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

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Running head: Molecular characterizations of *Vibrio parahaemolyticus*

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

Abstract

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

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

Keywords

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

Introduction

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

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

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

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

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

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

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

Serotyping of *V. parahaemolyticus*

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

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

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

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

PFGE typing of *V. parahaemolyticus* strains

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

In the present study, all the four *trh*+/*tdh*-and eight *trh*-/*tdh*- strains were subjected to PFGE analysis. Genomic DNA of *V. parahaemolyticus* was digested with 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***

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

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

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

Total DNA was extracted from bacterial cultures in Luria - Bertani (LB) broth with 2% NaCl (Sambrook *et al.* 1989). Presumptive identification of the isolates was performed 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 μg/ml^{-1} (Sigma).
219 PCR products were visualized under UV illumination (Alpha Innotech 2200 UV
220 transilluminator, San Leandro, California). The *tox*R, *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 μl containing 1X FastStart PCR master
228 mix (Roche, Indianapolis), 0.075 μmol l^{-1} each for *tlh and ttr* Internal Amplification
229 Control IAC primers (Integrated DNA Technologies), 0.25 μmol l^{-1} *tdh* primer, 0.3 μ
230 mol l^{-1} IAC, 0.15 μmol l^{-1} TaqMan *tlh* probe, 0.075 μmol l^{-1} each for TaqMan *tdh* and
231 TaqMan IAC probes (VIC) and 2.5 μl templete 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.

Serotyping and PFGE typing of *V. parahaemolyticus* strains

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

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

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Conflict of Interest

There is no conflict of interest.

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

Sample	Date	Seawater Temparature	No. of presumptive *V. parahaemolyticus* detected by conventional methods	No. of presence of gene *V.parahaemolyticus* by moleculer methods					
			Sample	Strains	Conventional PCR			Real time PCR	
					toxR	*tdh*	*trh*	*tlh*	*tdh*
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	-

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

Date	Source	Serotype	Genotype			PFGE	(Figure 2)
			toxR	*tdh*	*trh*		
October 2006	Fish (*Sarda sarda*)	01:K1	+	-	+	A	Line 2
October 2006	Fish (*Sarda sarda*)	01:K1	+	-	+	A	Line 3
October 2006	Fish (*Sarda sarda*)	01:K1	+	-	+	A	Line 4
April 2008	Fish (*Merlangius merlangus*)	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

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Figure Legends

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

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

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

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