Acta Aquatica Turcica
E-ISSN: 2651-5474
Home Page: https://dergipark.org.tr/actaquatr 18(3): 295-302, 2022 DOI: 10.22392/actaquatr.902891
Research Article Araştırma Makalesi

Determination of *tdh* and *trh* Positive *Vibrio alginolyticus* Isolates from Black Mussel (*Choromytilus meridionalis*) in Aegean Sea coast of Turkey

Türkiye'nin Ege Denizi kıyısındaki Kara Midye (*Choromytilus meridionalis*)'lerden *tdh* ve *trh* Pozitif *Vibrio alginolyticus* İzolatlarının Belirlenmesi

Meriç Lütfi Avsever[1*]

[1*]Manisa Celal Bayar University, Akhisar Vocation of High School, Plant and Animal Production Department, Manisa-Türkiye

Corresponding author: lutfiavsever@gmail.com

Received: 12.05.2021 Accepted: 14.12.2021 Published: 01.09.2022

How to Cite: Avsever, M. L. (2022). Determination of *tdh* and *trh* positive Vibrio alginolyticus isolates from Black Mussel (Choromytilus meridionalis) in the Aegean Sea coast of Turkey. Acta Aquatica Turcica, 18(3), 295-302. https://doi.org/10.22392/actaquatr.936723

Abstract: *Vibrio alginolyticus* is one of the important pathogens, especially found in bivalve mollusks and food poisoning in humans. The severity of food poisoning is directly proportional to the virulence genes of *V. alginolyticus*. Tdh-Related Hemolysin (*trh*) and Thermostable Direct Hemolysin (*tdh*) genes have an important place among the virulence genes found in *V. alginolyticus*. In this research, 17 *V. alginolyticus* were isolated from 17 orders (80.95%) of 21 sets of black mussels (*Choromytilus Meridionalis*) samples purchased from local divers in İzmir and Balıkesir regions. While *trh* gene was detected in 7 (42.17%) of 17 isolates, *tdh* gene was found in 6 (35.29%) and both *trh* and *tdh* genes were found in 2 (11.76%) isolates; no *trh* or *tdh* gene was found in 2 isolates (11.76%). The results of the study are also important in terms of public health. Black mussel is a product that is mainly consumed in coastal areas in Turkey and is mostly sold uncontrolled by mussel sellers. Vibrios with virulence genes can cause food poisoning, especially in summer. In addition, *V. alginolyticus* may be a *tdh-trh* reservoir for other vibrio species. To clarify this, more detailed research should be done with other vibrio species and other bivalve species.

Keywords
- *Vibrio alginolyticus*
- Black mussel
- *trh*
- *tdh*
- Turkey

Özet: *Vibrio alginolyticus*, özellikle çift kabuklu yumuşakçalarda bulunan ve insanlarda gıda zehirlenmesine neden olan önemli patojenlerden biridir. Gıda zehirlenmesinin şiddeti, *V. alginolyticus*'un virülans genleri ile doğru orantılıdır. *V. alginolyticus*' ta tespit edilmiş virulans genler içinde Tdh-İlişkili Hemolizin (*trh*) ve Termostabil Direkt Hemolizin (*tdh*) genleri önemli bir yer tutar. Bu çalışmada, İzmir ve Balıkesir yörelerindeki yerel dalgıçlardan satın alınan 21 takım (Her takımda yüz civarında midye olmak üzere) kara midye (*Choromytilus Meridionalis*) numunesinin 17 takımından (%80,95) 17 adet *V. alginolyticus* izole edildi. On yedi izolatın 7'sinde (%42,17) *trh* geni, 6'sında (%35,29) *tdh* geni, 2'sinde (%11,76) hem *trh* hem *tdh* geni bulunurken; 2 izolatta (%11,76) *trh* ya da *tdh* geni tespit edilmedi. Çalışma sonuçları halk sağlığı açısından da önemlidir. Kara midye, Türkiye'de ağırlıklı olarak kıyı bölgelerde tüketilen ve çoğunlukla kontrolsüz olarak seyyar midyeciler tarafından satılan bir üründür. Virulans genlere sahip vibriolar özellikle yaz aylarında gıda zehirlenmeleri meydana getirebilir. Ek olarak, *V. alginolyticus*, diğer vibrio türleri için *tdh-trh* rezervuarı olabilir. Bunu netleştirmek için diğer vibrio türleri ve diğer çift kabuklu türleri ile daha detaylı araştırmalar yapılmalıdır.

Anahtar kelimeler
- *Vibrio alginolyticus*
- Kara midye
- *trh*
- *tdh*
- Türkiye

1. INTRODUCTION

Vibrio alginolyticus is one of the most widespread *Vibrio* spp. to be found in seawater, bottom of the sea, fish, bivalve and can keep its virulence under adverse conditions (Benkahla et al., 2007; Covazzi et al., 2008; Karim et al., 2018). This microorganism causes conjunctivitis, wound infections, and gastroenteritis in humans (Osorio and Klose, 2000; Türk et al., 2011; Weils and Wendy, 2012). Pathogenicity is closely related to virulence genes (Hentschel et al., 2000; Zhangx and Austin, 2005; Chewdhury and Chewdhury, 2015). *tdh*-related hemolysin (*trh*) and thermostable direct hemolysin (*tdh*) genes are the notable virulence features in *V. alginolyticus* (Reina et al., 1995). After rupture of the erythrocyte membranes, hemolysis occurs and hemoglobin is released (Lida et al., 1997; Bej et al., 1999). Haemolysins are maked by many different microorganisms (*Vibrio* spp., *Pseudomonas* spp.) (Lida and Honda, 1997). Numerous studies claim that hemolysins are involved in disease pathogenesis (Osorio et al., 2000; Stalin and Srinivasan, 2016).

Vibrio alginolyticus is an important pathogen in marine aquaculture in South Asia and Europe (Lopes et al., 1993; Balebona et al., 1998). The detection of *V. alginolyticus* with pathogenic genes from bivalve is also notable for human health. Because bivalves are often eaten raw, salted, and undercooked (Elliott et al., 1992).

Although cases of food poisoning due to *V. alginolyticus* have been reported worldwide, there is limited information on virulence genes with these isolates. *tdh* and *trh* genes mostly were detected in *V. cholerae* and *V. parahaemolyticus* (Tada et al., 1992; Xie et al., 2005, González-Escalona et al., 2006; Gutierrez West et.al. 2013). And there are only a few studies on *tdh* (Gargouti et al., 2015) and *trh* (Gonzales-Escolana et al., 2006, Avsever, 2016) genes in *V. alginolyticus* available. Avsever (2016) found that *trh* positive *V. alginolitycus* isolates in bivalve molluscs in the Balıkesir and Ayvalık regions between 2007 and 2010. However, there is no report for *tdh* positive *V. alginolitycus* from bivalve mollusks in Turkey.

This study aims to fix the *trh*, *tdh* genes of *V. alginolyticus* isolates obtained from black mussel located in Turkey, to take attention the potential virulence gene transfer between other Vibrio species and *V. alginolyticus*, and to note that *V. alginolyticus* is an important foodborne bacteria.

2. MATERIAL and METHODS

In this study, 21 black mussels (*Choroytilus meridionalis*) sampling were performed by local divers in İzmir (n=15) and Balıkesir (n=6) Provinces. 100 mussel (relatively small) samples were taken in each sampling. Sample collection took place from 1 May to 31 August 2018, when shellfish collection was not prohibited. The mean water temperature during the sampling seasons was 21^{o}C ±2. The samples were delivered to the laboratory by a special car, in a cold chain, and quickly.

2.1. Bacterial isolation

In this study, isolation of *V. alginolitycus* was done in accordance with TS/TS ISO 8914 standard (1998). Each group of mussels was accepted as a sample. The mussel groups were crushed in separate sterile mortars and homogenized. 25 g of sample for each group from the homogenate was weighed and used in the bacteriological study. For pre-enrichment, 25 g of the homogenate from mussels in each sampling were placed in peptonized water containing 225 ml of 3% NaCl and incubated at 37 ° C for 18-24 hours. In line with the pre-enrichment medium, the line was plated with TCBS (Thiosulfate Citrate Bile Sucrose) agar. After incubation at 37 ° C for one day, DNA extract was obtained from the four yellow-colored colonies (2-3 mm in diameter), with flagellar moving, oxidase-positive, Gram-negative rod.

2.2. DNA isolation

DNA extraction was performed from the isolates (ATCC 17749, *V. alginolyticus*; ATCC 19264, *Vibrio anguillarum; V. parahaemolyticus* isolates positive for *trh* and *tdh* and 17 *V. alginolyticus*

suspicious isolates with a commercial DNA isolation kit (High pure, Germany) in accordance with the user manual.

2.3. Confirmation of *V. alginolyticus* isolates with PCR

In the confirmation of the isolates, the target gene was selected as gyrB and used the PCR method specified by Luo et al. (2008). ATCC 17749, *V. alginolyticus* was performed as the positive bacteria. *V. anguillarum* ATCC 19264 (568 bp.) was performed as the negative control. The primer sequences were presented in Table 1. The PCR formation was used in a 25 µl quantity containing 10x PCR buffer (2.50 µl), *Taq* DNA polymerase enzyme (5 U/µl) (0.40 µl) (MBI, Fermantas), 5 µl bacteria DNA, 50 mM $MgCl_2$ (1.25 µl), 0.4 µM Alg F1 (2 µl), 0.4 µM Alg R1 (2 µl), 10 mM dNTPs (dATP, dCTP, dGTP, dTTP) (0.63 µl) and 11.22 µl non-nuclease water. The amplification procedure occurred of an initial denaturation at 94°C for 4 min, 32 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 8 min. After PCR amplification, 4 µl of each product was added into 1.0% agarose gel, and electrophoresis was performed. Bands were visualized with designated equipment as 568 bp. As DNA size markers, 100 DNA Ladder (MBI, Fermentas) was performed.

Table 1. *gyrB* primers.

gyrB primers (Luo et al. (2008)	5'-CATCGTCGCCTGAAGTCG CTGT -3' (AlgR1), 5'-TCAGAGAAAGTTGAGCTAACGATT-3' (AlgF1).

2.4. Detection of *tdh* and *trh* genes using PCR

DNA samples from 17 *V. alginolyticus* isolates were researched separately (PCR 1 and 2) for *tdh* and *trh* genes with PCR. PCR studies were used with the primer pairs (in Table 2) according to the method reported by Cohen et al., 2007. *V. parahaemolyticus* isolates previously found positive for *trh* and *tdh* by Terzi et al., 2009 were performed as positive bacteria. Distilled water was performed as a negative control. Master-mix occurred 5 µl genomic DNA, 10x PCR buffer (2.50 µl), 50 mM $MgCl_2$ (1.25 µl), 10 mM dNTPs (dATP, dCTP, dGTP, dTTP) (0.63µl), 5 µM *TRH*-L primer (2.0 µl), 5 µM *TRH*-R primer (2.0 µl), *Taq* DNA polymerase (5 units/µl) (0.40 µl) (MBI, Fermantas), 11.22 µl non-nuclease water for PCR 1 (*trh*); 5 µl genomic DNA, 10x PCR buffer (2.50 µl), 50 mM $MgCl_2$ (1.25 µl),10 mM dNTPs (dATP, dCTP, dGTP, dTTP)(0.63 µl), 5 µM *TDH*-L primer(1 µl), 5µ M *TDH*-R primer (1 µl), *Taq* DNA polymerase (5 U/µl)(0.40 µl) (MBI, Fermantas), 13.22 µl non-nuclease water for PCR 2 (*tdh*). The reactions (PCR 1, 2) performed with an automated thermocycler were as follows: Initial denaturation at 94°C for 5min., followed by 40 cycles of denaturation at 94°C for 30 s., primer annealing at 58°C for 45 s. and primer extension at 68°C for 75 s. A final extension was performed at 68°C for 7min. PCR end-products were separated by electrophoresis on 2% (w⁄v) agarose gel (1 hour, 75 volts) was performed. As a DNA size marker, 100 DNA Ladder (MBI-Fermentas) was used. Bands were observed with suitable equipment as 250 and 373 bp.

Table 2. *trh, tdh* primers.

trh primers (Cohen et al., 2007)	5'-GGC TCA AAA TGG TTA AGCG-3' and 5'-CAT TTC CGC TCT CAT ATGC-3'
tdh primers (Cohen et al., 2007)	5'-CCA TCT GTC CCT TTT CCT GC-3' and 5'-CCA AAT ACA TTT TAC TTGG-3'

3. RESULTS

Vibrio alginolyticus was isolated from 17 of (80.95 %) sampling of black mussels. Six isolates (35.29 %) for *tdh* gene, 7 isolates (42.17 %) for *trh* gene, two isolates (11.76 %) for *trh* and *tdh* genes were

found positive. Two isolates (11.76 %) were negative for *trh* and *tdh* genes. Results are shown in Scale 3 and Figure 1,2,3.

Table 3. Results of the *gyrB, tdh and trh positive V. alginolyticus* isolates.

Black mussel groups	***gyrB* positive *V. alginolyticus* (80.95 %)**	***trh* positive (only) *V. alginolyticus* (42.17 %)**	***tdh* positive (only) *V. alginolyticus* (35.29 %)**	***tdh-trh* positive *V.alginolyticus* (11.76 %)**	***tdh-trh* negative *V. alginolyticus* (11.76 %)**
21	17	6	7	2	2

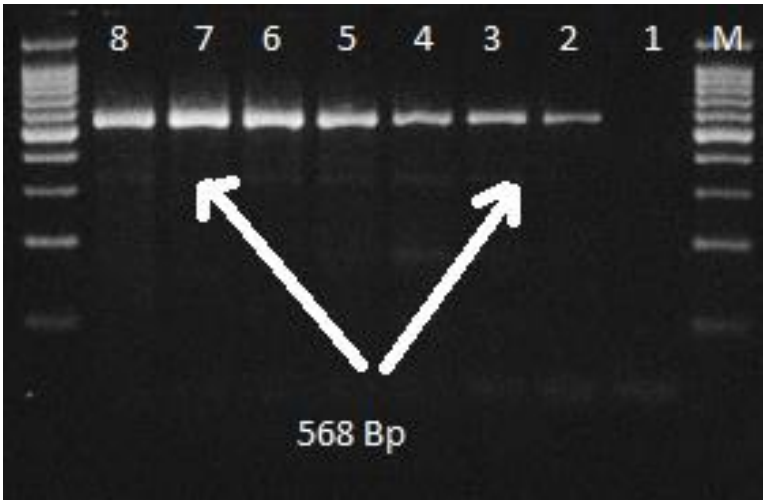

Figure 1. Confirmation of *gyrB* gene-positive *V. alginolyticus* isolates. Line 1: Negative control ATCC 19264, *Vibrio anguillarum*. Line 2: ATCC 17749, *V. alginolyticus*, Positive control,568 bp. Line 3-8: Isolates, 568 bp. M: Marker, 100 bp.

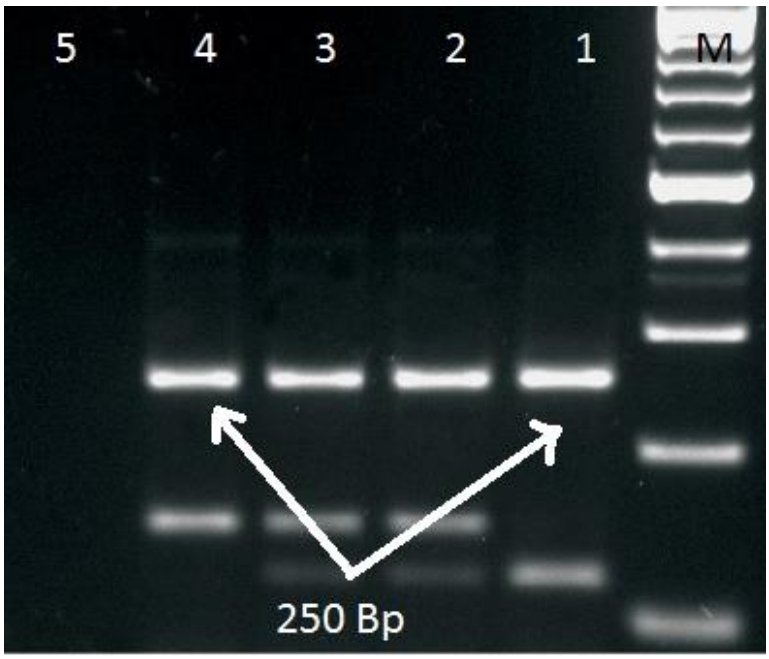

Figure 2. Detection of *trh* gene in *V. alginolyticus* isolates. Line 1: *trh* positive control (Terzi and Büyüktanır, 2009), 250 bp. Line 2, 3, 4: *trh* positive isolates, 250 bp. Line 5: Negative control distilled water. M: Marker 100 bp.

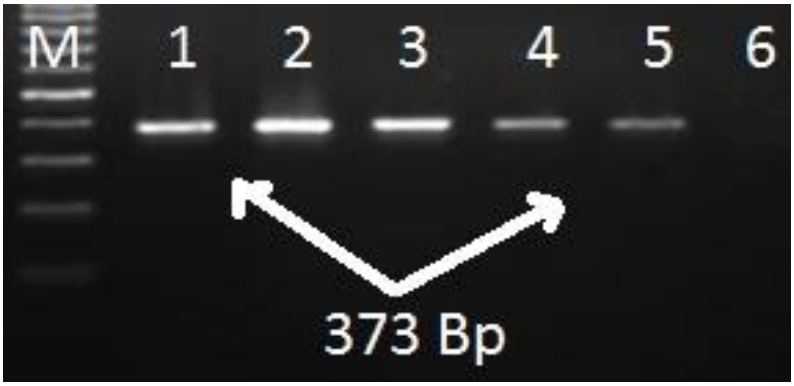

Figure 3: Detection of *tdh* gene in *V. alginolyticus* isolates. Line 1: *tdh* positive control (Terzi, Büyüktanır and Yurdusev, 2009), 373 bp. Line 2-5: *tdh* positive isolates. Line 6: Negative control distilled water. M: Marker 100 bp.

4. DISCUSSION

In this study, *Vibrio alginolyticus* was isolated from 17 of (80.95 %) sampling of black mussel. Seven isolates (42.17 %) for *trh* gene, six isolates (35.29 %) for *tdh* gene, and two isolates (11.76 %) for *trh* and *tdh* genes were found positive.

In the literature, there are studies on *Vibrio parahaemolyticus*, which has *trh - tdh* genes isolated from mussels but there are few studies (González-Escalona et al. 2006; Avsever, 2016) on investigating *trh - tdh* genes in *V. alginolyticus* bacteria isolated from mussels. So it does not seem possible to make a healthy comparison of isolation rates. Gargouti et al. (2015) isolated *V. alginolyticus* from two (20 %) of five Mantis Shrimp (*Oratosquilla oratoria*) samples collected from markets and found positive for the *tdh, trh* and *tox-R* genes from one (50%) of two isolates. In our study, while the isolation rate of *V. alginolyticus* was higher (80.95 %); *trh* (42.17 %) and *tdh* (35.29 %) rates were found to be relatively similar. The high isolation rate in Gargouti et. al (2015) may be due to the small number of samples used. On the other hand, Mustapha et al. (2012) isolated *V. alginolyticus* at a rate of (70 %) from shellfish, which is consistent with our study (80.95 %).

Avsever (2016) found *trh* positive *V. alginolyticus* (13.04 %) in the same region (Agean sea) between 2007-2010 from different bivalve mollusk species in Turkey. But *tdh* positive *V. alginolyticus* isolates were not reported. In this study, the *trh* positivity rate in the same region was found to be 42.17 %. This may be because the *trh* gene frequency is on the rise among Vibrio species. On the other hand, while *tdh* was not found in the study (Avsever, 2016), *tdh* positivity was found at a rate of 35.29 % in this study. This may suggest the transfer of *tdh* from other vibrios (especially *V. parahaemolyticus*) to *V. alginolyticus* (González-Escalona et al. 2006). However, further studies are needed before these can be said. Except for Avsever (2016), in Turkey, Terzi et al. (2009) noted *tdh-trh* positive *V. parahaemolyticus* in mussels from the Black Sea coast of Turkey.

Some studies have reported *V. alginolyticus* isolate carries virulence genes derived from other *Vibrio* species (Boyde et al., 2000). In America, during a *V. parahaemolyticus* outbreak, isolated *V. alginolyticus* which possessed and expressed a *trh* gene with 98% homology to the *trh2* gene of *V. parahaemolyticus* (Tada et al., 1992). *V. alginolyticus* and *V. parahaemolyticus* are highly homogeneous, with 99Æ8 and 61Æ7% similar nucleotides respectively (Osorio et al., 2000), especially because these two bacteria can exchange more often genetic material and can make each other *trh-tdh* genes in terms of the positive.

However, although there are common virulence genes between *V. alginolyticus* and *V. parahaemolyticus* and and *V. alginolyticus* might have a different virulence gene system and different pathogenic mechanism compared with *V. parahaemolyticus* although adhesion and hydrolytic activities

are also essential parameters for the infection and disease symptoms because of *V. alginolyticus* (Balebona et al., 1995).

Black mussels are eaten mainly in the Mediterranean and Aegean coastal regions of Turkey. This product, which is mostly sold uncontrolled by mobile mussels, poses a great danger, especially in the summer months. For this reason, the results of the study are also important in terms of public health. In addition, *V. alginolyticus* can be the tdh-trh reservoir for other Vibrio species. More detailed research with other vibrio species and other bivalve species should be done to clarify these.

ACKNOWLEDGEMENT

The authors thank the divers who contributed to the study.

FUNDING

No financial support was received in the conduct of this study.

CONFLICT of INTEREST

The authors declare that there are no financial interests or personal relationships that could affect this work.

AUTHOR CONTRIBUTIONS

MLA; Methodology: MLA; Performing the experiment: MLA; Data analysis: MLA; Article writing: MLA, Supervision: MLA. All authors approved the final draft.

ETHICAL STATEMENTS

Local Ethics Committee Approval was not obtained. Because mussels are invertebrates and ethics committee permission is not required.

DATA AVAILABILITY STATEMENT

The data used in this study are available on the Figshare platform with the DOI address https://doi.org/10.6084/m9.figshare.11815566.v1

REFERENCES

Ali, H., Rahman, M.M., Rico, A., Jaman, A., Basak, S.K., MahbubulIslam, M., Khan, N., Keus, H.J. & Mohan, C.V. (2018). An assessment of health management practices and occupational health hazards in tiger shrimp (*Penaeus monodon*) and freshwater prawn (*Macrobrachium rosenbergii*) aquaculture in Bangladesh. *Turkısh Journal Of Veterınary And Anımal Scıences*. 5, 10–19. https://doi.org/10.1016/j.vas.2018.01.002

Avsever, M.L. (2016). First report of *trh* positive *Vibrio alginolyticus* isolates from bivalve molluscs in Turkey. *Revue de Medecine Veterinaire*, 167(3), 65-70.

Balebona, M,C., Andreu, M.J., Bordas, M.A., Zorrilla, I., Moriñigo, M.A. & Borrego, J. (1998). Pathogenicity *of Vibrio alginolyticus* from cultured gilt-head sea bream (Sparus aurata, L.). *Applied and Environmental Microbiology Journal*. 64, 4269-4275. https://doi.org 10.1128/AEM.64.11.4269-4275.1998.

Bej, A.K., Patterson, D.P., Brasher, C.W., Vickery, M.C.L., Vickery, M.C., DJones, D.D. & Kaysner, C.A. (1999). Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh* and *trh*. *Journal of Microbiological Methods*, 36, 215- 225. https://doi.org10.1016/s0167-7012(99)00037-8

Benkahla-Nakbia, A., Besbesa, A., Chaieba, K., Rouabhia, M. & Bakhrouf, A. (2007). Survival of *Vibrio alginolyticus* in sea- water and retention of virulence of its starved cells. *Marine Environmental Research*. 46, 469-478 https://doi.org/10.1016/j.marenvres.2007.04.002

Boyde, F., Moyerk, E. & Shi, I. (2000). Infectious CTX phi and the Vibrio pathogenicity is land prophage in *Vibrio mimicus*: evidence for recent horizontal transfer between *V. mimicus* and *V. cholerae. Infection and Immunity - ASM Journals.* 68,1507-1513. https://doi.org 10.1128/IAI.68.3.1507-1513.2000

Chowdhury, G., Rahman, M.M., Mondal, M.N. & Hannan, A. (2015). Studies on pathogens associated with black spot disease of prawn and shrimp in Bangladesh. Confarence paper. https://www.researchgate.net/publication/284673047

Cohen, N., Karib, H., Said, J., Lemee, L., Guenole, A. & Quılıcı, M.L. (2007) Prévalence des vibrions potentiellement pathogènes dans les produits de la pêche commercialisés à Casablanca (Maroc). *Revue de Medecine Veterinaire*, 158, 562-568.

Covazzi-Harriague, A., Brino, M.D., Zampini, M., Albertelli, G., Pruzzo, C. & Misic, C. (2008). Vibrios in association with sedimentary crustaceans in three beaches of the northern Adriatic Sea (Italy). *Marine Pollution Bulletin*, 56, 574-579. https://doi.org/10.1016/j.marpolbul.2007.12.011

Elliott, E.L., Kaysnery, C.A. & Tamplin ML (1992.) *V. cholerae*, *V. vulnificus* and other Vibrio spp. In FDA Bacteriological Analytical Manual, 7th ed, AOAC International, Arlington.

González-Escalona, N., Blackstone. G.M. & Depaola, A. (2006). Characterization of a *Vibrio alginolyticus* Strain, isolated from Alaskan Oysters, Carrying a Hemolysin Gene Similar to the Thermostable Direct Hemolysin-Related Hemolysin Gene (*trh*) of Vibrio parahaemolyticus. *Applied and Environmental Microbiology*, 72, 7925-7929. https://doi.org 10.1128/AEM.01548-06

Gargouti, A.S; Ab-Rashid, M.N.K; Ghazali, M.F; Mitsuaki, N; Haresh, K.K; Radu, S. (2015). Detection of *tdh* and *trh* Toxic Genes in *Vibrio Alginolyticus* Strain from Mantis Shrimp (*Oratosquilla Oratoria*), *Journal of Nutrition & Food Sciences*, 5 (5) (2015), p. 1000405 https://doi.org.10.4172/2155-9600.1000405

Gutierrez West, C.K., Klein, S.L. & Lovell, H.R. (2013). High frequency of virulence factor genes *tdh*, *trh*, and *tlh* in *Vibrio parahaemolyticus* strains isolated from a pristine estuary, *Applied and Environmental Microbiology*. 79(7), 2247–2252. https://doi.org. 10.1128/AEM.03792-12

Hentschel, U., Stelnert, M. & Hacker J. (2000). Common molecular mechanism of symbiosis and pathogenesis. *Trends Microbiol.* 8, 226- 231.Karim, M.R., Uddin, M.N., Rahman, M.K & Uddin A. (2018). Microbiological study of costal shrimp aquaculture production system of Bangladesh. *Journal of Biology and Life Science*. 9(1). https://doi.org.10.1016/s0966-842x(00)01758-3.

Lida, T. & Honda, T. (1997). Hemolysin sproduced by vibrios. *Journal of Toxicology: Toxin Reviews*, 16, 215-227. 7 https://doi.org.10.3109/15569549709016457

Lopes, C.M., Rabadao, E.M., Ventura, C., Cunha, S.D., Côrte-Real, R. & Meliço-Silvestre, A.A. (1993). A Case of *Vibrio alginolyticus* Bacteremia and Probable Sphenoiditis Following a Dive in the Sea. *Clinical Infectious Diseases*, 17, 299-300. https://doi.org 10.1093/clinids/17.2.299

Luo, P. & Hu, C. (2008). *Vibrio alginolyticus gyrB* sequence analysis and *gyrB*-targeted PCR identification in environmental isolates. *Diseases of Aquatic Organisms*, 82, 209-216. https://doi.org 10.3354/dao01984

Mustapha S., Mustapha Em., Brahim B. & Nozha C. (2012). Characterization of Vibrio alginolyticus *trh* Positive From mediterranean Environment in Tamouda Bay (Morocco). *World Environment*, 2, 76-80. https://doi.org.10.5923/j.env.20120204.04

Osorio, CR. & Klose, K.E. (2000). A region of the transmembrane regulatory protein *toxR* that tethers the transcriptional activation domain to the cytoplasmic membrane displays wide divergence

among Vibrio species. *Journal Bacteriology*. 182, 526–528. https://doi.org.10.1128/jb.182.2.526-528.2000

Reina, J., Fernandez-Baca, V. & Lopez, A. (1995). Acute gastroenteritis caused by *Vibrio alginolyticus* in an immunocompetent patient. *Clinical Infectious Diseases*, 21, 1044-5. https://doi.org.10.1093/clinids/21.4.1044

Stalin, N. & Srinivasan, P. (2016). Characterization of Vibrio parahaemolyticus and its specific phage from shrimp pond in Palk Strait, South East coast of India. *Biologicals*, 44:526–533 https://doi.org.10.1016/j.biologicals.2016.08.003

Tada, J., Ohashi, T., Nishimura, N., Shirasaki, Y., Ozaki, O., Fukushima, S., Takano, J., Nishibuchi, M. & Takeda, Y. (1992). Detectionof 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, 477-487. https://doi.org.10.1016/0890-8508(92)90044-x

Terzi, G., Büyüktanır, Ö. & Yurdusev, N. (2009). Detection of the *tdh* and *trh* genes in *Vibrio parahaemolyticus* isolates in fish and mussels from middle black sea coast of Turkey. *Letters in Applied Microbiology*. 49,757-763 https://doi.org.10.1111/j.1472-765X.2009.02736.x

TS/TS ISO 8914 (1998). Microbiology-General Guidance for the detection of *vibrio parahaemolyticus*.

Türk, N., Avsever, M.L. & Ün, C. (2011). Investigation of *tdh* and *trh* genes in *Vibrio parahaemolyticus* isoletes from isolated Bivalve molluscs. *Bornova Veteriner Bilimleri Dergisi*. 33, 29-36.

Xie, Z.Y., Hu, C.G., Chen, C., Zhang, L.P. & Ren, C.H. (2005). Investigation of seven Vibrio virulence genes among *Vibrio alginolyticus* and *Vibrio parahaemolyticus* strains from the coastal mariculture systems in Guangdong, China. *Letters in Applied Microbiology*, 41, 202-207. https://doi.org/10.1111/j.1472-765X.2005.01688.x

Weils, L.S. & Wendy, W. (2012). Characterization of *Vibrio alginolyticus* isolated from white leg shrimp (*Litopenaeus vannamei*) with emphasis on its antibiogram and heavy metal resistance pattern. *Veterinarski arhiv*, 82, 221-227.

Zhangx, H. & Austin, B. (2005). Haemolysins in *Vibrio* species. *Journal of Applied Microbiology* . 98, 1011-1019. https://doi.org.10.1111/j.1365-2672.2005.02583.x