Electronic Journal of Biotechnology 18 (2015) 459–463

HOSTED BY

ELSEVIER

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research Article

Amplification of *tlh* gene in other Vibrionaceae specie by specie-specific multiplex PCR of *Vibrio parahaemolyticus*

Romina Yáñez [a], Roberto Bastías [a], Gastón Higuera [b], Oscar Salgado [c], Pantelis Katharios [d], Jaime Romero [b], Romilio Espejo [b], Katherine García [c,*]

[a] Laboratorio de Microbiología, Instituto de Biología, Pontificia Universidad Católica de Valparaíso, Av. Universidad 330, Curauma, Valparaíso, Chile
[b] Instituto de Nutrición y Tecnología de los Alimentos (INTA), Universidad de Chile, Av. El Líbano 5524, Macul, Santiago, Chile
[c] Instituto de Ciencias Biomédicas, Universidad Autónoma de Chile, Av. El Llano Subercaseaux 2801, San Miguel, Santiago, Chile
[d] Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research, Crete, Greece

ARTICLE INFO

Article history:
Received 30 July 2015
Accepted 23 September 2015
Available online 28 October 2015

Keywords:
Hemolysin
Multiplex PCR
Pathogen surveillance
Virulence factor
Vibrio parahaemolyticus

ABSTRACT

Background: The surveillance of *Vibrio parahaemolyticus* in the Chilean coast has been mainly performed by multiplex PCR amplification of three different hemolysin genes, which are specie-specific virulence factors. These genes are also employed in the determination of *V. parahaemolyticus* pathogenic load in seafood and for characterization of pathogenic strains associated to diarrhea cases in human. During environmental surveillance that we performed every summer, we occasionally observed a thermolabile hemolysin (*tlh*) PCR product of a slightly smaller size than expected, which was coincident with low loads of *V. parahaemolyticus* in the environment. In order to understand this observation, we probed the specificity of *tlh* primers for the detection of *V. parahaemolyticus* at different bacterial loads and DNA concentrations.
Results: Primers used for the detection of *V. parahaemolyticus* specific *tlh* amplified a slightly smaller *tlh* gene, which is found in *Vibrio alginolyticus* and other related strains. These amplicons were observed when *V. parahaemolyticus* was absent or in undetectable loads in the environment.
Conclusions: Surveillance of *V. parahaemolyticus* using *tlh* primers can be imprecise because amplification of a *V. parahaemolyticus* specific marker in *V. alginolyticus* and other related strains occurs. This situation complicates potentially the estimation of bacterial load in seafood, because do not ensure the correct identification of *V. parahaemolyticus* when his load is low. Additionally, it could complicate the tracking of outbreaks of *V. parahaemolyticus* infections, considering the genetic markers used would not be specie-specific.

1. Introduction

Vibrio parahaemolyticus is a halophilic gram negative bacterium widely distributed in marine environments and is recognized as the world's leading cause of gastroenteritis in humans due to raw or undercooked seafood [1]. Some strains are capable of causing disease and most of them are related to the pandemic serotype O3:K6 [2]. This serotype comprises a clonal complex originally observed in Southeast Asia that reached Antofagasta, northern Chile, in 1998 [3], and reached and remained in Region de Los Lagos, southern Chile since 2004. During this period, seafood consumption-related diarrhea increased drastically in Chile, because approximately 69% of the country's shellfish are produced in this region [4]. Filter-feeding bivalve, such as mussels, can concentrate pathogenic strains of *V. parahaemolyticus* resulting in bacterial loads capable of producing infection to the people that ingest them [5]. Consequently, virulent *V. parahaemolyticus* strains are clearly a concern for seafood safety, and hence, their reliable detection in mussels is crucial.

In Chile, surveillance of *V. parahaemolyticus* in seafood has been performed mainly by multiplex PCR (mPCR) [6] including amplification of thermolabile hemolysin (*tlh*), thermostable direct hemolysin (*tdh*) and *tdh*-related hemolysin (*trh*) genes [7]. The *tlh* gene encodes a phospholipase A2 [8], while its contribution to *V. parahaemolyticus* pathogenicity is not clear, the expression of *tlh* is upregulated under conditions mimicking the human intestine [9]. This gene is considered a specie-specific marker for *V. parahaemolyticus* and is frequently employed to identify this specie [10]. On the other hand, most of pathogenic strains additionally possess *tdh* and/or *trh* hemolysin genes, which are considered important markers of clinical strains [1,11].

Using mPCR, Fuenzalida et al. [2] observed *tlh* amplicons slightly smaller than expected (450 bp) derived from shellfish extracted in Antofagasta. Authors sequenced *tlh* amplicon and observed 91% similarity with *tlh* gene found in *Vibrio alginolyticus* [12] and 82%

* Corresponding author.
E-mail address: katherine.garcia@uautonoma.cl (K. García).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2015.09.007
0717-3458/

similarity with *V. parahaemolyticus tlh* gene. Smaller *tlh* amplicons were later observed in Puerto Montt, coinciding with lower loads of *V. parahaemolyticus* (geometric average <0.3 to 2 g^{-1}) than observed in previous years (geometric average <2 to 110 g^{-1}). To understand these observations and the potentially critical consequences for *V. parahaemolyticus* surveillance, we performed sampling to quantify the bacterial load in mussels using mPCR, including *tlh* primers. Additionally, we studied the specie-specificity of these primers for isolates obtained from Chile and we performed PCR at different loads of *V. parahaemolyticus* and *V. alginolyticus*, simultaneously.

2. Materials and methods

2.1. Sampling and load estimation

Mussel samples obtained in Quillaipe, Puerto Montt, were analyzed by mPCR as described previously [13]. Briefly, samples of shellfish soft tissue were enriched for *V. parahaemolyticus* in three-tube serial dilutions in alkaline peptone water for the assessment of bacterial load by the most probable number (MPN) method; tubes with bacterial growth were tested for *tlh*, *tdh* and *trh* by multiplex PCR [6]. PCR was performed using approximately 10 ng of total bacterial DNA per reaction tube. The *tlh* amplicons were visualized in 8% polyacrylamide gels and stained with silver nitrate. Total and pandemic *V. parahaemolyticus* loads were calculated according to the number of tubes positive for *tlh* and for *tdh* and *trh*, respectively.

2.2. Phenotypic characterization

According to the mPCR results, *tlh* positive enrichment tubes were plated on TCBS agar (Difco) and chromogenic agar CHROMagar *Vibrio* (CHROMagar Microbiology, Paris, France). Furthermore, purified colonies were characterized with Api20E kit for enteric bacteria (Biomerieux, Halzelwood, MO) and referred as A1 to A7. *V. alginolyticus* strains R4 and VA1 were isolated from Crete, Greece and were identified by BIOLOG GENIII and molecular methods (16s RNA sequence and PCR using collagenase gene primers) [14].

2.3. tlh amplification

PCR using *tlh* primers (Forward 5′-AAA GCG GAT TAT GCA GAA GCA CTG-3′, Reverse 5′-GCT ACT TTC TAG CAT TTT CTC TGC-3′) [6] was performed using *V. alginolyticus* strains R4, and VA1. As positive controls, reference *V. parahaemolyticus*, Vp222, RIMD 2210633 (VpKX) and Vp7 strains were used. All of these strains were previously confirmed by growth in CHROMagar *Vibrio*. Same conditions of mPCR amplification were used [6] and PCR products were visualized in 1% agarose gels and stained with ethidium bromide.

2.4. tlh restriction assay

To determine the sequence similarity of *tlh* PCR products, in silico restriction analysis was performed with Webcutter 2.0 (http://rna.lundberg.gu.se/cutter2/) using *tlh* sequence of *V. alginolyticus* (JF718655.1) and *V. parahaemolyticus* (JX262976.1). Of the tested enzymes, *Hha*I and *Mbo*I were selected because they showed clear differences between species. Experimental restriction analysis of *tlh* was performed using *Hha*I (Fermentas) and *Mbo*I (Fermentas) for *V. alginolyticus* and *V. parahaemolyticus* amplicons according to the manufacturer's instructions, and PCR products were visualized in 1% agarose gels and stained with ethidium bromide.

2.5. Sequencing and phylogenetic analysis

PCR product of *tlh* gene from colonies obtained from sampling (A1 to A7) along with *V. alginolyticus* R4 and ANw1 (Antofagasta's strain referred as X in [2]) were sequenced in Macrogen (Korea) and results were compared with *tlh* sequences obtained from Genbank [15] using BLAST [16]. Phylogenetic tree was constructed using MEGA5 software v5.2 [17] based on UPGMA method.

2.6. tlh PCR assay specificity

The strains *V. alginolyticus* R4 and *V. parahaemolyticus* Vp222 were selected to determine *tlh* primer specificity at different DNA concentrations and bacterial load of both species. For PCR with different concentrations of DNA, the genome was extracted with

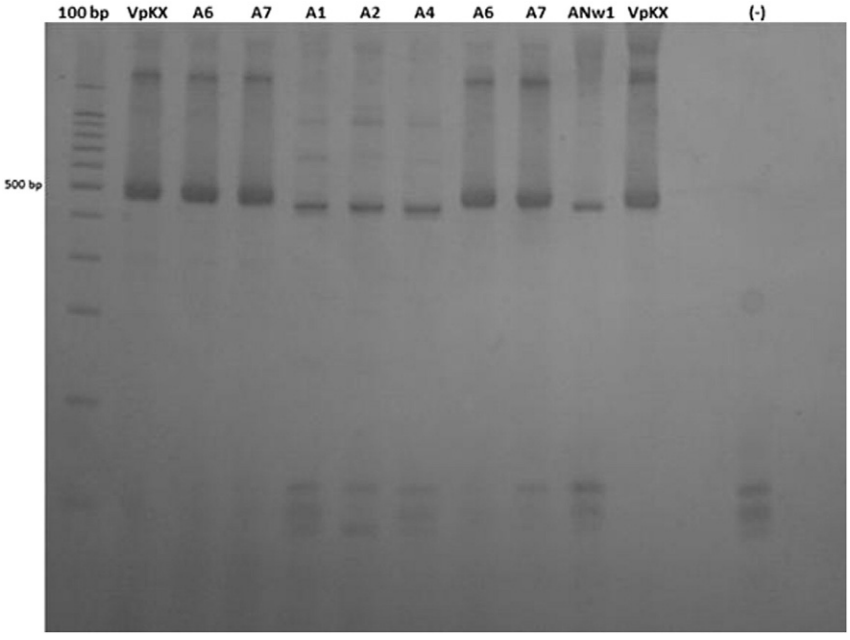

Fig. 1. *tlh* amplification. 8% polyacrylamide gel shows that in samples A1, A2 and A4 PCR amplification rendered a *tlh* amplicon slightly smaller than obtained with *bona fide V. parahaemolyticus* (A6 and A7). VpKX and ANw1 were used as positive control. Molecular weight, in the first line, corresponds to ladder 100 bp.

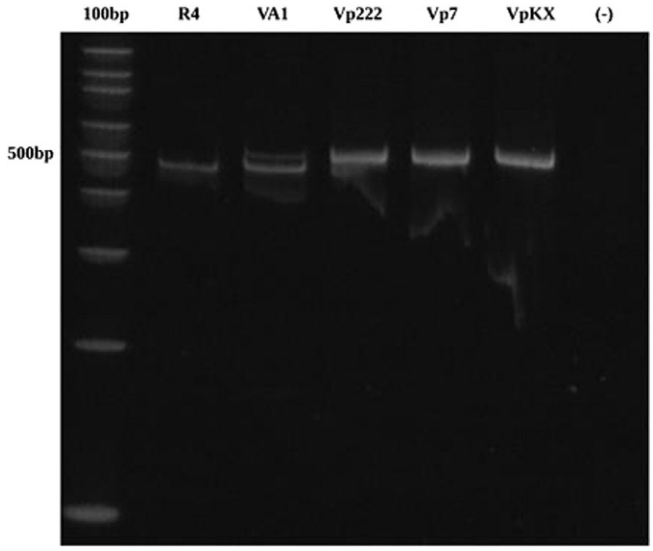

Fig. 2. *tlh* amplification in reference strains. 1% agarose gel shows amplicons of *tlh* in all strains. Two strains of *V. alginolyticus* (R4 and VA1, lanes 2 and 3, respectively) and three strains of *V. parahaemolyticus* (Vp222, Vp7 and VpKX, lanes 4, 5 and 6, respectively) were used. Molecular weight, in the first line, corresponds to ladder 100 bp.

Wizard Genomic DNA purification kit (Promega). The PCR was performed using DNA concentrations from 0.0001 ng to 30 ng of each bacterial strain and templates were mixed using proportional inverse concentration of genomic DNA. For PCR with different bacterial loads, cells were counted under microscope and serial dilutions were prepared from 10^0 to 10^5 total cells for each species. Six mixtures of R4 and Vp222 were performed using proportional inverse bacterial load. PCR was performed according amplification conditions mentioned in [6]. The *tlh* amplicons were visualized in 8% polyacrylamide gels and stained with silver nitrate or gel red as appropriate.

3. Results

3.1. Vibrio parahaemolyticus load estimation and isolates characterization

Of 28 samples analyzed, *tlh* was detected in 89% and all samples were negatives for *tdh* and *trh* genes. *Vibrio parahaemolyticus* load was estimated between 0.7 g^{-1} and 110 g^{-1}, with a geometric mean of 2.1 g^{-1}. *tlh* amplicons obtained from some of these samples (A1 to A5; size 440 bp) were slightly smaller than that obtained with *bona fide V. parahaemolyticus* (A6, A7; size 450 bp), as was reported previously in Antofagasta [2]. To confirm this observation, PCR with *tlh* primers was repeated for isolates A1, A2, A4, A6 and A7, using VpKX and ANw1 as positive control (Fig. 1). All the isolates with a smaller *tlh* amplicon showed phenotypic characteristics of *V. alginolyticus*: they were yellow and white colonies in TCBS and CHROMagar *Vibrio*, respectively, and Voges Proskauer positive and *ara* negative according Api20E. While isolates with expected *tlh* amplicon showed phenotypic characteristic from *V. parahaemolyticus*.

3.2. Analysis of tlh in V. alginolyticus strains

To corroborate that *tlh* amplicons were effectively amplified from *V. alginolyticus* or related isolates, *tlh* primers were used to perform PCR using strains R4 and VA1 isolated in Crete, Greece and *V. parahaemolyticus* Vp7, Vp222, and VpKX. Slightly size differences of approximately 10 bp were observed between *V. alginolyticus* and *V. parahaemolyticus* amplicons (Fig. 2), as was observed in isolates obtained during sampling (Fig. 1).

Additionally, restriction pattern analysis of *tlh* amplicon showed difference between *V. alginolyticus* R4 and VA1 strains and *V. parahaemolyticus* strains. This distinctiveness was observed using *Hha*I (Fig. 3A) or *Mbo*I (Fig. 3B) restriction enzymes, suggesting that *tlh* sequence were not equal in both species.

3.3. Sequencing and phylogenetic analysis of tlh gene from isolates obtained during sampling

According to the suggested differences between *tlh* sequences of the reference strains (Fig. 3), *tlh* of isolates from sampling (A1 to A7) was sequenced, including R4 and ANw1 strains as control. Additionally, other *tlh* sequences reported for other *Vibrio* were obtained from GenBank and considered for analysis. Phylogenetic tree showed that isolates with smaller *tlh* amplicons (A1 to A5) do not clustered with *V. parahaemolyticus* (Fig. 4). Of this smaller *tlh* amplicons, isolates A1, A2, and A3 were clustered with *V. alginolyticus* strains, including reference strain R4. Isolates A4 and A5 grouped with *Vibrio diabolicus* strains and formed a cluster with ANw1 strain, previously isolated in Antofagasta [2] (Fig. 4).

3.4. Specificity of tlh primers

As we mentioned before, our previous observations suggest that *tlh* primers could amplify a *tlh* gene from other *Vibrio* when

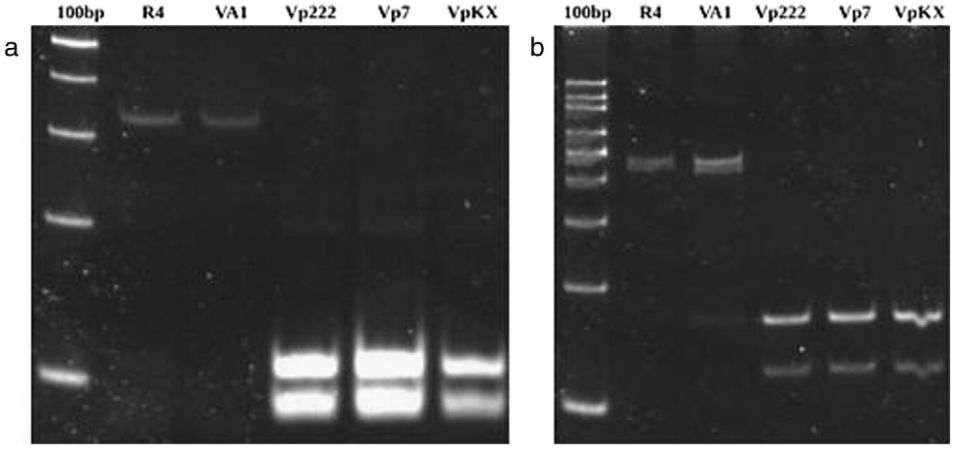

Fig. 3. Restriction pattern of *tlh* amplicons from *V. alginolyticus* and *V. parahaemolyticus* strains. Amplicons were cut with *Mha*I (a) or *Mbo*I (b). Molecular weight, in the first line, corresponds to ladder 100 bp.

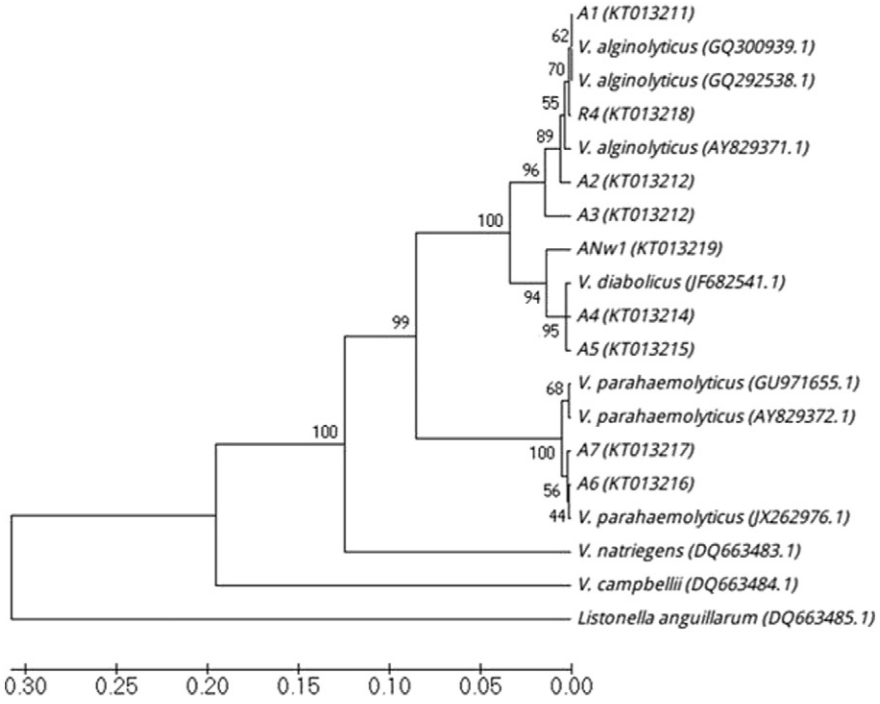

Fig. 4. Phylogenetic tree of *tlh* sequences constructed with the UPGMA method. Sequences from smaller *tlh* (isolates A1–A3 and R4) grouped with those *tlh* previously reported in *V. alginolyticus* or *V. diabolicus* (A4, A5 and ANw1). By contrast, sequences from *tlh* of expected size (isolates A6 and A7) grouped with those *tlh* previously reported in *V. parahaemolyticus*. Bootstrap values based on 1000 replications are shown at the nodes. The bottom scale denotes the degree of dissimilarity. Genbank accession number is showed in brackets. DQ663485.1 (*Listonella anguillarum*) is a GenBank synonym of *Vibrio anguillarum*.

loads of *V. parahaemolyticus* are lower. To prove this observation, we performed a simultaneously *tlh* amplification with different concentrations of DNA (Fig. 5, upper panel) or bacterial load (Fig. 5, bottom panel) from *V. alginolyticus* R4 and *V. parahaemolyticus* Vp222. The results show that at lower concentration of *V. parahaemolyticus* DNA (30 ng R4/0.0001 ng Vp222), the *tlh* from *V. alginolyticus* is amplified. A decrease in the DNA of *V. alginolyticus* and increased in the DNA of *V. parahaemolyticus* will lead to the amplification of both amplicons (Fig. 5, upper panel, lane 2) until the *tlh* from *V. alginolyticus* is no longer amplified (Fig. 5, upper panel, lanes 3–8). Similar results were observed when the template DNA was determined by different loads of cells. Polyacrylamide electrophoresis show that a lower *V. parahaemolyticus* load determine *tlh* amplification corresponding to *V. alginolyticus* (10^5 R4/10^0 Vp222) which is characterized by smaller size. Again, it is observed that when *V. parahaemolyticus* load is low, smaller *tlh* amplicon take place. Inversely, when *V. parahaemolyticus* load increased is observed *bona fide tlh* amplification (Fig. 5, bottom panel).

4. Discussion

Since *V. parahaemolyticus* reached Chile in 1998, the mPCR proposed by Bej and collaborators [6] has provided a high-throughput and convenient means to determine total and pathogenic *V. parahaemolyticus* numbers in shellfish. However, these last years, we observed that during surveillance when *V. parahaemolyticus* loads were lower in Chilean coasts, mPCR was clearly a subject to false positives because thermolabile hemolysin gene was amplified from other *Vibrio* species (*V. alginolyticus* and *V. diabolicus*).

The presence of *tlh* in other species of *Vibrio* is not novel and had already been previously reported. Wang et al. [18], showed that *tlh* is widespread in vibrios [18], including *V. alginolyticus*, *Vibrio harveyi*, *Vibrio fischeri*, *Vibrio mimicus*, *Vibrio natriegens*, *V. parahaemolyticus*, *Vibrio proteolyticus*, *Vibrio anguillarum*, and *Vibrio vulnificus* among others. In other study, Xie et al. [12] showed that *tlh* sequence data was recovered from non-*V. parahaemolyticus* strains, indicating that *tlh* gene sequences in *V. diabolicus*, *Vibrio tubiashii*-like, and *Photobacterium damselae* are highly similar to those in *V. parahaemolyticus*. Worryingly,

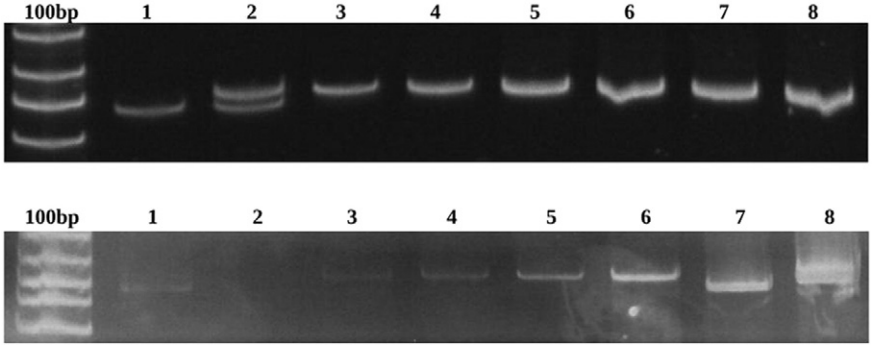

Fig. 5. 8% polyacrylamide gel. Upper panel: *tlh* amplification using different concentrations of DNA from *V. alginolyticus* R4 and *V. parahaemolyticus* Vp222. Proportions of DNA template start from 30 ng of R4 and 0.0001 ng of Vp222 in lane 1 to 0.0001 ng of R4 and 30 ng of Vp222 in lane 8. A 10 fold dilution of PCR product was loaded in lanes 4–8 due to oversaturation of gel images. Bottom panel: *tlh* amplification using different bacterial loads of R4 and Vp222. Cells load start from 10^5 of R4 and 10^0 of Vp222 in lane 1 to 10^0 of R4 and 10^5 of Vp222 in lane 6. Lane 7: DNA positive control for R4. Lane 8: DNA positive control for Vp222. Ladder 100 bp.

Xie also reported that *V. alginolyticus* strains often possess homologues of the *V. parahaemolyticus* and *V. cholerae* virulence genes such as *tox*R, and VPI [12], which suggests that *V. alginolyticus* maybe contain other known virulence genes of *V. parahaemolyticus*, besides *tlh*, potentially complicates the analysis employed during surveillance of *V. parahaemolyticus*, including pathogenic strains. Additionally, Klein and their coworkers [19] reported that virulence-related genes occurred at high frequencies in non-*V. parahaemolyticus*–Vibrionaceae species. In this study, *V. diabolicus* was of particular interest, as several strains were recovered, and the large majority (>83%) contained virulence-related genes [19].

Similarity sequence between different *Vibrio tlh* genes makes the specie distinction imprecise. Consequently, the use of *tlh* as marker for *V. parahaemolyticus* loads may lead to overestimation by including other *Vibrio* species carrying *tlh*. The level of overestimation may be variable among different systems and sample types. Considering that *tlh* detection is formally recommended to the detection of *V. parahaemolyticus* [20,21], the results of this study are critical for surveillance in coasts. It is essential to find additional specie-specific probes and perform supporting analysis, to find reliable markers for *V. parahaemolyticus* quantitative detection, and to avoid overestimation of loads and erroneous identification of this specie in the environment.

Conflict of interest

The authors declare that they have no conflicts of interest in the research.

Financial support

This research was supported by Fondecyt 11140257, Fondecyt 11140412, and Scientific Information Program/Fund for Scientific Journals Publishing, Year 2014, ID FP140010.

References

[1] Ceccarelli D, Hasan NA, Huq A, Colwell RR. Distribution and dynamics of epidemic and pandemic *Vibrio parahaemolyticus* virulence factors. Front Cell Infect Microbiol 2013;3:97. http://dx.doi.org/10.3389/fcimb.2013.00097.

[2] Fuenzalida L, Armijo L, Zabala B, Hernández C, Rioseco ML, Riquelme C, et al. *Vibrio parahaemolyticus* strains isolated during investigation of the summer 2006 seafood related diarrhea outbreaks in two regions of Chile. Int J Food Microbiol 2007;117:270–5. http://dx.doi.org/10.1016/j.ijfoodmicro.2007.03.011.

[3] González-Escalona N, Cachicas V, Acevedo C, Rioseco ML, Vergara J, Cabello F, et al. *Vibrio parahaemolyticus* diarrhea, Chile, 1998 and 2004. Emerg Infect Dis 2005;11:129–31. http://dx.doi.org/10.3201/eid1101.040762.

[4] Sernapesca (Servicio Nacional de Pesca y Acuicultura). Anuario Estadístico de Pesca. https://www.sernapesca.cl/index.php?option=com_content&view=article&id=1806:anuario-estadistico-de-pesca-2013; 2013. [Reviewed: July 17, 2015].

[5] DePaola A, Hopkins LH, Peeler JT, Wentz B, McPhearson RM. Incidence of *Vibrio parahaemolyticus* in U.S. coastal waters and oysters. Appl Environ Microbiol 1990;56:2299–302.

[6] Bej AK, Patterson DP, Brasher CW, Vickery MCL, Jones DD, Kaysner CA. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh* and *trh*. J Microbiol Methods 1999;36:215–25. http://dx.doi.org/10.1016/S0167-7012(99)00037-8.

[7] García K, Bastías R, Higuera G, Torres R, Mellado A, Uribe P, et al. Rise and fall of pandemic *Vibrio parahaemolyticus* serotype O3:K6 in southern Chile. Environ Microbiol 2013;15:527–34. http://dx.doi.org/10.1111/j.1462-2920.2012.02883.x.

[8] Zhang XH, Austin B. Haemolysins in *Vibrio* species. J Appl Microbiol 2005;98:1011–9. http://dx.doi.org/10.1111/j.1365-2672.2005.02583.x.

[9] Gotoh K, Kodama T, Hiyoshi H, Izutsu K, Park K, Dryselius R, et al. Bile acid-induced virulence gene expression of *Vibrio parahaemolyticus* reveals a novel therapeutic potential for bile acid sequestrants. PLoS One 2010;5:e13365. http://dx.doi.org/10.1371/journal.pone.0013365.

[10] Gutierrez CK, Klein SL, Lovell CR. High frequency of virulence factor genes *tdh*, *trh*, and *tlh* in *Vibrio parahaemolyticus* strains isolated from a pristine estuary. Appl Environ Microbiol 2013;79:2247–52. http://dx.doi.org/10.1128/AEM.03792-12.

[11] Su YC, Liu C. Vibrio parahaemolyticus: A concern of seafood safety. Food Microbiol 2007;24:549–58. http://dx.doi.org/10.1016/j.fm.2007.01.005.

[12] Xie ZY, Hu CQ, Chen C, Zhang LP, Ren CH. Investigation of seven *Vibrio* virulence genes among *Vibrio alginolyticus* and *Vibrio parahaemolyticus* strains from the coastal mariculture systems in Guangdong, China. Lett Appl Microbiol 2005;41:202–7. http://dx.doi.org/10.1111/j.1472-765X.2005.01688.x.

[13] Fuenzalida L, Hernández C, Toro J, Rioseco ML, Romero J, Espejo RT. *Vibrio parahaemolyticus* in shellfish and clinical samples during two large epidemics of diarrhoea in southern Chile. Environ Microbiol 2006;8:675–83. http://dx.doi.org/10.1111/j.1462-2920.2005.00946.x.

[14] Di Pinto A, Ciccarese G, Tantillo G, Catalano D, Forte VT. A collagenase-targeted multiplex PCR assay for identification of *Vibrio alginolyticus*, *Vibrio cholerae*, and *Vibrio parahaemolyticus*. J Food Prot 2005;68:150–3.

[15] Benson DA, Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW. GenBank. Nucleic Acids Res 2015;43:30–5. http://dx.doi.org/10.1093/nar/gku1216.

[16] Neumann RS, Kumar S, Shalchian-Tabrizi K. BLAST output visualization in the new sequencing era. Brief Bioinform 2013;15:484–503. http://dx.doi.org/10.1093/bib/bbt009.

[17] Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28:2731–9. http://dx.doi.org/10.1093/molbev/msr121.

[18] Wang SX, Zhang XH, Zhong YB, Sun BG, Chen JX. Genes encoding the *Vibrio harveyi* haemolysin (VHH)/thermolabile haemolysin (TLH) are widespread in vibrios. Acta Microbiol Sin 2007;47:874–81.

[19] Klein SL, Gutierrez West CK, Mejia DM, Lovell CR. Genes similar to the *Vibrio parahaemolyticus* virulence-related genes tdh, tlh, and vscC2 occur in other *Vibrionaceae* species isolated from a pristine estuary. Appl Environ Microbiol 2014;80:595–602. http://dx.doi.org/10.1128/AEM.02895-13.

[20] Nordstrom JL, Vickery MCL, Blackstone GM, Murray SL, DePaola A. Development of a Multiplex Real-Time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. Appl Environ Microbiol 2007;73:5840–7. http://dx.doi.org/10.1128/AEM.00460-07.

[21] Jones JL, Hara-Kudo Y, Krantz JA, Benner Jr RA, Smith A, Dambaugh T, et al. Comparison of molecular detection methods for *Vibrio parahaemolyticus* and *Vibrio vulnificus*. Food Microbiol 2012;30:105–11. http://dx.doi.org/10.1016/j.fm.2011.12.011.