APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2005, p. 3575–3580 Vol. 71, No. 7
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.7.3575–3580.2005

Seasonal Variation in Abundance of Total and Pathogenic *Vibrio parahaemolyticus* Bacteria in Oysters along the Southwest Coast of India

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The seasonal abundance of *Vibrio parahaemolyticus* in oysters from two estuaries along the southwest coast of India was studied by colony hybridization using nonradioactive labeled oligonucleotide probes. The density of total *V. parahaemolyticus* bacteria was determined using a probe binding to the *tlh* (thermolabile hemolysin) gene, and the density of pathogenic *V. parahaemolyticus* bacteria was determined by using a probe binding to the *tdh* (thermostable direct hemolysin) gene. Furthermore, the prevalence of *V. parahaemolyticus* was studied by PCR amplification of the *toxR*, *tdh*, and *trh* genes. PCR was performed directly with oyster homogenates and also following enrichment in alkaline peptone water for 6 and 18 h. *V. parahaemolyticus* was detected in 93.87% of the samples, and the densities ranged from <10 to 10^4 organisms per g. Pathogenic *V. parahaemolyticus* could be detected in 5 of 49 samples (10.2%) by colony hybridization using the *tdh* probe and in 3 of 49 samples (6.1%) by PCR. Isolates from one of the samples belonged to the pandemic serotype O3:K6. Twenty-nine of the 49 samples analyzed (59.3%) were positive as determined by PCR for the presence of the *trh* gene in the enrichment broth media. *trh*-positive *V. parahaemolyticus* was frequently found in oysters from India.

Vibrio parahaemolyticus is a halophilic bacterium that occurs in estuarine environments worldwide (5, 13, 14, 16, 18, 19). This organism was first discovered in Japan in 1950 in association with a food poisoning case (9). *V. parahaemolyticus* infection can cause gastroenteritis in humans, and the illness is most frequently associated with the consumption of raw or undercooked seafood and seafood recontaminated with the bacterium after cooking (31). *V. parahaemolyticus* accounts for about 70% of the gastroenteritis associated with seafood in Japan (14), and in India about 10% of the cases of gastroenteritis in patients admitted to the Infectious Diseases Hospital in Kolkata are due to *V. parahaemolyticus* (3). However, not all strains of *V. parahaemolyticus* are pathogenic. It has been demonstrated that the Kanagawa phenomenon, a beta-hemolysis in high-salt blood agar (Wagatsuma agar), is associated with most clinical strains but with very few environmental strains (32, 38). The hemolysis is due to the production of a thermostable direct hemolysin (TDH) (30). A TDH-related hemolysin (TRH) produced by a Kanagawa phenomenon-negative strain was discovered during investigation of an outbreak of gastroenteritis in the Maldives Islands in 1985 (10, 11). TDH and TRH, encoded by the *tdh* and *trh* genes, respectively, are considered major virulence factors in *V. parahaemolyticus* (31). It has been reported that more than 90% of clinical isolates but less than 1% of environmental isolates produce TDH (7, 22, 33). In recent years, the incidence of *V. parahaemolyticus* infection has been increasing in many parts of the world, and this has been attributed to the emergence of a new clone of the O3:K6 serotype carrying only the *tdh* gene (24). Although various serovars of the bacterium can cause infections, O3:K6 has been recognized as the predominant serovar responsible for most outbreaks worldwide since 1996 (24).

The ecology of *V. parahaemolyticus* has been studied mainly in temperate waters. The U. S. Food and Drug Administration (FDA) bacteriological manual (8) recommends the most-probable-number (MPN) method for enumeration of *V. parahaemolyticus* from food and water, but this method is cumbersome and the recovery of the organism is low (4). Detection of pathogenic *V. parahaemolyticus* is traditionally done by the Wagatsuma agar test for the Kanagawa reaction, which requires fresh human or rabbit blood and tends to give false-positive reactions (M. Nishibuchi, personal communication). There is no phenotypic test for TRH. In recent years, a number of researchers have drawn attention to the need for more sensitive and rapid techniques using molecular approaches for detection of *V. parahaemolyticus* in clinical samples, as well as in seafood. PCR-based methods that amplify regulatory *toxR* sequences (22), conserved sequences such as *gyrB* (34), conserved chromosomal sequences (16, 17, 23), and hemolysin sequences such as *tdh* (7, 20, 28, 32), *trh* (32), or *tlh* (thermolabile hemolysin) (2) have been used by various workers. Enumeration of *V. parahaemolyticus* from seafood are important in the context of current FDA guidelines which indicate that shellfish should contain less than 10,000 *V. parahaemolyticus* cells per g (25). Enumeration using radioactive DNA probes as well as nonradioactive DNA probes in colony hybridization have been described (25, 29, 37). DePaola et al. (6) determined the seasonal abundance of total and pathogenic *V. parahaemolyticus* bacteria in Alabama oysters using a colony hybridization technique. However, there are no quantitative data on total and pathogenic *V. parahaemolyticus* bacteria in tropical oysters. The present study was carried out to determine the abundance of total (tlh^+) and pathogenic (tdh^+) *V. parahaemolyticus* bacteria in oysters from two estuaries along the southwest

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coast of India during a 1-year period. Colony hybridization using an alkaline phosphatase-labeled oligonucleotide probe was used to study the levels of these organisms. For sensitive detection of the organisms, PCR targeting the *toxR*, *tdh*, and *trh* genes of *V. parahaemolyticus* was performed with oyster samples without enrichment and after 6 and 18 h of enrichment in alkaline peptone water (APW).

MATERIALS AND METHODS

Sample collection. Oysters (*Crassostrea madrasensis*) were harvested biweekly from two estuaries, Mulki (site 1) and Sasthan (site 2), which were about 100 km apart along the southwest coast of India. The study was conducted from January 2002 to December 2002. The temperature of the surface water was recorded at the time of sample collection using a calibrated thermometer. The salinities of the water at the sampling sites were estimated by titrimetry (1). The oysters were transported to the laboratory within 2 h of collection and processed for analysis.

Enumeration of *V. parahaemolyticus* by colony hybridization. Two alkaline phosphatase-labeled oligonucleotide probes described previously (25, 26) were used. A *tlh* (thermolabile hemolysin) probe that bound to the gene encoding the thermolabile hemolysin present in all *V. parahaemolyticus* strains was used to enumerate total *V. parahaemolyticus* bacteria, while a *tdh* (thermostable direct hemolysin) probe was used to enumerate pathogenic *V. parahaemolyticus* bacteria. The probes were supplied by DNA Technology A/S (Aarhus, Denmark). Colony hybridization was carried out as described in the FDA *Bacteriological Analytical Manual* (8). Briefly, the oysters were scrubbed and shucked, 200 to 250 g of oyster meat was homogenized in a sterile blender, and 1:1 and 1:10 dilutions of the shellfish homogenate were prepared in APW. Then 0.2 g of the 1:1 dilution and 100 μl of the 1:10 dilution were spread on plates containing T_1N_3 medium (1% tryptone, 3% NaCl, 2% agar, pH 7.4). To determine the *V. parahaemolyticus* level in seawater, a 0.1-ml aliquot was spread plated on T_1N_3 medium plates in duplicate and incubated at 37°C for 16 to 18 h. Colonies were lifted onto Whatman no. 541 filter paper, alkali lysis was performed, and the lysed cells were subjected to proteinase K digestion. The filter with the 1:1 dilution was used for hybridization with the *tdh* probe, and the filter with the 1:10 dilution was used for hybridization with the *tlh* probe. Hybridization was carried out at 54°C, after which the excess probe was washed with a standard sodium citrate-sodium lauryl sulfate solution at a stringent washing temperature (54°C). The hybridized filters were exposed to the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate–nitro blue tetrazolium (Sigma Aldrich) for development of a blue color. The number of blue spots with intensities similar to those on the control strips, each representing a colony, was determined. The number of positive colonies in the dilutions multiplied by the dilution factor gave the number in the original sample.

Detection of *V. parahaemolyticus* by a conventional method and PCR. Twenty-five grams of shucked oyster meat was homogenized in 225 ml of APW and incubated at 37°C for 18 h. A loopful of the enrichment broth was streaked on thiosulfate citrate bile salt sucrose (TCBS) agar (HiMedia, Mumbai, India). A minimum of five typical colonies were picked and subjected to a battery of biochemical tests for identification of *V. parahaemolyticus* (8). To prepare lysates for PCR, 1-ml aliquots of the enrichment broth were removed at 0, 6, and 18 h and centrifuged at a low speed (800 × *g*) (Biofuge, Heraeus, Germany) for 10 min to sediment the meat particles. The supernatant was then subjected to high-speed centrifugation at 8,000 × *g* for 10 min to pellet the bacteria. The deposit was resuspended in 100 μl of distilled water and lysed by heating it at 100°C for 10 min, snap cooled on ice, and centrifuged at 10,000 × *g* for 5 min. A 5-μl portion of the supernatant was used for the PCR.

PCR was performed to detect the presence of the *toxR* (22), *tdh* (32), and *trh* (32) genes of *V. parahaemolyticus* in enrichment broth obtained at 0, 6, and 18 h. A group-specific PCR (GS-PCR) for detection of the recently described pandemic clones of *V. parahaemolyticus* (24) was also performed for *tdh* gene-positive *V. parahaemolyticus* isolates obtained by colony hybridization. Strains NICED (*tdh*$^+$), AQ4037 (*trh*$^+$), and SY O3:K6 (*tdh*$^+$, O3:K6) were used as control strains in PCRs. A negative control to which sterile PCR grade water was added as the template was included for each reaction mixture. The PCR was performed with a 50-μl mixture consisting of 5 μl of 10× buffer (Bangalore Genei, Bangalore, India), each of the four deoxynucleoside triphosphates at a concentration of 200 μM, 25 pmol of each primer, and 1.5 U of *Taq* polymerase (Bangalore Genei, Bangalore, India). The PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide (0.5 μg/ml), and photographed using a gel documentation system (HeroLab, Weisloch, Germany).

TABLE 1. Levels of *V. parahaemolyticus* in oyster samples from site 1 (Mulki)

Date (day.mo.yr)	Water temp (°C)	Water salinity (ppt)	Fecal coliform count (MPN/100 g)	*V. parahaemolyticus* levels as determined by colony hybridization (CFU/g)	
				tlh	*tdh*
01.01.02	29.0	29.5	ND[a]	1.66×10^3	$<1.00 \times 10^1$
11.01.02	29.5	29.5	ND	3.50×10^3	$<1 \times 10^1$
15.01.02	30	30	ND	1.92×10^3	$<1 \times 10^1$
05.02.02	30	30	1.1×10^5	2.70×10^3	3.50×10^1
5.3.02	31.5	30	1.5×10^5	1.10×10^3	$<1 \times 10^1$
18.3.02	31.5	30	2.4×10^4	7.50×10^2	$<1 \times 10^1$
7.4.02	31	30.5	1.1×10^5	3.80×10^3	$<1 \times 10^1$
18.4.02	33	30.3	1.1×10^5	4.00×10^2	$<1 \times 10^1$
23.4.02	35	31.16	4.3×10^3	6.00×10^3	$<1 \times 10^1$
2.5.02	34	30.06	2.3×10^3	9.00×10^3	$<1 \times 10^1$
8.5.02	32.1	29.8	2.3×10^3	4.00×10^3	$<1 \times 10^1$
16.5.02	32	26.27	1.1×10^5	6.70×10^4	$<1 \times 10^1$
20.5.02	32	27	2.3×10^3	4.70×10^3	$<1 \times 10^1$
31.5.02	28.5	27.39	1.1×10^5	3.20×10^3	$<1 \times 10^1$
13.6.02	29.5	12.24	1.5×10^4	1.0×10^3	$<1 \times 10^1$
27.6.02	30.5	2.4	1.1×10^5	3.00×10^2	$<1 \times 10^1$
12.7.02	30	12.7	7.5×10^3	3.00×10^2	$<1 \times 10^1$
27.7.02	30.5	12.5	9.3×10^3	2.00×10^2	$<1 \times 10^1$
9.8.02	26.5	9.2	7.5×10^3	6.00×10^2	$<1 \times 10^1$
26.8.02	27	11.74	2.4×10^4	7.00×10^2	$<1 \times 10^1$
10.9.02	27.5	8.5	1.1×10^5	1.30×10^3	1.3×10^2
25.9.02	26	9.0	1.1×10^5	1.20×10^4	$<1 \times 10^1$
8.10.02	29	11.3	3.9×10^3	1.0×10^4	$<1 \times 10^1$
22.10.02	27	8.3	1.1×10^5	8.0×10^2	$<1 \times 10^1$
6.11.02	26	9.8	1.5×10^3	1.1×10^3	$<1 \times 10^1$
19.11.02	25.5	9.2	4.3×10^3	$<1 \times 10^2$	$<1 \times 10^1$
4.12.02	25	9.8	2.3×10^3	1.2×10^4	$<1 \times 10^1$
17.12.02	30	11.0	4.3×10^3	4.0×10^2	$<1 \times 10^1$
Avg	29.61	19.61		5.37×10^3	

[a] ND, not determined.

Statistical analysis. The relationship between *V. parahaemolyticus* levels and the salinity or temperature of water was analyzed by multiple regression using SYSTAT (Systat, Richmond, CA). The log values of the counts were used for statistical analysis. *V. parahaemolyticus* counts of <100 bacteria/g (nondetectable) were considered 100 bacteria/g and *tdh*$^+$ *V. parahaemolyticus* counts of <10 bacteria/g (nondetectable) were considered 10 bacteria/g for statistical purposes. Arithmetic means of counts were determined for each site to compare the counts for the two sites.

RESULTS

Enumeration of *V. parahaemolyticus* by colony hybridization. The levels of total (*tlh*$^+$) and pathogenic (*tdh*$^+$) V. *parahaemolyticus* bacteria in oysters in two estuaries along the southwest coast of India between January and December 2002 are shown in Tables 1 and 2. Overall, in the two estuaries, the average counts of *V. parahaemolyticus* ranged from 10^1 to 10^4 CFU/g. *V. parahaemolyticus* could be detected in 46 (93.8%) of the 49 samples collected from the two sites by the alkaline phosphatase-labeled oligonucleotide probe for *tlh*. The average level of *V. parahaemolyticus* in the oyster samples from site 1 was 5.4×10^3 CFU/g (Table 1). This is higher than the average level for site 2 (2.7×10^3 CFU/g) (Table 2), although the difference in the values for the two sites was not statistically significant. For site 1, the highest level (Table 1) of *V. parahaemolyticus* was in May (1.7×10^4 CFU/g) and the lowest level was in July (2.5×10^2 CFU/g). The corresponding data

TABLE 2. Levels of *V. parahaemolyticus* in oyster samples from site 2 (Sasthan)

Date (day.mo.yr)	Water temp (°C)	Water salinity (ppt)	Fecal coliform count (MPN/100 g)	*V. parahaemolyticus* levels as determined by colony hybridization (CFU/g)	
				tlh	*tdh*
23.4.02	34.0	28.83	2.30×10^2	8.0×10^2	$<1 \times 10^1$
2.5.02	33.5	29.02	2.38×10^2	1.2×10^3	$<1 \times 10^1$
8.5.02	32.1	29.5	9.3×10^2	9.0×10^3	$<1 \times 10^1$
16.5.02	31.0	23.64	$<3.0 \times 10^2$	3.1×10^4	$<1 \times 10^1$
20.5.02	28.5	28.0	4.30×10^3	7.0×10^2	$<1 \times 10^1$
30.5.02	28.0	20.63	4.60×10^4	2.2×10^3	$<1 \times 10^1$
5.6.02	30.0	16.82	1.1×10^5	7.3×10^3	2.0×10^1
13.6.02	30.5	12.99	4.6×10^4	1.2×10^3	4.0×10^1
27.6.02	29	1.24	4.3×10^3	1×10^2	$<1 \times 10^1$
12.7.02	29.5	11.24	4.3×10^3	1×10^2	$<1 \times 10^1$
27.7.02	27	9.8	2.1×10^4	2×10^2	$<1 \times 10^1$
9.8.02	28	8.7	1.1×10^5	1×10^2	$<1 \times 10^1$
28.8.02	25	7.4	2.4×10^4	$<1 \times 10^2$	$<1 \times 10^1$
10.9.02	28	8.49	2.9×10^3	6×10^2	$<1 \times 10^1$
25.9.02	26	9.0	2.1×10^4	6.4×10^2	4.0×10^1
8.10.02	29	12.0	1.1×10^5	3.75×10^2	$<1 \times 10^1$
1.10.02	27	9.5	1.5×10^3	3.0×10^2	$<1 \times 10^1$
6.11.02	28	12.0	2.3×10^3	4.2×10^2	$<1 \times 10^1$
9.11.02	25	9.0	4.3×10^3	$<1 \times 10^2$	$<1 \times 10^1$
4.12.02	26	10.0	9.3×10^3	2.5×10^2	$<1 \times 10^1$
7.12.02	31	14.0	1.1×10^5	6.0×10^2	$<1 \times 10^1$
Avg	28.86	14.84		2.7×10^3	

for site 2 were 8.8×10^3 CFU/g and 1.0×10^2 CFU/g in May and August, respectively (Table 2). The level of the organism was monitored in the seawater at the two sampling sites, and the values varied between 6×10^1 and $<1 \times 10^1$ CFU/ml (data not shown). Pathogenic *V. parahaemolyticus* bacteria could be detected in five samples (10.2%) of oysters from the two estuaries by colony hybridization using the alkaline phosphatase-labeled oligonucleotide probe for *tdh*. As shown in Table 1, one sample from site 1 obtained during February and one sample from site 1 obtained during September were positive for pathogenic *V. parahaemolyticus*, and the levels were 3.5×10^1 and 1.3×10^2 CFU/g, respectively. For site 2, pathogenic *V. parahaemolyticus* was detected in three samples, two collected during June and one collected in September, and the levels ranged from 2×10^1 to 4×10^1 CFU/g (Table 2). The *tdh*-positive isolates detected by hybridization were tested for the presence of the *trh* gene by PCR. None of the *tdh* gene-positive isolates possessed the *trh* gene.

GS-PCR was performed for the isolates obtained from the *tdh* probe-positive sample, as well as the APW enrichment lysates of this sample collected in June from site 2. The sample was positive as determined by GS-PCR, and it also yielded GS-PCR-positive *V. parahaemolyticus*. The strains isolated from this sample were later serotyped and confirmed to be O3:K6 pandemic clones of *V. parahaemolyticus*. The total *V. parahaemolyticus* counts were compared with environmental factors, such as water temperature, salinity, and the fecal coliform count. The surface water temperature at all stations recorded from January to December 2000 was between 35°C and 25°C. The average temperatures at site 1 and site 2 were 29.6°C and 28.8°C, respectively. The salinity of the surface water varied from 31 ppt to 0.76 ppt, and the average salinities were 19.6 ppt at site 1 and 14.8 ppt at site 2.

TABLE 3. Detection of *V. parahaemolyticus* from oyster samples by PCR[a]

Site	*n*	Gene	No. of samples positive as determined by PCR with enrichment broth (%)		
			0 h	6 h	18 h
1	28	*toxR*	3 (10.7)	14 (50.0)	26 (92.8)
		trh	0 (0)	10 (35.7)	17 (60.7)
		tdh	1 (3.5)	1 (3.5)	1 (3.5)
2	21	*toxR*	3 (14.28)	17 (80.9)	20 (95.23)
		trh	2 (9.52)	8 (38.09)	12 (57.14)
		tdh	2 (9.52)	0 (0)	0 (0)

[a] All the lysates were prepared from APW enrichment broth.

Water temperature and salinity did not significantly influence the total *V. parahaemolyticus* counts ($P < 0.05$). The abundance of total *V. parahaemolyticus* bacteria at the two sites did not correlate with water temperature (for site 1, $r = 0.16$; for site 2, $r = 0.31$). Similarly, there was no significant correlation between salinity and total *V. parahaemolyticus* counts (for site 1, $r = 0.13$; for site 2, $r = 0.38$). From the available data R^2 values of 0.39 and 0.16 were determined, indicating that 39% and 16% of the total *V. parahaemolyticus* density was attributed only to the difference in the water temperature and salinity. The fecal coliform counts were uniformly high throughout the year, and no correlation was observed between *tdh*-positive *V. parahaemolyticus* and the presence of fecal coliforms ($r = 0.13$).

Detection of *V. parahaemolyticus* in enrichment broth by PCR. PCR were performed with lysates prepared from oyster homogenates at zero time (without enrichment) and from homogenates enriched in APW for 6 h and 18 h. Primers specific for the *toxR*, *tdh*, and *trh* genes of *V. parahaemolyticus* were used in this study. *V. parahaemolyticus* was detected in 93.8% (46 of 49) of the samples after 18 h of enrichment by *toxR*-primed PCR. In 6.1% (3 of 49) of the samples, the *tdh* gene could be detected in zero-time samples (without enrichment), while the *tdh* gene could be detected after 6 and 18 h of enrichment in only 2.04% (1 of 49) of the samples. Fifty-nine percent (29 of 49) of the samples were positive for the *trh* gene after 18 h of enrichment (Table 3). The prevalence of *trh* gene-positive *V. parahaemolyticus* at the two sites was 60.7% for site 1 and 57.1% for site 2 (Table 3). As indicated in Table 3, the percentage of samples positive for *trh* increased with enrichment time.

Detection of *V. parahaemolyticus* by conventional methods. The 18-h oyster enrichment broth media were streaked on TCBS agar and incubated at 37°C for 24 h. Five typical green colonies were picked and subjected to a battery of biochemical tests to confirm the presence of *V. parahaemolyticus*. *V. parahaemolyticus* could be detected in 45 of 49 samples by the conventional method. Biochemically identified samples were further confirmed to be *V. parahaemolyticus* by *toxR* PCR. It was interesting that all 225 colonies picked from these samples were negative for the *tdh* gene by PCR.

DISCUSSION

V. parahaemolyticus is one of the major seafood-borne gastroenteritis-causing bacteria and is frequently associated with the consumption of improperly cooked seafood. Oysters have been reported to concentrate *Vibrio* spp. 100-fold compared with the amount found in the surrounding water through filtration (6). In this study, the level of the organism in oysters was examined by using alkaline phosphatase-labeled probes. Under the conditions of the assay (filters containing colonies from a 1:10 dilution), the minimum detectable limit was 10^2 CFU/g oyster meat. In 98.5% of the samples *V. parahaemolyticus* could be detected throughout the year (Tables 1 and 2). This is in contrast to temperate waters, in which the *V. parahaemolyticus* levels during the winter (December to March) are less than 100 bacteria/g and the organism is detected only after enrichment (6). In this study, the levels of *V. parahaemolyticus* were high during the dry season between January and May (peak levels, 6.7×10^4 CFU/g at site 1 and 3.1×10^4 CFU/g at site 2) and decreased during the postmonsoon months (2×10^2 CFU/g at site 1 and $<1 \times 10^2$ CFU/g at site 2). In tropical countries the seasonal cycle of the organism is correlated with the rainy and dry seasons; the lowest numbers are found in rainy months, and the highest numbers are found in the dry season (27). Our observations suggest a similar trend. At sites 1 and 2 the highest counts were recorded during the premonsoon months. From May to June, levels of 10^4 CFU/g were recorded for both estuaries. The average salinities during this period were 19.6 ppt and 14.8 ppt for site 1 and site 2, respectively (Tables 1 and 2). The temperatures ranged between 34 and 24°C during the sampling period. This study indicates that the level of *V. parahaemolyticus* in oysters in this region is around 10^3 CFU/g. The levels of *V. parahaemolyticus* crossed the hazardous limit of 10^4 bacteria per g at site 1 during May, when the temperature was 32°C and the salinity was 26.2 ppt, and in September and October, when the temperature was 26 to 29°C and the salinity was 9 to 11.3 ppt. At site 2, similar levels were recorded only once in the premonsoon month of May, when the temperature and salinity recorded were 31°C and 23.6 ppt, respectively.

Risk assessment of *V. parahaemolyticus* in seafood requires quantitative data on the organism, particularly the pathogenic strains. The FDA *Bacteriological Analytical Manual* MPN method (8) is most frequently used to enumerate *V. parahaemolyticus* in foods. In this MPN method biochemical techniques are used to identify isolates, and the method is time-consuming and labor-intensive. A colony hybridization procedure using alkaline phosphatase-labeled *tlh* and *tdh* probes is described in the FDA *Bacteriological Analytical Manual* (8). Nonradioactive labeled probes are sensitive and have the advantage of universal application, unlike radiolabeled probes. This study shows that colony hybridization could be a very useful method for enumeration of both total and pathogenic *V. parahaemolyticus* bacteria in tropical oysters.

Temperature has been found to be a major factor in both the seasonal and geographical distribution of *V. parahaemolyticus* in shellfish-growing areas of the temperate region (15, 21). DePaola et al. (6) noted that in Alabama oysters, the *V. parahaemolyticus* levels were <100 CFU/g during December to March. This study showed that in tropical waters the temperature fluctuates less throughout the year, while the salinity fluctuates more. *V. parahaemolyticus* was reported previously to be sensitive to salinities associated with freshwater (24), but Horie et al. (12) observed significantly high levels of *V. parahaemolyticus* (1.5×10^5 CFU/liter at salinities as low as 5 ppt). Hence, it is clear that low salinity per se is not necessarily detrimental to *V. parahaemolyticus* but may favor growth and survival if the water is rich in organic matter. This study showed that in tropical waters *V. parahaemolyticus* density does not seem to be correlated with temperature. Therefore, the linear regression model suggested by DePaola et al. (6) to predict the level of *V. parahaemolyticus* in oysters based on temperature and salinity data does not seem to be applicable to tropical oysters. Sewage and industrial drainage have been reported by several workers to affect the incidence of *V. parahaemolyticus*, and correlation with fecal coliforms has been suggested (36). However, no discernible correlation between fecal coliform counts and *V. parahaemolyticus* counts was noted for either of the two stations sampled in this study.

V. parahaemolyticus positive for the *tdh* gene could be detected in 5 of 49 samples (10.2%) using the *tdh* probe in colony hybridization (Tables 1 and 2). Previous studies indicated that only 1 to 2% of the environmental strains produce TDH or contain the *tdh* gene (5, 20). However, the recent study of DePaola et al. (6) indicated a higher prevalence (12.8%) of *tdh*-positive *V. parahaemolyticus* in Alabama oysters as determined by direct plating. The prevalence observed in our study is similar to this value. In our study fecal coliforms were used as an index of fecal contamination, and attempts were made to see whether there was any correlation between fecal coliform levels and the prevalence of *tdh*$^+$ *V. parahaemolyticus*. No significant correlation ($r = 0.14$) was observed. Also, the higher counts of *V. parahaemolyticus* did not correlate with the presence of *tdh*-positive *V. parahaemolyticus*. The occurrence of the *tdh* gene in the sample was sporadic; at site 1, the samples were positive during February and September, while at site 2, positive samples were obtained during June and September. As determined by GS-PCR, one sample from site 2 belonging to the pandemic serotype, O3:K6, was detected in June. The isolates obtained during other periods were GS-PCR negative. This suggests that both O3:K6 and other serotypes are present in oysters in this region. Infections caused by *V. parahaemolyticus* are usually associated with diverse serovars. Recently, the incidence of *V. parahaemolyticus* infection has been increasing in many parts of the world, and this has been attributed to the emergence of a new clone of the O3:K6 serotype carrying only the *tdh* gene (35). Infection due to this clone was confirmed in Asian countries first and subsequently in the United States (24). In this report we document the appearance of the *tdh*-positive, *trh*-negative, and urease-negative pandemic clone of *V. parahaemolyticus* (O3:K6). However, only one of the five samples (20%) possessing the *tdh* gene was confirmed to be a member of the O3:K6 serovar, and the detection of this gene in June supports the finding that the incidence of O3:K6 is high during June or during the onset of the monsoon season (26).

The direct plating and colony hybridization technique used in this study has limits of detection of 10 CFU/g for *tdh*$^+$ strains and 100 CFU/g for total *V. parahaemolyticus* bacteria. Table 3 shows the results obtained by PCR with enrichment

broth for the *toxR*, *tdh*, and *trh* genes after different intervals of enrichment. Forty-six of 49 samples were positive for *V. parahaemolyticus* as determined by the *toxR* PCR, and this result is similar to the results obtained with the colony hybridization technique. However, PCR results indicated a high prevalence of trh^+ *V. parahaemolyticus* in oysters. The number of trh^+ samples increased with enrichment, and after 18 h of enrichment, 60.7% of the samples from site 1 and 57.14% of the samples from site 2 were positive as determined by the *trh* PCR. On the other hand, only one sample from site 1 and two samples from site 2 were positive as determined by the *tdh* PCR. For site 2, two samples that were positive at zero time (before enrichment) were negative after enrichment. This could have been due to overgrowth of *tdh*-negative strains during the enrichment.

V. parahaemolyticus could be isolated from 45 of 49 samples by the conventional method, while the organism could be detected in one additional sample (46 samples) by colony hybridization and PCR, demonstrating the usefulness of the technique for detection of *V. parahaemolyticus* in oysters. The advantage of PCR over conventional isolation is its ability to distinguish virulent and avirulent strains; it also saves time, and hence molecular methods are valuable in such cases.

ACKNOWLEDGMENTS

We thank M. Nishibuchi, Center for Southeast Asian studies, Kyoto University, Kyoto, Japan, for serotyping the thermostable direct hemolysin-positive isolates.

This work was supported in part by grants from the Food and Agriculture Organization Nutrition Division, FAO Rome, for the purchase of oligonucleotide probes and reagents for colony hybridization used in this study and by the Indian Council of Medical Research (ICMR), New Delhi, India. This financial support is gratefully acknowledged.

REFERENCES

1. **American Public Health Association.** 1998. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, Washington, D.C.
2. **Bej, A. K., D. P. Patterson, C. W. Brasher, M. C. L. Vickery, D. D. Jones, and C. Kaysner.** 1999. Detection of total and hemolysin producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh*, and *trh*. J. Microbiol. Methods **36:**215–225.
3. **Deb, B. C., R. Sinha, S. P. De, P. G. Sengupta, S. M. Sikda, and A. Mondal.** 1975. Studies on *Vibrio parahaemolyticus* infection in Calcutta as compared to cholera infection. Prog. Diagn. Res. Trop. Dis. **19:**400–405.
4. **DePaola, A., L. H. Hopkins, and R. M. McPhearson.** 1988. Evaluation of four methods for enumeration of *Vibrio parahaemolyticus*. Appl. Environ. Microbiol. **54:**617–618.
5. **DePaola, A., L. H. Hopkins, J. T. Peeler, B. Wentz, and R. M. McPhearson.** 1990. Incidence of *Vibrio parahaemolyticus* in United States coastal waters and oysters. Appl. Environ. Microbiol. **56:**2299–2302.
6. **DePaola, A., J. L. Nordstrom, J. C. Bowers, J. G. Wells, and D. W. Cook.** 2003. Seasonal abundance of total and pathogenic *Vibrio parahaemolyticus* in Alabama oysters. Appl. Environ. Microbiol. **69:**1521–1526.
7. **Dilip, V., H. S. Kumar, Y. Kumar, I. Karunasagar, M. Nishibuchi, and I. Karunasagar.** 2003. Application of polymerase chain reaction for the detection of *Vibrio parahaemolyticus* associated with tropical seafoods and coastal environment. Lett. Appl. Microbiol. **36:**423–427.
8. **Food and Drug Administration.** 2004. Bacteriological analytical manual on line, 8th ed., revision A, 1998. [Online.] AOAC International, Arlington, Va. (http://www.cfsan.fda.gov/~ebam/bam-toc.html).
9. **Fujino, T., Y. Okuno, D. Nakada, A. Aoyama, K. Fukai, T. Mukai, and T. Ueho.** 1953. On the bacteriological examination of shellfish food poisoning. Med. J. Osaka Univ. **4:**299–305.
10. **Honda, S., I. Gato, I. Minematsu, N. Ikeda, N. Asano, M. Ishibashi, Y. Kinoshita, M. Nishibuchi, T. Honda, and T. Miwatani.** 1987. Gastroenteritis due to Kanagawa negative *V. parahemolyticus*. Lancet **i:**331–332.
11. **Honda, T., Y. Ni, and T. Miwatani.** 1988. Purification and characterization of a hemolysin produced by clinical isolates of Kanagawa phenomenon negative *V. parahemolyticus* related to the thermostable direct hemolysin. Infect. Immun. **56:**961–965.
12. **Horie, S., K. Sakeki, and T. Okuzami.** 1967. Quantitative enumeration of *Vibrio parahaemolyticus* in sea and estuarine waters. Bull. Jpn. Soc. Sci. Fish. **33:**126–130.
13. **Joseph, S. W., R. R. Colwell, and J. B. Kaper.** 1982. *Vibrio parahaemolyticus* and related halophilic vibrios. Crit. Rev. Microbiol. **10:**77–124.
14. **Kaneko, T., and R. R. Colwell.** 1973. Ecology of *Vibrio parahaemolyticus* in Chesapeake Bay. J. Bacteriol. **113:**24–32.
15. **Kaneko, T., and R. R. Colwell.** 1975. Incidence of *Vibrio parahaemolyticus* in Chesapeake Bay. Appl. Microbiol. **30:**251–257.
16. **Karunasagar, I., B. B. Nayak, and I. Karunasagar.** 1997. Rapid detection of pathogenic *Vibrio parahaemolyricus* from fish by PCR, p. 119–122. *In* T. Flegel and I. H. MacRae (ed.), Diseases of Asian aquaculture, vol. III. Asian Fisheries Society, Manila, The Philippines.
17. **Karunasagar, I., G. Sugumar., I. Karunasagar, and P. J. A. Reilly.** 1996. Rapid PCR method for detection of Kanagawa positive *Vibrio parahemolyticus* in seafoods. Int. J. Food Microbiol. **31:**317–323.
18. **Karunasagar, I., I. Karunasagar, M. N. Venugopal, and C. N. Nagesha.** 1987. Survival of *Vibrio parahaemolyticus* in estuarine water, seawater and in association with clams. Syst. Appl. Microbiol. **9:**316–319.
19. **Karunasagar, I., M. N. Venugopal., I. Karunasagar, and K. Segar.** 1986. Evaluation of methods for enumeration of *Vibrio parahaemolyticus* from seafoods. Appl. Environ. Microbiol. **52:**583–585.
20. **Kelly, M. T., and D. Stroh.** 1988. Temporal relationship of *Vibrio parahaemolyticus* in patients and the environment. J. Clin. Microbiol. **26:**1754–1756.
21. **Kiiyukia, C., K. Venkatesswaran, I. M. Navarroo, H. Nakano, H. Kawakami, and H. Hashimoto.** 1989. Seasonal distribution of *Vibrio parahaemolyticus* serotypes along oyster beds in Hiroshima coast. J. Fac. Appl. Biol. Sci. **28:**49–61.
22. **Kim, Y. B., J. Okuda, C. Matsumoto, N. Takahashi, S. Hashimoto, and M. Nishibuchi.** 1999. Identification of *Vibrio parahaemolyticus* strains at the species level by PCR targeted to the *tox* R gene. J. Clin. Microbiol. **37:**1173–1177.
23. **Lee, C. Y., S. F. Pan, and C. H. Chen.** 1995. Sequence of a cloned pR72H fragment and its use for detection of *Vibrio parahaemolyticus* in shellfish with the PCR. Appl. Environ. Microbiol. **61:**1311–1317.
24. **Matsumota, C., J. Okuda, M. Ishibashi, M. Iwanaga, P. Garg, T. Rammamurthy, H. C. Wong, A. DePaola, Y. B. Kim, M. J. Albert, and M. Nishibuchi.** 2000. Pandemic spread of an O3:K6 clone of *Vibrio parahaemolyticus* and emergence of related strains evidenced by arbitrarily primed PCR and *toxRS* sequence analyses. J. Clin. Microbiol. **38:**578–585.
25. **McCarthy, S. A., A. DePaola, D. W. Cook, C. A. Kaysner, and W. E. Hill.** 1999. Evaluation of alkaline phosphatase- and digoxigenin-labeled probes for detection of the thermolabile hemolysin (*tlh*) gene of *Vibrio parahaemolyticus*. Lett. Appl. Microbiol. **28:**66–70.
26. **McCarthy, S. A., A. DePaola, C. A. Kaysner, W. E. Hill, and D. W. Cook.** 2000. Evaluation of non-isotopic DNA hybridization methods for detection of the *tdh* gene of *Vibrio parahaemolyticus*. J. Food Prot. **63:**1660–1664.
27. **Neumann, D. A., H. Benemon, E. Hubster, N. Thi Nhu Tuan, and L. Tie-Van.** 1972. *Vibrio parahaemolyticus* in the Republic of Vietnam. Am. J. Trop. Med. Hyg. **22:**464–470.
28. **Nishibuchi, M., and J. B. Kaper.** 1995. Thermostable Direct hemolysin gene of *Vibrio parahaemolyticus*: a virulence gene acquired by a marine bacterium. Infect. Immun. **63:**251–256.
29. **Nishibuchi, M., M. Ishibashim, Y. Takeda, and J. B. Kaper.** 1985. Detection of thermostable direct hemolysin gene and related DNA sequences in *Vibrio parahaemolyticus* and other *Vibrio* spp by DNA colony hybridization test. Infect. Immun. **49:**481–486.
30. **Raimondi, F., J. P. Kao, C. Fiorentini, A. Fabbri, G. Donelli, N. Gasparini, A. Rubino, and A. Fasano.** 2000. Enterotoxicity and cytotoxicity of *Vibrio parahaemolyticus* thermostable direct hemolysin in vitro systems. Infect. Immun. **68:**3180–3185.
31. **Rippey, S. R.** 1994. Infectious diseases associated with molluscan shellfish consumption. Clin. Microbiol. Rev. **7:**419–425.
32. **Tada, J., T. Ohashi, N. Nishimura, Y. Shirasaki, H. Ozaki, S. Fukushima, J. Takano, M. Nishibuchi, and Y. Takeda.** 1992. Detection of the thermostable direct hemolysin gene (*tdh*) and the thermostable direct hemolysin-related hemolysin gene (*trh*) of *Vibrio parahaemolyticus* by polymerase chain reaction. Mol. Cell. Probes **6:**477–487.
33. **Thompson, C. A., and C. Vanderzant.** 1976. Serological and hemolytic characteristics of *Vibrio parahaemolyticus* from marine sources. J. Food Sci. **41:**150–151.
34. **Venkateswaran, K., N. Dohmoto, and S. Harayama.** 1998. Cloning and nucleotide sequence of the *gyrB* gene of *Vibrio parahaemolyticus* and its application in detection of this pathogen in shrimp. Appl. Environ. Microbiol. **64:**681–687.
35. **Vuddhakul, V., A. Chowdhury, V. Laohaprertthisan, P. Pungrasamee, P.**

Patararungrong, M. Ishibashi, C. Matsumoto, and M. Nishibuchi. 2000. Isolation of a pandemic O3:K6 clone of a *Vibrio parahaemolyticus* strains from environmental and clinical sources in Thailand. Appl. Environ. Microbiol. **66:**2685–2689.

36. **Watkins, W. D., and V. J. Cabelli.** 1985. Effect of fecal pollution on *Vibrio parahaemolyticus* densities in an estuarine environment. Appl. Environ. Microbiol. **49:**1307–1313.
37. **Yamamoto, K., T. Honda, T. Miwatani, S. Takamatsukuri, and S. Shibata.** 1991. Enzyme labeled oligonucleotide probes for the detection of thermostable direct hemolysin (TDH) and TDH related hemolysin (TRH) of *Vibrio parahaemolyticus*. Can. J. Microbiol. **38:**410–416.
38. **Zen Yoji, H., H. Hitokoto, S. Morozumi, and R. A. Le Clair.** 1971. Purification and characterization of a hemolysin produced by *Vibrio parahaemolyticus*. J. Infect. Dis. **123:**665–667.