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Short Communication

Molecular identification of *Vibrio* species in waste water urban effluent of the Persian Gulf: Implications for shrimp industry

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Introduction

Litopenaeus vannamei is a species of shrimp that is cultivated throughout the Persian Gulf, Bushehr province. It has been recognized as the pioneer of the shrimp industry in the country with 7% of shrimp cultivation (Mohsen & Yang, 2021).There are 10 sites and 290 shrimp farms in Bushehr province, with 4,800 hectares of earthen ponds and one and a half million shrimp larvae stored there (FAO, 2018).

Vibriosis is one of the most important problems in the aquaculture of shrimp. Growth, propagation, and aquaculture industry due to greater use of natural resources also disturb the natural balance of life. Samocha and Lawrence (1995) states that there are two general considerations regarding the positive and negative effects of these changes on the ecosystem , and Hafezi *et al.* (2020) believes that the uncontrolled development of these industries could have devastating effects on coastal ecosystems. Barg (1992) argues that breeding has a positive impact on the environment and its development can have positive effects such as job creation, increasing income, helping to rebuild indigenous reserves in the area. Among the factors that are considered in the field of breeding and rearing is the physical and

chemical quality of the water entering the pools, the outlet water (effluent) as well as the study of the possible effects of the effluent on the marine environment receiving it. Waters off farms (sewage), including switching waters during the breeding period, mud pools, and water washing after harvest their Due to the high consumption of nutrients especially proteins as well as fertilizers during the growing season, the effluents contain large amounts of organic and inorganic minerals with little soluble oxygen. Pool And because of their entry into the open waters (seas), there are likely to be changed in the marine environment that can be attributed to the risk of high nitrification, high fertility with increased early production, and possibly high planktonic blooms, reduced oxygen and increased numbers of micro-organisms. (Raven *et al*, 2011). with the beginning of the breeding industry in Iran, the issue of its development and the capacity of the Persian Gulf as a source of wastewater has been raised. Finally, since 1998, the impact of aquaculture on the environment has been considered and studies have been carried out in the region continuously. Boushehr hills and batch in other breeding areas. The results of these researches have shown that farms of Helle (1377-1381), (Paighambari and Daliri, 2012) with contamination load during sampling period are not polluting factor for marine environment but due to the existing ambiguities, it was suggested that this research be continued over several years. Wastewater is domestic effluent, water from institutions stormwater, and other types of urban run-off. Wastewater composition characterized by turbidity, temperature, pH, dissolved oxygen, chlorine, suspended solids, nutrients, toxic substances, and biological or microbial contents. Industrial and commercial activities show some wastewater-related contaminants. These factors also subsequently affect the discharge patterns of the wastewater and could impact the chemical and microbial status of treated wastewater final effluent, in turn impacting the surrounding water bodies. One of the dominant bacterial species in seawater is a Gram-negative bacterium from the family *Vibrionaceae*. Vibrio species (Chaijarasphong *et al*, 2020). are part of the natural micro flora of wild and cultured shrimps (Kongnum *et al*, 2012) and become opportunistic pathogens when natural defense mechanisms are suppressed (Brock and Lightner, 1990) It is widely distributed in culture facilitates and some cause gastrointestinal infections (Barbieri *et al.*, 1999). Vibrio infection occurs mainly in reproductive centers. One of the most important bacterial diseases associated with aquaculture as vibriosis. This is, a bacterial disease that is responsible for mortality in shrimp cultivation worldwide (Balcázar *et al*; 2010) Vibriosis is caused by a number

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of Vibrio species of bacteria, including *V. harveyi, V. vulnificus, V. parahaemolyticus, V. alginolyticus, V. penaeicida* (Brock and Lightner, 1990) opportunistic pathogens for mass mortalities of cultured shrimp that can be triggered by the stressful environment. Also, excessive consumption of antibiotics in the aquaculture industry has caused severe economic loss and the establishment of antibiotic-resistant bacteria. Vibrio-infections occur frequently in hatcheries, and frequently in shrimp reared in ponds (Barbieri *et al.*, 1999). The incidence of the disease occurs when environmental factors trigger the rapid proliferation of bacteria; these bacteria are present in normal conditions with low levels of shrimp in the blood and tolerated by the animal (Aguirre-Guzmán *et al.*, 2001). Physical barrier to pathogens trying to penetrate the outer surface of the crustaceans, as well as the anterior and posterior intestines. Vibrio spp. the bacteria associated with the disease shell and can enter through wounds into the exoskeleton (Jiravanichpaisal and Miyazaki, 1994; Alday-Sanz *et al*,. 2002). Vibriosis syndromes include oral and enteric vibriosis, appendage and cuticular vibriosis, localized vibriosis of wounds, shell disease, systemic vibriosis, and septic hepatopancreatitis (Chaijarasphong *et al*, 2020). The gill is covered with a thin shell, so it is more sensitive to bacterial penetration, but its surfaces are cleaned by seto branchs. Aquatic intestines are not covered with skin, so it is the most prone point for penetration of pathogens in water, blood, and other sediments (Martinez-Diaz *et al.*, 2013). The objective of the present study is the identification of Vibrio species in wastewater urban effluent of the Persian Gulf.

Materials and methods

Sample collection

Water samples were collected from two main wastewater outlet channel in Bushehr province to the Persian Gulf and were transferred to the Microbiology laboratory of the Shrimp Research Center in the vicinity of dry ice bags.

Bacterial identification

Identification of bacterial isolates was performed by phenotypic, biochemical and molecular tests (PCR and sequencing).

Molecular tests

The 16S rRNA gene of identified bacteria was amplified (Table 1 for conditions and master mix) using specific primers including forward: 5-GAGTTTGATCCTGGCTCAG-3 and reverse: 5-ACGGGCGGTG+GTRC-3 by PCR (Mirbakhsh and *et al*., 2014). After that PCR was performed for the amplification of the 16S rDNA gene, the gene was cloned into *Escherichia coli* DH5α after insertion into the pTZ57R/T cloning vector and after replication of plasmid; it was extracted

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and was purified by enzymatic digestion. Plasmid extraction was conducted based on silica column (spin) alkaline lysis with IBRC Plasmid DNA Extraction kit (Mini-Prep) Cat NO. MBK0051 according to the instructions of the manufacturer. In addition, sequencing was performed according to instructions by ABI 3730xl kit manufacturer purchased from GATC-Biotech (Table 1).

Table 1: PCR reaction conditions and thermal profile for the amplification of 16S rDNA gene.

PCR component	Amount	Thermal profile
10X Taq Buffer	0.5 µl	94°C (5min)
dNTP Mix,2mM each	1.0 µl	94°C (1min)
27F	0.5 µl	62°C (40s)
1392R	0.5 µl	72°C (80s)
25Mm MgCl2	1.5µl	72°C (10min
Template DNA	50-100 ng	
Taq DAN Polymerase	0.5 µl	4°C (1hr)
Water, nuclease-free	up to 50 µl	
Total Volume	50 µl	

Antibiotic susceptibility test

The antibiotic susceptibility test was performed according to that advised by clinical and laboratory standards institute (CLSI) version 2014. The antibiotic disks including ampicillin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, gentamicin, imipenem, and tetracycline were employed.

Results

Bacterial isolates

Obtained colonies were identified by phenotypic biochemical and molecular tests as followings: green fluirescent colonies on TCBS medium, oxidase-positive, glucose fermentation in aerobic and anaerobic conditions, and fermentation of mannitol, sucrose, sorbitol, rhamnose, cellobiose, and consumption of gelatin. Further phenotypic tests including growth in salt (0-10%), and 42°C, and positive results for lysine-decarboxylase and ornithine-decarboxylase and MR were observed.

Molecular identification

the PCR was performed for the amplification of 16S rDNA gene. The gene was cloned into *Escherichia coli* DH5α and after replication of plasmid, it was extracted and the gene was pured by enzymatic digestion. Next the replicated pTZ57R/T cloning vector was extracted and digested and the product was sequenced.

Based on the results of 16srDNA molecular analysis of strain isolated using BLAST and EZ TAXON DATABASE, this bacterium belonged

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to Gamma-proteobacteria, *Vibrionacea* family, and *Vibrio* genus and was 100% similar to *V. alginolyticus*, so the results of the software were recorded the strain SeqID-10 in the World GenBank, and its nucleotide sequence was as followings (Fig. 1):

1 aaagaggggg accttcgggc ctctcgcgtc aggatatgcc taggtgggat tagctagttg
61 gtgaggtaag ggctcaccaa ggcgacaatc cctagctggt ctgagaggat gatcagccac
121 actggaactg agacacggtc cagactccta cgggaggcag cagtggggaa tattgcacaa
181 tgggcgcaag cctgatgcag ccatgccgcg tgtgtgaaga aggccttcgg gttgtaaagc
241 actttcactc gtgaggaagg tagtgtagtt aatagctgca ttatttgacg ttagcgacag
301 aagaagcacc ggctaactcc gtgcccccag ccgcggtaat acggagggtg cgagcgttaa
361 tcggaattac tgggcgtaaa gcgcatgcaa gtggtttgtt aagtcagatg tgaaagcccg
421 gggctcaacc tcggaatagc atttgaaact ggcagactag agtactgtag agggggggtag
481 aatttccgcg cgtagcggtg aaatgcgata tacatctgaa ggaataccgc tggcgaaagt
541 ccgcccccccg gacagattct gacactctga tatgaaaaag tgggtgatca aaaaggaata
601 cactgctctg tgtatttccg gccttaaaaa aacatctacc tggcaggttt

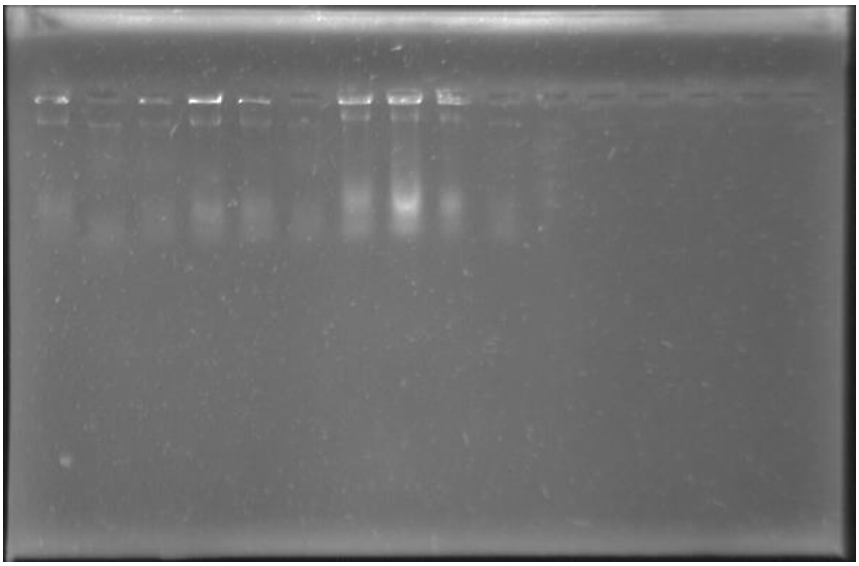


Figure 1: the PCR product of 16S rDNA gene amplified.

Discussion

Urban and industrial effluent discharge to the seawater accompanied with some problems. These problems have threated for the environmental, food safety, health human, and aquatic. Since most hatcher centers and farms of shrimp take water directly from seawater, it is possible to transfer any contamination to the system. Vibriosis is one of the most important diseases in the shrimp industry. Among Vibrio species known to be associated with shrimp disease, *V. harveyi* (Mirbakhsh *et al*., 2014), *V. parahaemolyticus*, *V. alginolyticus*, and *V. vulnificus* are most important. These agents cause a lot of economic losses annually. One of the most important preventative proceedings is to provided specific pathogenic free of seawater.

Given the development of the shrimp industry over the past twenty years, the risk of the spread of some diseases has

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always threatened the industry. One of the most important pathogenic factors is the bacterial agents belonging to the *Vibrionaceae* family that cause vibriosis in the *Penaeidae* family shrimp and reduce growth and survival in breeding centers and shrimp farms (Martinez-Diaz *et al.*, 2013; De Schryver *et al.*, 2014). *V. harveyi*, *V. alginolithicus*, *V. parahaemolyticus*, *V. volnificus*, *V. damsella*, and *V. pennsace* are among the most important pathogenic species of the *Vibrionaceae* family in the Penaeidae shrimp family (Swain *et al.*, 2009; Kandasamy *et al.*, 2013). In addition to reducing the growth and survival rate of Penaeida shrimp family, these pathogens have made always irreparable damage to the industry of propagation and breeding of shrimp (Govindasamy *et al.*, 2019). Water contamination with these pathogens may lead to an uncompesate outcome in shrimp production. Therefore, the accurate identification and control of the mentioned factors have always been one of the main concerns of shrimp breeding centers. By the results of biochemical/phenotypic and molecular tests, bacterial species *V. alginolyticus* (MK641453.1) was identified and isolated according to World Bank No. MK641453.1, *V. alginolyticus (MK641453.1)* was similarly isolated in India with phenotypic tests, and also genotyping was performed by 16S rRNA sequence analysis with the ability to produce PHB (Gómez *et al.*, 2005). However, currently, various methods, including the use of antiseptic chemicals and broad-spectrum antibiotics such as oxytetracycline, are used to control and treat the bacterial contaminations, but due to the occurrence of drug resistance and the persistence of these compounds in shrimp tissues, transferred by contaminated water, the consumption of synthetic antimicrobials has been restricted in this industry (Quiroz-Guzmán *et al.*, 2018).

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