. The most-probable-number (MPN) method (2) and a PCR method for *tdh* gene detection were used for quantitative analysis, and the isolation method employing a chromogenic agar medium (12) combined with selective enrichment was used for qualitative analysis. We examined primarily molluscan shellfish, because mollusks are filter feeders that accumulate bacteria in aquatic environments and thus are likely be the source of *V. parahaemolyticus* infection. The sediments of rivers near the coast in Japan were also examined for the prevalence of TDH-producing strains. To investigate whether the strains isolated in this study belong to the pandemic clone, their characteristics were compared with those of the pandemic strains isolated previously from clinical sources in various countries.

MATERIALS AND METHODS

Samples for detection of *V. parahaemolyticus*. Seafood samples were collected in the north region (four prefectures), the central region (four prefectures), and

the south region (five prefectures) of Japan from June to October 2001. Unless otherwise specified, the seafoods were packed in polyethylene bags and kept in styrene foam boxes with ice. Short-necked clam (*Tapes japonica*), scallop (*Patinopecten yessoenisis*), Japanese abalone (*Haliotis discus*), sea urchin (*Echinoidea* sp.), rock oyster (*Crassostrea nippona*), spiny top shells (*Turbo cornutus*), Japanese razor shell (*Solen strictus*), underlined fig shell (*Ficus subintermedius*), pen shell (*Atrina pectinata japonica*), Tokobushi abalone (*Sulculus diversicolor aquatilis*), Japanese hard clam (*Meretrix lusoria*), Japanese oyster (*Crassostrea gigas*), and hen clam (*Mactra sulcatari*) samples were purchased from nurseries. The shells were removed aseptically, and the meat was subjected to examination. The meat of hen clams sold without shells at a fresh seafood market was also examined. Common freshwater clams (*Corbicula leana*) were purchased from retail shops and examined without removing the shells. Horse mackerel (*Trachurus japonicus*) purchased from retail shops and markets were cut into several portions, and the head with the gills and surface skin was used as the test sample.

Procedure for analysis of *tdh*-positive *V. parahaemolyticus* in seafood. Qualitative and quantitative analyses of *tdh*-positive *V. parahaemolyticus* in seafood were carried out as shown schematically in Fig. 1. In the quantitative analysis with an MPN method, a 25-g portion of test sample was mixed with 225 ml of alkaline peptone water (APW; Nissui Co., Tokyo, Japan) in a stomacher bag, which was then gently homogenated with hands. Either 10 ml, 1 ml, or 0.1 ml of the homogenate and 1 ml or 0.1 ml of a 10^{-2} dilution of the homogenate were

inoculated into 10 ml of APW in triplicate and incubated at 35 to 37°C for 18 h. One-milliliter portions of each APW culture were inoculated into 10 ml of salt polymyxin broth (SPB; Nissui Co.) and incubated for 18 h at 35 to 37°C, and then 1 ml of the SPB culture was inoculated into 10 ml of fresh SPB and incubated for 6 h at 35 to 37°C. A portion of the third enrichment culture in SPB was plated onto CHROMagar Vibrio (CV) agar (CHROMagar, Paris, France) (12) for detection of total *V. parahaemolyticus* by isolation and biochemical identification. Another 1-ml portion of the third enrichment culture served as the material for PCR analysis for detection of *tdh*-positive *V. parahaemolyticus*.

For qualitative analysis, the remaining portion of the sample homogenate in APW was incubated at 35 to 37°C for 18 h, and then two further steps of enrichment culture were conducted in the same manner as for the quantitative analysis except that only one tube was used for each enrichment step. During the course of the three-step enrichment cultures, the first enrichment culture in APW was screened for the presence of *tdh*-positive *V. parahaemolyticus* by the PCR method. If the PCR analysis showed a positive reaction, portions of the third enrichment culture were plated directly and also after the immunomagnetic separation step (described below) onto CV agar for isolation and identification of *tdh*-positive *V. parahaemolyticus*.

In the previous study, we found that repeated enrichment with fresh medium improved detection of *V. parahaemolyticus* in seafood (12). Preliminary experiments showed that TDH-producing *V. parahaemolyticus* was isolated from all of five seawater samples by the three-step enrichment, whereas that was isolated from two out of five samples by single-step enrichment, indicating that the three-step enrichment procedure is more effective in detection of TDH-producing *V. parahaemolyticus* than the one-step enrichment procedure.

Procedure for isolation of *tdh*-positive *V. parahaemolyticus* from river sediments. The following three-step enrichment procedure was employed for isolation of *tdh*-positive *V. parahaemolyticus* from river sediments. Each sample (10 g) was added to 100 ml of tryptic soy broth (TSB; Difco, Detroit, Mich.) containing 2% NaCl and cultured for 6 h at 36°C. Ten milliliters of the culture was further cultured with 100 ml of SPB for 18 h at 36°C. The SPB culture (0.5 ml) was again cultured with 10 ml of SPB for 6 h at 36°C. Preliminary experiments showed that the three-step enrichment procedure was superior to the one-step enrichment procedure with SPB; *tdh* was detected by PCR in four out of eight river water samples by the three-step enrichment, whereas *tdh* was detected in two out of eight samples by the one-step enrichment. After inoculation of the culture onto thiosulfate citrate bile salts sucrose (Nissui Co.) and incubation for 18 h at 36°C, colonies suspected as *V. parahaemolyticus* were confirmed for production of TDH on Wagatsuma agar, which is described below.

Identification of *V. parahaemolyticus* on CV agar. After inoculation of the enrichment culture, the CV agar plate was incubated at 37°C for 18 h. Purple colonies on the CV agar plate were presumptively identified as *V. parahaemolyticus* and inoculated into triple sugar iron medium (Eiken Co, Tokyo, Japan), nutrient broth (Difco), and nutrient broth supplemented with 8% NaCl. Test strains showing alkaline slant and acid butt reactions in the triple sugar iron medium, and, in nutrient broth, no growth without and growth with 8% NaCl were identified as *V. parahaemolyticus*.

Immunomagnetic separation of K6 strains and detection of TDH production. Since investigations of recent outbreaks have indicated that O3:K6 is a major serotype of TDH-producing *V. parahaemolyticus*, immunomagnetic separation of K6 strains was performed to improve isolation of TDH-producing organisms (23). A 1-ml portion of the enrichment culture was added to 25 μl of immunomagnetic beads coated with antibody against the *V. parahaemolyticus* K6 antigen (Denka Seiken, Tokyo, Japan). According to the manufacturer's instructions, the beads were incubated, separated, and then suspended in 0.1 ml of washing buffer. Then 20 ml of the suspension was streaked onto CV agar. After the agar was incubated at 37°C for 18 h, purple colonies presumptively identified as *V. parahaemolyticus* were examined for TDH production with Wagatsuma agar medium for the Kanagawa phenomenon.

To prepare Wagatsuma agar medium, melted basal medium [yeast extract, 5 g/liter; Bacto-peptone, 10 g/liter; sodium chloride, 70 g/liter; D-(−)-mannitol, 5 g/liter; agar, 15 g/liter; crystal violet, 1 mg/liter; dipotassium hydrogen phosphate, 5 g/liter (pH 7.5)] (Kyokuto Pharmaceutical Ind. Co., Tokyo, Japan) was mixed with a 20% solution of washed human blood (100 ml/liter). The test strains were inoculated onto the Wagatsuma agar plate. After incubation for 18 h at 35 to 37°C, the strain showing a clear zone around the colony was examined for TDH production by an immunological method with anti-TDH antibody. The agglutination test with a reversed passive latex agglutination assay kit (KAP-RPLA; Denka Seiken) was performed according to the manufacturer's specifications. Finally, the presence of the *tdh* gene in the TDH-producing strains was confirmed by the PCR method described below.

Serotyping. Serovars of the strains isolated from CV agar and identified as *V. parahaemolyticus* were determined with a serotyping kit (Denka Seiken) according to the manufacturer's instruction.

PCR. Enrichment cultures at the first step in APW and third step in SPB and the SPB culture of the isolated strain grown for 18 h at 37°C served as the materials for the PCR assay for *tdh* detection. One milliliter of the test culture was centrifuged at 5,000 × *g* for 10 min. After the supernatant was removed, the pellet was resuspended in 0.1 ml of sterilized distilled water and heated at 100°C for 5 min. After centrifugation at 10,000 × *g* for 10 min, the supernatant was transferred to a new tube and stored at 4°C or −20°C until the PCR assay.

PCR for detection of the *tdh* gene was performed with primer 1 (5′-GGTAC TAAATGGCTGACATC) and primer 2 (5′-CCACTACCACTCTCATATGC) as described by Tada et al. (22). Briefly, the PCR mixture (50 μl) consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01 mM EDTA, 0.1 mM dithiothreitol, 0.05% Tween 20, 0.05% Nonidet P-40, 5% glycerol, 0.2 mM each of the four deoxynucleoside triphosphates (dNTP mixture; Takara, Ohtsu, Japan), and 0.5 U of *Taq* polymerase (Takara *Ex Taq*; Takara). The amplification conditions were set at one cycle of 96°C for 5 min, followed by 35 cycles of amplification consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min, followed by one cycle of 72°C for 7 min.

For detection of the *toxR* gene and detection of the pandemic strains by GS-PCR, the test strains were grown in tryptic soy broth containing 2% NaCl at 37°C for 18 h. One milliliter of the culture was centrifuged at 5,000 × *g* for 10 min. After the supernatant was removed, the pellet was resuspended in 0.1 ml of sterile distilled water and heated at 100°C for 5 min. After centrifugation at 10,000 × *g* for 10 min, the supernatant was transferred to a new tube and stored at 4°C or −20°C. The PCRs for *toxR* detection and for GS-PCR were carried out following the methods described by Kim et al. (16) and by Matsumoto et al. (17), respectively.

AP-PCR. AP-PCR was carried out according to the method described by Okuda et al. (20) with primer 2 (5′-GTTTCGCTCC) and primer 4 (5′-AAGA GCCCGT).

PFGE analysis. For pulsed-field gel electrophoresis (PFGE) analysis, the test strain was grown to mid-log phase (optical density of 0.9 to 1.0 at 600 nm) in Luria-Bertani broth containing 1% NaCl. Bacterial cells were harvested by centrifugation (3,000 × *g*), washed, mixed with 2% agarose, and dispensed into a plug mold as described by Albert et al. (1) except that low-melting-point agarose (Bethesda Research Laboratories, Gaithersburg, Md.) was used. The agarose plug was treated with lysozyme solution, deproteinated, and digested with 50 U of *Not*I restriction enzyme as described by Chowdhury et al. (9). The digested DNA fragments were separated by the contour-clamped homogeneous electric field method on a CHEF-DRIII system (Bio-Rad Laboratories, Hercules, Calif.) in 1% agarose (pulse field certified agarose; Bio-Rad Laboratories) with 0.5× TBE buffer (44.5 mM Tris-HCl, 44.5 mM boric acid, 1.0 mM EDTA [pH 8.0]). The running conditions were 6 V/cm at 14°C for 24 h at a field angle of 120°, and switch times were 1 to 18 s for 12 h and 3 to 80 s for 12 h. Following electrophoresis, the gels were stained with ethidium bromide (10 μg/ml) for 30 min, destained in distilled water for 1 h, and photographed with a UV transilluminator.

The dendrogram was constructed from the PFGE profiles according to an unweighted pair-group method with arithmetic average (UPGMA) clustering analysis with Dendron software, version 3 (Solltech Inc., Oakdale, La.).

RESULTS

Qualitative analysis: incidence of total and TDH-producing *V. parahaemolyticus* in seafood. Three hundred and twenty-nine seafood samples belonging to 13 species of molluscan shellfish (bivalves) and two other seafoods were purchased in various regions in Japan between June and October 2001. The presence of total *V. parahaemolyticus* was examined for 173 of 329 samples (Table 1). The unknown region means that the samples were purchased at the Central Market in Tokyo but their exact harvest locations could not be identified. Ninety-five percent (*n* = 165) of the 173 samples were positive. All 329 samples were examined for *tdh*-positive *V. parahaemolyticus* in the qualitative analysis (Table 1). PCR analysis of the first enrichment culture in APW of the seafoods revealed the presence of the *tdh* gene in 33 samples (10.0%): 8 of 137 samples

TABLE 1. Detection of *tdh*-positive and total *V. parahaemolyticus* in seafood samples in Japan in 2001

| Region of Japan | Seafood | No. of samples | | | | |
| | | *tdh*-positive *V. parahaemolyticus* | | | Total *V. parahaemolyticus* | |
		Total	*tdh* positive[a]	Positive for TDH-producing strains[b]	Total	*V. parahaemolyticus* isolated
North	Rock oyster	78	7	6	1	1
	Scallop	29	0	NT[c]	NT	NT
	Japanese abalone	18	0	NT	NT	NT
	Hen clam	7	1	0	4	4
	Sea urchin	3	0	NT	NT	NT
	Spiny top shell	1	0	NT	NT	NT
	Japanese oyster	1	0	NT	1	0
	Total	137	8	6	6	5
Central	Hen clam	97	8	2	76	72
	Short-neck clam	32	7	2	30	30
	Horse mackerel	9	0	NT	8	7
	Common freshwater clam	3	0	NT	3	3
	Total	141	15	4	117	112
South	Horse mackerel	8	0	NT	7	6
	Spiny top shell	6	1	0	6	5
	Short-neck clam	4	1	0	4	4
	Common freshwater clam	2	0	NT	2	2
	Japanese hard clam	2	0	NT	2	2
	Japanese razor shell	1	0	NT	1	1
	Underlined fig shell	1	0	NT	1	1
	Japanese oyster	1	0	NT	1	1
	Pen shell	1	0	NT	1	1
	Tokobushi abalone	1	1	0	1	1
	Total	27	3	0	26	24
Unknown	Hen clam	24	7	1	24	24
Total		329	33	11	173	165

[a] APW enrichment culture directly examined by the PCR method.
[b] TDH-producing strains were actually isolated.
[c] NT, not tested.

from the north region, 15 of 141 samples from central region, 3 of 27 samples from the south region, and 7 of 24 samples from the unknown region. TDH-producing *V. parahaemolyticus* was isolated from 11 of 33 *tdh*-positive samples.

Quantitative analysis: MPN of total and *tdh*-positive *V. parahaemolyticus* in seafood samples. In a preliminary study, we confirmed the effectiveness of the PCR for the MPN method. The MPN of *tdh*-positive *V. parahaemolyticus* was determined by PCR and plating methods with short-neck clam samples which had been inoculated with *tdh*-positive *V. parahaemolyticus*. The PCR method gave 2.5-fold lower MPN than the plating method in 1 out of 10 samples, but both methods gave the same MPN values for nine samples, indicating that the two methods have similar sensitivities. Furthermore, a *tdh*-negative (10^7 CFU/0.1 ml) and a *tdh*-positive (10^2, 10^3, or 10^4 CFU/0.1 ml) strain of *V. parahaemolyticus* were inoculated into the enrichment cultures of short-neck clams and scallops, which contained background bacteria at concentrations of 10^2 to 10^3 CFU/ml, and PCR for *tdh* detection was performed. *tdh* was detected in all the samples by the three-step enrichment procedure, demonstrating that *tdh*-positive *V. parahaemolyticus* can be detected in the presence of a large population of *tdh*-negative *V. parahaemolyticus*.

Accordingly, the number of *V. parahaemolyticus* was esti-

mated by the MPN method described in the Materials and Methods section for 173 samples spanning more than 12 species of molluscan shellfish and one other seafood (details not shown). The total number of *V. parahaemolyticus* ranged from <3 to >110,000 MPN/10 g (Fig. 2). Sixteen of 173 samples were *tdh* positive in the qualitative analysis, and therefore these *tdh*-positive samples were analyzed for the MPN of *tdh*-positive *V. parahaemolyticus* (Fig. 2). The numbers of *tdh*-positive *V. parahaemolyticus* were <3 MPN/10 g for two hen clam, five short-neck clam, and one Tokobushi abalone sample, 4 to 15 MPN/10 g for four hen clam, one spiny top shell, and two short-neck clam samples, and 93 MPN/10 g for one short-neck clam sample.

The ratio of *tdh*-positive and total *V. parahaemolyticus* ranged widely (93 of 4,300 to <3 of 46,000) (Fig. 2). The number of *tdh*-positive *V. parahaemolyticus* was not exactly in parallel to that of total *V. parahaemolyticus*. However, the incidence of *tdh*-positive *V. parahaemolyticus* tended to be high in samples contaminated with relatively high levels of total *V. parahaemolyticus*. The *tdh* gene was detected in 14 of 55 samples (25.5%) contaminated with total *V. parahaemolyticus* at equal to or greater than 100 MPN/10 g, whereas only 3 (2.5%) of 118 samples contaminated with total *V. parahaemolyticus* of less than 100 MPN/10 g were *tdh* positive.
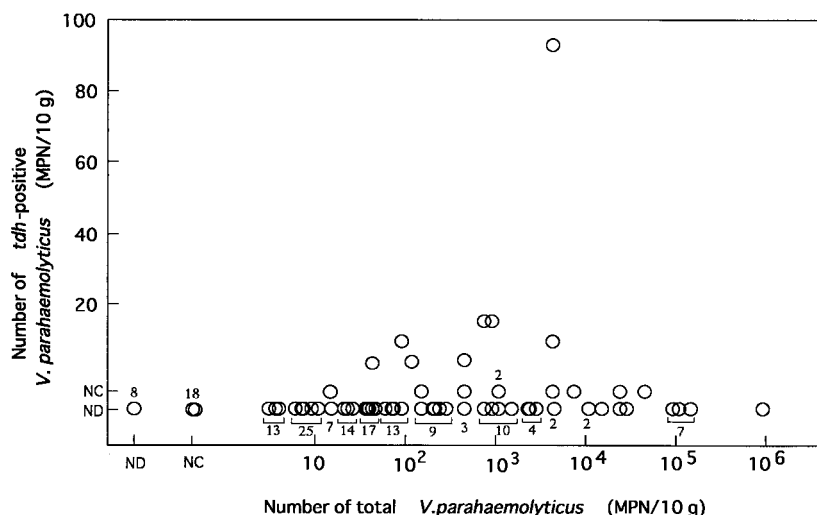
FIG. 2. Relationship between total number of *V. parahaemolyticus* organisms and number of *tdh*-positive *V. parahaemolyticus* organisms in seafood. Each circle indicates the number in each sample. ND, *tdh* gene not detected by qualitative analysis (<1 CFU/25 g), NC, *tdh* gene not detected by quantitative analysis (<3 MPN/10 g) but detected by qualitative analysis (>1 CFU/25 g).

Effect of clam storage at 30°C for 3 h on detection of *tdh* gene. Whether storage of clam samples at a high temperature before examination improves *tdh* detection was examined. Twenty-two batches of hen clam samples were purchased in the markets between August and October. Two portions were sampled from each of the 22 hen clam samples and subjected to qualitative and quantitative analyses. A portion was transferred immediately from an iced styrene foam box into APW enrichment broth warmed to 37°C, while the other portion of

the clam sample was stored at 30°C for 3 h before the APW enrichment step. The *tdh* gene was detected in three samples by the routine procedure without storage at 30°C for 3 h and in seven samples by the procedure with storage at 30°C for 3 h (Table 2). In all samples except for sample 7, the MPN of total *V. parahaemolyticus* increased remarkably after storing the samples at 30°C for 3 h.

Characteristics of selected isolates. The strains isolated from CV agar of the seafood samples and TDH-producing and

TABLE 2. Effect of storage at 30°C for 3 h on detection of *tdh* gene and the MPN of total and *tdh*-positive *V. parahaemolyticus* in hen clam samples

Sample no.	Sampling date in 2001	Without storage at 30°C for 3 h			After storage at 30°C for 3 h		
		tdh gene	MPN/10 g		*tdh* gene	MPN/10 g	
			Total *V. parahaemolyticus*	*tdh*-positive *V. parahaemolyticus*		Total *V. parahaemolyticus*	*tdh*-positive *V. parahaemolyticus*
1	August 13	−	43	NT[a]	+	>110,000	15
2		−	43	NT	+	46,000	<3
3		+	1,100	<3	+	>110,000	<3
4		−	23	NT	−	9,300	NT
5		+	4,300	<3	+	>110,000	240
6	August 20	−	93	NT	−	2,100	NT
7		−	750	NT	+	460	7
8	September 10	−	43	NT	+	46,000	20
9		−	150	NT	−	24,000	NT
10		−	93	NT	−	29,000	NT
11		−	93	NT	−	9,300	NT
12	September 17	−	21	NT	−	210	NT
13	September 25	−	4	NT	−	23	NT
14		−	<3	NT	−	24,000	NT
15		−	21	NT	−	24,000	NT
16		+	43	3	+	3,800	NT
17	October 1	−	4	NT	−	210	NT
18		−	9	NT	−	1,100	NT
19		−	<3	NT	−	210	NT
20		−	7	NT	−	21,000	NT
21		−	<3	NT	−	>1,500	NT
22	October 9	−	9	NT	−	240	NT

[a] NT, not tested.

TABLE 3. Characteristics of selected strains of *V. parahaemolyticus*

Class	Strain no.	Sample	Origin[a]	Date of isolation	Serotype	*toxR* gene	*tdh* gene[b]	GS-PCR	AP-PCR pattern[c] obtained with: Primer 2	Primer 4	PFGE profile[d]
Japanese environmental samples	VP1152	Rock oyster	N	24 July 2001	O3:K6	+	+	+	a	i	H
	VP1153	Rock oyster	N	24 July 2001	O3:K6	+	+	+	a	i	F
	VP1219	Rock oyster	N	7 Aug. 2001	O3:K6	+	+	+	a	i	F
	VP1280	Rock oyster	N	30 Aug. 2001	O3:K6	+	+	+	a	i	E
	VP1281	Rock oyster	N	30 Aug. 2001	O3:K6	+	+	+	a	i	E
	VP1282	Rock oyster	N	30 Aug. 2001	O3:K6	+	+	−	c	ii	ND[e]
	1001A44	Hen clam[f]	N	17 Sep. 2001	O3:K6	+	−	+	b	i	J
	1001A46	Hen clam[f]	N	17 Sep. 2001	O3:K6	+	−	+	b	i	K
	VPF01-13	Hen clam	C	6 Aug. 2001	O3:K6	+	+	+	a	i	ND
	VP80	Hen clam	C	20 Aug. 2001	O3:K6	+	+	+	a	i	F
	VPF01-5	Short-neck clam	C	16 July 2001	O3:K6	+	+	+	a	i	ND
	19-13	Hen clam	U	14 Aug. 2001	O3:K6	+	+	+	a	i	F
	VPF00-18	Short-neck clam	C	16 Oct. 2000	O3:K6	+	+	+	a	i	G
	VPF00-10	Short-neck clam	C	2 Oct. 2000	O3:K6	+	+	+	a	i	E
	APCC VP 00157	Sediment of river N	N	July 2001	O1:K25	+	+	+	a	i	O
	APCC VP 00190	Sediment of river N	N	Aug. 2001	O4:K68	+	+	+	a	i	P
	APCC VP 00030	Sediment of river A	N	Dec. 2000	O3:K6	+	−	+	b	i	L
	APCC VP 00031	Sediment of river K	N	Dec. 2000	O3:K6	+	−	+	b	i	M
	APCC VP 9810	Sediment of river N	N	Aug. 1998	O3:K6	+	+	+	a	i	I
Reference strains[g]	FIHES98V1-32-4	Clinical specimen	Japan	1998	O3:K6	+	−	+	b	i	A
	JKY-VP6	Clinical specimen	Japan	1998	O3:K6	+	+	+	a	i	B
	VP-2	Clinical specimen	Korea	1998	O3:K6	+	+	+	a	i	C
	BE98-2062	Clinical specimen	USA	1998	O3:K6	+	+	+	a	i	D
	KX-V225	Clinical specimen	Int.travel.	1996	O3:K6	+	+	+	a	i	E
	AN-8373	Clinical specimen	Bangladesh	1998	O3:K6	+	+	+	a	i	E
	VP-81	Clinical specimen	India	1996	O3:K6	+	+	+	a	i	E
	DOH958-15	Clinical specimen	Taiwan	1997	O3:K6	+	+	+	a	i	E
	97LVP2	Clinical specimen	Laos	1997	O3:K6	+	+	+	a	i	E
	VP-47	Clinical specimen	Thailand	1998	O3:K6	+	+	+	a	i	E
	VP-67	Clinical specimen	Thailand	1999	O3:K6	+	+	+	a	i	E
	4917	Clinical specimen	Thailand	1999	O1:K25	+	+	+	a	i	N
	5095	Clinical specimen	Thailand	1999	O4:K68	+	+	+	a	i	P

[a] N, northern Japan; C, central Japan; U, unknown; Int. travel., International traveler to Thailand.
[b] Ability to produce TDH was confirmed by detection of TDH by reversed-phase passive latex agglutination test.
[c] The patterns are shown in Fig. 3.
[d] The profiles are shown in Fig. 4A.
[e] ND; not determined because of DNA degradation.
[f] From the same sample.
[g] Matsumoto et al. (17) and M. Nishibuchi (unpublished data).

-nonproducing strains isolated from the river sediment (see the Materials and Methods section for the latter) in Japan were examined for the O:K serovar. Seventeen O3:K6, one O4:K68, and one O1:K25 strain, each isolated from different samples, and the reference strains of the pandemic clone were characterized to examine their relatedness (Table 3). All strains were confirmed to be *V. parahaemolyticus* by detecting the species-specific *toxR* gene. Only one strain isolated from rock oyster was negative in the GS-PCR test. Of the strains isolated from Japanese seafood and the river sediment, four strains were atypical in that they lacked the ability to produce TDH but were GS-PCR positive.

The test strains were compared by two genetic fingerprinting techniques. In the AP-PCR analysis with primer 2, the test strains were classified into three types, arbitrarily designated a, b, and c (Fig. 3; Table 3). The TDH-producing and GS-PCR-positive strains and TDH-nonproducing and GS-PCR-positive strains isolated from Japanese seafood and river sediment belonged to types a and b, respectively. The TDH-producing and GS-PCR-negative O3:K6 strain belonged to type c.

In the AP-PCR analysis with primer 4, the test strains were classified into two types, arbitrarily designated i and ii (Fig. 3;
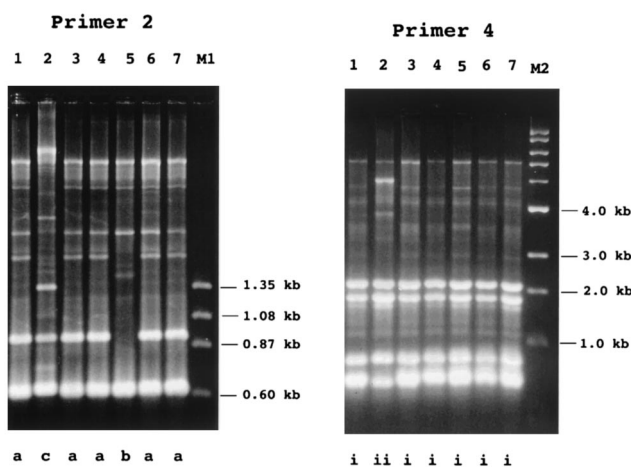
FIG. 3. Representative AP-PCR patterns exhibited by selected strains. The results obtained with primer 2 and primer 4 are shown as indicated. Lanes: 1, VP1281; 2, VP1282; 3, VPFO1-5; 4, APC-CVP9810; 5, 1001A46; 6, VPFOO-10; 7, VP-81; M1, φX174 DNA digested with *Hae*III; M2, 1-kb DNA ladder. Arbitrarily designated AP-PCR patterns are indicated at the bottom. The test strains and their AP-PCR patterns are listed in Table 3.
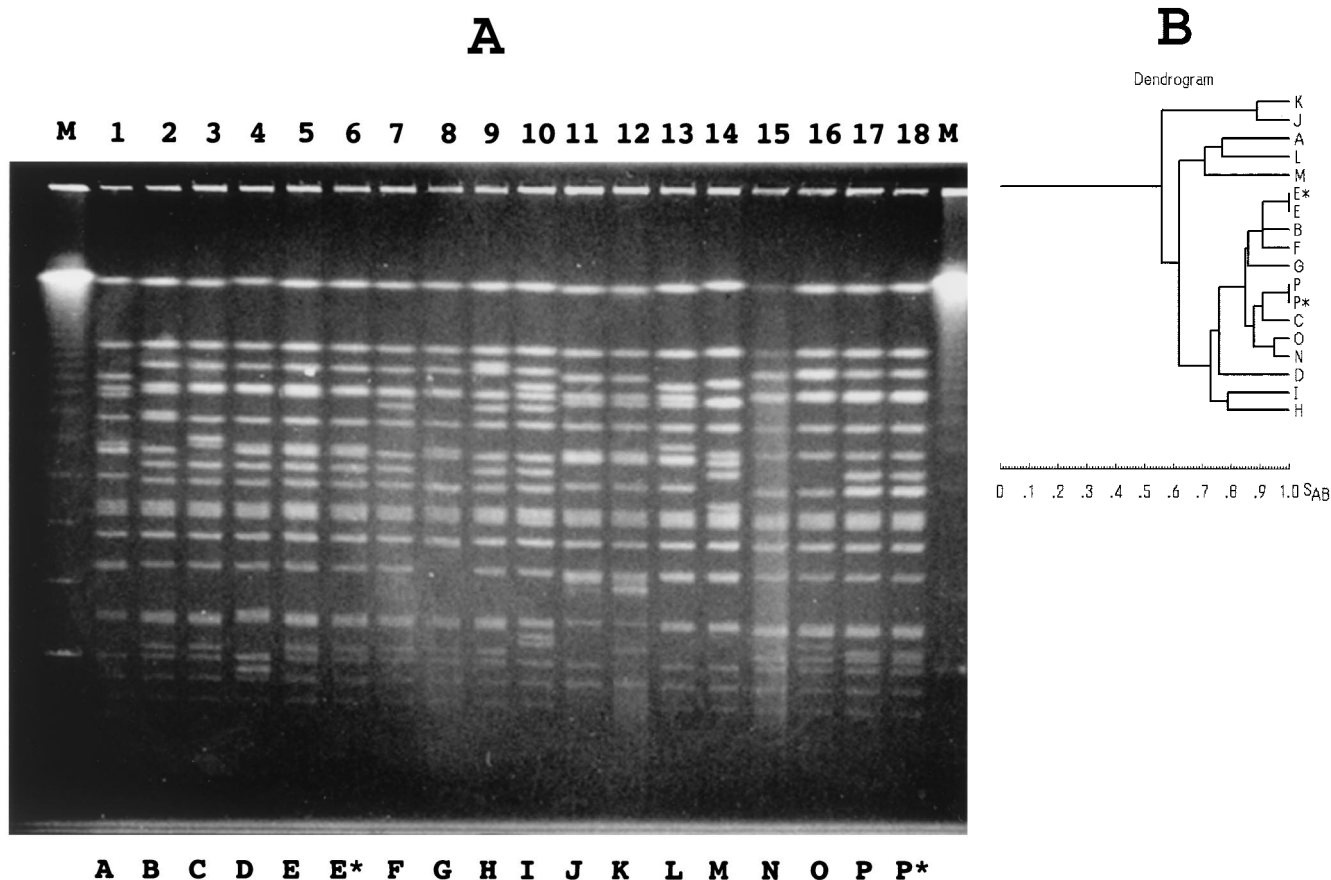
FIG. 4. PFGE profiles of selected strains of *V. parahaemolyticus* and their relations. (A) PFGE profiles of *Not*I-digested genomic DNAs. The profiles were considered different if they differed by one or more DNA fragment. The profiles were classified into 15 profiles, arbitrarily designated A through P (indicated at the bottom). The asterisk denotes the profile obtained from reference strains. Lanes: M, molecular size markers (DNA size standard maker, lambda ladder; Bio-Rad); 1, FIHES98V1-32-4; 2, JKY-VP6; 3, VP-2; 4, BE98-2062; 5, VP1280; 6, KX-V225; 7, VP80; 8, VPF00-18; 9, VP1152; 10, APCC VP 9810; 11, 1001A44; 12, 1001A46; 13, APCC VP 00030; 14, APCC VP 00031; 15, 4917; 16, APCC VP 00157; 17, APCC VP 00190; 18, 5095. Test strains and their PFGE profiles are listed in Table 3. (B) Dendrogram constructed from the 15 PFGE profiles shown in panel A. The scale for the similarity coefficient is indicated as S_{AB}.

Table 3). The TDH-producing and GS-PCR-negative O3:K6 strain belonged to type ii, but the other strains belonged to type i.

PFGE profiles of the *Not*I-digested DNA of the test strains were obtained except that those of three strains were not available because of DNA degradation. All available profiles were from GS-PCR-positive strains. These profiles were classified into 15 patterns, arbitrarily designated A to O (Fig. 4A; Table 3). The reference pandemic O3:K6 strains isolated in 1996 to 1999 exhibited one of the five (A to E) profiles. Four isolates from Japanese seafood showed profile E. However, the other seafood isolates and river sediment isolates belonging to the TDH-producing O3:K6 group showed PFGE profiles (F to I) that were different from those of the reference strains. The distribution of the PFGE profiles of the seafood and river sediment isolates did not correlate with their location of isolation.

TDH-nonproducing O3:K6 isolates exhibited PFGE profile A and J to M patterns. These profiles were different from those of TDH-positive isolates. The PFGE profiles of O4:K68 and

O1:K25 strains isolated from the river sediment (O and P) were the same as or resembled those of the reference strains of the same serovars (N and P) (Table 3; Fig. 4A).

The dendrogram analysis of the PFGE profiles (Fig. 4B) revealed that TDH-negative isolates (profiles A, J, K, L, and M) were rather distant from TDH-positive isolates (all other profiles) and that the TDH-positive strains were closely related to each other regardless of the serovar.

DISCUSSION

Various non-*V. parahaemolyticus* bacteria distributed in the coastal environment can grow in enrichment media and on selective agar media commonly used for isolation of *V. parahaemolyticus*. In addition, both virulent and avirulent strains of *V. parahaemolyticus* inhabit the coastal environment. Previous studies showed that the proportion of virulent strains of *V. parahaemolyticus* in the coastal environment is very low (10, 15). These make it difficult to isolate virulent strains of *V. parahaemolyticus* from environmental samples such as seafood,

coastal water, and sediments (13). To overcome such difficulties, we used a three-step enrichment procedure, a PCR method for detection of the *tdh* gene, and new chromogenic agar plates highly specific for *V. parahaemolyticus* (13). As a result, we detected the *tdh* gene in 33 (10.0%) of 329 seafood samples (mostly molluscan shellfish) and isolated TDH-producing strains of *V. parahaemolyticus* from 3.3% of the samples. The result that TDH-producing *V. parahaemolyticus* was isolated from only one third of *tdh*-positive samples suggests that the method for isolation of TDH-producing *V. parahaemolyticus* needs to be improved.

The difficulty in isolating TDH-producing colonies on agar plates may be due at least in part to the fact that the relative proportion of TDH-producing *V. parahaemolyticus* to total *V. parahaemolyticus* is extremely low in some samples. A study on the prevalence of *V. parahaemolyticus* in oysters in the United States suggested that the ratio of *tdh*-positive *V. parahaemolyticus* to total *V. parahaemolyticus* ranged from 1:5.6 to 1:64 (11), while the trial to detect TDH-producing *V. parahaemolyticus* in foods in Japan revealed only one TDH-producing colony in 1,369 *V. parahaemolyticus*-like colonies (19). In this study, the *tdh* gene was not detected even in the samples containing high levels of total *V. parahaemolyticus* (10^4 to 10^6 MPN/10 g; Fig. 2). Therefore, the number of total *V. parahaemolyticus* does not appear to correlate directly with the number or with the presence of TDH-producing *V. parahaemolyticus*.

Storing samples at 30°C for 3 h prior to the initial enrichment step resulted in an increase of the detection rate of *tdh*-positive *V. parahaemolyticus* with increases in the number of total *V. parahaemolyticus* (Table 2). This suggests that there may be some conditions in hen clam that are more favorable for the growth or resuscitation of *tdh*-positive *V. parahaemolyticus* than the enrichment condition used in this study. Therefore, there is some room to improve the enrichment conditions for isolation of *tdh*-positive *V. parahaemolyticus*. The increase in the detection rate of *tdh*-positive *V. parahaemolyticus* at a relatively high temperature may explain the increase in *V. parahaemolyticus* infection in the summer season in Japan (14). Avoiding consumption of raw shellfish with a large number of total *V. parahaemolyticus* may be effective in reducing the incidence of *V. parahaemolyticus* infection, although raw shellfish might not always contain adequate numbers of TDH-producing *V. parahaemolyticus* to cause food poisoning.

Characterization of the strains representing seafood and sediment isolates revealed for the first time that the pandemic strains are distributed throughout the Japanese coastal environment. Of 19 strains examined, 14 strains were *tdh* positive and GS-PCR positive and showed the same AP-PCR profiles as the reference strains of the pandemic clone. These 14 strains were thus judged to be typical pandemic strains. These strains belonged not only to the O3:K6 serovar but also to two other pandemic serovars (O4:K68 and O1:K25). High-resolution analysis by PFGE showed that three of the O3:K6 pandemic strains isolated from the Japanese environment were indistinguishable from the O3:K6 pandemic strains prevalent in various countries (Table 3, profile E). This result suggests that the O3:K6 strains with profile E may belong to the ancestral pandemic clone. Other O3:K6 pandemic strains isolated from the Japanese environment showed PFGE profiles F to I. These profiles and the PFGE profiles of other pandemic strains of

O3:K6 serovar (profiles B to D), O4:K68 serovar (profile P), and O1:K25 serovar (profiles N and O) were closely related to profile E (Fig. 4B).

The present results suggest that the strains showing profiles other than E may have diverged from the ancestral clone (profile E). In contrast, *tdh*-negative and GS-PCR-positive O3:K6 strains showed profiles (A, J, K, L, and M) that were rather distant from the other profiles (Fig. 4B). The strains showing these profiles were also differentiated from *tdh*-positive pandemic strains by the AP-PCR pattern. These strains seem to be a variants that diverged from the ancestor of the current pandemic strains. They may have lost the *tdh* gene at some point during adaptation to the environment and are no longer virulent.

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