



*Citation for published version:*

Terzi Gulel, G & Martinez-Urtaza, J 2016, 'Molecular characterizations of *Vibrio parahaemolyticus* in seafood from the Black Sea, Turkey', *Letters in Applied Microbiology*, vol. 62, no. 6, pp. 494-500.  
<https://doi.org/10.1111/lam.12579>

*DOI:*

[10.1111/lam.12579](https://doi.org/10.1111/lam.12579)

*Publication date:*

2016

*Document Version*

Peer reviewed version

[Link to publication](#)

This is the peer reviewed version of the following article: G. Terzi Gulel J. Martinez-Urtaza (2016) Molecular characterizations of *Vibrio parahaemolyticus* in seafood from the Black Sea, Turkey. *Letters in Applied Microbiology*. 62(6), which has been published in final form at 10.1111/lam.12579. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.

**University of Bath**

**Alternative formats**

If you require this document in an alternative format, please contact:  
[openaccess@bath.ac.uk](mailto:openaccess@bath.ac.uk)

**General rights**

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

**Take down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

1 **Molecular Characterizations of *Vibrio Parahaemolyticus* in Seafood from the Black**  
2 **Sea, Turkey**

3

4 Goknur Terzi Gulel <sup>1\*</sup>, Jaime Martinez Urtaza <sup>2</sup>

5 1 Department of Food Hygiene and Technology, Faculty of Veterinary Medicine,

6 Ondokuz Mayıs University, 55139, Samsun, Turkey

7 2 The Milner Center for Evolution, Department of Biology and Biochemistry,

8 University of Bath, United Kingdom

9 **Running head:** Molecular characterizations of *Vibrio parahaemolyticus*

10

11 **Significance and Impact of the Study:** *V. parahaemolyticus* is the most prevalent food  
12 poisoning bacterium associated with seafood consumption. The number of infections is  
13 increasing worldwide and are being reported in areas with no previous incidence. The  
14 present study provides the first instance of the occurrence of *V. parahaemolyticus* strains  
15 with virulence traits in the Black Sea, contributing to gain a better understanding about  
16 potential risk associated with this pathogen in the region.

17

18 **Abstract**

19 *Vibrio parahaemolyticus* is a marine bacterium that is considered as one of the major  
20 causes of bacterial food-borne outbreaks at a global scale. A total of 114 samples  
21 including mussel ( $n=42$ ), seawater ( $n=22$ ) and fish ( $n=50$ ) samples were collected and  
22 subjected to investigation. *V. parahaemolyticus* was detected in 45 (39%) of 114

---

\* Correspondence

Goknur Terzi Gulel, Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Ondokuz Mayıs University, 55139, Samsun, Turkey. E-mail: goknurterzi@yahoo.com

23 samples with a occurrence in mussel, seawater and fish samples of 76%, 40.9% and 8%,  
24 respectively. A total of 96 isolates were positive for the species-specific genes *toxR* and  
25 *tlh* and confirmed as *V. parahaemolyticus*. Presence of the virulence marker gene *tdh*  
26 was not identified in any of the strains investigated; however four of strains were  
27 positive for the *trh* gene. Serological analysis of eight randomly selected *trh*-negative  
28 isolates identified three different serotypes: O4:K untypeable (KUT), O2:KUT,  
29 O3:KUT. Conversely, all four *trh*-positive strains belonged to a single serotype (O1:K1)  
30 and share an undistinguishable genetic profile by PFGE analysis, suggesting the  
31 existence of a dominant clone for the *trh*- positive strains in the region.

## 32 **Keywords**

33 *V. parahaemolyticus*, seafoods, *tdh* and *trh* genes, PFGE, real time PCR, Black Sea

34

## 35 **Introduction**

36 *Vibrio parahaemolyticus* is a halophilic, Gram-negative bacterium that occurs in marine  
37 and coastal environments worldwide (Twedt 1989). It causes gastroenteritis in humans  
38 following consumption of raw or undercooked seafood (DePaola *et al.* 1990). The  
39 thermostable direct hemolysin (TDH) and the thermostable direct hemolysin-related  
40 hemolysin (TRH) are considered the most important virulence factors in *V.*  
41 *parahaemolyticus*. TDH and TRH are encoded by the *tdh* and *trh* genes, respectively  
42 (Honda *et al.* 1988). TDH causes beta hemolysis on Wagatsuma agar containing human  
43 erythrocytes which is called Kanagawa phenomenon (KP) and TRH is associated with  
44 KP-negative strains (Miyamoto *et al.* 1969). It has been reported that more than 90% of  
45 clinical isolates of *V. parahaemolyticus* are KP-positive, while only 1 to 2% of the  
46 strains of environmental origin are KP- positive (Nishibuchi and Kaper 1995).

47 *V. parahaemolyticus* has been frequently isolated from seawater samples (Haley  
48 *et al.* 2014). In the Black Sea region of Turkey, fishing is an important industry, and  
49 seafoods are consumed in high quantities. The Turkish Statistical Institute (Turkish  
50 Statistical Institute 2015) has reported that the quantity of caught sea fish and mussel  
51 was 295,767 tons/year and 29,000 tons/year, respectively in 2013. *V. parahaemolyticus*  
52 causes sporadic cases of gastroenteritis in the Black Sea (Libinzon *et al.* 1981). In the  
53 Georgian coast of the Black Sea, *V. parahaemolyticus* were detected from water and  
54 plankton samples however all isolates were found negative for *tdh*, *trh*, and the  
55 Kanagawa Phenomenon (Haley *et al.* 2014). In the Middle Black Sea coast of Turkey,  
56 *V. parahaemolyticus* isolates were found positive for *tdh* and/or *trh* gene from fish and  
57 mussel samples by multiplex PCR (Terzi *et al.* 2009).

58 Despite the growing number of studies on presence of pathogenic *V.*  
59 *parahaemolyticus* in seafood worldwide, little information has been obtained in the  
60 molecular characterization of *V. parahaemolyticus* in seafood of the Black Sea coast.  
61 The objectives of this study were (i) to investigate the presence of *V. parahaemolyticus*  
62 in mussel, fish and seawater samples in Samsun coastal zone of the Black Sea Region;  
63 ii) to determine the presence of potentially virulent strains; iv) to identify the  
64 serotype and genetic profiles of *V. parahaemolyticus* isolated from seafood and  
65 seawater.

66

## 67 **Results and discussion**

### 68 **Presence of *V. parahaemolyticus* in seafood samples**

69 One hundred and fourteen seafood samples were investigated for the presence of *V.*  
70 *parahaemolyticus* (Table 1). Overall, presence of *V. parahaemolyticus* strains was

71 identified in 45 samples (39%). *V. parahaemolyticus* was detected most frequently in  
72 mussel samples with 76% (32/42) of positive samples, followed by seawater and fish  
73 samples with 40.9% (9/22) and 8% (4/50) respectively. The highest occurrence  
74 observed in mussels was probably associated with the characteristic filter-feeding of  
75 these organisms and place of collection in coastal areas close to residential sites.  
76 Several previous studies have reported variable incidences for the presence of *V.*  
77 *parahaemolyticus* in seafoods worldwide. In this study, a similar incidence of *V.*  
78 *parahaemolyticus* was identified in samples in previous study (Cabrera-Garcia *et al.*  
79 2004). Environmental conditions in the areas investigated in this study ranged from 13.5  
80 to 16.7 ppt of salinity and seawater temperatures of 10.6-20.5°C, which are favourable  
81 for the presence and growth of *V. parahaemolyticus* according to previous studies (Terzi  
82 *et al.* 2009).

83

#### 84 **Confirmation of *V. parahaemolyticus* by conventional PCR and real-time PCR**

85 A total of 107 presumptive *V. parahaemolyticus* colonies with the characteristic mauve  
86 color on CHROMagar Vibrio were isolated from 45 samples. All the presumptive *V.*  
87 *parahaemolyticus* colonies were subjected to confirmation by conventional PCR by  
88 amplification of the 368 bp region of the regulatory gene *toxR* (Figure 1) and  
89 investigation of presence of species-specific *tlh* gene by real time PCR (Table 1). PCR  
90 analysis confirmed 96 of 107 strains (89%) as *V. parahaemolyticus* (*toxR* and *tlh*  
91 positive). Of the 96 *V. parahaemolyticus* strains, 72 (75%) were from mussel samples,  
92 16 (16.6%) were from seawater, and eight (8.3%) were from fish samples. There was no  
93 difference between results obtained by conventional PCR and real time PCR. There are  
94 some advantages and disadvantages of both methods; real time PCR is a highly sensitive,

95 efficient, fast, low contamination risk technique and it does need the use of ethidium  
96 bromide to stain the PCR products after gel electrophoresis. However probes used  
97 should be specially designed and are not cost effective (Wong and Medrano 2005).  
98 Conventional PCR is low sensitivity, resolution, poor precision, end point detection is  
99 time consuming and it provides size-based discrimination only. Disadvantages include  
100 the need to run agarose gels with the potential for cross contamination between samples  
101 and experiments (Basra 2006). In TaqMan®-style real time PCR, specificity and  
102 reproducibility higher than SYBR Green dye system. SYBR Green assay is that the dye  
103 is nonspecific, which can generate false positive signals if nonspecific products or  
104 primer dimers are present in the assay (Cao and Shockey 2012).

105

#### 106 **Presence of virulence genes in the strains of *V. parahaemolyticus***

107 Presence of the major virulence factors for *V. parahaemolyticus*, the thermostable direct  
108 hemolysin (*tdh*) and the *tdh*-related hemolysin (*trh*), was investigated in all the 96  
109 strains isolated in this study. Despite the high occurrence of *V. parahaemolyticus*,  
110 presence of *tdh* gene was not detected in any of the strains (Table 1). Only four of the  
111 96 strains showed amplification of the 250 bp region of the *trh* gene, all them isolated  
112 from fish samples (Figure 1). A low occurrence of potentially pathogenic isolates of  
113 *V. parahaemolyticus* in environmental samples has been reported in previous  
114 investigations carried out Europe. In the Atlantic coast of Spain, Martinez-Urtaza *et al.*  
115 (2008), identified the presence of the *tdh* gene in only two of the 194 investigated in  
116 mussel samples. In Italy, *tdh*-positive strains were identified in three of nine mussel  
117 samples, although no *trh*-positive isolates were found (Di Pinto *et al.*, 2008).

118

## 119 Serotyping of *V. parahaemolyticus*

120 In the current study, serological analysis of the O and K antigens were performed on all  
121 four *trh*-positive strains and eight randomly selected *trh*-negative isolates of *V.*  
122 *parahaemolyticus* representative of the different samples analysed (fish, mussel and  
123 seawater). Six different *V. parahaemolyticus* serotypes were identified. All the four *trh*+  
124 strains belonged to a single serotype (O1:K1) (Table 2), whereas among the remaining  
125 strains three were O4:K untypeable (KUT), two O2:KUT, and the other three were  
126 O3:K30, O3:KUT and O11:KUT, respectively.

127 Serotypes of *V. parahaemolyticus* isolates from clinical sources typically show  
128 some level of consistency at regional level, such as the case of the pandemic serogroup  
129 O3:K6 in Asia (Okuda *et al.* 1997) the O4:K11 in Spain (Martinez-Urtaza *et al.* 2004),  
130 or the O4:K12 in the Pacific Northwest region of the USA (Turner *et al.* 2013).  
131 However, *V. parahaemolyticus* strains isolated from the environment usually show a  
132 high level of variability in terms of serotype and genotype (Hernandez-Diaz *et al.*  
133 2015). A similar situation was found in the present study, where all the strains positive  
134 for the virulence marker *trh* and potentially pathogenic belonged to a single serotype,  
135 O1:K1, although they were isolated from different fish species (*Sarda sarda* and  
136 *Merlangius merlangus*). *V. parahaemolyticus* serotype O1:K1 has been reported in  
137 clinical and environmental samples in different countries such as China (Li *et al.* 2014),  
138 Japan (Obata *et al.* 1996) and Calcutta (Chowdhury *et al.* 2000). Our study represents  
139 the first evidence of presence of potentially virulent *V. parahaemolyticus* strains from  
140 seafood in Middle Black Sea Region of Turkey and although no cases of *V.*  
141 *parahaemolyticus* have been reported in Turkey or in neighbouring regions so far. The  
142 identification of *trh*+ populations in environmental samples may represent a risk for

143 public health in the future, in particular under scenarios of warming in coastal areas  
144 providing more suitable conditions for the presence and abundance of this organisms  
145 (Baker-Austin *et al.* 2013).

146 *V. parahaemolyticus* strains isolated from environmental sources and foods  
147 rarely carry the *tdh* and *trh* genes, or both, and strains bearing *tdh* or *trh* genes usually  
148 represent less than 3% of all *V. parahaemolyticus* strains isolated from the environment  
149 in many studies (DePaola *et al.* 2000). Unlike clinical strains, environmental  
150 populations of *V. parahaemolyticus* typically show a high level of heterogeneity both at  
151 serotype and genetic levels (Ellingsen *et al.* 2008). Similar high serodiversity was  
152 observed in the present study among the isolates lacking the virulence markers, with the  
153 presence of five serotypes among the eight isolates characterised.

154

#### 155 **PFGE typing of *V. parahaemolyticus* strains**

156 PFGE analysis has been successfully applied for the routine subtyping of many  
157 pathogenic bacteria in an epidemiological context (CDC 2013). Other molecular typing  
158 techniques, such as DNA fingerprinting, REP-PCR, ribotyping and multi-locus  
159 sequence typing, have been equally used for assessing the diversity of strains belonging  
160 to different virulotypes (Paydar *et al.* 2013). PFGE typing has finally become used as  
161 the standard method, due to the high levels of sensitivity, specificity and  
162 reproducibility of this technique (CDC 2013).

163 In the present study, all the four *trh*<sup>+</sup>/*tdh*- and eight *trh*-/*tdh*- strains were  
164 subjected to PFGE analysis. Genomic DNA of *V. parahaemolyticus* was digested with  
165 the restriction enzymes *NotI*. Three isolates obtained from mussel showed poorly



166 resolved PFGE patterns and were omitted for further analysis. PFGE analysis of the  
167 remaining nine strains identified six different PFGE profiles (Figure 2).

168 All the four *trh+/tdh-* strains isolated from fish samples showed an  
169 undistinguishable profile and were grouped in a single PFGE type (A) (Figure 2). Three  
170 of four *trh+/tdh-* strains indistinguishable by PFGE were obtained from three samples of  
171 fish (*Sarda sarda*) collected from the same place, whereas the additional *trh+/tdh-* strain,  
172 isolated from a different fish species (*Merlangius merlangus*) and collected two years  
173 later, showed an identical serotype and indistinguishable PFGE profile. This finding  
174 represents a preliminary evidence of the existence of a dominant genetic variant  
175 prevailing among potentially pathogenic variants of *V. parahaemolyticus* in  
176 environment sources in this region of Turkey. A parallel situation was found in other  
177 studies carried out in different areas of the world, where isolates with virulence traits  
178 have been shown high levels of homogeneity (Rodriguez-Castro *et al.* 2010).

179 The other six *trh-/tdh-* isolates obtained from seawater and mussel showed  
180 unrelated profiles. The high degree of diversity has been typically reported for *trh-/tdh-*  
181 isolates all around the world as found in the present study. This pattern of heterogeneity  
182 among non-pathogenic populations of *V. parahaemolyticus* has been reported in strains  
183 obtained from imported crab, crawfish, snail, shrimp and fish originating from several  
184 different geographic regions in Asia (Wong *et al.* 1996). Tsai *et al.* (2013) also  
185 identified strains genetically more variable in the environment isolates (water, sediment,  
186 oyster and clam) than those of clinical isolates (Center for Disease Control, Taiwan).

187

## 188 **Material and methods**

### 189 **Isolation and identification of *V. parahaemolyticus***

190 A total of 114 seafood and seawater samples consisted of fish ( $n=50$ ), mussel ( $n=42$ )  
191 and seawater samples ( $n=22$ ) were collected from Samsun region ( $41.2903^{\circ}$  N,  
192  $36.3336^{\circ}$  E) at the Middle Black Sea coast of Turkey between 2006 and 2010. Fish  
193 samples included: *Engraulis encrasicolus* ( $n=14$ ), *Merlangius merlangus* ( $n=12$ ),  
194 *Trachurus trachurus* ( $n=12$ ) and *Sarda sarda* ( $n=12$ ) which were obtained from  
195 different station, mussel (*Mytilus galloprovincialis*) samples (each consisting of ten  
196 mussels) were collected from rock by divers.

197 The isolation and identification of *V. parahaemolyticus* was performed as  
198 recommended by International Organization for Standardization 8914 (ISO 1990).  
199 Briefly, 25 g of samples were homogenized in 225 ml<sup>-1</sup> alkaline peptone water (APW)  
200 (2% NaCl, pH 8.6) (Merck, Germany) and incubated at 37°C for 6–8 h. A loopful of  
201 enriched broth culture was streaked on the surface of  
202 CHROMagar™ *Vibrio* (CHROMagar, Paris, France) and plates were incubated at 37°C  
203 for 24 h. At least three typical mauve colonies were picked from  
204 CHROMagar™ *Vibrio*. Additional biochemical identification tests were carried out  
205 according to FDA (1998). The salinity of the seawater samples were determined by  
206 titrimetry according to AOAC (2000), and pH was measured by using a digital pH-  
207 meter (Inolab-pH730, Weilheim, Germany). Seawater temperature values during the  
208 sample collection periods were obtained from Turkish State Meteorological Service.

209

#### 210 **Conventional PCR assay for the detection of *toxR*, *tdh* and *trh* genes**

211 Total DNA was extracted from bacterial cultures in Luria - Bertani (LB) broth with 2%  
212 NaCl (Sambrook *et al.* 1989). Presumptive identification of the isolates was performed  
213 using the *V. parahaemolyticus* species-specific genes *toxR* primers according to Kim *et*

214 *al.* (1999). Additionally, the presence of virulence related genes *tdh* and *trh* were  
215 investigated by PCR protocols, as previously described by Tada *et al.* (1992). The PCR  
216 reactions were carried out in PCR System 2720 thermal cycler (Applied Biosystems,  
217 Foster City, California, USA). PCR products were separated by electrophoresis on 1.5%  
218 agarose gel in TBE (Sigma) and stained with ethidium bromide at 0.5  $\mu\text{g}/\text{ml}^{-1}$  (Sigma).  
219 PCR products were visualized under UV illumination (Alpha Innotech 2200 UV  
220 transilluminator, San Leandro, California). The *toxR*, *tdh* and *trh* genes were visualized  
221 at 368, 251 and 250 bp, respectively. The reference strains AQ4037 (*trh+*, *tdh-*) and  
222 ATCC43996 (*tdh+*, *trh-*) were used as positive controls in PCR assays.

223

#### 224 **Real time PCR assay for the detection of *tdh* and *tlh* genes**

225 Detection of *tlh* and *tdh* genes of *V. parahaemolyticus* was confirmed by real time PCR  
226 according the procedure described by Nordstrom *et al.* (2007). Real time PCR  
227 amplification was performed in a volume of 25  $\mu\text{l}$  containing 1X FastStart PCR master  
228 mix (Roche, Indianapolis), 0.075  $\mu\text{mol l}^{-1}$  each for *tlh* and *ttr* Internal Amplification  
229 Control IAC primers (Integrated DNA Technologies), 0.25  $\mu\text{mol l}^{-1}$  *tdh* primer, 0.3  $\mu$   
230  $\text{mol l}^{-1}$  IAC, 0.15  $\mu\text{mol l}^{-1}$  TaqMan *tlh* probe, 0.075  $\mu\text{mol l}^{-1}$  each for TaqMan *tdh* and  
231 TaqMan IAC probes (VIC) and 2.5  $\mu\text{l}$  template DNA.

232 Real time PCR thermal cycling was performed using the AB Applied  
233 Biosystems 7300 Real Time PCR System (California, USA). The optimal cycling  
234 conditions consisted of a 95°C initial hold for 10 min to denature the DNA and followed  
235 by 40 cycles of amplification, with each amplification cycle consisting of denaturation  
236 at 95°C for 5 s followed by a combined primer annealing/extension step at 59 °C for 45  
237 s.

238 **Serotyping and PFGE typing of *V. parahaemolyticus* strains**

239 All the *trh*-positive strains and a selection of *trh* and *tdh*-negative strains were selected  
240 and subjected to characterization by serotyping and PFGE analysis. All the strains were  
241 serotyped using antisera (Denka; Seiken Corp., Tokyo, Japan) in terms of  
242 lipopolysaccharide (O) and capsular (K) serotypes as previously described Suthienkul *et*  
243 *al.* (1995).

244 Analysis of PFGE was conducted according to the PulseNet USA protocol with  
245 minor modifications (Parson *et al.* 2007; Martinez-Urtaza *et al.* 2004). *V.*  
246 *parahaemolyticus* was digested with the restriction enzyme *NotI*. A *Salmonella* serotype  
247 Braenderup strain (H9812) was chosen as the universal size standard and restricted with  
248 *XbaI*. PFGE was performed on CHEF DRIII system by using the following conditions:  
249 running conditions of 6.0 V/cm for 18 h at 14°C, a pulse times of 2 to 40 s. After the  
250 electrophoresis, the gels were stained with ethidium bromide (Sigma, St. Louis, MO)  
251 and destained in distilled water.

252

253 **Acknowledgements**

254 The authors would like to thank Veronica Blanco Abad, Ronnie Gavilan and Alba  
255 Rodriguez Castro (Instituto de Acuicultura, Universidad de Santiago de Compostela,  
256 Spain) for the support with the characterization of the strains.

257

258 **Conflict of Interest**

259 There is no conflict of interest.

260

261

262

263 **References**

- 264 AOAC (2000) Official Methods of Analysis 937.09. Salt (Chlorine as Sodium Chloride)  
265 in Seafood, 17th edn. Arlington, VA: Association of Official Analytical Chemists.
- 266 Baker-Austin, C., Trinanés, J.A., Taylor, N.G.H., Hartnell, R., Siitonen A. and  
267 Martínez-Urtaza, J. (2013) Emerging *Vibrio* risk at high latitudes in response to ocean  
268 warming. *Nat Clim Change* **3**, 73–77.
- 269 Basra, A.S. (2006) Handbook of Seed Science and Technology. p.660, Food Products  
270 Pres, Binghamton, NY.
- 271 Cabrera-García, M.E., Vázquez-Salinas, C. and Quinones-Ramírez, E.I. (2004)  
272 Serologic and molecular characterization of *Vibrio parahaemolyticus* strains  
273 isolated from seawater and fish products of the gulf of Mexico. *Appl Environ*  
274 *Microbiol* **70**, 6401–6406.
- 275 Cao, H. and Shock, J.M. (2012) Comparison of TaqMan and SYBR Green qPCR  
276 Methods for Quantitative Gene Expression in Tung Tree Tissues. *J Agric Food*  
277 *Chem* **60**, 12296–12303.
- 278 CDC (2013) Pulsed-field Gel Electrophoresis (PFGE). Accessible at  
279 <http://www.cdc.gov/pulsenet/pathogens/pfge.html> Accessed 30 January 2016
- 280 Chowdhury, N.R., Chakraborty, S., Ramamurthy, T., Nishibuchi, M., Yamasaki, S.,  
281 Takeda, Y. and Nair, G.B. (2000) Molecular evidence of clonal *Vibrio*  
282 *parahaemolyticus* pandemic strains. *Emerg Infect Dis* **6**, 631–636.
- 283 Depaola, A., Hopkins, L.H., Peeler, J.T., Wentz, B. and McPhearson, R.M.  
284 (1990) Incidence of *Vibrio parahaemolyticus* in U.S. coastal waters and  
285 oysters. *Appl Environ Microbiol* **56**, 2299–2302.
- 286 Depaola, A., Kaysner, C.A., Bowers, J. and Cook, D.W. (2000) Environmental  
287 investigations of *Vibrio parahaemolyticus* in oysters after outbreaks in  
288 Washington, Texas, and New York (1997 and 1998). *Appl Environ Microbiol* **66**,  
289 4649–4654.
- 290 Di Pinto, A., Ciccacese, G., De Corato, R., Novello, L. and Terio, V. (2008) Detection  
291 of pathogenic *Vibrio parahaemolyticus* in southern Italian shellfish. *Food Control*  
292 **19**, 1037–1041.
- 293 Ellingsen, A.B., Jørgensen, H., Wagley, S., Monshaugen, M. and Rørvik, L.M. (2008)  
294 Genetic diversity among Norwegian *Vibrio parahaemolyticus*. *J Appl Microbiol*  
295 **105**, 2195–2202.
- 296 FDA (1998) *Bacteriological Analytical Manual*, 8th edn. Revision A, AOAC  
297 International, USA: Food and Drug Administration.
- 298 Haley, B.J., Kokashvili, T., Tskshvediani, A., Janelidze, N., Mitaishvili, N., Grim,  
299 J.C., Constantin De Magny, G., Chen, A.J., Taviani, E., Eliashvili, T.,  
300 Tediashvili, M., Whitehouse, C.A., Colwell, R.R. and Huq, A. (2014) Molecular  
301 diversity and predictability of *Vibrio parahaemolyticus* along the Georgian coastal  
302 zone of the Black Sea. *Front Microbiol* **5**, 1–9.
- 303 Hernández-Díaz, L.D.J., Leon-Sicairos, N., Velázquez-Roman, J., Flores-Villaseñor,  
304 H, Guadron-Llanos, A.M., Martínez-García, J.J., Vidal, J.E. and Canizalez-  
305 Roman, A (2015) A pandemic *Vibrio parahaemolyticus* O3:K6 clone causing  
306 most associated diarrhea cases in the Pacific Northwest coast of Mexico. *Front*  
307 *Microbiol* **24**, 221.
- 308 Honda, T., Ni, Y.X. and Miwatani, T. (1988) Purification and characterization of a  
309 hemolysin produced by a clinical isolate of Kanagawa phenomenon-negative

310 *Vibrio parahaemolyticus* and related to the thermostable direct hemolysin. *Infect*  
311 *Immun* **56**, 961–965.

312 ISO (1990) International Organization for Standardization, 8914. Microbiology-General  
313 Guidance on Methods for the Detection of *Vibrio parahaemolyticus*. Geneva,  
314 Switzerland: ISO.

315 Kim, Y.B., Okuda, J., Matsumoto, C., Takahashi, N., Hashimoto, S. and Nishibuchi,  
316 M. (1999) Identification of *Vibrio parahaemolyticus* strains at the species level by  
317 PCR targeted to the *toxR* gene. *J Clin Microbiol* **37**, 1173–1177.

318 Li, Y., Xie, X., Shi, X., Lin, Y. Qiu, Y., Mou, J., Chen, Q., Lu, Y., Zhou, L., Jiang, M.,  
319 Sun, H., Ma, H., Cheng, J., and Hu, Q (2014) *Vibrio parahaemolyticus*, Southern  
320 Coastal Region of China, 2007–2012. *Emerg Infec Dis* **20**(4), 685-688.

321 Libinzon, A.E., Brudnyi, R.A., Nagornaia, A.F., Demina, A.I. and Krasnova, N.V.  
322 (1981) Halophilic Black Sea vibrios and their role in human pathology. *Zh*  
323 *Mikrobiol Epidemiol Immunobiol* **2**, 97–101.

324 Martinez-Urtaza, J., Lozano-Leon, A., Depaola, A., Ishibashi, M., Shimada, K.,  
325 Nishibuchi, M. and Liebana, E. (2004) Characterization of pathogenic *Vibrio*  
326 *parahaemolyticus* isolates from clinical sources in Spain and comparison with  
327 Asian and North American pandemic isolates. *J Clin Microbiol* **42**, 4672–4678.

328 Martinez-Urtaza, J., Lozano-Leon, A., Varela-Pet, J., Trinanes, J., Pazos, Y. and  
329 Garcia-Martin, O. (2008) Environmental determinants of the occurrence and  
330 distribution of *Vibrio parahaemolyticus* in the Rias of Galicia, Spain. *Appl*  
331 *Environ Microbiol* **74**, 265–274.

332 Miyamoto, Y., Kato, T., Obara, Y., Akiyama, S., Takizawa, K. and Yamai, S. (1969) In  
333 vitro hemolytic characteristic of *Vibrio parahaemolyticus*: its close correlation  
334 with human pathogenicity. *J Bacteriol* **100**, 1147–1149.

335 Nishibuchi, M. and Kaper, J.B. (1995) Thermostable Direct hemolysin gene of *Vibrio*  
336 *parahaemolyticus*: A virulence gene acquired by a marine bacterium. *Infect*  
337 *Immun* **63**, 2093–2099.

338 Nordstrom, J.L., Vickery, M.C., Blackstone, G.M., Murray, S.L. and Depaola, A.  
339 (2007) Development of a multiplex real-time PCR assay with an internal  
340 amplification control for the detection of total and pathogenic *Vibrio*  
341 *parahaemolyticus* bacteria in oysters. *Appl Environ Microbiol* **73**, 5840–5847.

342 Obata, H., Kai, A., Sekiguchi, K., Matsushita, S., Yamada, S., Itoh, T., Ohta, K.  
343 and Kudoh, Y. (1996) Detection of the *trh* gene in *Vibrio parahaemolyticus*  
344 isolated from overseas travellers' diarrhea and their biochemical characteristics.  
345 *Kansenshogaku Zasshi* **70**, 815–820.

346 Okuda, J., Ishibashi, M., Hayakawa, E., Nishino, T., Takeda, Y., Mukhopadhyay, A.K.,  
347 Garg, S., Bhattacharya, S.K., Nair, G.B. and Nishibuchi M. (1997) Calcutta, India,  
348 and isolation of strains from the same clonal group from Southeast Asian travelers  
349 arriving in Japan. *J. Clin. Microbiol* **35**, 3150–3155.

350 Parsons, M.B., Cooper, K.L., Kubota, K.A., Pühr, N., Simington, S., Calimlim, P.S.,  
351 Schoonmaker-Bopp, D., Bopp, C., Swaminathan, B., Gerner-Smidt, P. and Ribot,  
352 E.M. (2007) PulseNet USA standardized pulsed-field gel electrophoresis protocol  
353 for subtyping of *Vibrio parahaemolyticus*. *Foodborne Pathog Dis* **4**, 285–292.

354 Paydar, M., Teh, C.S.J. and Thong, K.L. (2013) Prevalence and characterisation of  
355 potentially virulent *Vibrio parahaemolyticus* in seafood in Malaysia using  
356 conventional methods, PCR and REP-PCR. *Food Control* **32**, 13–18.

357 Rodriguez-Castro, A., Ansede-Bermejo, J., Blanco-Abad, V., Varela-Pet, J., Garcia-  
358 Martin, O. and Martinez-Urtaza, J. (2010). Prevalence and genetic diversity of  
359 pathogenic populations of *Vibrio parahaemolyticus* in coastal waters of Galicia,  
360 Spain. *Environ Microbiol Rep* **2**, 58–66.

361 Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory*  
362 *Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory  
363 Press, New York.

364 Suthienkul, O., Ishibashi, M., Iida, T., Nettip, N. and Supavej, S., Eampokalap, B.,  
365 Makino, M. and Honda, T. (1995) Urease production correlates with possession of  
366 the *trh* gene in *Vibrio parahaemolyticus* strains isolated in Thailand. *J Infect Dis*  
367 **172**, 1405–1408.

368 Tada, J., Ohashi, T., Nishimura, N., Shirasaki, Y., Ozaki, H., Fukushima, S., Takano, J.,  
369 Nishibuchi, M. and Takeda, Y. (1992) Detection of the thermostable direct  
370 hemolysin gene (*tdh*) and the thermostable direct hemolysin-related hemolysin  
371 gene (*trh*) of *Vibrio parahaemolyticus* by polymerase chain reaction. *Mol Cell*  
372 *Probe* **6**, 477–487.

373 Terzi, G., Buyuktanir, O. and Yurdusev, N. (2009) Detection of the *tdh* and *trh* genes in  
374 *Vibrio parahaemolyticus* isolates in fish and mussels from Middle Black Sea  
375 Coast of Turkey. *Lett Appl Microbiol* **49**, 757–763.

376 Tsai, S.E., Jong, K.J., Tey, Y.H., Yu, W.T., Chiou, C.S., Lee, Y.S. and Wong, H.C.  
377 (2013) Molecular characterization of clinical and environmental *Vibrio*  
378 *parahaemolyticus* isolates in Taiwan. *Int J Food Microbiol* **165**, 18–26.

379 Turkish Statistical Institute (2015) Seafood Statistics. Ankara, Turkey. Accessible at  
380 [http://www.tuik.gov.tr/PreTablo.do?alt\\_id=1005](http://www.tuik.gov.tr/PreTablo.do?alt_id=1005) Accessed 27 October 2015.

381 Turner, J.W., Paranjpye, R.N., Landis, E.D., Biryukov, S.V., González-Escalona, N.,  
382 Nilsson, W.B. and Strom, M.S. (2013) Population structure of clinical and  
383 environmental *Vibrio parahaemolyticus* from the Pacific Northwest coast of the  
384 United States. *PLoS One* **8**, e55726.

385 Twedt, R.M. (1989) *V. parahaemolyticus*. In: *Foodborne Bacterial Pathogens* (Doyle,  
386 M.P., ed.) pp. 543–562, Marcel Dekker, New York.

387 Wong, H.C., Lu, K.T., Pan, T.M., Lee, C.L. and Chi Shih, D.Y. (1996) Subspecies  
388 typing of *Vibrio parahaemolyticus* by Pulsed-Field Gel Electrophoresis. *J Clin*  
389 *Microbiol* **34**, 1535–1539.

390 Wong, M.L. and Medrano, J.F. (2005) Real-time PCR for mRNA quantitation.  
391 *BioTechniques* **39**, 75–85.

392  
393  
394  
395  
396  
397  
398  
399  
400  
401  
402  
403

404 **Table 1** Occurrence and distribution of *toxR*, *tlh*, *tdh* and *trh* genes among *Vibrio*  
 405 *parahaemolyticus* strains

Sample	Date	Seawater Temperature	Sample	No. of presumptive <i>V. parahaemolyticus</i> detected by conventional methods	No. of presence of gene <i>V. parahaemolyticus</i> by molecular methods				
					Strains	Conventional PCR			Real time PCR
					<i>toxR</i>	<i>tdh</i>	<i>trh</i>	<i>tlh</i>	<i>tdh</i>
Mussel (n=42)	May, 2010 June, 2010	13.2 °C 18.9 °C	32 (76%)	83	72	-	-	72	-
Sea water (n=22)	June, 2009	20.5 °C	9 (40.9%)	16	16	-	-	16	-
Fish (n=50)	October, 2006 April, 2008	20.7 °C 10.6 °C	4 (8%)	8	8	-	4	8	-

406  
407  
408  
409  
410

**Table 2** Some of *V. parahaemolyticus* strains of different serotypes and PFGE pattern

Date	Source	Serotype	Genotype			PFGE	(Figure 2)
			<i>toxR</i>	<i>tdh</i>	<i>trh</i>		
October 2006	Fish ( <i>Sarda sarda</i> )	01:K1	+	-	+	A	Line 2
October 2006	Fish ( <i>Sarda sarda</i> )	01:K1	+	-	+	A	Line 3
October 2006	Fish ( <i>Sarda sarda</i> )	01:K1	+	-	+	A	Line 4
April 2008	Fish ( <i>Merlangius merlangus</i> )	01:K1	+	-	+	A	Line 5
1 June 2009	Sea water	O11:KUT	+	-	-	B	Line 6
6 June 2009	Sea water	O3: KUT	+	-	-	C	Line 7
1 June 2009	Sea water	O3:K30	+	-	-	D	Line 9
1 June 2009	Sea water	O4:KUT	+	-	-	E	Line 10
7 May 2010	Mussel	O2:KUT	+	-	-	F	Line 11
19 May 2010	Mussel	O4: KUT	+	-	-	G	data not shown
22 May 2010	Mussel	O2:KUT	+	-	-	H	data not shown
9 June 2010	Mussel	O4: KUT	+	-	-	I	data not shown

411  
412  
  
413  
  
414  
  
415



416 **Figure Legends**

417 **Figure 1** Representative results of PCR products analysed by agarose gel  
418 electrophoresis for *toxR* and *trh* gene (+) isolates.

419 (a): Line M: 100-bp DNA ladder; lanes 1-8: *toxR* gene (+) seafood isolates; line 9:  
420 positive control (ATCC 43996 strain), line 10: negative control. (b): line M: 100-bp  
421 DNA ladder; line 1-4: *trh* gene (+) seafood isolates, line 5-8: *trh* gene (-) isolates, line 9:  
422 positive control (*Vibrio parahaemolyticus* AQ4037), line 10: negative control

423

424 **Figure 2** PFGE patterns of *NotI*-digested DNA from the selected *V. parahaemolyticus*  
425 isolates examined in this study

426 Line 1: *Salmonella* Braenderup H9812 strain; lines 2-3-4: *trh*<sup>+</sup> strains isolated from  
427 fish (*Sarda sarda*); line 5: *trh*<sup>+</sup> strain isolated from fish (*Merlangius merlangus*); line  
428 6,7: seawater isolates; line 8: *Salmonella* Braenderup H9812 strain; line 9,10: seawater  
429 isolates; line 11: mussel isolates.

430

431

432

433

434

435

436

437