Vibrio parahaemolyticus in shellfish and clinical samples during two large epidemics of diarrhoea in southern Chile

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Summary

Large epidemics of diarrhoea associated with seafood consumption and *Vibrio parahaemolyticus* occurred during the austral summers of 2004 and 2005 in the environs of Puerto Montt, Chile (41°29′S 72°24′W). There are no reports of *V. parahaemolyticus* infections before 2004 in this region, their absence being explained by the low ocean temperatures which seldom reach 16°C. We analysed *V. parahaemolyticus* obtained from shellfish and clinical samples during epidemics. Isolates were examined using conventional protocols and an improved method for restriction enzyme analysis using total bacterial DNA which permits direct genome restriction enzyme analysis by conventional gel electrophoresis (DGREA) with a similar discrimination index as restriction fragment length polymorphism-pulsed field gel electrophoresis (RFLP-PFGE). Analysis of clinical samples showed that the epidemics were caused by the *V. parahaemolyticus* O3:K6 pandemic clonal group. On the other hand, analysis of shellfish samples during both epidemics showed that 53% contained *V. parahaemolyticus* (3–93 g^{-1}). Detailed analysis of 50 positive shellfish samples showed that only three contained detectable levels of the pandemic clone. Most *V. parahaemolyticus* isolates obtained from shellfish corresponded to non-pandemic clones differentiated into 14 groups by DGREA. In summary, the causative agent during epidemics was only a minor component of a small but diverse population of *V. parahaemolyticus* in shellfish.

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Introduction

Large epidemics of diarrhoea associated with raw seafood consumption occurred during austral summers of 2004 and 2005 in the environs of Puerto Montt and its surroundings in southern Chile (41°S 72°W, approximately). In 2004, there were approximately 1500 cases. In 2005, approximately 3600 clinical cases were reported in the same region (Olea *et al*., 2005), but in this year, the epidemic extended to the whole country with approximately 11 000 cases reported in other parts of Chile. It is likely that seafood from the Puerto Montt region caused most of the cases observed in the rest of Chile because this region produces about 80% of the seafood consumed in the large cities. Before 2004, *Vibrio parahaemolyticus* infections were relatively infrequent in Chile, particularly in the region of Puerto Montt (González-Escalona *et al*., 2005). This was explained by the low surface temperature of seawater in this region (11–16°C year around; http://www.shoa.cl/cendoc-jsp/index.jsp), as the incidence of *V. parahaemolyticus* is strongly correlated with water temperature (Kaneko and Colwell, 1973; Joseph *et al*., 1982; Chiou *et al*., 2000). An earlier outbreak occurring mostly in the northern city of Antofagasta (23°39′S 70°24′W) between November 1997 and March 1998 caused approximately 300 clinical cases (Cordova *et al*., 2002). However, this earlier outbreak was not of major concern because average seawater temperature is 5°C higher in this region than in Puerto Montt (http://www.shoa.cl/cendoc-jsp/index.jsp). The 1997–1998 and 2004 epidemics were caused by the *V. parahaemolyticus* O3:K6 pandemic clone that emerged in Southeast Asia in 1996 (González-Escalona *et al*., 2005). 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 *trh* and urease gene (Suthienkul *et al*., 1995). The clonal nature of these pandemic strains has been ascertained by the close similarity of the patterns obtained by either genome restriction fragment length polymorphism-pulsed field gel electrophoresis (RFLP-PFGE) (Wong *et al*., 2000) or arbitrarily primed polymerase chain reaction (AP-PCR)

(Okuda *et al*., 1997; Matsumoto *et al*., 2000), and by multilocus sequence typing (MLST) (Chowdhury *et al*., 2004). To study the characteristics of the epidemics in Chile in detail, we analysed seafood and clinical samples for the presence of the pandemic strain. In order to process large number of samples involved in this analysis, we applied a simple restriction enzyme analysis (REA) method to differentiate *V. parahaemolyticus* clones. This REA method differentiates isolates according the pattern of a fraction of the DNA fragments obtained after hydrolysis with a restriction enzyme that have been separated by conventional polyacrylamide gel electrophoresis. Similar approaches for direct REA of total bacterial DNA have been previously employed to compare strains of several bacterial species (Bjorvatn *et al*., 1984; Gerner-Smidt *et al*., 1996; Djordjevic *et al*., 1999), but these methods have not been generally adopted, probably because of the lack of a suitable protocol that could guarantee unambiguous and reproducible results. Our protocol solved this problem by using an improved combination of techniques to differentiate *V. parahaemolyticus* isolates. We found that the pandemic strain was responsible for the diarrhoeal outbreak in 2005 (as was the case in 2004), but that this strain was only a minor component of a highly diverse *V. parahaemolyticus* population in shellfish.

Results

Analysis of V. parahaemolyticus *isolates by direct genome restriction enzyme analysis (DGREA)*

Clonal groups were differentiated by REA of the total extracted bacterial DNA using the improved protocol described in *Experimental procedures*. This protocol includes digestion of bacterial DNA with a six-base restriction endonuclease that generates 30–40 fragments of sizes ranging from 2500 to 500 bp, separation of the fragments by polyacrylamide gel electrophoresis and visualization with silver nitrate staining. Once the conditions for analysis were standardized, *V. parahaemolyticus* isolates previously assigned to different groups by RFLP-PFGE were analysed to test the discriminatory capability of the method. Isolates belonging to the O3:K6 pandemic clonal group were clearly distinguished in a cohesive group (Fig. 1A). The results obtained by direct genome restriction enzyme analysis (DGREA) were very similar to those observed by RFLP-PFGE (Fig. 1B). Comparison of the pattern between the 13 strains shown in Fig. 1A and B yielded the same index of discriminatory power (0.90) (Hunter and Gaston, 1988).

Analysis of clinical isolates obtained during the epidemics

Analyses of clinical isolates of *V. parahaemolyticus* from the epidemic in 2004 had shown that they corresponded to the clonal group that emerged in Southeast Asia in 1996 (González-Escalona *et al*., 2005). Analyses of 40 clinical isolates from the 2005 epidemic showed them all to possess the antigens and genes characteristic of the pandemic clone. They were all O3:K6 serovar, *tlh*, *tdh* and *orf8* positive and *trh* negative. Direct genome restriction enzyme analysis showed that the 40 isolates corresponded to the O3:K6 pandemic clone (Fig. 2A). Their specific clonal origin was confirmed in 10 of these isolates by the more conventional RFLP-PFGE method (Fig. 2B). The clinical isolates displayed the same pattern observed in the Southeast Asian strain RIMD2210633 (VpKX) when analysed by both DGREA and RFLP-PFGE.

Pandemic V. parahaemolyticus *in shellfish during epidemic periods*

Data on *V. parahaemolyticus* in seafood of the southern coast of Chile were scarce because diarrhoea associated to its presence was uncommon before 2004. In 309 shellfish samples analysed since January 2004, *V. parahaemolyticus* was detectable almost exclusively during the summer months (Fig. 3). In 204 shellfish samples obtained during the midst of the epidemics in January–March, 2004 and 2005, 108 (53%) contained *V. parahaemolyticus*. However, among 51 samples analysed in greater detail, only three contained the pandemic strain responsible of the epidemics, one from 2004 and two from 2005. The O3:K6 pandemic clonal nature of the isolates obtained from these three samples was shown by their possession of *tlh*, *tdh* and *orf8*, the absence of *trh*, and the characteristic pandemic DGREA pattern. Two of these isolates from the same shellfish sample showed a few additional bands in an otherwise typical pandemic DGREA pattern (Fig. 4A, lane 109.5).

Load and diversity of V. parahaemolyticus *in seafood during the epidemics*

Twenty-five shellfish samples containing *V. parahaemolytics* from 48 samples collected during the summer of 2005 were examined in further detail. They were collected in the eastern region of the Seno de Reloncaví, in Quillaipe and La Arena (Fig. 5), where the number of outbreaks was apparently larger and the outbreaks were longer lasting. The average monthly seawater temperature in these areas was 18.3°C and 19.2°C, respectively, some 1.5–2.5°C higher than that measured at the official weather station (http://www.shoa.cl/cendoc-jsp/index.jsp). The load of *V. parahaemolyticus* in these samples (determined by the most probable number) yielded an average geometric density of 9.4 g^{-1} with a range from 3 to 93 g^{-1}. In general, two colonies of *V. parahaemolyticus* from each sample were examined by DGREA. However, in 10 of

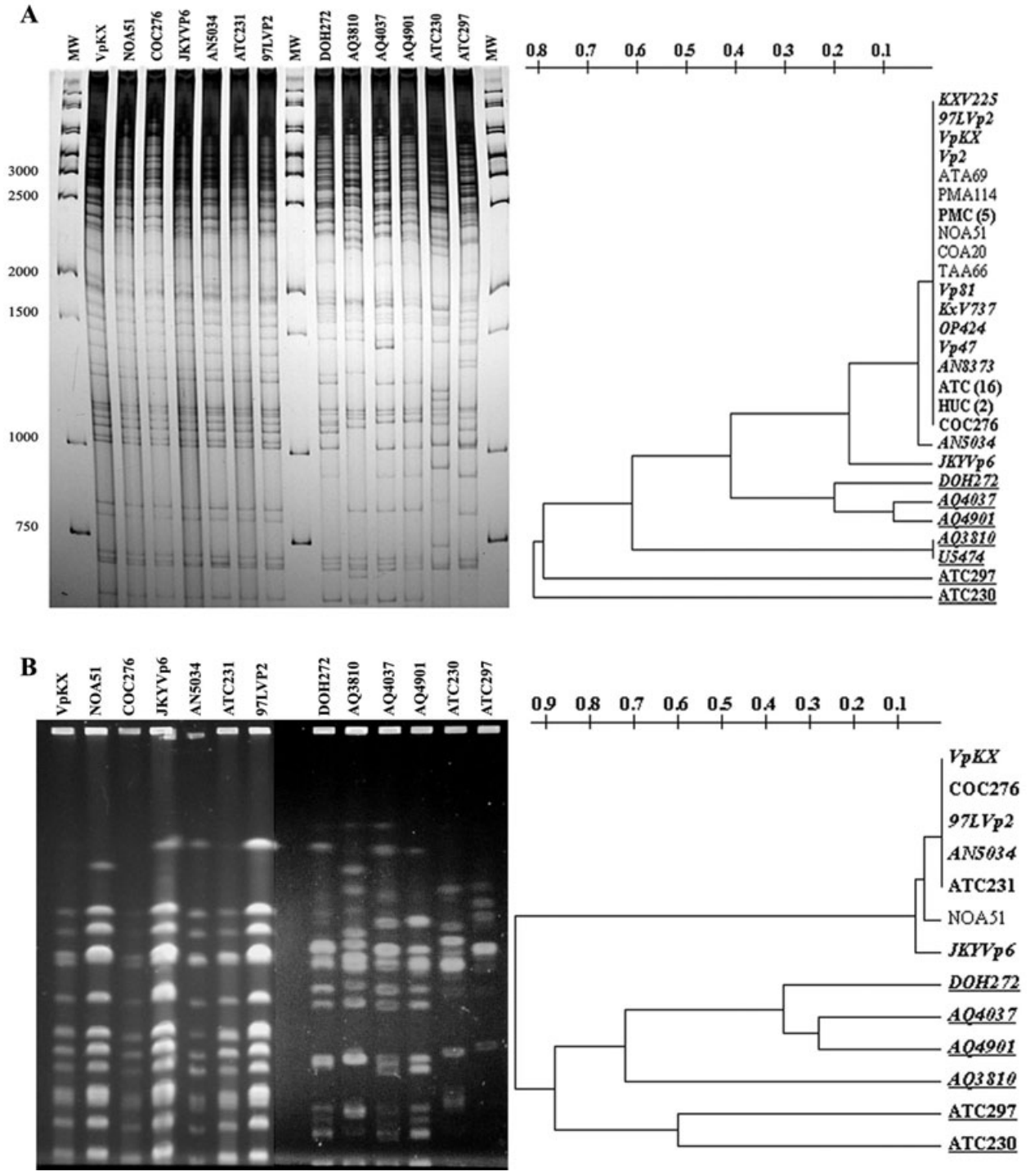

Fig. 1. Restriction enzyme analysis by DGREA (A) and RFLP-PFGE (B) of clonal and non-clonal pandemic strains of *Vibrio parahaemolyticus*. Dendrograms illustrating the clusters of the patterns by dissimilarity are shown on the right.
A. Direct genome restriction enzyme analysis obtained with NaeI; gel shows representative strains for every observed pattern.
B. Restriction fragment length polymorphism-pulsed field gel electrophoresis; gel shows the patterns for the same strains exposed in (A).
MW corresponds to molecular weight marker GeneRuler 1 kb DNA Ladder (Fermentas, Hanover, MD). ATC, HUC and PMC correspond to isolates from Chilean clinical cases; numbers within brackets indicate the number of isolates analysed in each group. Southeast Asian strains are shown in cursive; non-clonal strains are underlined.

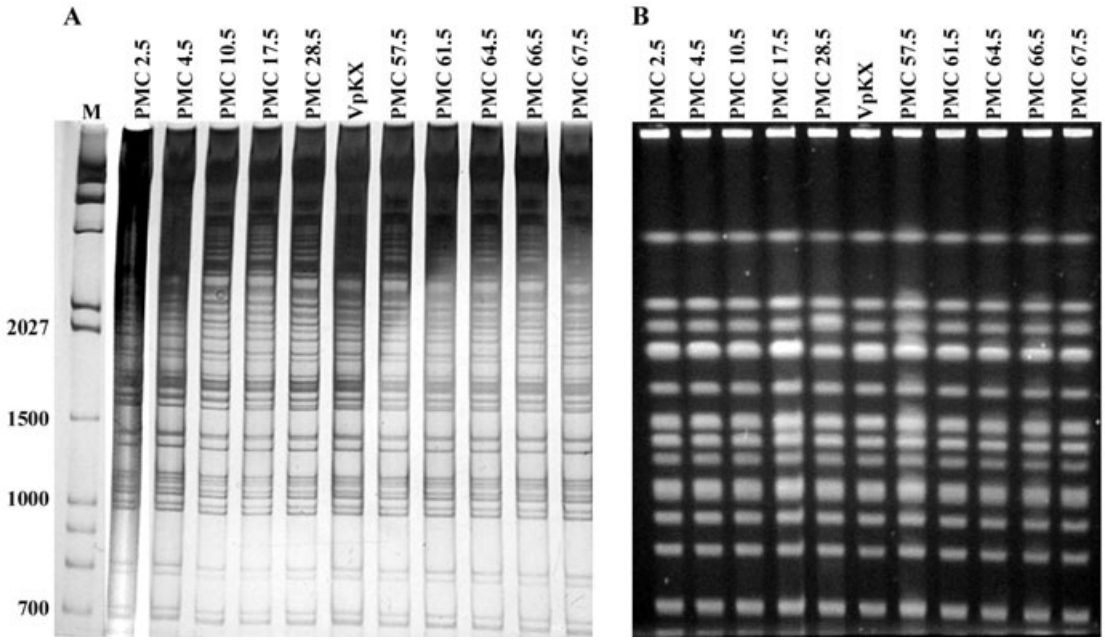

Fig. 2. Restriction enzyme analysis of *Vibrio parahaemolyticus* isolates obtained from clinical cases observed in the summer of 2005.
A. Restriction analysis by DGREA.
B. Restriction fragment length polymorphism obtained by PFGE for the same isolates shown in (A).
VpKX corresponds to the Southeast pandemic strain RIMD2210633; M to the molecular weight marker consisting of λ DNA/HindIII fragments combined with BenchTop 100 bp Ladder.

these shellfish samples, a larger number (10–30 colonies per sample) was examined by DGREA. Shellfish samples from 2005 which were found to contain pandemic clone isolates were only observed in samples subjected to this more extensive analysis. One of these samples contained eight pandemic clone colonies of 18 colonies analysed, the other four of 30 colonies analysed.

Analysis of the non-pandemic *V. parahaemolyticus* isolates obtained from shellfish in 2004 and 2005 by DGREA distinguished 14 groups. Ten groups were observed in 2005 and 11 in 2004; four were present in both summers (Fig. 4). These four groups and the pandemic group are labelled with asterisks in this figure. None of the non-pandemic *V. parahaemolyticus* isolates contained the pathogenicity associated genes *tdh* or *trh*.

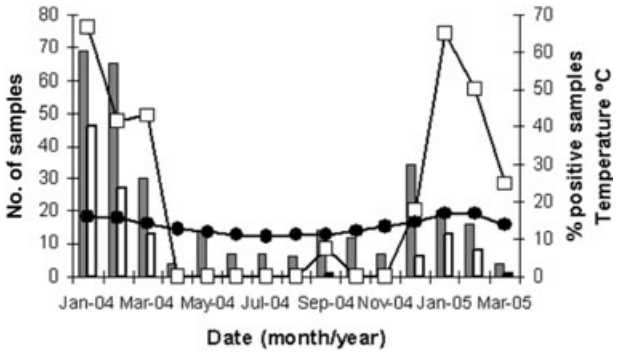

Fig. 3. *Vibrio parahaemolyticus* in molluscs obtained from Seno de Reloncaví and temperature of seawater. Grey bars, number of analysed samples; white bars, number of samples with *V. parahaemolyticus*; □, percentage positive samples; ●, temperature of seawater.

Discussion

The large epidemics of diarrhoea observed during the last two austral summers in Puerto Montt, Chile, seem to be directly related to the introduction of the O3:K6 serovar pandemic strain to this region (González-Escalona *et al*., 2005 and this work). The extent of the epidemics suggests that this strain has proliferated very successfully in this region in spite of the fact that water surface temperature seldom exceeds 16°C according to official records (http://www.shoa.cl/cendoc-jsp/index.jsp). However, our own observations indicate that seawater temperatures may reach average monthly temperatures of slightly over 19°C in some sectors characterized by intensive shellfish cultivation and extraction. This higher temperature range has been found critical for *V. parahaemolyticus* proliferation (Kaneko and Colwell, 1973). A similar situation may be developing in some regions in the northern hemisphere. Outbreaks of diarrhoea caused by *V. parahaemolyticus* were observed during the boreal summer of 2004 in Alaska (Nart, 2004), where as in the south of

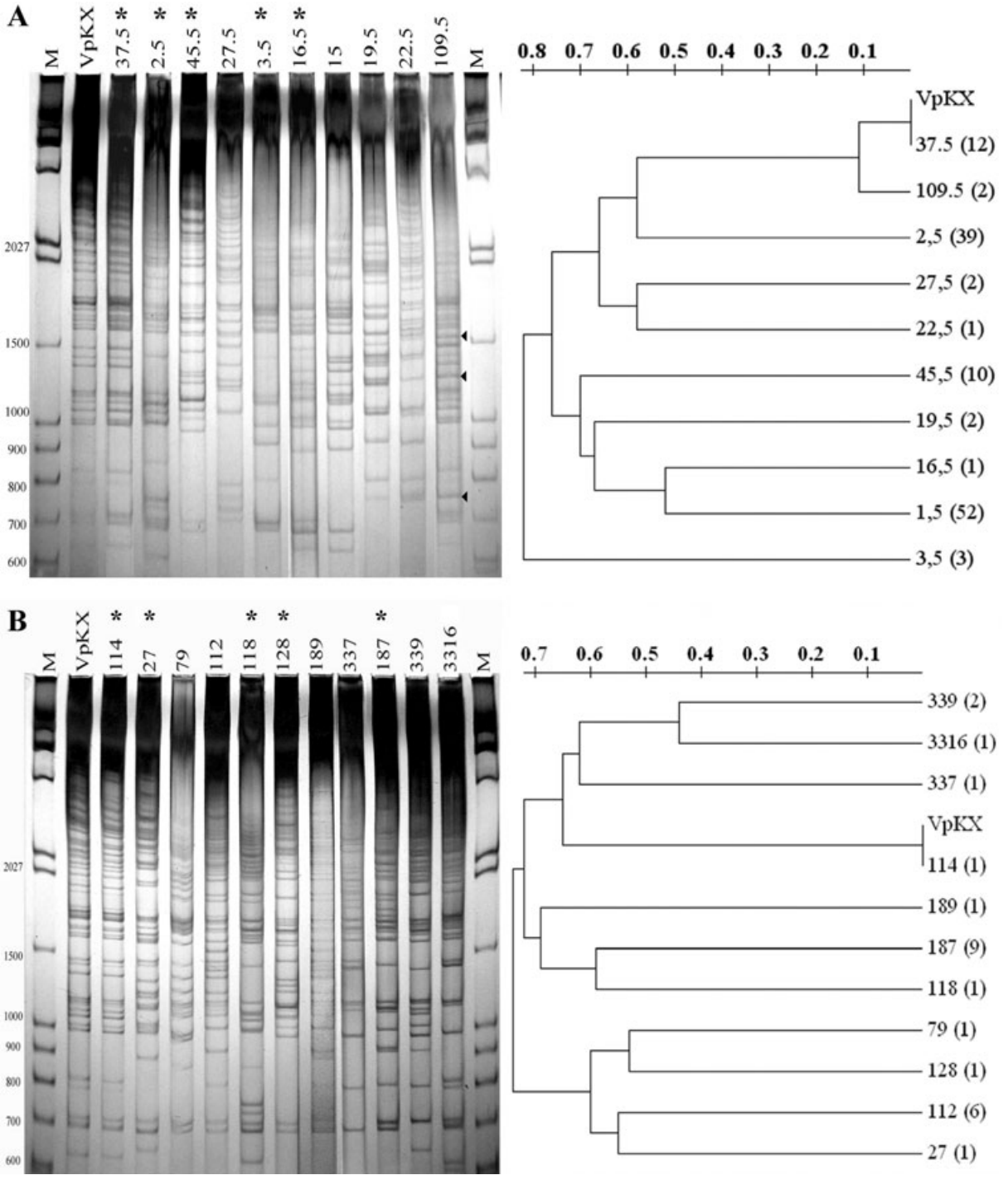

Fig. 4. Direct genome restriction enzyme analysis (DGREA) of *Vibrio parahaemolyticus* isolates obtained from shellfish collected in the summers of 2005 (A) and 2004 (B), and dendrograms illustrating the clusters of the patterns by dissimilarity. Gels show representative strains for every observed pattern. Arrows in (A) indicate extra bands in pattern of pandemic strain 109.5. The four groups found in both summers are labelled with asterisks. The pandemic group corresponding to the pattern in lane 37.5 (A) and 114 (B) is also labelled. Dendrogram shows the clustering of the analysed colonies. Numbers within brackets indicate the number of analysed colonies clustering in each group. VpKX corresponds to the Southeast pandemic strain RIMD2210633; M to the molecular weight marker consisting of λ DNA/HindIII fragments combined with BenchTop 100 bp Ladder.

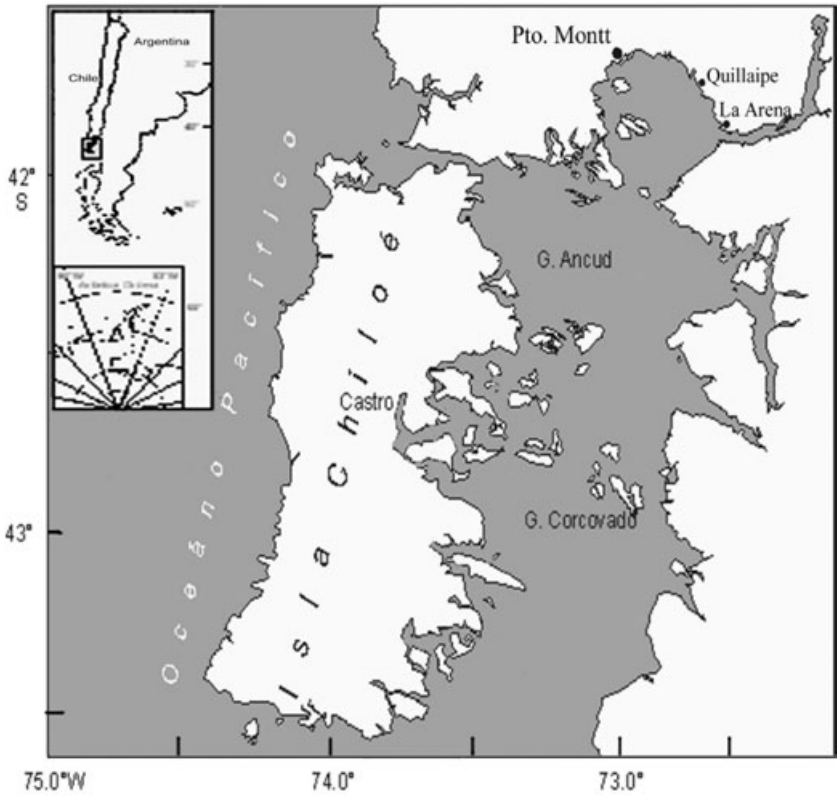

Fig. 5. Map showing the location of Puerto Montt and surrounding region. Seno de Reloncaví corresponds to the bay of Puerto Montt.

Chile, seawater temperatures are low and *V. parahaemolyticus* infection is very rare. In the present study we found that more than 50% of the shellfish obtained during the epidemics contained *V. parahaemolyticus* but their bacterial load was much lower than that reported from other regions of the world (Chiou *et al*., 2000; DePaola *et al*., 2000; Cabrera-Garcia *et al*., 2004). Furthermore, only 6% of the shellfish samples containing *V. parahaemolyticus* had detectable levels of the pathogenic agent. The observation that the pathogenic strains constitute a minor fraction of the *V. parahaemolyticus* loads has also been reported in other regions of the world (DePaola *et al*., 2000; Alam *et al*., 2003; Hara-Kudo *et al*., 2003). Our observation, however, shows that this is also true for the aetiological agent in the midst of a large epidemic. *Vibrio parahaemolyticus* loads and the presence of genes associated with pathogenicity are important factors in the risk management of diarrhoea associated to seafood consumption. The effect of *V. parahaemolyticus* loads especially has been continuously reassessed (CODEX Committee on Food Hygiene, 2003) and our observations suggest that this reassessment should be continued. The production of a large epidemic by consumption of shellfish with the observed low *V. parahaemolyticus* loads could be explained by a very low infective dose of the pandemic strain (Yeung *et al*., 2002) and/or by a low recovery of this strain with the analytical procedures employed as is the case with some pathogenic strains of *Vibrio cholerae* (Faruque *et al*., 2004). A low recovery could be also due to the prevalence of viable but non-culturable forms of the pandemic strain (Bates and Oliver, 2004).

The REA method used in the present study, DGREA, incorporates an improved combination of techniques. Direct genome restriction enzyme analysis is relatively rapid compared with RFLP-PFGE, gives highly reproducible results and can be easily implemented with equipment available in any modern microbiology laboratory. It is likely that it could be also employed to differentiate clones of other bacterial species. Among its potential applications is tracing the source of food contamination and hospital infections.

The use of DGREA permitted discrimination of different clones of *V. parahaemolyticus*, and showed that *V. parahaemolyticus*, although in low abundance in shellfish of the Puerto Montt region, consists of a highly diverse population comprising at least 16 different groups. Among these, only two groups corresponded to the O3:K6 pandemic clone. One of these groups comprised only two isolates, obtained from the same sample, which contained a few additional bands in the DGREA pattern. They may constitute a mutant version of the original pandemic clone arrived in Chile. Diversity between isolates of the pandemic clonal group is not unusual. It has been observed

between isolates obtained in Southeast Asia by RFLP-PFGE (Bag *et al*., 1999; Chowdhury *et al*., 2000; Hara-Kudo *et al*., 2003) and by serotyping (Bhuiyan *et al*., 2002). Diversity was also found among some of the Southeast Asian isolates tested by DGREA (Fig. 1). The existence of these variants provides key pieces for the study of bacterial evolution in the midst of clonal expansion. The observation of only six of the 16 clonal groups in shellfish in both 2004 and 2005 suggests that the diversity within this species could be larger than that observed in these two years, or that there is a significant temporal variation of the prevalent strains. In summary, our observations indicate that even during the midst of the epidemics in Chile, the causal agent was a minor component of a small but diverse *V. parahaemolyticus* population in shellfish.

Experimental procedures

Shellfish and clinical samples

Seafood samples were collected in the coastal region of Seno de Reloncaví (Fig. 5) between January 2004 and March 2005. Most samples consisted of small mussels, *Mytilus chilensis* (72.6%), small amounts of clams, *Venus antiqua* (6.8%), and oysters, *Tiostrea chilensis* (6.4%). In collection sites close to Quillaipe and La Arena (Fig. 5), seawater temperature was measured every day with digital thermometers during the months of collection. Shellfish were packed in polyethylene bags and kept in styrene foam boxes with ice until analysis. Detection of *V. parahaemolyticus* was performed not later than 4 h 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, 2004). Briefly, 50 g of 10 animals was homogenized in 450 ml of PBS dilution water. The homogenate was seeded at different concentrations into a series of three tubes containing alkaline peptone water (APW). After incubation overnight at 37°C the surface of the medium in the tubes showing bacterial growth was streaked onto TCBS plates. After incubation overnight at 37°C, green or bluish colonies were purified and identified by API-20E for enterobacteria (BioMerieux, Halzelwood, MO) according to the manufacturer's instructions. Three colonies were usually examined. In 48 samples examined in detail, the *V. parahaemolyticus* load was calculated applying three-tube MPN tables (Kaysner and DePaola, 2004). In some of these samples 10–30 colonies were examined and stored for further analysis. Colonies derived from different APW enrichment tubes were chosen when more than one tube was positive.

Clinical isolates of *V. parahaemolyticus* were obtained from rectal swabs of 40 patients with acute diarrhoea seeking medical attention during January and February, 2005, as previously described (González-Escalona *et al*., 2005).

Bacterial strains

Vibrio parahaemolyticus 2210633 (VpKX) was obtained from the Research Institute for Microbial Diseases, Osaka University, Japan (RIMD). Clonal and non-clonal strains from Southeast Asia were kindly provided by Dr Mitsuaki Nishibuchi of the Center for Southeast Asian Studies, Kyoto University. The Southeast Asian pandemic clonal strains were: AN5034, KXV737, KXV225, VP2, VP47, VP81, OP424, 97LVP2, JKYVP6 and AN8373. The Southeast Asian pandemic non-clonal strains were: U5474, AQ3810, AQ4901, AQ4037 and DOH272. The following Chilean *V. parahaemolyticus* isolates had been previously characterized (González-Escalona *et al*., 2005). The Chilean pandemic clonal strains were: ATC208, ATC211, ATC213, ATC214, ATC216, ATC217, ATC218, ATC219, ATC220, ATC222, ATC223, ATC224, ATC225, ATC227, ATC231, ATC232, COC276, HUC265, HUC275, PMC33, PMC48, PMC49, PMC65, PMC66, NOA 51, COA20, TAA66, ATA69 and PMA114. The Chilean non-pandemic clonal strains were: ATC230 and ATC297.

Phenotype characterization

Phenotype characterization was performed by API-20NE (Bio Merieux, Halzelwood, MO) according to the manufacturer's instructions. The determination of the O and K antigens of the *V. parahaemolyticus* strains was performed by slide agglutination with rabbit antisera obtained from Seiken (Denka Seiken, Japan) as described by the supplier. K serovar was exclusively determined for K 1, 25, 26, 41, 56, 6, 8, 12, 68 and 46.

Genotype characterization

Bacterial DNA was extracted from overnight cultures in Luria–Bertani broth-3% NaCl using the Wizard Genomic DNA Purification kit (Promega Madison, WI). DNA concentration was assessed by the intensity of the DNA band after agarose gel electrophoresis and staining with ethidium bromide. Known amounts of λ DNA were used as a standard. Polymerase chain reaction assays were performed using approximately 10 ng per reaction tube. Amplifications of the different markers were performed as previously described: *tlh*, *tdh* and *trh* (Bej *et al*., 1999), *orf8* (Laohaprertthisan *et al*., 2003), *toxRS/new* (Matsumoto *et al*., 2000).

Direct genome restriction enzyme analysis (DGREA) and RFLP-PFGE

For selection of an appropriate restriction enzyme for DGREA the size and number of restriction fragments generated by commercially available enzymes from the *V. parahaemolyticus* was initially calculated. The EMBOSS Restrict Program (http://emboss.source-forge.net/apps/restrict. html) was employed using the genome sequence reported by Makino *et al*. (2003). These results were used to estimate the pattern obtained after electrophoresis in 7.5% polyacrylamide gel. Only fragments between sizes of 500 bp and 2500 bp, the size range well resolved in this gel, were included. According to this analysis, NaeI seemed the most appropriate restriction enzyme. DNA was extracted as described above from 1.0 ml of an overnight culture and suspended in 50 μl of TE (Tris 0.01 M, EDTA 0.001 M, pH 8.0). Aliquots (10 μl) of DNA from each strain were digested with 5 U of NaeI (Promega, Mad-

ison, WI) according to the manufacturer's instructions and incubated at 37°C for 2 h. Samples were subsequently incubated with Proteinase K at a final concentration of 0.2 mg ml^{-1} for 1 h at 37°C, to hydrolyse the restriction enzyme and the bovine serum albumin present in the buffer. Approximately 7 μl of each digestion product was then mixed with 2 μl of loading dye buffer (Sambrook and Russell, 2001) and resolved by electrophoresis through 7.5% polyacrylamide gels. Electrophoresis was performed in gels $8 \times 7 \times 0.1$ cm run for 3 h at 100 V, except for the case shown Fig. 1 when the gel was $16 \times 18 \times 0.1$ cm and the electrophoresis was run for 18 h at 90 V. Bands were visualized by silver nitrate staining (Espejo and Escanilla, 1993). Restriction fragment length polymorphism-pulsed field gel electrophoresis was performed using NotI (Hara-Kudo *et al*., 2003) except that a CHEF-DRII system (Bio-Rad Laboratories, Hercules, CA) was used for electrophoresis.

Restriction patterns and dendrograms

For construction of the dendrogram, bands with similar and different migration were distinguished and then identified by their relative migration in the gel, using the navigator tool of Adobe Photoshop. The generated data were employed to construct a similarity matrix, calculated using the Nei and Li coefficient (Nei and Li, 1979). This matrix was finally used to obtain the dendrogram applying WPGM in Treecom (Van de and DeWachter, 1994).

Discrimination index

The 'Hunter–Gaston discrimination index' was calculated according to the following equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{N} a_j$$

where D is the index of discriminatory power, a_j is the number of strains in the population which are indistinguishable from the jth strain, and N is the number of strains in the population (Hunter and Gaston, 1988).

Acknowledgements

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