Detection of *Tcpa* and *Ctxb* Virulence Genes in Isolates from Surface Waters and Salt Waters in Golestan, Iran

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ABSTRACT

Background and Objective: *Vibrio* is a genus of bacteria that are widely distributed in aquatic environments. The genus includes several important pathogens that endanger farm animals and humans who ingest seafood or water contaminated with the bacteria. Virulence of *Vibrio* spp. is regulated by *ctxAB* and *tcpA* genes. The aim of this study was to determine the prevalence of *Vibrio spp*., and *tcpA* and *ctxB* virulence genes in isolates from surface water and salt water samples collected from Golestan Province, Iran.

Methods: Overall, 115 water samples were collected from the Caspian Sea coast, lagoons and rivers in the Golestan Province. The samples were filtered by membrane filtration method, and enriched in alkaline peptone water with 1% NaCl. The isolates were grown on TCBS agar, and identified by biochemical tests. Presence of the *tcpA* and *ctxB* virulence genes was investigated by polymerase chain reaction.

Results: In this study, *Vibrio alginolyticus* was the predominant species (38%) isolated from the seawater and surface water samples, followed by *Vibrio parahaemolyticus* (23%), *Vibrio harvei* (15%), *Vibrio fluvialis* (14%) and *Vibrio damsela* (10%). The virulence genes were not detected in any of the isolates found in the study.

Conclusion: This study indicates that *V. alginolyticus* is the most prevalent *Vibrio* spp. in surface water and seawater samples collected from the Golestan Province, Iran.

Keywords: Environmental *Vibrio*, Surface water, *ctx B* gene, *tcpA* gene.

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INTRODUCTION

Vibrio species are gram-negative, motile, facultative anaerobic, oxidase positive and non-spore-forming bacilli. These bacteria are usually halophile and need salt for optimized growth, while some species are unable to grow without salt (1,2). Clinically significant *Vibrio* spp. are divided into two categories: dangerous organisms such as *Vibrio cholerae*, and low-risk organisms such as *Vibrio alginolyticus* (3). Cholera is an acute infectious disease, mainly transmitted through ingestion of food and water contaminated with *V. cholerae*. The main virulence factors of *V. cholera* are cholera toxin and toxin-coregulated pilus, which are encoded by the *ctxAB* and *tcpA* genes, respectively (4-6). *V. cholerae* is usually spread through infected humans, healthy carriers and environmental spp. that act as reservoirs for the pathogenic genes. The World Health Organization has classified Iran as a cholera-endemic country (7). Other significant non-cholera environmental *Vibrios* include *Vibrio parahaemolyticus* (causes gastroenteritis), *Vibrio vulnificus* (causes primary septicemia), *Vibrio alginolyticus (*causes cellulitis, bacteremia and wound infection), *Vibrio mimicus* (causes cholera-like infection) and *Vibrio fluvialis* (causes diarrhea) (8-10). The aim of this study was to determine the prevalence of *Vibrio* spp., and detect *tcpA* and *ctxB* virulence genes in isolates from surface water and salt water samples collected from the Golestan Province, Iran.

MATERIAL AND METHODS

Salt water samples were collected from different coastal areas of the Caspian Sea in the Golestan Province between August and September 2013. First, 80 salt water samples were collected in sterile glasses from depth of 30 cm, in an area 500 meters beyond the seashores. The samples were collected from Bandar Torkaman, Bandar Gaz, Bandar Nokandeh and Gomishan. In addition, 35 surface water samples were collected. First, 500 ml of the water sample was filtered by membrane filtration method. The membrane was then vortexed with 10 mM phosphate buffered saline (pH=7.4) and transferred to alkaline peptone containing 1% NaCl for enrichment for 16-18 hours. After culture on TCBS agar, the plates were incubated for 24-48 hours at 37°C. In order to identify genus and species, a set of standard biochemical tests including oxidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, triple sugar iron agar, and growth in 0% and 6% NaCl were performed (11).

DNA extraction was performed using phenol-chloroform-isoamyl alcohol method (12). Amplification reaction solution with a final volume of 50 µl contained 1U Taq polymerase, 5 µl 10x PCR buffer, 4 µl 50mM $MgCl_2$, 0.8 µl of each deoxynucleoside triphosphate, 1µl of each primer (20 pmol) and 5 µl of sample.

Amplification program for the *tcpA* gene started with initial denaturation at 94 ^{o}C for 5 min, followed by 35 cycles at 94 °C for 45 sec, annealing at 54 °C for 45 sec, elongation at 72 °C for 45 sec and final extension at 72 °C for 20 min.

The annealing temperature for detection of the *ctxB* gene was 55 °C, and final extension was done at 72 °C for 10 min. The products were electrophoresed on 1.5% agarose gel for 90 min at 90V. The PCR products were then visualized by ethidium bromide staining to evaluate the presence of *tcpA* and *ctxB* genes. Genomic DNA of *V. cholerea* ATCC62013 was used as positive control for the *tcpA* and *ctxB* genes. The primers used in this study are shown in Table 1.

RESULTS

Of 115 water samples collected, 100 *Vibrio* isolates were detected including 38 *V. alginolyticus*, 23 *V. parahaemolyticus*, 15 *V. harvei*, 14 *V. fluvialis* and 10 *V. damsel* isolates. *V. alginolyticus* was the most frequent strain isolated (Table 2).

Moreover, 74 *Vibrio* spp. were isolated from salt water samples consisting of 26 (35.14%) *V. alginolyticus*, 18 (24.32%) *V. parahaemolyticus*, 12 (16.21%) *V. harvei*, 11 (14.86%) *V. fluvialis* and 7 (9.4%) *V. damsel* isolates. Overall, 26 *Vibrio* strains were isolated from the surface water samples, including 12 (46.15%) *V. alginolyticus*, 5 (19.23%) *V. parahaemolyticus*, 3 (11.54%) *V. harvei*, 3 (11.54%) *V. fluvialis* and 3 (11.54%) *V. damsel isolates*.

No band related to the *tcpA* and *ctxB* genes was found in gel electrophoresis, which indicated that none of the isolates contained these virulence genes (Figures 1 and 2).

Table 1- Sequences of the primers used for the detection of the *tcpA* and *ctxB genes*

Target gene	Primer	Product size	Reference
tcpA	5' GAAGAAGTTTGTAAAAGAAGAACAC3' F	417bp	(13)
	5' GAAAGGACCTTCTTTCACGTTG3' R		
ctxB	5' GGTTGCTTCTCATCATCGAACCAC 3' F	460bp	(14)
	5' GATACACATAATAGAATTAAGGATG 3' R		

Table 2- Prevalence of Vibrio spp. in salt water and surface water samples collected from the Golestan Province

Species	Prevalence
V. alginolyticus	38%
V. parahaemolyticus	23%
V. harvei	15%
V. fluvialis	14%
V. damsela	10%

Figure 1- Agarose gel electrophoresis of the PCR product for detection of the *tcpA* gene. M: 100bp DNA ladder, C+: positive control, C-: Negative control, 1-4: Samples

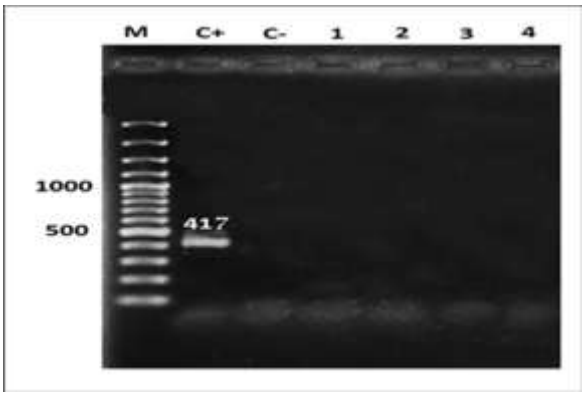
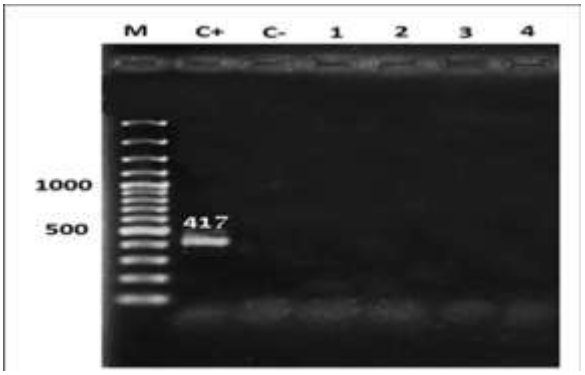


Figure 2- Agarose gel electrophoresis of the PCR product for detection of the *ctxB* gene. M: 100bp DNA ladder, C+: positive control, C-: Negative control, 1-4: Samples



DISCUSSION

In this study, 5 *Vibrio* species including *V. alginolyticus, V. parahaemolyticus, V. fluvialis, V. harveyi* and *V.damsela* were isolated from the northern shores of the Caspian Sea and a number of international lagoons along the coastline. All the isolated strains are human pathogens except for *V. harveyi*. It has to be noted that *V. damsela* is able to infect fish as well as humans. Pathogenicity of *V. harveyi* was only reported in a shrimp called *Penaeusmondo* (15).

The most prevalent species found in the study was *V. alginolyticus*, a halophilic human pathogen that can cause wound infections. The highest frequency of *V. alginolyticus* was observed in salt waters of Bandar Torkaman (38.4%) and surface waters of Alagol lagoon (33%), respectively. In a study by Zorrilla et al. in Spain, 34 *V. alginolyticus* isolates were detected in cultured fish, which is consistent with the findings of the present study (16). *V. parahaemolyticus* is a halophilic bacterium that usually spreads in marine environments, and causes gastroenteritis, vomiting, diarrhea, and fever in humans. This bacterium was the second most prevalent isolate (23%) found in Bandar Torkaman, Bandar Gaz, Bandar Nokandeh and Gomishan. The frequency of *V. parahaemolyticus* was highest in surface waters of Alagol and Ajigol lagoons (40%) and in seawaters of Bandar Torkaman and Gomishan (33.4%). A study in Nigeria studied 120 seafood and seawater samples and found *V. parahaemolyticus* in 18.9% of the seafood samples, indicating the impact of *Vibrio* on the life of aquatic animals in seawaters (17). Another study in Nigeria reported the presence of *V. parahaemolyticus* in 21% and 23.9% of

the seawater samples and shells, respectively. These results are consistent with the findings of the present study (18).

We found *V. harveyi* in 15% of the samples collected from this region. The bacterium usually infects fish and some shrimp species such as *Penaeus monodon*, which are of great importance in fishery industry. *V. harveyi* was mostly frequent in seawaters of Gomishan (75%). Consistent with our findings, Rosaanna et al. found *V. harveyi* in 14% of seawater samples and 4% of shells (19). However, a study in China reported higher frequencies for *V. harveyi* compared to our study (20).

The frequency of *V. fluvialis* was 14% in the present study. This bacterium can cause wound infection, gastroenteritis and sepsis syndrome. The frequency of *V. fluvialis* was highest in Bandar Torkaman and Gomishan (36%). Onyedikachukwu et al. reported the frequency of *V. fluvialis* as 18.31% in seawater samples, which is higher compared to our study (18). In a study in Italy, Sechi et al. studied the distribution of *V. cholerae* virulence genes in environmental *Vibrios*. They reported the presence of *V. fluvialis* in 3.84% of seawater samples, which is lower compared to our study (21).

V. damsela was the least prevalent isolate (10%) in our study. The frequency of this bacterium in was highest (43%) in seawater samples from Bandar Torkaman. Since Bandar Torkaman is a fishery center in this region, isolation of *V. damsel* is of great importance. Croocki et al. studied the prevalence of *Vibrio* spp. in oysters and seawater samples and found *V. damsel* in 10.88% of the seawater samples, which is consistent with our findings (22). In another study in Iran, Halakoo et al. isolated *V. damsel* from 1% of seawater samples collected from coastal areas of the Caspian Sea, which is similar to the results of the present study (23).

V. vulnificus is a deadly and fatal species that can cause septicemia, wound infection and gastroenteritis in humans. This microorganism was not detected in any of the samples collected in the present study. This bacterium is usually present in waters with temperature of 8-31^{o}C and salinity of 1-34 g/lit. Thus, the surface waters are not salty enough for this organism to survive (24). *V. cholera* and *V. mimicus* were not found in seawater and surface water samples collected in the present study. This could be due to the high salinity of the water in the Caspian Sea and lagoons. Similarly, *V. cholerae* and *V. mimicus* were not isolated from the samples collected from waterfalls, which could be attributed to water temperature and lack of contamination with wastewater.

We also investigated the presence of *tcpA* and *ctxB* virulence genes in *Vibrio* spp. isolated from the samples. However, the virulence genes were not detected in any of the isolates. Similar to the results of the present study, a study in Italy found no virulence gene in environmental *Vibrio* spp. (21). Study of Ruwandeepika et al. in Belgium also found no virulence gene among 48 *V. harveyi* isolates (25). Study of Ren et al. in China reported the presence of the *ctxB* gene in 6 of 31 environmental *V. alginolyticus* isolates from seawater and fish samples. However, the *ctxA and tcpA* genes were not detected in any of the isolates. These results are partially consistent with the findings of our study (26).

CONCLUSION

The pathogenic *V. alginolyticus* is the most prevalent *Vibrio spp*. among the isolates found in surface water and seawater samples of the Golestan Province. The *Ctxb* and *tcpA* virulence genes are not present in the isolates found in the areas studied.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES

1. Thompson FL, Iida T, Swings J. *Biodiversity of vibrios. Microbiology and molecular biology reviews.* 2004; 68(3): 403-31. DOI:10.1128/MMBR.68.3.403-431.2004

2. Janda J, Powers C, Bryant R, Abbott S. *Current perspectives on the epidemiology and pathogenesis of clinically significant Vibrio spp.* Clinical Microbiology Reviews. 1988; 1(3): 245-67.

3. Austin B. *Vibrios as causal agents of zoonoses.* Veterinary microbiology. 2010;140(3):310-7. DOI:10.1016/j.vetmic.2009.03.015.

4. Pal P. *Role of cholera toxin in Vibrio cholerae infection in humans-A Review.* International Letters of Natural Sciences. 2014; 22: 22-32.

5. Faruque SM, Kamruzzaman M, Meraj IM, Chowdhury N, Nair GB, Sack RB, et al. *Pathogenic potential of environmental Vibrio cholerae strains carrying genetic variants of the toxin-coregulated pilus pathogenicity island.* Infection and immunity. 2003; 71(2): 1020-5. PMCID:PMC145385.

6. Faruque SM, Nair GB. *Molecular ecology of toxigenic Vibrio cholerae.* Microbiology and immunology. 2002; 46(2): 59-66.

7. Daniels NA, Shafaie A. *A review of pathogenic Vibrio infections for clinicians.* Infections in medicine. 2000; 17(10): 665-85.

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

9. Mukherji A, Schroeder S, Deyling C, Procop GW. *An Unusual Source of Vibrio alginolyticus–Associated Otitis: Prolonged Colonization or Freshwater Exposure?* Archives of Otolaryngology–Head & Neck Surgery. 2000; 126(6): 790-1.

10. Hossain A, Haider K, Huq M, Zaman A. *Studies on the factors affecting the haemolysin production of Vibrio mimicus isolated from clinical and environmental sources.* Transactions of the Royal Society of Tropical Medicine and Hygiene. 1988; 82(2): 337-9.

11. Alsina M, Blanch AR. *A set of keys for biochemical identification of environmental Vibrio species.* Journal of applied bacteriology. 1994; 76(1): 79-85.

12. Abd-Elghany SM, Sallam KI. *Occurrence and molecular identification of Vibrio parahaemolyticus in retail shellfish in Mansoura, Egypt.* Food control. 2013; 33(2): 399-405.

13. Sharma A, Chaturvedi AN. *Prevalence of virulence genes (ctxA, stn, OmpW and tcpA) among non-O1 Vibrio cholerae isolated from fresh water environment. I*nternational journal of hygiene and environmental health. 2006; 209(6): 521-6.

14. Rajpara N, Vinothkumar K, Mohanty P, Singh AK, Singh R, Sinha R, et al. *Synergistic effect of various virulence factors leading to high toxicity of environmental V. cholerae Non-O1/Non-O139 isolates lacking ctx gene: comparative study with clinical strains.* PloS one. 2013; 8(9): e76200.

15. Harris L, Owens L, Smith S. *A selective and differential medium for Vibrio harveyi.* Applied and environmental microbiology. 1996; 62(9): 3548-50.

16. Zorrilla I, Moriñigo M, Castro D, Balebona M, Borrego J. *Intraspecific characterization of Vibrio alginolyticus isolates recovered from cultured fish in Spain.* Journal of applied microbiology. 2003; 95(5): 1106-16.

17. Adebayo-Tayo B, Okonko I, John M, Odu N, Nwanze J, Ezediokpu M. *Occurrence of potentially pathogenic Vibrio species in Sea foods obtained from Oron Creek.* Advances in Biological Research. 2011; 5(6): 356-65.

18. Eyisi OA, Nwodo UU, Iroegbu CU. *Distribution of Vibrio species in shellfish and water samples collected from the Atlantic coastline of south-east Nigeria.* Journal of health, population, and nutrition. 2013; 31(3): 314-320.

19. Cavallo RA, Stabili L. *Presence of vibrios in seawater and Mytilus galloprovincialis (Lam.) from the Mar Piccolo of Taranto (Ionian Sea).* Water Research. 2002; 36(15): 3719-26.

20. Bai F, Pang L, Qi Z, Chen J, Austin B, Zhang X-H. *Distribution of five vibrio virulence-related genes among Vibrio harveyi isolates.* The Journal of general and applied microbiology. 2008; 54(1): 71-8.

21. Sechi LA, Duprè I, Deriu A, Fadda G, Zanetti S. *Distribution of Vibrio cholerae virulence genes among different Vibrio species isolated in Sardinia, Italy.* Journal of applied microbiology. 2000; 88(3): 475-81.

22. Croci L, Serratore P, Cozzi L, Stacchini A, Milandri S, Suffredini E, et al. *Detection of Vibrionaceae in mussels and in their seawater growing area.* Letters in Applied Microbiology. 2001; 32(1): 57-61.

23. Amirmozafari N, Forohesh H, Halakoo A. *Occurrence of pathogenic vibrios in coastal areas of Golestan province in Iran.* Archives of Razi Institute. 2005; 60(1): 33-44.

24. Høi L, Larsen J, Dalsgaard I, Dalsgaard A. *Occurrence of Vibrio vulnificus biotypes in Danish marine environments.* Applied and environmental microbiology. 1998; 64(1): 7-13.

25. Ruwandeepika H, Defoirdt T, Bhowmick P, Shekar M, Bossier P, Karunasagar I. Presence of typical and atypical virulence genes in vibrio isolates belonging to the Harveyi clade. Journal of applied microbiology. 2010;109(3):888-99. DOI:10.1111/j.1365-2672.2010.04715.x.

26. Ren C, Hu C, Jiang X, Sun H, Zhao Z, Chen C, et al. *Distribution and pathogenic relationship of virulence associated genes among Vibrio alginolyticus from the mariculture systems.* Molecular and cellular probes. 2013; 27(3): 164-8. DOI:10.1016/j.mcp.2013.01.004.