Isolation and molecular characterization of toxigenic *Vibrio parahaemolyticus* from the coastal water in the Eastern Province of Saudi Arabia

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Background. *Vibrio parahaemolyticus* is recognized globally as a cause of foodborne gastroenteritis and its widely disseminated in marine and coastal environment throughout the world. The main aim of this study was conducted to investigate the presence of toxigenic *V. parahaemolyticus* in costal water in the Eastern Province of Saudi Arabia by using immunomagnetic separation (IMS) in combination with chromogenic Vibrio agar medium and PCR targeting *toxR* gene of species level and virulence genes. **Methods.** A total of 192 seawater samples were collected from 5 locations and enriched in alkaline peptone water (APW) broth. One-milliliter portion from enriched samples in APW were mixed with an immunomagnetic beads (IMB) coated with specific antibodies against *V. parahaemolyticus* polyvalent K antisera and separated beads with captured bacteria streaked on thiosulfate citrate bile salts sucrose (TCBS) agar and CHROMagar Vibrio (CaV) medium. **Results.** Of the 192 examined seawater samples, 19.8% and 22.9% were positive for *V. parahaemolyticus*, producing green and mauve colonies on TCBS agar and CaV medium, respectively. Among 120 isolates of *V. parahaemolyticus* isolated in this study, three (2.5%) and 26 (21.7%) isolates of *V. parahaemolyticus* isolated without and with IMB treatment tested positive for the toxin regulatory (*tox*R) gene, respectively. Screening of the confirmed *tox*R gene-positive isolates revealed that 17.5% and 2.5% were positive for the thermostable direct hemolysin (*tdh*) encoding gene in strains isolated with IMB and without IMB treatment, respectively. None of the *V. parahaemolyticus* strains tested positive for the thermostable related hemolysin (*trh*) gene. In this study, we found that the CaV medium has no advantage over TCBS agar if IMB concentration treatment is used during secondary enrichment steps of environmental samples. The enterobacterial repetitive intergenic consensus (ERIC)-PCR DNA fingerprinting analysis revealed high genomic diversity, and 18 strains of *V. parahaemolyticus* were grouped and identified into four identical ERIC clonal group patterns. **Conclusions.** The presented study reports the

first detection of *tdh* producing *V. parahaemolyticus* in coastal water in the Eastern Province of Saudi Arabia.

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Abstract

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Methods. A total of 192 seawater samples were collected from 5 locations and enriched in alkaline peptone water (APW) broth. One-millimeter portion from enriched samples in APW were mixed with an immunomagnetic beads **(**IMB) coated with specific antibodies against *V. parahaemolyticus* polyvalent K antisera and separated beads with captured bacteria streaked on thiosulfate citrate bile salts sucrose (TCBS) agar and CHROMagar Vibrio (CaV) medium.

Results. Of the 192 examined seawater samples, 19.8% and 22.9% were positive for *V. parahaemolyticus*, producing green and mauve colonies on TCBS agar and CaV medium, respectively. Among 120 isolates of *V. parahaemolyticus* isolated in this study, three (2.5%) and 26 (21.7%) isolates of *V. parahaemolyticus* isolated without and with IMB treatment tested positive for the toxin regulatory (*tox*R) gene, respectively. Screening of the confirmed *tox*R gene-positive isolates revealed that 17.5% and 2.5% were positive for the thermostable direct hemolysin (*tdh*) encoding gene in strains isolated with IMB and without IMB treatment, respectively. None of the *V. parahaemolyticus* strains tested positive for the thermostable related hemolysin (*trh*) gene. In this study, we found that the CaV medium has no advantage over TCBS agar if IMB concentration treatment is used during secondary enrichment steps of environmental samples. The enterobacterial repetitive intergenic consensus (ERIC)-PCR DNA fingerprinting analysis revealed high genomic diversity, and 18 strains of *V. parahaemolyticus* were grouped and identified into four identical ERIC clonal group patterns.

Conclusions. The presented study reports the first detection of *tdh* producing *V. parahaemolyticus* in coastal water in the Eastern Province of Saudi Arabia.

Keywords: coastal environment; immunomagnetic beads; PCR; *Vibrio parahaemolyticus*

Introduction

Vibrio parahaemolyticus is a halophilic bacterium that is abundant in marine and estuarine environments (*Kalburge et al., 2014*). The highest abundance of *V. parahaemolyticus* is in sediment and benthic environments (*Böer et al., 2013*). *V. parahaemolyticus* is also present in various types of marine seafood and organisms, such as shrimps, mollusks, oysters, fish, crabs, lobsters, mussels, and zooplankton (*DePaola et al., 2003; Su and Liu, 2007; Julie et al., 2010; Letchumanan et al., 2014*). However, the growth of *V. parahaemolyticus* has an absolute salt requirement for survival and is capable of growth at 1 to 9% NaCl (*Whitaker et al., 2010; Kalburge et al., 2014*). The presence of *V. parahaemolyticus* in the environment widely varies according to differences in geographical locations and environmental factors, such as temperature and salinity (*Parveen et al., 2008; Johnson et al., 2012*). Several studies have reported an association between the isolation of *V. parahaemolyticus* and a higher temperature of seawaters (*Blackwell and Oliver, 2008*). In the Chesapeake Bay, USA, the detection of *V. parahaemolyticus* was rare and difficult until the temperature reached 19℃ or above (Kaneko and Colwell, 1973). Also, during the winter season, *V. parahaemolyticus* will survive in the sediment; it usually appears in the water column at the end of spring or the beginning of the summer season (Julie et al., 2010). On the other hand, the salinity of the seawater affects the presence or absence of *V. parahaemolyticus* in the environment (*Johnson et al., 2010*).

The most pathogenic virulence factors of *V. parahaemolyticus* are thermostable direct hemolysin (TDH), and TDH-related hemolysin (TRH) (*Tada et al., 1992*), but the underlying mechanism of these proteins in human infection remains unknown (*Broberg et al., 2011; Ceccarelli et al., 2013*). *V. parahaemolyticus* can cause wide-scale infection transmitted through the consumption of raw or undercooked contaminated seafood, usually during the warmer months (*Baker-Austin et al., 2017*). The infection takes between 4 to 24 h; then, the symptoms begin to appear and self-resolve within 48 to 72 h. However, three significant medical conditions can be caused by *V. parahaem*olyticus: acute gastroenteritis, wound infection, and septicemia (*Nair et al., 2007*). Acute gastroenteritis appears with abdominal pain, diarrhea, vomiting, nausea, and headache with fever, as well as sometimes with bloody diarrhea (*Li et al., 2016*). Wound infection is commonly detected in fishermen with a small wound occurring at the time of fishing in contaminated seawater; usually, the infected person will suffer from cellulitis, though in some cases, the infection will progress to severe necrotizing fasciitis (*Hlady and Klontz,*

1996). Very few cases of *V. parahaemolyticus* lead to septicemia, which might be fatal to a person with an underlying medical problem, including immunocompromised patients, such as those with cancer or liver diseases (*Jia et al., 2016*).

V. parahaemolyticus is commonly isolated from Asian regions because of the nature of the food consumed in these countries. Therefore, outbreaks start in various countries in Asia, such as Japan, India, and China (*Hara-Kudo et al., 2001; Yonekita et al., 2020)*. The main cause of outbreaks in Asian regions has been reported as the consumption of contaminated seafood (*Jacxsens et al., 2009; Yonekita et al., 2020*). Globally and compared with other foodborne illnesses, *V. parahaemolyticus* infections have been increasing and have become the leading cause of seafood bacterial infections (*Martinez-Urtaza et al., 2010; Abanto et al., 2020*). The US Centers for Disease Control and Prevention (CDC) estimated that the average annual incidence of all *Vibrio* infections increased by 54% during 2006–2017 (*Marder Mph et al., 2018*), and *V. parahaemolyticus* was responsible for the highest number of infections (*Newton et al., 2014*). In the United States, *V. parahaemolyticus* is responsible for more than 35,000 human infections per year, and in China, since 1990, *V. parahaemolyticus* has been registered as the leading cause of foodborne infections (*Scallan et al., 2011; Liu et al., 2011*).

In the summer of 2004, in Alaska, 14 passengers were infected on a cruise trip after consuming raw oysters (*McLaughlin et al., 2005*). The largest outbreak of *V. parahaemolyticus* was in the summer of 2012 on a cruise boat in Spain; 100 out of 114 passengers were infected. After a laboratory investigated, they found different genes and reported the first presence of *V. parahaemolyticus* strains carrying both the *tdh*+ and *trh*+ pathogenicity genes (*Martinez-Urtaza et al., 2016*).

Not all *V. parahaemolyticus* strains are pathogenic, only those expressing *tdh* that encodes the *tdh* or *trh* genes (*Tada et al., 1992; Ceccarelli et al., 2013*; *Saito et al., 2015*). Therefore, the objectives of the present study were (i) to isolate *V. parahaemolyticus* from coastal water by using IMBs in samples treatment to concentrate bacteria after the enrichment process; (ii) to confirm all isolates of *V. parahaemolyticus* to species level by using PCR targeted to the *tox*R gene; (iii) to examine all *tox*R gene-positive isolates for the presence of the *tdh* and *trh* genes using PCR; and (iv) to genotype all isolates of *V. parahaemolyticus tox*R gene-positive isolated from different locations along the coast of Eastern Province of Saudi Arabia for relative genetic similarity by using enterobacterial repetitive intergenic consensus (ERIC)-PCR.

MATERIALS AND METHODS

Study design and sample collection

In the present study, a total of 192 sea surface water samples were collected from five different sites along the coast of the Eastern Province of Saudi Arabia between March 2018 and May 2018. All samples were collected in sterile 500 ml screw-cap bottles from: (i) 40 samples from Al aziziyah beach (AZB); (ii) 39 samples from corniche Al-Khobar (KBC); (iii) 38 samples from corniche Al-Khobar front (KBF); (iv) 36 samples from Dammam beach (DMB); and 39 samples from Half-Moon beach (HMF). During sample collection from each location, the temperature and pH of surface seawater were measured using a Multi-Parameter Water Quality Meter (YSI-50 series, Horiba, USA). Seawater samples were transported after collection to the microbiology research laboratory at Imam Abdurrahman bin Faisal University (IAU) and processed immediately to test for the presence of *V. parahaemolyticus.*

Enrichment process

All samples were treated by adding 25 ml of the seawater sample into 225 ml enrichment medium of alkaline peptone water broth (APW) supplemented with 3% NaCl and incubated at 37℃ for 24 h. On the second day, a loop full of each enriched sample was streaked on Thiosulfate citrate bile salts sucrose (TCBS) agar (Oxoid, UK) and CHROM agar (CHROM, France) and incubated at 37℃ for 24 h.

Immunomagnetic beads (IMB) separation of *V. parahaemolyticus*

The concentration of suspected *V. parahaemolyticus* in enriched samples in APW were done using commercially available magnetic beads coated with antibodies against *V. parahaemolyticus* polyvalent K antisera groups I to IX (Denka Seiken, Tokyo, Japan). The magnetic bead was prepared as previously described with modifications (*Tanaka et al., 2014*). Briefly, 1 ml from each enriched sample in APW supplemented with 3% NaCl was inoculated into tryptic soy broth (TSB) with 2% NaCl for second enrichment and incubated at 37℃ for 24 h. Then, 1 ml was transferred from the second enrichment samples into 1.5 ml tube and mixed with 20 µl of IMB specific to *V. parahaemolyticus*. All mixed tubes with IMB were gently inverted and incubated for 45 min at room temperature. The magnetic concentrator rack was used to separate the beads with captured bacteria from enriched samples and washed three times with

phosphate buffer saline (PBS). Finally, the bead-aggregated bacterium were resuspended in 50 µl of PBS and spread on TCBS and CaV agar and incubated at 37℃ for 24 h. Finally, the results of isolated *V. parahaemolyticus* with and without IMB treatment could be compared.

DNA template preparation

DNA extraction was done for all selected colonies on TCBS and CaV agar isolated with and without IMB as described elsewhere (*Elhadi et al., 2018*). Briefly, 1 ml of an overnight test culture in Luria Bertani (LB) broth was transferred into a 1.5 ml tube and centrifuged at 10,000 rpm for 2 min, and the supernatant discarded. The obtained pellet was suspended in sterilized distilled water and boiled at 100°C for 15 min. Then the tube was centrifuged at 12,000 rpm for 5 min, and the supernatant was transferred to a new tube and stored at -20°C until use.

Identification of *V. parahaemolyticus* to species level using PCR targeted to the *tox*R gene

The confirmation of *V. parahaemolyticus* to species level was performed using PCR targeted to the *tox*R gene, as described previously (*Kim et al., 1999*) [35]. All isolates of *V. parahaemolyticus* isolated with and without IMB were screened for *tox*R gene amplicon (size 368 bp) using primer sequence, as indicated in Table 1. A positive *V. parahaemolyticus* (ATCC 17802) and negative of *V. alginolyticus* (ATCC 17749) control were included in each PCR run.

Detection of virulence gene markers

All *tox*R-positive isolates of *V. parahaemolyticus* recovered on TCBS and CaV agar with and without IMB were tested for the presence or absence of tdh and trh virulence gene markers following a previously described protocol *(Tada et al., 1992)*. Briefly, the total volume of the reaction was 25 µl, consisting of 12.5 µl of GoTaq Green Master Mix (Promega, USA), 2 µl of DNA template, 8.5 µl nuclease-free water (Promega, USA), and 2 µl of forward and reverse primers (Invitrogen, Japan) (Table 1). The positive controls of *V. parahaemolyticus* ATCC 17802, *V. parahaemolyticus* AQ3815, and *V. parahaemolyticus* AQ4037 were used in each PCR control for the *tox*R, tdh, and trh genes, respectively. Amplification of both the 251 and 250 bp region for the *tdh* and *trh* genes were performed following the described conditions by *Tada et al.* (1992): 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. Finally, 10 µl of

amplified products were separated using electrophoresis in 1.5% agarose gels stained with ethidium bromide in 1X Tris borate EDTA buffer (Promega, USA).

Molecular typing analysis

*V. parahaemolyticus tox*R positive isolates were fingerprinted using enterobacterial repetitive intergenic consensus (ERIC)-PCR as described elsewhere. Briefly, ERIC-PCR was performed using two repetitive primer set sequences, ERIC1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3'), as described previously (*Versalovic et al., 1991*). ERIC-PCR was performed in a volume of 25 µl containing 12.5 µl of GoTaq Green Master Mix (Promega, USA), 3 µl of DNA template, 2 µl of ERIC primer, and 7.5 µl nuclease-free water. The PCR reactions were performed using a Bio-Rad T100 thermocycler (Bio-Rad, USA) as follows: 4 min at 94˚C, followed by 35 cycles of 94˚C for 1 min, 52˚C for 1 min and 65˚C for 1 min, with a final extension at 65˚C for 10 min. The ERC-PCR fingerprint patterns obtained by electrophoresis were analyzed by GelJ software (*Heras et al., 2015*). The dendrogram was constructed with the unweighted average pair group method (UPGMA) with a band position tolerance of 1%.

Results

Physical parameters of water

Seawater temperature values ranged from 25°C to 31°C during the sampling events from February to May 2018 (Table 2). The highest water temperature was documented during April 2018 at Half-Moon beach (HMF), while the lowest value was logged in February at Alaziziyah beach (AZB). The water pH values ranged from 7.35 to 8.46. The highest pH was recorded during April 2018 in Dammam corniche (DMC), while the lowest was recorded during March 2018 in Alkhubar corniche (KBC) (Table 2). The highest number of positive samples for *V. parahemolyticus* was recorded in Half-Moon beach, and the seawater pH and temperature values were 8.22 and 31°C (Table 2).

Immunomagnetic bead (IMB) separation of *V. parahaemolyticus*

In this study, samples were considered positive for *V. parahaemolyticus* based on the preliminary appearance of green and mauve colonies on TCBS and CaV agar isolated with IMB and without

IMB enrichment treatment. Among the examined samples, the highest number of positive samples for *V. parahaemolyticus* was detected in samples enriched with IMB and the lowest in samples enriched without IMB (Table 2). The highest distribution rate of positive samples for *V. parahaemolyticus* isolated with IMB on CaV medium was found at HMF (48.7%) and AZB beach (37.5%) as shown in Table 2.Whereas the highest positive samples rates for *V. parahaemolyticus* isolated with IMB on TCBS agar was 43.7% and 27.5% from HMF and AZB beaches, respectively (Table 1). Among the total of 192 seawater samples that were enriched in alkaline peptone water broth and processed without using IMB in secondary enrichment, only three (1.6%) and five (2.6%) samples were reported positive for *V. parahaemolyticus* on TCBS agar and CHROMagar Vibrio, respectively (Table 2). After enrichment with IMB, the rate number of positive samples was 19.8% and 22.9% for *V. parahaemolyticus* on TCBS and CaV agar from all locations, respectively (Table 2). A total of 48 and 58 isolates of *V. parahaemolyticus* were isolated on TCBS and CaV agar with IMB, respectively (Table 2). Among the five locations, the highest number of *V. parahaemolyticus* isolates were isolated from HMF (Table 2). The abundance of *V. parahaemolyticus* isolates were recovered on TCBS and CaV agar from samples examined from all locations after using IMB in the secondary enrichment process (Table 2). In this, we found that the use of IMB in secondary enrichment of examined seawater samples could successfully recover typical colonies of *V. parahaemolyticus* on CaV and TCBS agar, outperforming when samples were plated on CaV and TCBS agar without the use of IMB in secondary enrichment.

Confirmation of *V. parahaemolyticus* to species level using PCR targeted to the *tox*R gene

To confirm the identification of *V. parahaemolyticus* more accurately to species level and for comparison purposes, a total of 120 isolates of *V. parahaemolyticus* isolated using IMB were subjected to PCR with species-specific primers (Table 1). Among 120 isolates of *V. parahaemolyticus*, three (2.5%) and 26 (21.7%) isolates of *V. parahaemolyticus* isolated without and with IMB were positive for the *tox*R gene as judged by amplification of a 368 bp fragment (Fig. 1).

Detection of virulence gene markers

Among the overall *tox*R gene-positive *V. parahaemolyticus*, 24 (20%) amplified the 251 bp *tdh* fragment and the highest number of tdh positive isolates was detected from HMF (Fig. 2). As presented in Table 3, 21 (17.5%) of the *tdh* gene-positive isolates of *V. parahaemolyticus* were isolated with IMB enrichment, and only three (2.5%) of the *tdh* gene-positive isolates of *V. parahaemolyticus* were isolated without IMB enrichment. None of the *V. parahaemolyticus* isolates tested positive for the *trh* gene (Table 3).

Molecular typing

Among 29 isolates of *tox*R gene-positive *V. parahaemolyticus*, 24 isolates were genotyped using ERIC-PCR DNA fingerprinting analysis and generated high genomic diversity among *V. parahaemolyticus* isolates. The ERIC primer sets produced four to ten fingerprint bands and ranged between 100 to 1200 bp (Fig. 3). Of the 24 genotyped isolates of *V. parahaemolyticus*, 18 isolates were grouped and identified into four ERIC identical clonal group patterns (Cluster 1, 2, 3, and 4), while a similarity cutoff value of 100% was applied and six isolates have shown a single cluster (SC) (Table 4 and Fig. 4). Among the four clusters, cluster-2 was comprised of the highest number of *V. parahaemolyticus* isolates with identical clonal origin isolated from AZB, HMF, and KBC corniche between March and April 2018 (Fig. 4).

Discussion

The results of this study are in agreement with several studies conducted worldwide that have reported the isolation of *V. parahaemolyticus* from the marine environment and surface water during sampling events at temperatures ranging from 10℃ and 39℃. The minimum and maximum pH values for the growth of *V. parahaemolyticus* in the environment were reported to be 4.8 and 11, respectively (*Food and Drug Administration, 2020*). The reported pH in this study are within the optimum range of pH, between 5 and 8.6 (*Whitaker et al., 2010; Mudoh et al., 2014*). Effectual methods for isolation and identification of *V. parahaemolyticus* from clinical, food, and environmental samples are required to speed identification and minimize the risk of infection (*Canizalez-Roman et al., 2011*). Our study was able to confirm the isolation of *V. parahaemolyticus* in all examined seawater samples from all locations along the coast while

using IMB in the secondary enrichment process (Table 2). To achieve these results, two different enrichment in APW broth without and with IMB were spread on CaV and TCBS agar for isolation of *V. parahaemolyticus*. In this study, we found the use of IMB in secondary enrichment increased the number of positive samples for *V. parahaemolyticus* while using both selective media (Table 2). However, the number of positive samples detected with the use of IMB in secondary enrichment on TCBS agar and CaV medium was 38 (19.8%) and 44 (22.9%), respectively (Table 2). The number positive for *V. parahaemolyticus* without using IMB in secondary enrichment was low in TCBS agar, and CaV medium was three (1.6%) and five (2.6%) respectively (Table 2). Therefore, our study disagrees with findings on the unsatisfactory performance of TCBS agar in selecting *V. parahaemolyticus* and other *Vibrio* spp. from environmental samples (*Fabbro et al., 2010; Di Pinto et al., 2011*). In this study, we found that CaV medium has no advantage over TCBS agar if IMB is used in secondary enrichment of environmental samples.

Consequently, our study used PCR amplification targeted to the *toxR* gene, by which the identity of 2.5% and 21.7% isolates of *V. parahaemolyticus* isolated without and with IMB on TCBS agar and CHROMagar Vibrio was confirmed to the species level, respectively (Table 3). Therefore, our study found that the coupling of IMB in secondary enrichment of environmental samples with *toxR* PCR assay is a reliable method for the detection of *V.* parahaemolyticus (*Kim et al., 1999*). Indicators of the potential pathogenicity of *V. parahaemolyticus* is the presence of *tdh* and *trh* genes. Almost all clinically isolated strains of *V. parahaemolyticus* possess hemolytic activity attributed to these two genes (*Ceccarelli et al., 2013*). The effect of TDH on intestinal and epithelial cells is crucial for the biological activities, like diarrhea, during *V. parahaemolyticus* infection (*Shimohata et al., 2010*). Also, *trh* works in an analogous pattern to TDH (*Raghunath, 2014*). To the best of our knowledge, this study represents the first report of the detection of *tdh*-positive *V. parahaemolyticus* strains from the coastal environment in the Eastern Province of Saudi Arabia.

The highest percentage of *tdh* positivity was 17.5% from the total isolates detected among *V. parahemolyticus* isolated with IMB enrichment, while the lowest percentage of *tdh* positive isolates was 2.5% detected in *V. parahaemolyticus* isolated without IMB enrichment (Table 3). The none or low detection of *tdh* positive *V. parahaemolyticus* isolated with major known cultural methods: enrichment in APW broth (without IMB enrichment), plating on selective agar

(TCBS and CHROMagar Vibrio), identification of suspected colonies using biochemical tests and PCR, therefore these results are in agreement with our previous studies (*Elhadi et al., 2018; Ghenem et al., 2018*). The detection of TDH positive strains of *V. parahaemolyticus* in the costal environment of the Eastern Province of Saudi Arabia is a pressing concern that has several impacts and requires instant attention. First, the fact that these strains are potentially toxigenic should prompt the healthcare facilities to monitor all bacterial gastroenteritis in clinical samples for the presence of *V.* parahaemolyticus (*Jun et al., 2012*). Second, incidences where pathogenic *V. parahaemolyticus* was held responsible for contaminating seafood produce and not only causing outbreaks of the infection, but also costing the industry enormous economic losses have been documented (*Fuenzalida et al., 2006; Thongjun et al., 2006; Johnson et al., 2010*). Thus, the results of this study emphasize the continuous monitoring of seafood products' safety. The ERIC-PCR clusters indicate that the isolates could have originated from the same clonal lineage of *V. parahaemolyticus*. These results agreed with our previous study (*Elhadi et al., 2018*) and are consistent with *Marshall et al* (1999), who reported that ERIC-PCR was a useful method for evaluating genetic and epidemiological relationships among *V. parahaemolyticus*.

Conclusions

The study concluded that, both TCBS and CHROMagar Vibrio are suitable selective media for isolation of *V. parahaemolyticus* if IMBs are used in the enrichment process of environmental water samples. Therefore, the use of IMB will separate *V. parahaemolyticus* from Vibrio and other non-Vibrio species in environmental samples and improve the isolation level of *V. parahaemolyticus*. This study also concludes that CHROMagar Vibrio has no advantage over TCBS agar if the enriched sample is treated with IMBs coated with specific polyvalent K antisera antibodies for immuno-concentration of *V. parahaemolyticus*. The isolation of TDH positive *V. parahaemolyticus* in this study identifies a public health risk and indicates there is a possibility of the spreading of this gene in the marine environment. The results of this study confirm *V. parahaemolyticus* can survive and adapt in different environments, including those with high temperatures.

Acknowledgements

The authors would like to thank Emeritus Professor Mitsuaki Nishibuchi of the Center of Southeast Asian Studies, Kyoto University for useful information and support.

References

Abanto M, Gavilan RG, Baker-Austin C, Gonzalez-Escalona N, Martinez-Urtaza J. 2020. Global expansion of Pacific Northwest *Vibrio parahaemolyticus* sequence type 36. *Emerging Infectious Diseases*. **26(2)**:323 DOI 10.3201/eid2602.190362.

Alipour M, Issazadeh K, Soleimani J. 2014. Isolation and identification of *Vibrio parahaemolyticus* from seawater and sediment samples in the southern coast of the Caspian Sea. *Comparative Clinical Pathology* 23(1):129-133 DOI 10.1007/s00580-012-1583-6.

Baker-Austin C, Trinanes J, Gonzalez-Escalona N, Martinez-Urtaza J. 2017. Non-cholera vibrios: the microbial barometer of climate change. *Trends in Microbiology*. **25(1)**:76-84 DOI 10.1016/j.tim.2016.09.008.

Blackwell KD, Oliver JD. 2008. The ecology of *Vibrio vulnificus*, *Vibrio cholerae*, and *Vibrio parahaemolyticus* in North Carolina estuaries. *The Journal of Microbiology*. **46(2)**:146-153 DOI 10.1007/s12275-007-0216-2.

Böer SI, Heinemeyer EA, Luden K, Erler R, Gerdts G, Janssen F, Brennholt N. 2013. Temporal and spatial distribution patterns of potentially pathogenic *Vibrio* spp. at recreational beaches of the German North Sea. *Microbial Ecology* **65(4)**:1052-1067 DOI 10.1007/s00248-013-0221-4.

Broberg CA, Calder TJ, Orth K. 2011. *Vibrio parahaemolyticus* cell biology and pathogenicity determinants. *Microbes and Infection* **13(12-13)**:992-1001. DOI 10.1016/j.micinf.2011.06.013.

Canizalez-Roman A, Flores-Villaseñor H, Zazueta-Beltran J, Muro-Amador S, León-Sicairos N. 2011. Comparative evaluation of a chromogenic agar medium–PCR protocol with a conventional method for isolation of *Vibrio parahaemolyticus* strains from environmental and clinical samples. *Canadian journal of Microbiology* **57(2)**:136-142 DOI 10.1139/w10-108.

Ceccarelli D, Hasan NA, Huq A, Colwell RR. 2013. Distribution and dynamics of epidemic and pandemic *Vibrio parahaemolyticus* virulence factors. *Frontiers in Cellular and Infection Microbiology* **3**:97 DOI 10.3389/fcimb.2013.00097.

DePaola A, Nordstrom JL, Bowers JC, Wells JG, Cook DW. 2003. Seasonal abundance of total and pathogenic *Vibrio parahaemolyticus* in Alabama oysters. *Applied and Environmental Microbiology* **69(3)**:1521-1526. DOI 10.1128/aem.69.3.1521-1526.2003.

Di Pinto A, Terio V, Novello L, Tantillo G. 2011. Comparison between thiosulphate-citrate-bile salt sucrose (TCBS) agar and CHROMagar Vibrio for isolating *Vibrio parahaemolyticus*. *Food Control* **22(1)**:124-127 DOI:10.1016/j.foodcont.2010.06.013.

Elhadi N, Nishibuchi M. 2018. Characterization of *Vibrio parahaemolyticus* isolated from coastal water in Eastern Province of Saudi Arabia. *Malaysian Journal of Microbiology* **14(1)**:1-9 DOI 10.21161/mjm.96316.

Elhadi N. 2018. Clonal relationship among the *Vibrio parahaemolyticus* isolates from coastal water in Saudi Arabia. *The Egyptian Journal of Aquatic Research* **44(2)**:131-137 DOI 10.1016/j.ejar.2018.04.002.

Fabbro C, Cataletto B, Del Negro P. 2010. Detection of pathogenic *Vibrio parahaemolyticus* through biochemical and molecular-based methodologies in coastal waters of the Gulf of Trieste (North Adriatic Sea). *FEMS Microbiology Letters* **307(2)**:158-164 DOI 10.1111/j.1574-6968.2010.01969.x.

Fuenzalida L, Hernández C, Toro J, Rioseco ML, Romero J, Espejo RT. 2006. *Vibrio parahaemolyticus* in shellfish and clinical samples during two large epidemics of diarrhoea in southern Chile. *Environmental Microbiology* **8(4)**:675-683. DOI 10.1111/j.1462-2920.2005.00946.x.

Ghenem L, Elhadi N. 2018. Isolation, molecular characterization, and antibiotic resistance patterns of *Vibrio parahaemolyticus* isolated from coastal water in the Eastern Province of Saudi Arabia. *Journal of Water and Health* **16(1)**:57-69 DOI 10.2166/wh.2017.361.

Hara-Kudo Y, Sugiyama K, Nishina T, Saitoh A, Nakagawa H, Ichihara T, Konuma H, Hasegawa J, Kumagai S. 2001. Detection of TDH-producing *Vibrio parahaemolyticus* O3: K6 from naturally contaminated shellfish using an immunomagnetic separation method and chromogenic agar medium. Kansenshogaku zasshi. The *Journal of the Japanese Association for Infectious Diseases* **75(11)**:955-60. DOI 10.11150/kansenshogakuzasshi1970.75.955.

Heras J, Domínguez C, Mata E, Pascual V, Lozano C, Torres C, Zarazaga M. 2015. GelJ–a tool for analyzing DNA fingerprint gel images. *BMC Bioinformatics* 16(1):1-8. DOI 10.1186/s12859-015-0703-0 (2015).

Hlady WG, Klontz KC. 1996. The epidemiology of Vibrio infections in Florida, 1981–1993. *Journal of Infectious Diseases* **173(5)**:1176-83 DOI 10.1093/infdis/173.5.1176.

Jacxsens L, Kussaga J, Luning PA, Van der Spiegel M, Devlieghere F, Uyttendaele M. 2009. A microbial assessment scheme to measure microbial performance of food safety management systems. *International Journal of Food Microbiology* **134(1-2)**:113-125 DOI 10.1016/j.ijfoodmicro.2009.02.018.

Jia L, Lin C, Gao Z, Qu M, Yang J, Sun J, Chen H, Wang Q. 2016. Prevalence and factors associated with different pathogens of acute diarrhea in adults in Beijing, China. *The Journal of Infection in Developing Countries* **10(11)**:1200-1207 DOI 10.3855/jidc.6831.

Johnson CN, Bowers JC, Griffitt KJ, Molina V, Clostio RW, Pei S, Laws E, Paranjpye RN, Strom MS, Chen A, Hasan NA. 2012. Ecology of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in the coastal and estuarine waters of Louisiana, Maryland, Mississippi, and Washington (United States). *Applied and Environmental Microbiology* **78(20)**:7249-7257 DOI 10.1128/AEM.01296-12.

Johnson CN, Flowers AR, Noriea NF, Zimmerman AM, Bowers JC, DePaola A, Grimes DJ. 2010. Relationships between environmental factors and pathogenic vibrios in the northern Gulf of Mexico. *Applied and Environmental Microbiology* **76(21)**:7076-7084 DOI 10.1128/AEM.00697-10.

Julie D, Solen L, Antoine V, Jaufrey C, Annick D, Dominique HH. 2010. Ecology of pathogenic and non-pathogenic *Vibrio parahaemolyticus* on the French Atlantic coast. Effects of temperature, salinity, turbidity and chlorophyll a. *Environmental Microbiology* 12(4):929-37 DOI 10. 111/j.1462-2920.2009.02136.x.

Jun JW, Kim JH, Choresca Jr CH, Shin SP, Han JE, Han SY, Chai JY, Park SC. 2012. Isolation, molecular characterization, and antibiotic susceptibility of *Vibrio parahaemolyticus* in Korean seafood. Foodborne Pathogens and Disease **9(3)**:224-31 DOI 10.1089/fpd.2011.1018.

Kalburge SS, Whitaker WB, Boyd EF. 2014. High-salt preadaptation of *Vibrio parahaemolyticus* enhances survival in response to lethal environmental stresses. *Journal of Food Protection* **77(2)**:246-53 DOI 10.4315/0362-028X.JFP-13-241.

Kaneko T, Colwell RR. 1973. Ecology of *Vibrio parahaemolyticus* in Chesapeake bay. *Journal of Bacteriology* **113(1)**:24-32 DOI 10.1128/JB.113.1.24-32.1973.

Kim YB, Okuda JU, Matsumoto C, Takahashi N, Hashimoto S, Nishibuchi M. 1999. Identification of *Vibrio parahaemolyticus* strains at the species level by PCR targeted to the toxR gene. *Journal of Clinical Microbiology* **37(4)**:1173-1177 DOI 10.1128/JCM.37.4.1173-1177.1999.

Larsen AM, Rikard FS, Walton WC, Arias CR. 2015. Temperature effect on high salinity depuration of *Vibrio vulnificus* and *V. parahaemolyticus* from the Eastern oyster (Crassostrea virginica). *International Journal of Food Microbiology* **192**:66-71 DOI 10.1016/j.ijfoodmicro.2014.09.025.

Letchumanan V, Chan KG, Lee LH. 2014. *Vibrio parahaemolyticus*: a review on the pathogenesis, prevalence, and advance molecular identification techniques. *Frontiers in Microbiology* **5**:705 DOI 10.3389/fmicb.2014.00705.

Li J, Xue F, Yang Z, Zhang X, Zeng D, Chao G, Jiang Y, Li B. 2016. *Vibrio parahaemolyticus* strains of pandemic serotypes identified from clinical and environmental samples from Jiangsu, China. *Frontiers in Microbiology* **7**:787 DOI 10.3389/fmicb.2016.00787.

Liu J, Bai L, Li W, Han H, Fu P, Ma X, Bi Z, Yang X, Zhang X, Zhen S, Deng X. 2018. Trends of foodborne diseases in China: lessons from laboratory-based surveillance since 2011. Frontiers of Medicine 12(1):48-57 DOI 10.1007/s11684-017-0608-6.

Marder EP, Griffin PM, Cieslak PR, Dunn J, Hurd S, Jervis R, Lathrop S, Muse A, Ryan P, Smith K, Tobin-D'Angelo M. 2018. Preliminary incidence and trends of infections with pathogens transmitted commonly through food—foodborne diseases active surveillance network, 10 US Sites, 2006–2017. *Morbidity and Mortality Weekly Report* **67(11)**:324 DOI 10.1111/ajt.15412.

Marshall S, Clark CG, Wang G, Mulvey M, Kelly MT, Johnson WM. 1999. Comparison of Molecular Methods for Typing *Vibrio parahaemolyticus*. Journal of Clinical Microbiology. **37(8)**:2473-2478 DOI 10.1128/JCM.37.8.2473-2478.1999.

Martinez-Urtaza J, Bowers JC, Trinanes J, DePaola A. 2010. Climate anomalies and the increasing risk of *Vibrio parahaemolyticus* and *Vibrio vulnificus* illnesses. *Food Research International* **43(7)**:1780-90 DOI 10.1016/ j.foodres.2010.04.001.

Martinez-Urtaza J, Powell A, Jansa J, Rey JL, Montero OP, Campello MG, López MJ, Pousa A, Valles MJ, Trinanes J, Hervio-Heath D. 2016. Epidemiological investigation of a foodborne outbreak in Spain associated with US West Coast genotypes of *Vibrio parahaemolyticus*. *Springerplus* **5(1)**:1-8 DOI 10.1186/s40064-016-1728-1.

McLaughlin JB, DePaola A, Bopp CA, Martinek KA, Napolilli NP, Allison CG, Murray SL, Thompson EC, Bird MM, Middaugh JP. 2005. Outbreak of *Vibrio parahaemolyticus* gastroenteritis associated with Alaskan oysters. *New England Journal of Medicine* **353(14)**:1463-70 DOI 10.1056/NEJMoa051594.

Mudoh M, Parveen S, Schwarz J, Rippen T, Chaudhuri A. 2014. The effects of storage temperature on the growth of *Vibrio parahaemolyticus* and organoleptic properties in oysters. *Frontiers in Public Health* **2**:45 DOI 10.3389/fpubh.2014.00045.

Nair GB, Ramamurthy T, Bhattacharya SK, Dutta B, Takeda Y, Sack DA. 2007. Global dissemination of *Vibrio parahaemolyticus* serotype O3: K6 and its serovariants. Clinical microbiology reviews. **20(1)**:39-48 DOI 10.1128/CMR.00025-06.

Newton AE, Garrett N, Stroika SG, Halpin JL, Turnsek M, Mody RK. 2014. Notes from the field: increase in *Vibrio parahaemolyticus* infections associated with consumption of Atlantic Coast shellfish—2013. *MMWR Morb Mortal Wkly Rep* **63**:335-336. PMCID PMC5779391.

Parveen S, Hettiarachchi KA, Bowers JC, Jones JL, Tamplin ML, McKay R, Beatty W, Brohawn K, DaSilva LV, DePaola A. 2008. Seasonal distribution of total and pathogenic *Vibrio parahaemolyticus* in Chesapeake Bay oysters and waters. *International Journal of Food Microbiology* **128(2)**:354-61 DOI 10.1016/j.ijfoodmicro.2008.09.019.

Raghunath P. 2015. Roles of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) in *Vibrio parahaemolyticus*. *Frontiers in microbiology* **22**;5:805. DOI 10.3389/fmicb.014.00805.

Saito S, Iwade Y, Tokuoka E, Nishio T, Otomo Y, Araki E, Konuma H, Nakagawa H, Tanaka H, Sugiyama K, Hasegawa A.2015. Epidemiological Evidence of Lesser Role of Thermostable Direct Hemolysin (TDH)–Related Hemolysin (TRH) Than TDH on *Vibrio parahaemolyticus* Pathogenicity. *Foodborne Pathogens and Disease* **12(2)**:131-138. doi: 10.1089/fpd.2014.1810.

Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. 2011. Foodborne illness acquired in the United States—major pathogens. *Emerging Infectious Diseases* **17(1)**:7 DOI 10.3201/eid1701.p11101.

Shimohata T, Takahashi A. 2010. Diarrhea induced by infection of *Vibrio parahaemolyticus*. *The Journal of Medical Investigation* **57(3, 4)**:179-182 DOI 10.2152/jmi.57.179.

Su YC, Liu C. 2007. *Vibrio parahaemolyticus*: a concern of seafood safety. Food microbiology. **24(6)**:549-558. DOI10.1016/j.fm.2007.01.005.

Tada J, Ohashi T, Nishimura N, Shirasaki Y, Ozaki H, Fukushima S, Takano J, Nishibuchi M, Takeda Y. 1992. Detection of the thermostable direct hemolysin gene (tdh) and the thermostable direct hemolysin-related hemolysin gene (trh) of *Vibrio parahaemolyticus* by polymerase chain reaction. *Molecular and Cellular Probes* **6(6)**:477-87. doi: 10.1016/0890-8508(92)90044-x.

Tanaka N, Iwade Y, Yamazaki W, Gondaira F, Vuddhakul V, Nakaguchi Y, Nishibuchi M. 2014. Most-Probable-Number Loop-Mediated Isothermal Amplification–Based Procedure Enhanced with K Antigen–Specific Immunomagnetic Separation for Quantifying tdh+ Vibrio parahaemolyticus in Molluscan Shellfish. *Journal of Food Protection* **77(7)**:1078-85 DOI 10.4315/0362-028X.JFP-13-536.

Thongjun J, Mittraparp-Arthorn P, Yingkajorn M, Kongreung J, Nishibuchi M, Vuddhakul V. 2013. The trend of *Vibrio parahaemolyticus* infections in Southern Thailand from 2006 to 2010. *Tropical Medicine and Health* **41(4)**:151-6 DOI 10.2149/tmh.2013-06.

Food and Drug Administration. 2020. Fishery products hazards and controls guidance, 2020. *Available at https://www.fda.gov/food/seafood-guidance-documents-regulatory-information/fish-and-fishery-products-hazards-and-controls* (Accessed on 14 July 2020).

Versalovic J, Koeuth T, Lupski R. 1991. Distribution of repetitive DNA sequences in eubacteria and application to finerpriting of bacterial genomes. *Nucleic Acids Research* **19(24)**:6823-6831 DOI 10.1093/nar/19.24.6823.

Whitaker WB, Parent MA, Naughton LM, Richards GP, Blumerman SL, Boyd EF. 2010. Modulation of responses of *Vibrio parahaemolyticus* O3: K6 to pH and temperature stresses by growth at different salt concentrations. *Applied and Environmental Microbiology* **76(14)**:4720-9. DOI 10.1128/AEM.00474-10.

Yonekita T, Morishita N, Arakawa E, Matsumoto T. 2020. Development of a monoclonal antibody for specific detection of *Vibrio parahaemolyticus* and analysis of its antigen. *Journal of Microbiological Methods* **173**:105919 DOI 10.1016/j.mimet.2020.105919.

572 **Yoon KS, Min KJ, Jung YJ, Kwon KY, Lee JK, Oh SW. 2008.** A model of the effect of
573 temperature on the growth of pathogenic and nonpathogenic *Vibrio parahaemolyticus* isolated
574 from oysters in Korea. *Food Microbiology* **25(5)**:635-41 DOI 10.1016/j.fm.2008.04.007.
575

Table 1(on next page)

Primer used in this study

Table 1 Primer used in this study.

Primer specificity	Primer sequence	Amplicon size (bp)	Annealing temperature (^{0}C)	Reference
***tox*R**	Forward: 5'-GTCTTCTGACGCAATCGTTG-3' Reverse: 5'-ATACGAGTGGTTGCTGTCATG-3'	368	63	(*Kim et al., 1999*)
tdh	Forward: 5'-CCACTACCACTCTCATATGC-3' Reverse: 5'-GGTACTAAATGGCTGACATC-3'	251	55	(*Tada et al., 1992*)
trh	Forward: 5'-GGCTCAAAATGGTTAAGCG-3' Reverse: 5'-CATTTCCGCTCTCATATGC-3'	250	58	(*Tada et al., 1992*)

Table 2(on next page)

Distribution of *V. parahaemolyticus* isolated with and without IMB from different locations

Table 2 Distribution of *V. parahaemolyticus* isolated with and without IMB from different locations.

Location	No. of samples	Date of collection	Surface seawater		Number of positive samples (%)			
					Isolated without IMB on		Isolated with IMB on	
			Temp (°C)	pH	TCBS agar	CaV	TCBS agar	CaV
AZB	40	4 March 2018	25	8.18	3 (7.5)	5 (12.5)	11(27.5)	15 (37.5)
KBC	39	25 March 2018	27.5	7.35	0	0	6 (15.4)	6 (15.4)
KBF	38	8 April 2018	28	8.18	0	0	2 (5.3)	2 (5.3)
HMF	39	22 April 2018	31	8.22	0	0	17 (43.6)	19 (48.7)
DMC	36	5 May 2018	28	8.46	0	0	2 (5.6)	2 (5.6)
TOTAL	192				3 (1.6)	5 (2.6)	38 (19.8)	44 (22.9)

Table 3(on next page)

Confirmation of *V. parahaemolyticus* isolated with and without IMB treatment targeting species (*tox*R) and virulence gene (*tdh*/*trh*) markers by using PCR

Table 3 Confirmation of *V. parahaemolyticus* isolated with and without IMB treatment targeting species (*tox*R) and virulence gene (*tdh*/*trh*) markers by using PCR.

Location	No. of tested isolates	Number of *V. parahaemolyticus* isolates					
		Isolated without IMB and positive for:			Isolated with IMB and positive for:		
		*tox*R gene	*tdh* gene	*trh* gene	*tox*R gene	*tdh* gene	*trh* gene
AZB	45	3	3	0	4	3	0
KBC	17	0	0	0	1	1	0
KBF	6	0	0	0	0	0	0
HMF	48	0	0	0	19	15	0
DMC	4	0	0	0	2	2	0
Total (%)	120	3 (2.5%)	3 (2.5%)	0	26 (21.7%)	21 (17.5%)	0

Table 4(on next page)

Molecular characterization of *V. parahaemolyticus* isolated with and without IMB treatment

Table 4 Molecular characterization of *V. parahaemolyticus* isolated with and without IMB treatment.

No	Strain code	Sample location	Isolation date	*tox*R gene	Virulence gene		ERIC type
					tdh	*trh*	
1	VP-7	AZB	4 March 2018	+	+	-	ET-4
2	VP-31-A	AZB	4 March 2018	+	+	-	ND*
3	VP-31-B	AZB	4 March 2018	+	-	-	ET-2
4	VP-32	AZB	4 March 2018	+	+	-	ET-2
5	VP-36	AZB	4 March 2018	+	+	-	SC$^{\Psi}$
6	VP-37	AZB	4 March 2018	+	+	-	ET-2
7	VP-49	KBC	25 March 2018	+	+	-	ET-2
8	VP-118	HMF	22 April 2018	+	+	-	ET-4
9	VP-121	HMF	22 April 2018	+	+	-	ET-2
10	VP-123-A	HMF	22 April 2018	+	-	-	ET-2
11	VP-123-B	HMF	22 April 2018	+	+	-	ET-4
12	VP-123-C	HMF	22 April 2018	+	+	-	ET-2
13	VP-125-A	HMF	22 April 2018	+	-	-	SC
14	VP-125-A	HMF	22 April 2018	+	+	-	ET-4
15	VP-126	HMF	22 April 2018	+	+	-	ND
16	VP-129-A	HMF	22 April 2018	+	+	-	ET-3
17	VP-129-B	HMF	22 April 2018	+	-	-	ET-4
18	VP-130-A	HMF	22 April 2018	+	-	-	ET-3
19	VP-130-A-B	HMF	22 April 2018	+	+	-	SC
20	VP-132	HMF	22 April 2018	+	+	-	SC
21	VP-133	HMF	22 April 2018	+	+	-	ND
22	VP-134	HMF	22 April 2018	+	+	-	ET-1
23	VP-137	HMF	22 April 2018	+	+	-	ET-1
24	VP-145	HMF	22 April 2018	+	+	-	ET-1
25	VP-151	HMF	22 April 2018	+	+	-	ET-1
26	VP-152	HMF	22 April 2018	+	+	-	SC
27	VP-153	HMF	22 April 2018	+	+	-	ND
28	VP-165	DMC	5 May 2018	+	+	-	SC
29	VP-166	DMC	5 May 2018	+	+	-	ND

ND*, not determined; SC$^{\Psi}$, a single cluster.

Figure 1

PCR amplification of the toxR gene. Lane 1, molecular weight marker (100 bp DNA ladder; Promega); 2, V. parahaemolyticus ATCC 17802 (positive control); 3, negative control; 4–18, tested isolates.

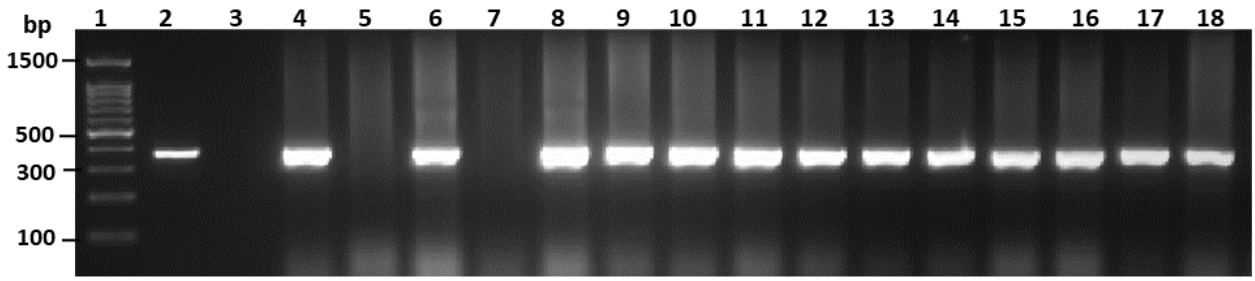


Figure 1 Representative electrophoresis image of PCR amplification of the *tox*R gene (368 bp fragment). Lane 1, molecular weight marker (100 bp DNA ladder; Promega); 2, *V. parahaemolyticus* ATCC 17802 (positive control); 3, negative control; 4–18, tested isolates for *tox*R gene.

Figure 2

Representative of PCR amplification of the tdh gene (251 bp fragment). Lane 1, molecular weight marker; 2, V. parahaemolyticus AQ3815 (positive control); 3, negative control; 4–20, positive isolates

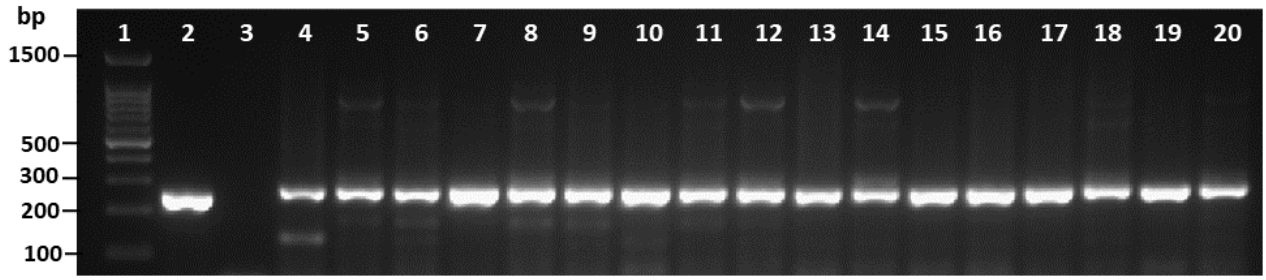


Figure 2 Representative electrophoresis image of PCR amplification of the *tdh* gene (251 bp fragment). Lane 1, molecular weight marker (100 bp DNA ladder; Promega); 2, *V. parahaemolyticus* AQ3815 (positive control); 3, negative control; 4–20, positive isolates for *tdh* gene isolated in this study.

Figure 3

Amplified DNA fingerprints produced by ERIC-PCR. Lane 1, 100 bp DNA ladder; from lane 2 to 20 representative isolates of V. parahaemolyticus isolated in this study

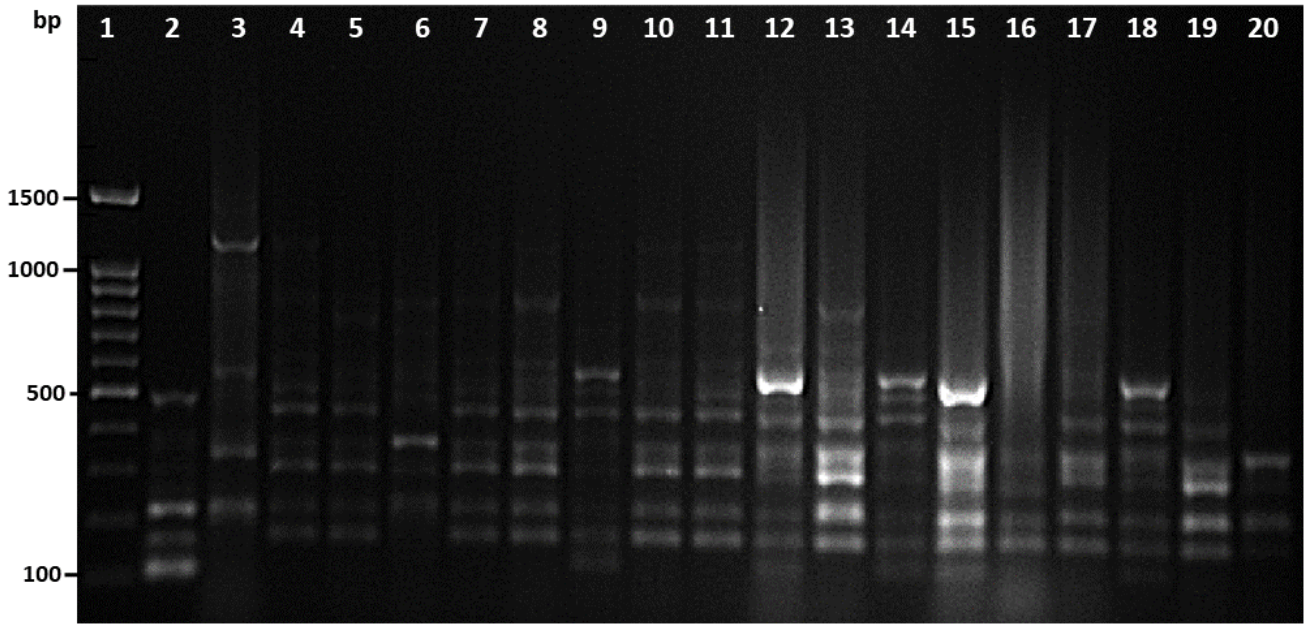
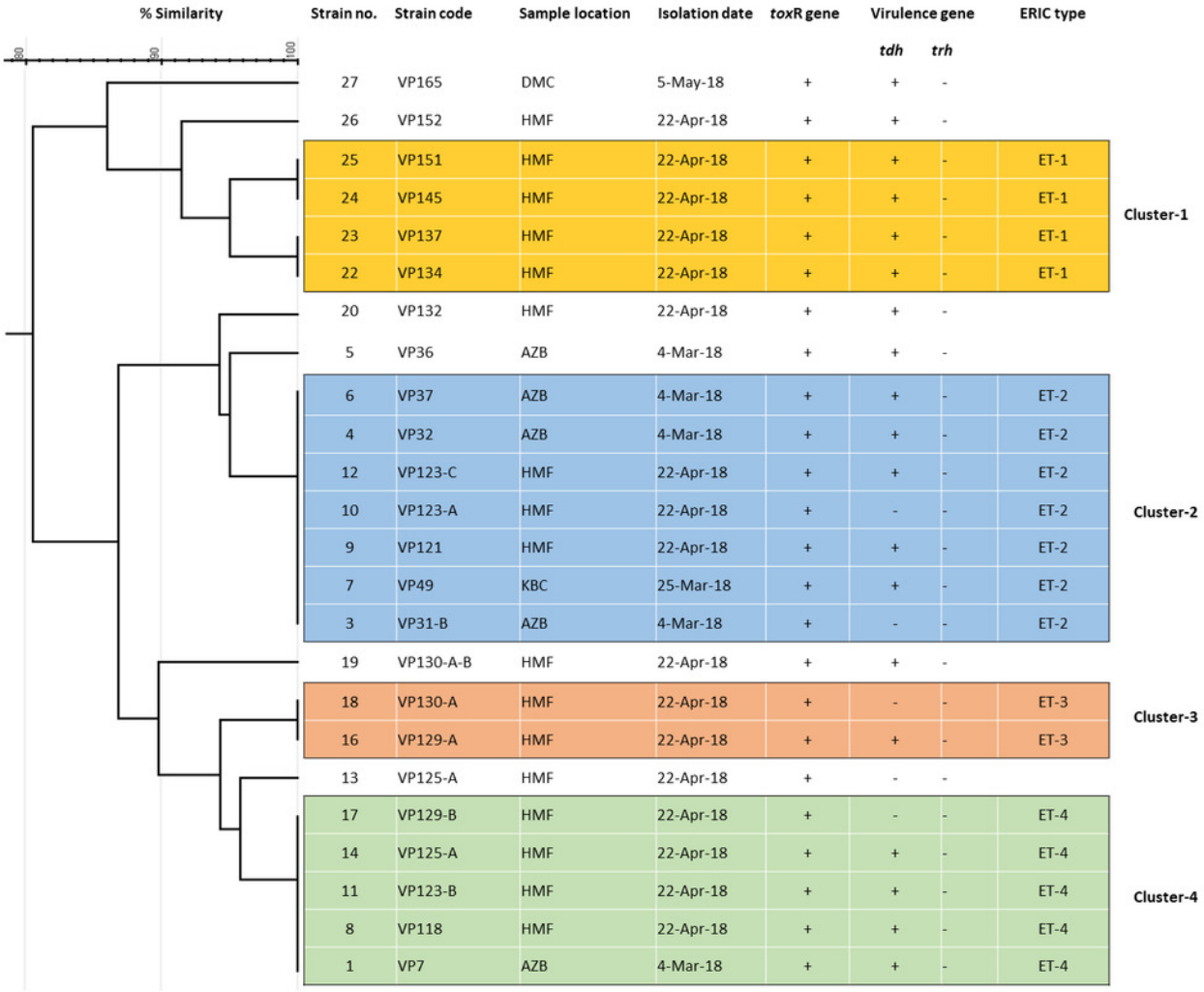


Figure 3 Amplified DNA fingerprints produced by ERIC-PCR. Lane 1, 100 bp DNA ladder; from lane 2 to 20 representative isolates of *V. parahaemolyticus* isolated in this study.

Figure 4

ERIC-PCR dendrogram of toxR and tdh gene-positive strains of V. parahaemolyticus isolated from coastal water in this study. Cluster 1 to 4 denotes identical clonal groups of V. parahaemolyticus strains



Strain no.	Strain code	Sample location	Isolation date	*toxR* gene	Virulence gene		ERIC type	
					tdh	*trh*		
27	VP165	DMC	5-May-18	+	+	-		
26	VP152	HMF	22-Apr-18	+	+	-		
25	VP151	HMF	22-Apr-18	+	+	-	ET-1	Cluster-1
24	VP145	HMF	22-Apr-18	+	+	-	ET-1	
23	VP137	HMF	22-Apr-18	+	+	-	ET-1	
22	VP134	HMF	22-Apr-18	+	+	-	ET-1	
20	VP132	HMF	22-Apr-18	+	+	-		
5	VP36	AZB	4-Mar-18	+	+	-		
6	VP37	AZB	4-Mar-18	+	+	-	ET-2	Cluster-2
4	VP32	AZB	4-Mar-18	+	+	-	ET-2	
12	VP123-C	HMF	22-Apr-18	+	+	-	ET-2	
10	VP123-A	HMF	22-Apr-18	+	-	-	ET-2	
9	VP121	HMF	22-Apr-18	+	+	-	ET-2	
7	VP49	KBC	25-Mar-18	+	+	-	ET-2	
3	VP31-B	AZB	4-Mar-18	+	-	-	ET-2	
19	VP130-A-B	HMF	22-Apr-18	+	+	-		
18	VP130-A	HMF	22-Apr-18	+	-	-	ET-3	Cluster-3
16	VP129-A	HMF	22-Apr-18	+	+	-	ET-3	
13	VP125-A	HMF	22-Apr-18	+	-	-		
17	VP129-B	HMF	22-Apr-18	+	-	-	ET-4	Cluster-4
14	VP125-A	HMF	22-Apr-18	+	+	-	ET-4	
11	VP123-B	HMF	22-Apr-18	+	+	-	ET-4	
8	VP118	HMF	22-Apr-18	+	+	-	ET-4	
1	VP7	AZB	4-Mar-18	+	+	-	ET-4	

Figure 4 ERIC-PCR dendrogram of *tox*R and *tdh* gene-positive strains of *V. parahaemolyticus* isolated from coastal water in this study. Cluster 1 to 4 denotes identical clonal groups of *V. parahaemolyticus* strains