Original Article

Characterization of *Vibrio parahaemolyticus* strains isolated in Chile in 2005 and in 2007

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Abstract
Introduction: *Vibrio* (*V.*) *parahaemolyticus* has endemically established in Chilean sea shores, causing outbreaks every year, with an important number of cases. In order to know the genetic relationship, genotype dominance and antibiotic resistance of isolates obtained from two outbreaks, this study characterized 110 strains isolated from environmental and clinical samples in years 2005 and 2007 in Chile.
Methodology: Genotyping was performed by determination of PFGE profiles, and pandemic group and integrons were screened by PCR. Antimicrobial susceptibility was studied by the disk diffusion method.
Results: High antibiotic susceptibility frequency was found, mainly among 2007 isolates, except to ampicillin, cephalothin, cefoxitin, cefpodoxime, amikacin, streptomycin and kanamycin. Strains belonging to the pandemic group in clinical isolates account for 88% in 2005, decreasing to 66% in 2007 and among environmental isolates were detected in 20% of the strains from 2005, rising to 36% in 2007. In 2005, nine different PFGE profiles were identified, with 78% of the strains corresponding to a single clone. In 2007, sixteen different PFGE profiles were detected, with 61% of the strains included into a sole clone. The same clone was prevalent in both years. None of class 1, 2, 3 and SXT integrases genes was detected; however, the superintegron integrase gene (*intIA*) was present in almost all strains.
Conclusions: These results suggest the persistence and dominance of a unique PFGE clone of *V. parahaemolyticus* during 2005 and 2007, and the absence of genetic elements that capture antibiotic resistance genes described in other species of *Vibrio*.

Key words: pandemic group; PFGE; *V. Parahaemolyticus*; integron; Chile

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Introduction

V. parahaemolyticus is a natural inhabitant of marine environments and can increase its population according to seasonal variations of temperature. A small percentage of the environmental population is pathogenic to humans, causing fever, vomiting, diarrhoea and nausea when ingested by consuming raw or undercooked seafood. The clinical manifestations are self-limited without antibiotic treatment [1]. *V. parahaemolyticus* has become endemic in some geographic areas of Chile since 2004, where consumption of seafood is common, and there are thousands of cases every summer season [1]. In the 1990s, serotype O3:K6 spread quickly across the world and has been described as a pandemic strain responsible for diseases in countries from four continents [2]. Studies of the pandemic serotype have shown a close correlation with markers such as the presence of thermostable direct hemolysin (TDH) toxin coded by *tdh*, which is responsible for diarrhoea caused by the electrolyte imbalance in enterocytes, and presence of a specific sequence in *toxRS* operon encoding transmembrane proteins that regulate virulence associated genes [3]. Pulsed field gel electrophoresis (PFGE) has revealed a cluster of strongly related genomic patterns among the pandemic group, and currently, approximately 14 different serotypes are included within the pandemic group [4].

The genomic composition of the genus *Vibrio* is dynamic, resulting in a high level of complexity. *Vibrio* possesses two chromosomes and superintegrons, systems able to capture, integrate and express genes. Superintegrons, named for their large size, carry mainly metabolism-associated genes, and a fully functional integrase which rearranges genes in order to adapt [5]. In addition, there have been frequent descriptions of different classes of resistance integrons in *Vibrio cholerae*, smaller and harbouring diverse antibiotic resistance genes (ARG). A class 1

integron bearing a trimethoprim resistance gene was already identified in *V. parahaemolyticus* [6]. A newer and larger integrative and conjugative genetic element also carrying ARG, called SXT element, has been detected in most of the *V. cholerae* epidemic strains [7] and in two *V. parahaemolyticus* strains [8]. The presence of any of these genetic elements promote mobilisation across different species, which has important implications for rapid evolution and dissemination of antibiotic resistance or virulence genes, depending on environmental selection, and can be directly or indirectly harmful to human health [9].

This work characterised strains of *V. parahaemolyticus* isolated from environmental and clinical samples in Chile during the years 2005 and 2007, according to their genotypes (PFGE profiles), relationship to the pandemic group, and the integrons they possess. The overall aim was to evaluate the genetic status regarding the exchange of antimicrobial resistance elements in strains intoxicating Chilean population, and whether a bigger concern should be taken into account if potential dangerous new genotypes emerge.

Methodology

Strains

A total of 110 strains of *V. parahaemolyticus* were isolated from clinical (patients' stool samples) and environmental (water surface samples) origins during outbreaks in 2005 (n = 60) and 2007 (n = 50) from several regions of Chile, and provided by the Instituto de Salud Pública de Chile. Identification was performed by biochemical tests [10], according to recommendations of Global Foodborne Infections Network (GFN-PAHO/WHO) for *V. parahaemolyticus*. All strains were regularly grown in LB broth or LB agar (Oxoid, Cambridge, England), supplemented with 1% NaCl, and incubated at 37°C for 24-48 hours.

Antimicrobial susceptibility test

The disk diffusion method was used to screen antimicrobial susceptibility in all isolates, following recommendations from the Clinical and Laboratory Standards Institute [11]. Susceptibility breakpoints were used according to *V. cholerae* or *Enterobacteriaceae*. Antimicrobials tested and respective disk power were as follows: kanamycin (KAN 30 µg), gentamicin (GEN 10 µg), amikacin (AMK 30 µg), streptomycin (STR 10 µg), cefoxitin (FOX 30 µg), cephalothin (CEF 30 µg), cefpodoxime (CPD 10 µg), cefotaxime (CTX 30 µg), ampicillin (AMP 10 µg), tetracycline (TET 30 µg), ciprofloxacin (CIP 5 µg), nalidixic acid (ANX 30 µg), sulfonamides (SUL 250 µg), trimethoprim (TMP 5 µg), sulfametoxazol-trimethoprim (SXT 25 µg), chloramphenicol (CLO 30 µg), and florfenicol (FLO 30 µg).

Polymerase chain reaction

DNA was obtained by resuspending 3-5 colonies in 200 µL of 5%- Chelex solution (BioRad, Hercules, CA, USA) and 2.5 µL of proteinase K (20 mg/mL, Invitrogen, Carlsbad, CA, USA). Samples were incubated at 56ºC for 45 minutes, and then at 100ºC for 8 minutes. A 2 minute centrifugation was done at 12,000 g to obtain DNA in the supernatant [12]. Identification of the pandemic group was performed amplifying by PCR the genes *tdh* and the new sequence of *toxRS* operon [13]. Presence of integrases from integron class 1 [14], class 2 [15], class 3 [16], and SXT element [17] was also detected by PCR. Control strains used for integrons were *Proteus mirabilis* UC44 (*intI*1^{+}, *intI*2^{+}) and *Serratia marcescens* AK9373 (*intI*3^{+}), kindly given by Dr. Y. Arakawa (Nagoya University, Japan), and recombinant *Escherichia coli* (int_{SXT}^{+}), kindly given by Dr. M. Colombo (Universitá di Roma, Italy). Primers were designed in this study to detect the presence of *V. parahaemolyticus* superintegron integrase gene, *intIA*, InVpF 5'-CCTGCACCTCTCTCAATTACG-3' and InVpR 5'-GCATATGCTTACTCGCCATT-3'.

Pulsed field gel electrophoresis

The genetic relationship among the strains was established by macrorestriction with enzyme *Sfi*I and pulsed field gel electrophoresis (PFGE) with minor modifications according to the CDC PulseNet Standardized protocol [4]. Shortly, colonies of each strain were suspended in 2 mL of suspension buffer (100 mM Tris, 100 mM EDTA, pH 8.0) and adjusted to 0.9 absorbance at 610 nm wavelength. Next, 400 µL of the suspension was mixed with 20 µL of proteinase K (20 mg/mL) and 400 µl of 1% molten agarose (Seakem Gold), forming plugs into the pockets of plug molds to solidify. Plugs were submerged in 5 mL lysis buffer (50 mM Tris, 50 mM EDTA, pH 8.0, 1% Sarcosyl), and incubated in a shaking water bath for 1 h at 54°C. Two washes with water and four washes with 1X TE buffer were performed, and at the end, plugs were kept in 1X TE buffer at 4°C until use. The macrorestriction was

performed by adding, per piece (2 mm) of plug, 200 µL of the following mixture: 176.75 µL of water, 20 µL of buffer, 2 µL of BSA (20 mg/mL), 1.125 µL of *Sfi*I (40 U/µL). The digestion was incubated for 4 h at 37°C and the mixture was removed, then 200 µL of 0.5X TBE buffer was added for maintenance. Plugs were run on 1% agarose gel in 0.5X TBE buffer in a PFGE equipment (CHEF DR-III, Bio-Rad, Hercules, CA, USA) adjusted to conditions: initial pulse: 10 seconds, final pulse 35 seconds, Voltage: 200 V, Run Time: 18 h. Band patterns analyses were performed with BioNumerics (Applied Maths NV, Sint-Martens-Latem), and pairwise similarity indices were calculated using the Dice coefficient. A genotype was defined with a cut-off 85% similarity.

Results

Antimicrobial susceptibility

High susceptibility percentages were detected, close to 100%, for drugs such as florfenicol, chloramphenicol, sulfonamides, trimethoprim, sulfamethoxazole-trimethoprim association, tetracycline, nalidixic acid, ciprofloxacin, cefotaxime and gentamicin. On the other hand, a high percentage of strains were resistant to streptomycin, ampicillin, kanamycin, amikacin, cefoxitin, cephalothin and cefpodoxime (Figure 1).

Pandemic group

Among the 50 strains of clinical origin isolated in 2005, positive amplification for genes, *tdh* and *toxRS*, characteristic for strains belonging to pandemic group, was observed in 88%. Furthermore, 8% only gave *tdh* positive amplifications, and 4% of isolates did not carry the *tdh* gene at all. Meanwhile, of the 39 clinical isolates from year 2007, a decrease to 66.7% amplified for both pandemic group genes, but isolates positive only for *tdh* increased to 20.5%, while 12.9% did not possess *tdh* (Figure 2).

Among strains from environmental origin, only 20% of 2005 strains had *tdh* and *toxRS* genes and, 80% did not carry *tdh*. For year 2007 environmental isolates, 36% indicated to carry both pandemic group genes, and 9% only *tdh*. A decrease to 54.6% was noticed in strains not bearing *tdh* (Figure 3).

Integrons

No amplification by PCR was obtained for classes 1, 2, 3 and SXT integrases genes in any of the strains studied. In contrast, the *intI*A (superintegron integrase gene) was amplified in 96% of the clinical strains and 80% of the environmental strains isolated in 2005. Strains isolated in 2007 showed 82% amplification in both clinical and environmental origin. Sequencing of *intIA* PCR fragment (approx. 850 bp) in 10 randomly chosen strains showed nucleotide changes in about 29 bases among strains of *V. parahaemolyticus*, which resulted in only three amino acid variations at positions 39, 183 and 259, with phenylalanine and leucine as variants in the first two positions and asparagine and serine in the third position (data not shown). All five strains harboring phenylalanine residues (in positions 39 and 183) belonged to the same clone (A1 PFGE profile) and

Figure 1. Antimicrobial susceptibility percentages of all *V. parahaemolyticus* strains according to their year of isolation

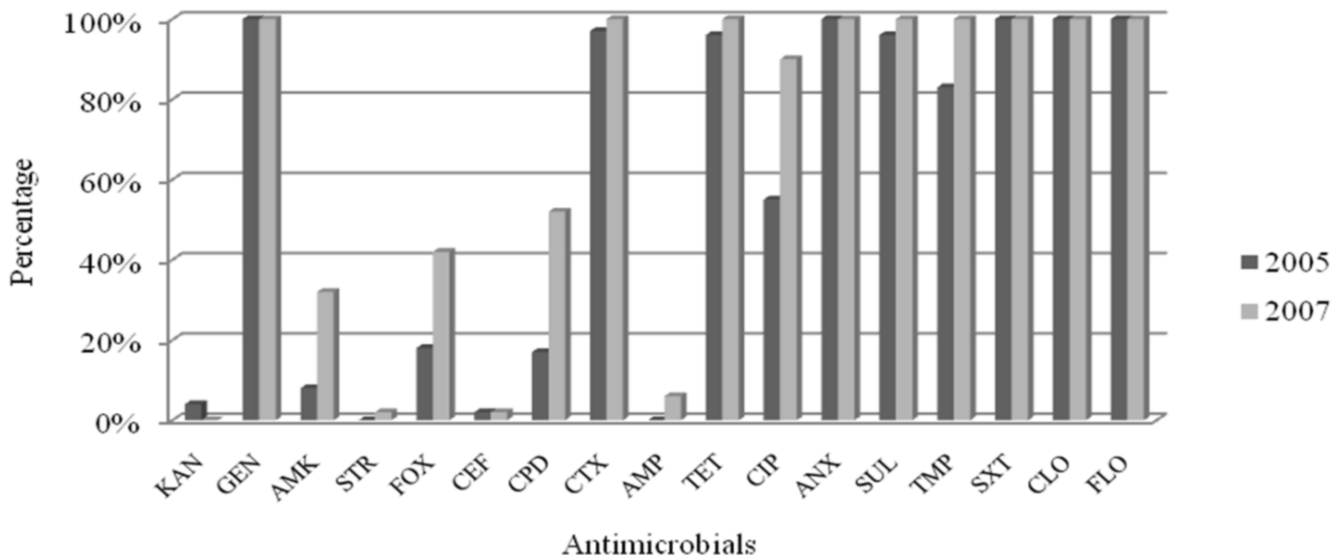

Figure 2. Distribution of genes characterizing pandemic group in clinical *V. parahaemolyticus* strains according to their year of isolation

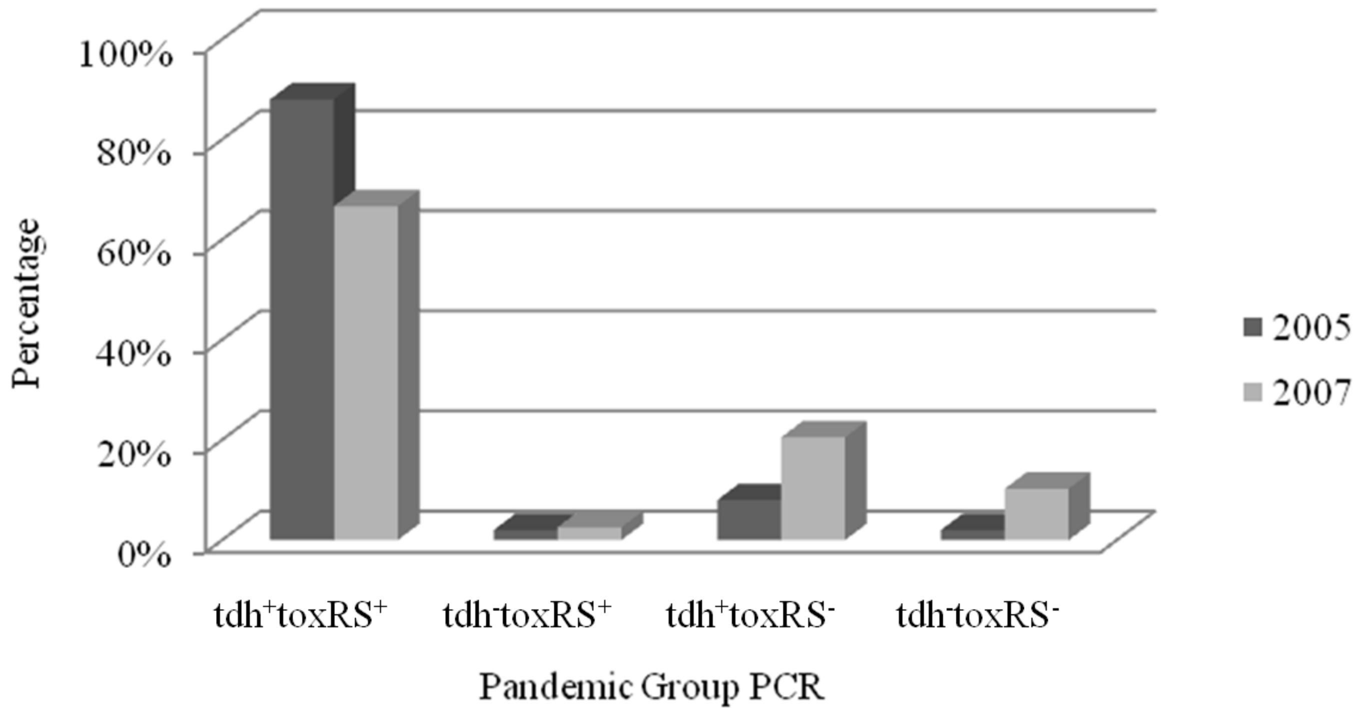

Figure 3. Distribution of genes characterizing pandemic group in environmental *V. parahaemolyticus* strains according to their year of isolation

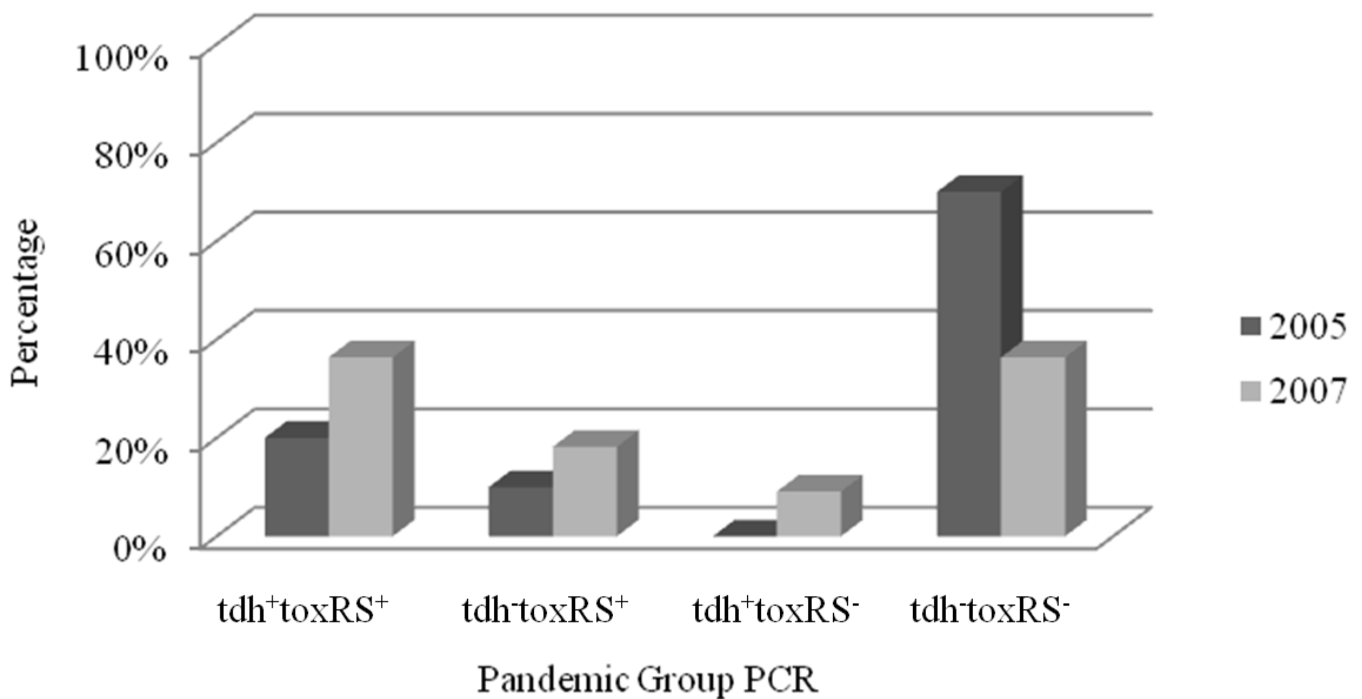

Table 1. PFGE profiles and molecular features of strains of *V. parahaemolyticus* isolated in 2005

Genotype	Profile	Nr strains	*tdh*	*toxRS*	Pandemic Clon	Origin type (n)	Region of Origin (n)
A	A1	44	+	+	+	Environmental (1) Clinical (43)	Araucanía (1) Maule (4), Biobío (10) Valparaíso (4), Los Lagos (8), Metropolitana (11), Los Ríos (1),Araucanía (3), L.B.O (2)
		1	-	+	-	Clinical (1)	Valparaíso (1)
		1	+	-	-	Clinical (1)	Metropolitana (1)
	A3	1	+	+	+	Clinical (1)	Biobío (1)
B	B1	1	+	+	+	Environmental (1)	Biobío (1)
		2	-	-	-	Environmental (2)	Biobío (1), Valparaíso (1)
D	D1	1	-	-	-	Environmental (1)	Valparaíso (1)
F	F1	1	-	-	-	Environmental (1)	Valparaíso (1)
G	G1	1	-	+	-	Environmental (1)	Valparaíso (1)
H	H1	2	-	-	-	Clinical (2)	Arica y Parinacota (1), Biobío (1)
J	J2	1	-	+	-	Environmental (1)	Los Lagos (1)
		1	-	-	-	Environmental (1)	Los Lagos (1)
L	L1	2	+	-	-	Clinical (2)	Arica y Parinacota (1) Tarapacá (1)

L.B.O: Libertador Bernardo O´Higgins

were positive for the pandemic group markers.

Pulsed field gel electrophoresis

PFGE profiles obtained from 105 analyzed strains are shown in Tables 1 and 2. Dendrograms were made from PFGE profiles, clustered as genotypes with similarity over 85%, and randomly assigned with letters from A to P. In total, 24 different PFGE profiles were detected, clustered as 16 genotypes. Strains isolated in 2005 showed a total presence of nine different PFGE profiles, of which 78% corresponded to a single profile. Four different PFGE profiles were detected in clinical strains, and 90% of them corresponded to profile A1. Six different PFGE profiles were detected in environmental strains in this year, and the most frequent profile was B1 in 33% of the strains. Among strains from 2007, a total of 16 different PFGE profiles were obtained, of which 61% matched a single profile. Ten different PFGE profiles were detected in clinical strains, 71% of which belonged to clone A1, and seven different PFGE profiles, with profiles N1 and A1 as the most frequent clones (27%), were found in environmental strains.

Discussion

Ampicillin resistance in *V. parahaemolyticus* strains is widespread in the world, but is not a characteristic of the genus or the marine environment itself [18, 19]. There is a specific mechanism of resistance to this natural antimicrobial agent, but it does not confer high levels of resistance as a mechanism suspected to be encoded in genes located on the integrons. Two single component efflux systems of the MATE (Multidrug And Toxic compound Extrusion) family have been described in *V. parahaemolyticus*, recognizing substrates such as norfloxacin, ethidium bromide, kanamycin and streptomycin [20], which explains in part the low susceptibility to the latter two drugs. High susceptibility to tetracycline, quinolones such as ciprofloxacin, and nalidixic acid, inhibitors of folate pathway as sulfonamides, trimethoprim, sulfamethoxazole-trimethoprim association, and phenicols as chloramphenicol and florfenicol, is the general rule, and confirmed in different parts of the world [1,18,19].

A decrease in the pandemic group was observed from 2005 to 2007 in clinical strains. This result is in agreement with the observations of other studies evaluating other markers from pandemic group of strains of *V. parahaemolyticus* isolated in Chile in 2005, 2006 and 2007 [21,22]. In addition, a strong

Table 2. PFGE profiles and molecular features of strains of *V. parahaemolyticus* isolated in 2007

Genotype	Profile	nr strains	*tdh*	*toxRS*	Pandemic Clon	Origin type (n)	Region of Origin (n)
A	A1	24	+	+	+	Environmental (3) Clinical (21)	Biobío (2), Metropolitana (1) Biobío (7), Metropolitana (6),Maule (4), Araucanía (1),Los Lagos (2), Coquimbo (1)
		1	+	-	-	Clinical (1)	Araucanía (1)
		1	-	+	-	Clinical (1)	Metropolitana (1)
		2	-	-	-	Clinical (2)	Biobío (1), Metropolitana (1)
	A2	1	+	+	+	Environmental (1)	Biobío (1)
	A4	2	+	+	+	Clinical (2)	Biobío (2)
C	C1	1	-	-	-	Environmental (1)	Coquimbo (1)
E	E1	1	-	-	-	Clinical (1)	Biobío (1)
I	I1	1	+	-	-	Clinical (1)	Coquimbo (1)
	I2	1	-	-	-	Clinical (1)	Araucanía (1)
J	J1	1	+	-	-	Environmental (1)	Biobío (1)
K	K1	1	+	-	-	Clinical (1)	Coquimbo (1)
	K2	1	+	-	-	Clinical (1)	Biobío (1)
L	L2	1	+	-	-	Clinical (1)	Coquimbo (1)
	L3	1	+	-	-	Clinical (1)	Metropolitana (1)
M	M1	1	+	-	-	Clinical (1)	Coquimbo (1)
N	N1	1	-	-	-	Environmental (1)	Metropolitana (1)
		2	-	+	-	Environmental (2)	Metropolitana (2)
O	O1	1	-	-	-	Environmental (1)	Coquimbo (1)
P	P1	1	-	-	-	Environmental (1)	Biobío (1)

correlation between the presence of the TDH toxin gene and the pathogenicity of *V. parahaemolyticus* was established; thus 96% and 87% of the clinical strains isolated in 2005 and 2007, respectively, carried this gene. There have been reports of pathogenic strains not having TDH toxin as the main virulence factor, but another toxin, namely Toxin Related Haemolysin (TRH), is also able to cause diarrhoea in humans [23]. In this study, by default it could be suspected that strains not amplifying for *tdh*, could harbour *trh* due to its clinical isolation, meaning they are isolated from patients affected by gastroenteritis. Comparing both years, a decrease in pathogenicity is also notable due to TDH toxin in year 2007. On the other hand, a much higher percentage than has historically been established of the pandemic group was observed among environmental strains isolated in 2005 and 2007; about 1% of environmental strains are pathogenic [6]. Nevertheless, more recently, high prevalence of the environment-originated pandemic group has been reported in the Unites States (12.5%), India (6%) and China (3%) [24,25,26]. Probably pathogenic *V. parahaemolyticus* has become endemic; having found a niche in the large coastline of Chile favoured by the current weather and sea conditions.

Absence of resistance integrons of classes 1, 2, and 3 among strains was closely related to the marine environment origin of this bacterium, as well as the absence of recommendations of antimicrobial treatment against this pathogen, leading to no selective pressure for this type of genetic element in the clinical settings. Primers used to screen integrase from the SXT^{MO10} element [17] are useful to detect others families of SXT elements as well, due to the conservation of this integrase in SXT families described so far [8]. The BLAST search with

primers [INT1 int (+) GCTGGATAGGTTAAGGGCGG and INT2 int (-) CTCTATGGGCACTGTCCACATTG] confirmed that both primers align with SXT element integrase or integrating conjugative element of *Vibrio* spp. and other bacteria. Nonetheless, there were no positive amplifications for this element in the studied strains. Antimicrobial susceptibility profiles did not indicate the presence of SXT element or integrons, because no resistance to trimethoprim or sulfamethoxazole or high level resistance to other antimicrobials were detected, respectively. However, other studies have detected SXT without a relevant antimicrobial resistance [8]. Previous identification of the SXT element in two strains of *V. parahaemolyticus* in Mozambique demonstrates how geographic coexistence increases probabilities of genetic exchange from *V. cholerae* to *V. parahaemolyticus* [8]. Fortunately, in Chile, *V. cholerae* has not been isolated since a small outbreak in 1991 [27]. The superintegron, despite not yet being associated with antibiotic resistance genes, has been associated with an ancestral connection with resistance integrons. It is also known to possess an integrase enzymatically active with substrates that extend its range of structural recognition sites of VCR (*Vibrio cholerae* repeats), so it has the potential capacity to incorporate exogenous DNA [5,9]. Variations in integrase IntIA have not yet been correlated to functionality changes, and two out of three amino acid changes described in this study have been already reported in the database of chromosome sequences of strain CIP 75.2T (accession number AY014399) isolated in 1951, which is highly likely to be non-pandemic [9], as well as strain RIMD 2210633 (accession number NC_004603) isolated in 1996 which is confirmed as being the pandemic O3:K6 [28]. Indeed, the same phenylalanine substitutions were previously identified in the pandemic O3:K6 clone, and in this study, and they also correlated with the dominant PFGE profile A1 and pandemic group markers, raising the question whether this amino acid change in superintegron integrase provides any virulence or adaptation advantage to the pandemic group, and if it is well conserved in such strains. Detection of *intIA* in almost all strains grants the potentiality for recruitment and retention of useful gene cassettes for adaptation to environmental conditions or pathogenicity, as has happened with multi-resistance integrons in hospital settings.

The total number of different PFGE profiles found in clinical isolates of *V. parahaemolyticus* was significantly lower than those identified in similar studies in other countries, and the percentage of clinical isolates belonging to the same clone was extremely higher in Chile compared to the percentages in other studies, providing evidence for the highest dominance of a single clone [18,29]. A clear higher polyclonality, *i.e.*, number of different PFGE profiles detected according to the number of strains tested, was established in the environmental strains compared to the clinical strains, despite a lower number of environmental strains being tested. The number of different PGFE profiles among environmental strains was proportionally similar to that of other countries [30]. Comparing total profiles between both years, an increased polyclonality was observed in 2007, which was associated with a decrease in the percentage of the prevalent clone. Clone A1 was the most prevalent, and was present in both years in clinical and environmental isolates, which could be explained by the ability of this species to enter into a dormant state called viable but non-culturable (VBNC) in response to some type of natural stress [31] and therefore persist in unfavourable conditions such as winter and then re-emerge in summer. In addition to clone A1, other clones from the entire genotype A are expected to be part of the pandemic group as well. Given the positivity of markers used in this study and the close similarity of their PFGE patterns, they could be integrated by different serotypes. Interestingly, profile B1, which is not genetically related to the pandemic group, also had pandemic group markers, and may represent a new genotype with pandemic potential.

Given the dominance of a single clone in *V. parahaemolyticus* intoxications in the Chilean population, more studies should be conducted to analyze the genetic and environmental optimal features that allow the exceptional survival and fitness of clone A1. However, the polyclonality shift from year 2005 to 2007 could be a cyclic variation helped by environmental conditions, such as ocean warming caused by El Niño Southern Oscillation (ENSO). This climatic phenomenon triggered slight increases in sea surface temperatures in the Pacific Ocean during the summers of 2005 and 2007; however, the temperature increase was greater in 2007 than that in 2005 [32], possibly affecting some genotypes' survival. Alternatively, this shift could be the beginning of a steady change through time that

needs to be followed in order to prevent endemic establishment of other genotypes of *V. parahaemolyticus*.

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