Vibrio parahaemolyticus strains isolated during investigation of the summer 2006 seafood related diarrhea outbreaks in two regions of Chile

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Abstract

Nine hundred cases of seafood related diarrhea were reported in the region of Puerto Montt, Chile during the austral summer of 2006. This is the continuation of the large outbreaks associated with the consumption of seafood containing the *Vibrio parahaemolyticus* serovar O3:K6 pandemic clonal group that arose last decade in Chile. The initial outbreaks occurred during the summer of 1998 in Antofagasta (23°39′S 70°24′W). Subsequently, outbreaks there were rare, but since 2004 outbreaks have been frequent farther south in Puerto Montt (41°29′S 72°24′W). The large outbreaks in Puerto Montt and their rarity in Antofagasta is atypical because the seawater temperature at Puerto Montt is 5°C lower than at Antofagasta and the presence of *V. parahaemolyticus* in seafood has been associated with higher water temperatures. To better understand the role of seafood in outbreak occurrences in these regions, we analyzed the *V. parahaemolyticus* populations in clinical cases and shellfish from Puerto Montt during diarrhea outbreaks in 2006 and in shellfish from Antofagasta, where no cases were observed. Enrichment culture from shellfish yielded no *V. parahaemolyticus* from samples from the north, but its presence was detected in 80% of the samples from the south. Grouping of the *V. parahaemolyticus* isolates by the fragment restriction pattern of their DNA showed that all pathogenic (*tdh*+) isolates obtained from Puerto Montt shellfish corresponded to the serovar O3:K6 South East Asian pandemic clone, while the non-pathogenic (*tdh*−) isolates corresponded to at least six discrete groups. The possible causes for the disappearance of the pandemic strain from the north and its persistence in the south are discussed.

Keywords: *Vibrio parahaemolyticus*; Shellfish; Chile; Outbreaks; Bacterial communities

1. Introduction

Vibrio parahaemolyticus is a marine bacterium that can be found in edible marine animals. The *V. parahaemolyticus* found in seawater are diverse and only a few of the strains found in seafood can cause diarrhea in consumers. (Alam et al., 2003; DePaola et al., 2000, 2003a,b; Fuenzalida et al., 2006; Hara-Kudo et al., 2003). Pathogenicity in *V. parahaemolyticus* has been correlated with the presence of two well-characterized haemolysins, thermostable direct haemolysin (TDH) and TDH-related haemolysin (TRH; Miyamoto et al., 1980; Honda et al., 1990). Before 1996 there were no clear associations of particular *V. parahaemolyticus* strains with infections. However, since then, many infections in different parts of the world have been associated with a serovar O3:K6 clone originally observed in Southeast Asia in 1996 (Okuda et al., 1997). Most isolates of this clone exhibit a unique sequence within the *toxRS* operon (*toxRS*/new) (Matsumoto et al., 2000), and possess a unique open reading frame, orf8 (Nasu et al., 2000), corresponding to an associated filamentous phage. Other common properties of pandemic isolates are the presence of the structural *tdh* gene and the absence of both the *trh* and urease genes found in other *V. parahaemolyticus* strains (Suthienkul et al., 1995). The clonal nature of these pandemic strains has been ascertained by the close similarity of the patterns obtained by genome restriction fragment length polymorphism-pulsed field gel electrophoresis

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(RFLP–PFGE; Wong et al., 2000), arbitrarily primed polymerase chain reaction (AP-PCR; Matsumoto et al., 2000; Okuda et al., 1997) and direct genome restriction enzyme analysis (DGREA; Fuenzalida et al., 2006), and also by multilocus sequence typing (MLST; Chowdhury et al., 2004b).

Seafood-borne diarrhea was rare in Chile until this clone caused outbreaks in Antofagasta (23°39′S 70°24′W) and surrounding areas in 1998, with approximately 300 clinical cases (Cordova et al., 2002; Gonzalez-Escalona et al., 2005). Since then, clinical cases have been largely absent from this region and less than 10 cases per year have been observed from 2004 to 2006 (Olea et al., 2005; http://epi.minsal.cl/epi/html/Actualidad/Vibrio.htm). However, from 2004 onward the same clone has caused large outbreaks in the environs of Puerto Montt (41°29′S 72°24′W) in southern Chile (Fuenzalida et al., 2006; Gonzalez-Escalona et al., 2005). There were approximately 1500 and 3600 reported cases in 2004 and 2005, respectively. For 2006 there were 900 reported cases (Olea et al., 2005; http://epi.minsal.cl/epi/html/Actualidad/Vibrio.htm).

Before 2004, *V. parahaemolyticus* infections were very rare in Puerto Montt, despite the fact that consumption of raw seafood is common practice (Gonzalez-Escalona et al., 2005). This was thought to be due to the low surface temperature of seawater in this region, which is 11 to 16°C year around (http://www.shoa.cl/cendoc-jsp/index.jsp), since the incidence of *V. parahaemolyticus* is strongly correlated with water temperature (Chiou et al., 2000; Kaneko and Colwell, 1973). The large outbreaks observed in the south obviously contrast with their absence in the north. The decline of outbreaks in Antofagasta is not unprecedented. In the Khanh Hoa province of Vietnam, infection by the pandemic clone that was causing 49% of diarrhea cases from 1996 onward abruptly stopped in November 1999 (Chowdhury et al., 2004a; Tuyet et al., 2002). A similar situation was observed in Galveston Bay, TX where this clone has not been found since it caused the largest *V. parahaemolyticus* outbreak ever reported in the United States in 1998 (DePaola et al., 2000). In Chile, however, the situation is peculiar, because the outbreaks disappeared in the north but have continued in the south where the seawater is 5°C colder (http://www.shoa.cl/cendoc-jsp/index.jsp). In an effort to understand this situation, we studied the outbreaks in the summer of 2006, analyzing *V. parahaemolyticus* in the clinical cases in Puerto Montt and in popularly consumed shellfish in both Puerto Montt and Antofagasta.

2. Methods

V. parahaemolyticus RIMD 2210633 (VpKX) was obtained from the Research Institute for Microbial Diseases, Osaka University, Japan. Isolates obtained in the summers of 2004 and 2005 have been previously described (Fuenzalida et al., 2006).

V. parahaemolyticus from clinical cases of 2006 were obtained from rectal swabs of 19 patients with acute diarrhea associated with seafood consumption who sought medical attention during January and February at the Hospital Regional de Puerto Montt. Analysis of the isolates was performed as previously described (Fuenzalida et al., 2006; Gonzalez-Escalona et al., 2005).

Seafood samples were collected at Puerto Montt and Antofagasta during January and February 2006. Samples from Puerto Montt were 80% small mussels (*Mytilus chilensis*) and 20% clams of the species *Venus antiqua*. Samples from Antofagasta were all clams of the species *Protothaca thaca*. The samples were collected directly at the growing sites and processed no later than 4h after collection. Enrichment, isolation, enumeration, screening and confirmation were performed as described in the Bacteriological Analytical Manual of the US Food and Drug Administration (Kaysner and DePaola, 1998). Briefly, 50g of 10–15 animals were homogenized in 450ml of phosphate buffered saline. The homogenate was seeded at different concentrations into three series of tubes containing alkaline peptone water (APW), starting with tubes containing 1g of homogenized soft tissue. After incubation overnight at 37°C each tube showing bacterial growth was tested by PCR amplification for the presence of *tlh*, *tdh* and *trh* as described (Bej et al., 1999), except that instead of DNA extraction for PCR the APW cultures were simply boiled as described by Blackstone et al. (2003). Total and pathogenic *V. parahaemolyticus* loads were calculated from most probable numbers determined by reference to MPN tables (Kaysner and DePaola, 1998) and the observed PCR positive dilutions of *tlh* or *tdh*.

For bacterial isolation, 0.1ml of fluid from the surfaces of the medium in APW tubes showing bacterial growth was streaked onto thiosulfate citrate bile salts sucrose (TCBS) plates and incubated overnight at 37°C. Ten green colonies were purified from each sample and identified by API-20E for enterobacteria (BioMerieux, Halzelwood, MO) according to the manufacturer's instructions. Isolates were genotyped by direct genome

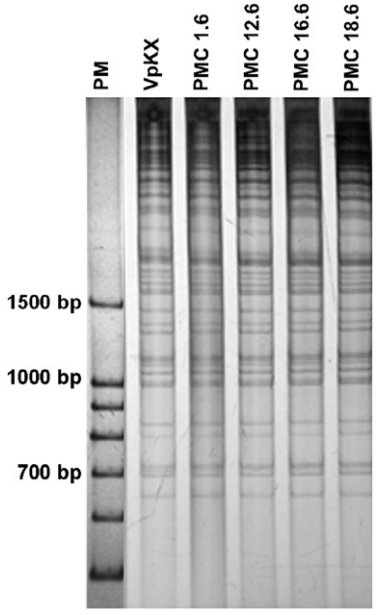

Fig. 1. Direct genome restriction enzyme analysis (DGREA) with *Nae*I of clinical isolates obtained during the outbreaks of January and February 2006 in Puerto Montt. PM, 100 bp size ladder; VpKX, O3:K6 southeast pandemic isolate, PMC 1.6, 12.6, 16.6 and 18.6, clinical isolates obtained the austral summer of 2006.

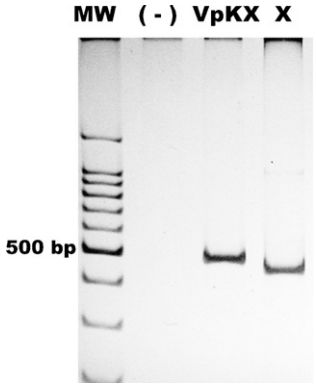

Fig. 2. Polyacrylamide gel electrophoresis of the products obtained by PCR amplification of *tlh* from APW enrichment cultures. MW, 100 bp size ladder; (−) negative control; VpKX, O3:K6 southeast pandemic isolate; X, APW enrichment culture from Antofagasta samples.

restriction enzyme analysis (DGREA) as described by Fuenzalida et al. (2006).

Amplification and sequencing of the highly variable region of the 16S rRNA between nucleotides 357–518 (*E. coli* numbering) were performed as previously described (Moreno et al., 2002). Sequencing of the product obtained after amplification for *tlh* was performed using the same primers employed for amplification (Bej et al., 1999). The sequences of *tlh* and 16S rRNA that were obtained have been deposited in GenBank (DQ660364_660367).

3. Results

3.1. V. parahaemolyticus in clinical cases of 2006

PCR amplification of the DNA from *V. parahaemolyticus* isolates from 19 clinical cases showed that every isolate was *orf8*, *tlh* and *tdh* positive and *trh* negative, like the pandemic clonal strains (Bej et al., 1999; Gonzalez-Escalona et al., 2005; Laohaprertthisan et al., 2003). DGREA analysis showed that the clinical isolates gave the same pattern observed for the Southeast Asian strain RIMD2210633 (VpKX) (Fig. 1).

3.2. V. parahaemolyticus in shellfishes from Antofagasta and Puerto Montt

While 17 and 10 of 20 samples from Puerto Montt were positive for total (*tlh*+) and pathogenic (*tdh*+) *V. parahaemolyticus*, respectively, none of the 20 samples from Antofagasta was positive for either of these markers. *trh* was not detected in any of the analyzed samples. *V. parahaemolyticus* numbers ranged from 15 to 5500 and 0 to 38 bacteria/g for total (*tlh*+) and pathogenic (*tdh*+) *V. parahaemolyticus*, respectively. In general *tdh*+ samples gave higher *tlh* titers than *tdh*− samples (data not shown). Samples from Antofagasta gave an amplicon after PCR with the primers for *tlh*, but this showed a slightly higher electrophoretic migration than the product obtained with confirmed *V. parahaemolyticus* strains (Fig 2). The numbers of bacteria in samples containing this target were between 10^4 and 10^5 per gram of soft tissue, i.e. 10 to 100 times higher than the load of *V. parahaemolyticus* in samples from the south. The sequence of the PCR product from the samples from Antofagasta showed closer similarity (91%) with a *tlh* found in *V. alginolyticus* (Xie et al., 2005) than with that of *V. parahaemolyticus* (82%).

In samples from Puerto Montt, green presumptive *V. parahaemolyticus* colonies were frequently the most abundant colonies on TCBS plates but samples from Antofagasta gave TCBS plates overcrowded with yellow and black colonies. Only three green colonies were recovered after screening about 100 of the latter. To recognize the most abundant bacterial colonies in samples from the north three of each of, green, yellow or black colonies were characterized by sequencing the variable 16S rRNA region between nucleotides 357 and 518 (*E. coli* numbering). The sequence obtained for the green colonies

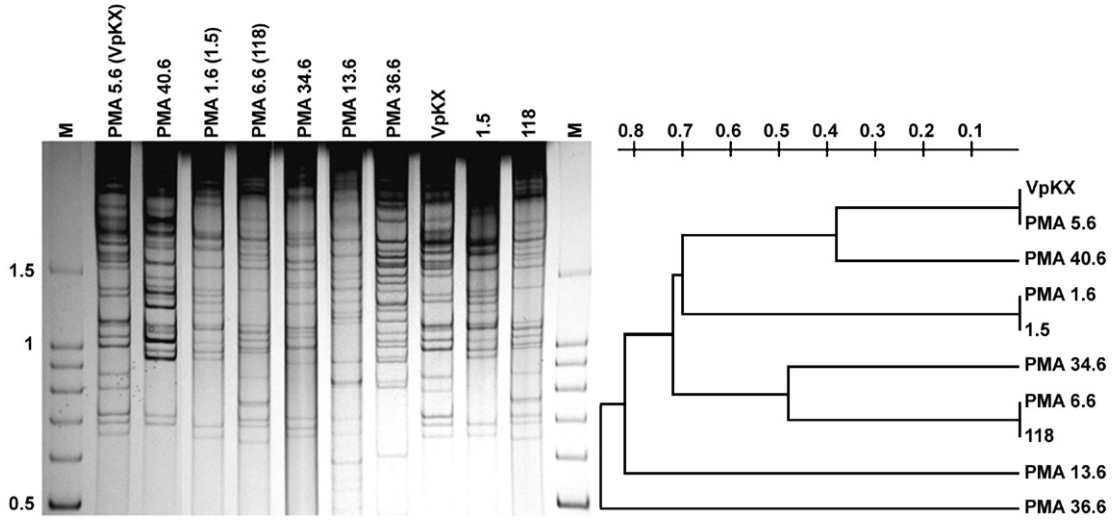

Fig. 3. Direct genome restriction enzyme analysis (DGREA) with *Nae*I of *Vibrio parahaemolyticus* isolates obtained from shellfish collected in the summer of 2006 and dendrogram illustrating the clusters of the patterns by dissimilarity. The gel shows representative strains for every observed pattern. Patterns observed in previous years are indicated with the name assigned to that group at that time between parentheses. The patterns from isolate VpKX and the isolates obtained in previous years, PMA1.5 and PMA118 are shown in the last lanes on the right.

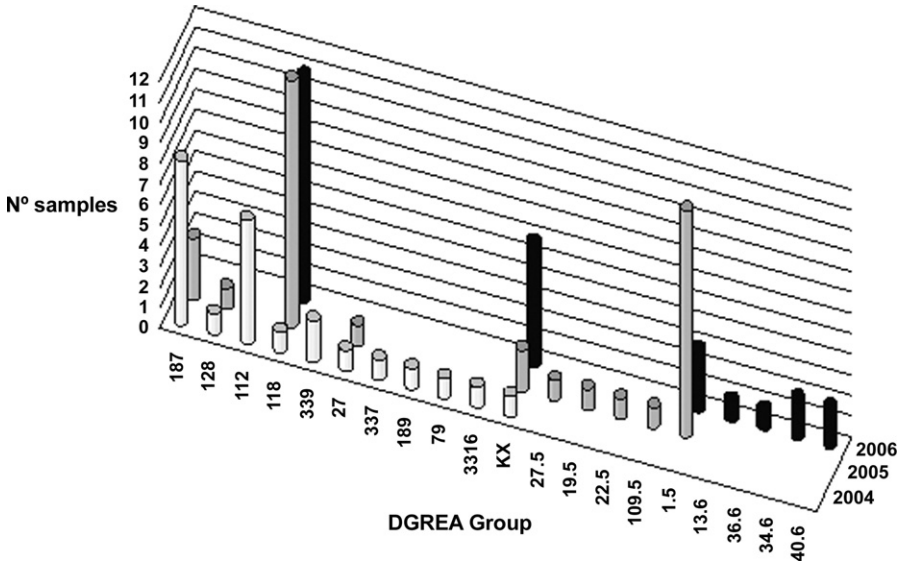

Fig. 4. Histogram of seafood samples containing *Vibrio parahaemolyticus* of the different DGREA groups observed during the summers of 2004, 2005, and 2006.

showed only 95% similarity to the 16S rRNA of *V. parahaemolyticus*. Sequences obtained from yellow and black colonies were more closely related to *V. harveyi* (98%) or *Shewanella* spp. (97%), respectively, than to *V. parahaemolyticus.*

3.3. Diversity of V. parahaemolyticus in shellfish from Puerto Montt

tdh+ isolates were only obtained from six of the 10 *tdh*+ samples appearing positive by PCR of their APW enrichment cultures. The 17 *tlh*+ *tdh*+ isolates obtained from the *tdh*+ samples and the 41 *tlh*+ *tdh*− isolates selected from the 17 *tlh*+ samples were genotyped by DGREA. The 17 *tdh*+ isolates were identical to the pandemic Southeast Asian strain RIMD2210633 (VpKX), whereas the 41 *tdh*− isolates clustered into six different groups (Fig. 3). Each of the seven DGREA patterns found in 2006 was compared to those described in previous years, and when the same or similar pattern was found the matching was confirmed by electrophoresis of the isolates' restricted DNA in the same gel. Only three of the seven patterns found in 2006 (including VpKX) had been observed in the two previous years. They corresponded to patterns VpKX and 118 observed in both 2004 and 2005, and to pattern 1.5 observed in 2005 (Fuenzalida et al., 2006). Overall, including VpKX, 20 patterns have been observed since 2004. Fig. 4 combines data of the summer of 2006 with data previously reported in part to show the number of shellfish samples containing each of the groups observed each year since 2004.

4. Discussion

Our results show that the outbreaks caused by the pandemic Southeast Asia clone continued for the third consecutive year in the Puerto Montt region. These outbreaks are apparently due to the persistence of pathogenic *V. parahaemolyticus* in most of the shellfish extracted from this region. The pandemic clone seems to be the only pathogen in the shellfish population since all other isolates examined lacked the *tdh* and *trh* genes present in pathogenic strains. Additionally, none of the other DGREA groups were found among the isolates obtained from clinical cases (Fuenzalida et al., 2006). On the other hand, the absence of clinical cases in Antofagasta is best explained by the disappearance of the pandemic strain that caused the outbreaks in 1998 from shellfish in the region. Not only was the pathogenic strain undetectable (<0.3 MPN/g) in these shellfish, but the total load of bona fide *V. parahaemolyticus* was below the detection limit, though the load of *Vibrio*-like bacteria was 10 to 100 times larger in samples from Antofagasta than in those from Puerto Montt. However, total and pathogenic *V. parahaemolyticus* may have been more prevalent than observed in this study because of a possible inhibition which has been sometimes observed for PCR of APW enrichment culture tubes containing 1 g of tissue.

The larger bacterial load in shellfish from Antofagasta is probably due to the higher temperature of the seawater in this region. The absence of *V. parahaemolyticus* could be due to their displacement due to competition by the high load of *Vibrio*-like bacteria. These bacteria corresponded mainly to the genera *Vibrio* and *Shewanella*. Bacteria of this last genus degrade sulfur-containing amino acids and produce volatile sulfides including H_2S (Gram and Huss, 1996), which is probably responsible for the black color of the abundant colonies observed on the TCBS plates. None of the isolates gave the *tlh* amplification product observed with the DNA from APW enrichment cultures, but it is likely that this came from some of the yellow colonies since the presence of *tlh* has also been detected in some *V. alginolyticus* strains (Xie et al., 2005).

The absence of *V. parahaemolyticus* in the north contrasts with their presence in 85% of the shellfish from the south. The percentage of *tdh*+ samples (50%) found in Puerto Montt in 2006 was much higher than the 6% observed during the summers of 2004 and 2005 (Fuenzalida et al., 2006), but this observation may be explained by the use of a more sensitive

procedure this time. In the summers of 2004 and 2005 the pathogenic strains from, on average, three *V. parahaemolyticus* colonies per sample were tested by PCR amplification. The direct detection of these markers in the enrichment cultures employed this time probably involved testing of a much larger number of strains of *V. parahaemolyticus*. The total *V. parahaemolyticus* load of 15 to 5500 bacteria per gram of shellfish soft tissue found in samples from Puerto Montt was not much lower than that reported for samples of shellfish from other, more temperate regions, despite the fact that the seawater temperature never increases above 18 °C during the summer (Alam et al., 2003; Deepanjali et al., 2005; DePaola et al., 2000, 2003a; Hara-Kudo et al., 2003). From the ratio between the MPN values for *tdh*+ samples (2 to 38 bacteria/g) and *tlh*+ samples (240 to 5500 bacteria/g) in samples with detectable *tdh*, a rough estimate of 1 *tdh*+ *V. parahaemolyticus* per 100 total *V. parahaemolyticus* seems realistic.

DGREA allows obtaining the patterns of restriction fragments generated from the bacterial DNA. This method discriminates among strains by the patterns of 500–2000 bp size fragments generated by direct restriction of the total bacterial DNA, with similar discriminatory power to RFLP–PFGE. According to the DGREA analysis, the *V. parahaemolyticus* load in shellfish from Puerto Montt is mainly composed of organisms of at least 20 groups. Only 7 to 11 groups are present in detectable amounts each summer and only a few persist in detectable amount more than one summer. Among these last groups is the pandemic clone, which has persisted for three consecutive years. This capacity to persist in shellfish–and maybe in other hosts or habitats–more effectively than other *V. parahaemolyticus* clones may be one cause for its successful worldwide spread.

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References

Alam, M.J., Miyoshi, S., Shinoda, S., 2003. Studies on pathogenic *Vibrio parahaemolyticus* during a warm weather season in the Seto Inland Sea, Japan. Environmental Microbiology 5, 706–710.

Bej, A.K., Patterson, D.P., Brasher, C.W., Vickery, M.C., Jones, D.D., Kaysner, C.A., 1999. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of tlh, tdh and trh. Journal of Microbiological Methods 36, 215–225.

Blackstone, G.M., Nordstrom, J.L., Vickery, M.C., Bowen, M.D., Meyer, R.F., DePaola, A., 2003. Detection of pathogenic *Vibrio parahaemolyticus* in oyster enrichments by real time PCR. Journal of Microbiological Methods 53, 149–155.

Chiou, C.S., Hsu, S.Y., Chiu, S.I., Wang, T.K., Chao, C.S., 2000. *Vibrio parahaemolyticus* serovar O3:K6 as cause of unusually high incidence of food-borne disease outbreaks in Taiwan from 1996 to 1999. Journal of Clinical Microbiology 38, 4621–4625.

Chowdhury, A., Ishibashi, M., Thiem, V.D., Tuyet, D.T.N., Van Tung, T., Chien, B.T., von Seidlein, L., Canh, D.G., Clemens, J., Trach, D.D., Nishibuchi, M., 2004a. Emergence and serovar transition of *Vibrio parahaemolyticus* pandemic strains isolated during a diarrhea outbreak in Vietnam between 1997 and 1999. Microbiology and Immunology 48, 319–327.

Chowdhury, N.R., Stine, O.C., Morris, J.G., Nair, G.B., 2004b. Assessment of evolution of pandemic *Vibrio parahaemolyticus* by multilocus sequence typing. Journal of Clinical Microbiology 42, 1280–1282.

Cordova, J.L., Astorga, J., Silva, W., Riquelme, C., 2002. Characterization by PCR of *Vibrio parahaemolyticus* isolates collected during the 1997–1998 Chilean outbreak. Biological Research 35, 433–440.

Deepanjali, A., Kumar, H.S., Karunasagar, I., Karunasagar, I., 2005. Seasonal variation in abundance of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters along the southwest coast of India. Applied and Environmental Microbiology 71, 3575–3580.

DePaola, A., Kaysner, C.A., Bowers, J., Cook, D.W., 2000. Environmental investigations of *Vibrio parahaemolyticus* in oysters after outbreaks in Washington, Texas, and New York ,1997 and 1998. Applied and Environmental Microbiology 66, 4649–4654.

DePaola, A., Nordstrom, J.L., Bowers, J.C., Wells, J.G., Cook, D.W., 2003a. Seasonal abundance of total and pathogenic *Vibrio parahaemolyticus* in Alabama oysters. Applied and Environmental Microbiology 69, 1521–1526.

DePaola, A., Ulaszek, J., Kaysner, C.A., Tenge, B.J., Nordstrom, J.L., Wells, J., Puhr, N., Gendel, S.M., 2003b. Molecular, serological, and virulence characteristics of *Vibrio parahaemolyticus* isolated from environmental, food, and clinical sources in North America and Asia. Applied and Environmental Microbiology 69, 3999–4005.

Fuenzalida, L., Hernandez, C., Toro, J., Rioseco, M.L., Romero, J., Espejo, R.T., 2006. *Vibrio parahaemolyticus* in shellfish and clinical samples during two large epidemics of diarrhoea in southern Chile. Environmental Microbiology 8, 675–683.

Gonzalez-Escalona, N., Cachicas, V., Acevedo, C., Rioseco, M.L., Vergara, J.A., Cabello, F., Romero, J., Espejo, R.T., 2005. *Vibrio parahaemolyticus* diarrhea, Chile, 1998 and 2004. Emerging Infectious Diseases 11, 129–131.

Gram, L., Huss, H.H., 1996. Microbiological spoilage of fish and fish products. International Journal of Food Microbiology 33, 121–137.

Hara-Kudo, Y., Sugiyama, K., Nishibuchi, M., Chowdhury, A., Yatsuyanagi, J., Ohtomo, Y., Saito, A., Nagano, H., Nishina, T., Nakagawa, H., Konuma, H., Miyahara, M., Kumagai, S., 2003. Prevalence of pandemic thermostable direct hemolysin-producing *Vibrio parahaemolyticus* O3:K6 in seafood and the coastal environment in Japan. Applied and Environmental Microbiology 69, 3883–3891.

Honda, T., Ni, Y.X., Hata, A., Yoh, M., Miwatani, T., Okamoto, T., Goshima, K., Takakura, H., Tsunasawa, S., Sakiyama, F., 1990. Properties of a hemolysin related to the thermostable direct hemolysin produced by a Kanagawa phenomenon negative, clinical isolate of *Vibrio parahaemolyticus*. Canadian Journal of Microbiology 36, 395–399.

Kaneko, T., Colwell, R.R., 1973. Ecology of *Vibrio parahaemolyticus* in Chesapeake Bay. Journal of Bacteriology 113, 24–32.

Kaysner, C.A., DePaola, A., 1998. *Vibrio cholerae*, *V. parahaemlyticus*, *V. vulificus*, and other *Vibrio* spp. Bacteriological Analytical Manual. 8th ed., rev A. Food and Drug Administration, Washington. Chap.9.

Laohaprertthisan, V., Chowdhury, A., Kongmuang, U., Kalnauwakul, S., Ishibashi, M., Matsumoto, C., Nishibuchi, M., 2003. Prevalence and serodiversity of the pandemic clone among the clinical strains of *Vibrio parahaemolyticus* isolated in southern Thailand. Epidemiology and Infection 130, 395–406.

Matsumoto, C., Okuda, J., Ishibashi, M., Iwanaga, M., Garg, P., Rammamurthy, T., Wong, H.C., DePaola, A., Kim, Y.B., Albert, M.J., Nishibuchi, M., 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. Journal of Clinical Microbiology 38, 578–585.

Miyamoto, Y., Obara, Y., Nikkawa, T., Yamai, S., Kato, T., Yamada, Y., Ohashi, M., 1980. Simplified purification and biophysicochemical characteristics of Kanagawa phenomenon-associated hemolysin of *Vibrio parahaemolyticus*. Infection and Immunity 28, 567–576.

Moreno, C., Romero, J., Espejo, R.T., 2002. Polymorphism in repeated 16S rRNA genes is a common property of type strains and environmental isolates of the genus *Vibrio*. Microbiology 148, 1233–1239.

Nasu, H., Iida, T., Sugahara, T., Yamaichi, Y., Park, K.S., Yokoyama, K., Makino, K., Shinagawa, H., Honda, T., 2000. A filamentous phage associated with recent

pandemic *Vibrio parahaemolyticus* O3:K6 strains. Journal of Clinical Microbiology 38, 2156–2161.

Okuda, J., Ishibashi, M., Hayakawa, E., Nishino, T., Takeda, Y., Mukhopadhyay, A.K., Garg, S., Bhattacharya, S.K., Nair, G.B., Nishibuchi, M., 1997. Emergence of a unique O3:K6 clone of *Vibrio parahaemolyticus* in Calcutta, India, and isolation of strains from the same clonal group from Southeast Asian travelers arriving in Japan. Journal of Clinical Microbiology 35, 3150–3155.

Olea, A.M., González, C., Chiu, M., Vallebuona, C., Labraña, M., Martiniello, F., 2005. Brote de gastroenteritis por *Vibrio parahaemolyticus* en Chile. Revista Chilena de Salud Pública 9, 51–53.

Suthienkul, O., Ishibashi, M., Iida, T., Nettip, N., Supavej, S., Eampokalap, B., Makino, M., Honda, T., 1995. Urease production correlates with possession of the *trh* gene in *Vibrio parahaemolyticus* strains isolated in Thailand. Journal of Infectious Diseases 172, 1405–1408.

Tuyet, D.T., Thiem, V.D., von Seidlein, L., Chowdhury, A., Park, E., Canh, D.G., Chien, B.T., Van Tung, T., Naficy, A., Rao, M.R., Ali, M., Lee, H., Sy, T.H., Nichibuchi, M., Clemens, J., Trach, D.D., 2002. Clinical, epidemiological, and socioeconomic analysis of an outbreak of *Vibrio parahaemolyticus* in Khanh Hoa Province, Vietnam. Journal of Infectious Diseases 186, 1615–1620.

Wong, H.C., Liu, S.H., Wang, T.K., Lee, C.L., Chiou, C.S., Liu, D.P., Nishibuchi, M., Lee, B.K., 2000. Characteristics of *Vibrio parahaemolyticus* O3:K6 from Asia. Applied and Environmental Microbiology 66, 3981–3986.

Xie, Z.Y., Hu, C.Q., Chen, C., Zhang, L.P., Ren, C.H., 2005. Investigation of seven *Vibrio virulence* genes among *Vibrio alginolyticus* and *Vibrio parahaemolyticus* strains from the coastal mariculture systems in Guangdong, China. Letters in Applied Microbiology 41, 202–207.