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Investigation of toxogenic saxitoxin cyanobacteria by PCR method in Hormozgan province, Persian Gulf, Iran

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Abstract

Introduction: Toxic cyanobacteria include Anabaena flos-aquae, Microcystis aeruginosa, Oscillatoria, and Planktotherix. Cyanotoxins fall into four categories: neurotoxin, hepatotoxin, cytotoxin, dermototoxin .The more critical toxins in Cyanobacteria are saxitoxin. It affects the nervous system and respiration. The cyanobacteria produce toxins that put water health at risk. This poison is one of the neurotoxins that is transmitted through the nervous system. The best-known poison is the paralysis of crustaceans (PST). Materials and Methods: This Study aims to identify saxitoxin-producing cyanobacteria in Hormozgan in the Persian Gulf rapidly. Twenty water samples were collected from different stations in the Persian Gulf. DNA extracted by modified DNG method kit. The standard DNA toxogenic strains of Anabaena circinalis (AWQC131C) optimized polymerase chain reaction (PCR) tests. They subsequently evaluated them for specificity and sensitivity. Amplicon in R57PTZ plasmid for sequencing and T-positive control A cloning method optimized the PCR test. Results: Nine out of twenty samples were collected at different stations observed. This study shows the stations located in the estuaries (Tiab, Darsarokh, Jalabi) contain saxitoxin-producing cyanobacteria. Conclusion: In the estuary areas, there is a lot of urban and industrial sewage, which increases harmful cyanobacteria, and with the increase of cyanobacteria, fish die, fishing, economic problems, and pollution increase. Contamination of crustaceans and fish has caused human poisoning, so we must prevent sewage from entering the estuary to avoid the rise of cyanobacteria and toxin production.

Keywords: *Saxitoxin, Cyanobacteria, Hormozgan, Persian Gulf*

Introduction

Cyanobacteria produce many side metabolites with antibiotic, antifungal, and cytotoxic effects[1]. Blooms of cyanobacteria in lakes, rivers, and estuaries cause problems such as producing dangerous toxins in water[1, 2]. These toxins cause taste and smell in the water beneath the secondary metabolites and cause disease and even death in humans[3, 4]. Toxic cyanobacteria include *Anabaena flos-aquae*, *Microcystis aeruginosa*, *Oscillatoria* , and *Planktotherix*. Cyanotoxins fall into four categories: neurotoxin, hepatotoxin, cytotoxin, dermototoxin[5] [6, 7]. Human, industrial, and agricultural wastewater activities contain many nutrients. They are essential factors in propagating harmful algae blooms [8]. A paralyzing shellfish poison (PSP) blocks the sodium channels in the nerve cells.[5]One of the more common neurotoxins is saxitoxin.[9] [10]The toxin blocks the nervous connections and causes death by paralysis of the respiratory muscles, contrary to the symptoms of its intoxication by digestion and consumption of crustaceans.[11]One way to diagnose cyanobacteria and their toxins is through PCR.[12, 13] In this study, the molecular detection of saxitoxin that produced cyanobacteria is considered a high level of toxins produced in the Persian Gulf due to human pollution.

Materials and Methods

First, we selected twenty stations in the western, central, and eastern parts of the Hormozgan and were collected samples (Figure 1).

Figure1.Satellite imagery of stations- Persian Gulf.

The geographical latitude and longitude for these stations are indicated in Table 1.

Sampling stations	latitude	longitude	Sampling stations	latitude	longitude
S1	26°53'50.5"	56°22'53.6"	S11	27°04'26.1"	56°48'13.6"
S2	26°53'0"	56°24'300"	S12	27°05'110"	56°49'242"
S3	26°53'235"	56°23'570"	S13	27°05'220"	56°50'100"
S4	26°55'0"	56°27'500"	S14	27°04'26.4"	56°43'51.8"
S5	26°56'53.9"	56°30'43.7"	S15	27°05'32.4"	56°38'5304"
S6	26°58'500"	56°35'0"	S16	27°06'28.8"	56°33'27.4"
S7	27°0'01.0"	56°34'58.9"	S17	27°08'15.5"	56°28'56.7"
S8	27°0'58.9"	56°39'39"	S18	27°09'09.7"	56°22'01.2"
S9	27°01'37"	56°43'17"	S19	27°09'09.9"	56°22'01.4"
S10	27°02'240"	56°48'000"	S20	27°10'44.6"	56°19'10.8"

Table1. Geographic latitude and longitude of sampling stations of Persian Gulf.

The surface water from each station takes a dangling modifier. Then, they were immediately placed in a box containing ice freeze and transferred to the laboratory in dark weather at a temperature of 4°C. Each sample was centrifuged at 12000 rpm for 5 minutes, the supernatant was removed, and the pellet was collected and mixed with 100µl of deionized water. The samples were then held at -22°C for 24 hours, and finally, the DNA was extracted from the samples with a DNG extraction kit.[14]. DNG-Plus kits are used for DNA extraction based on the protocol of the kit.[8, 15].

Optimization of the PCR test for each primer pair was achieved using a standard DNA strain of *Anabaena circinalis* (code number: AWQC131C) and general primers (CYA359F, CYA78IR) for cyanobacteria detection[15], and the specific primers (sxtAF,sxtAR) for Saxitoxin-coding gene[16]. Furthermore, The specificity and sensitivity of the molecular detection test were also assessed [5] [5]. PCR test using DNA extracted from standard strains *Anabaena circinalis* AWQC131C optimized with the primers listed (Table 2).

Gene	**Primer sequences 5′→3′**	**Product Size of PCR(bp)**
CYA359 F	GGGGAATYTTCCGCAATGGG	487
CYA781 R	GACTACWGGGGTATCTAATCCCWTT	
sxtAF	AGGTCTTCTTGACTTGCATCCAA	602
sxtAR	AACCGGCGACATAGATGATA	

Table 2. Universal primers for cyanobacteria detection and the primers specific for Saxitoxin-coding gene

As shown in Figure 2-A, Electrophoresis of the PCR product appeared in a specific band of the intended Amplicon at 602 bp in length, which was slightly higher than the 500bp size marker for saxitoxin. As shown in Figure 2-B, the 487 bps long was visible for cyanobacteria-specific primers.

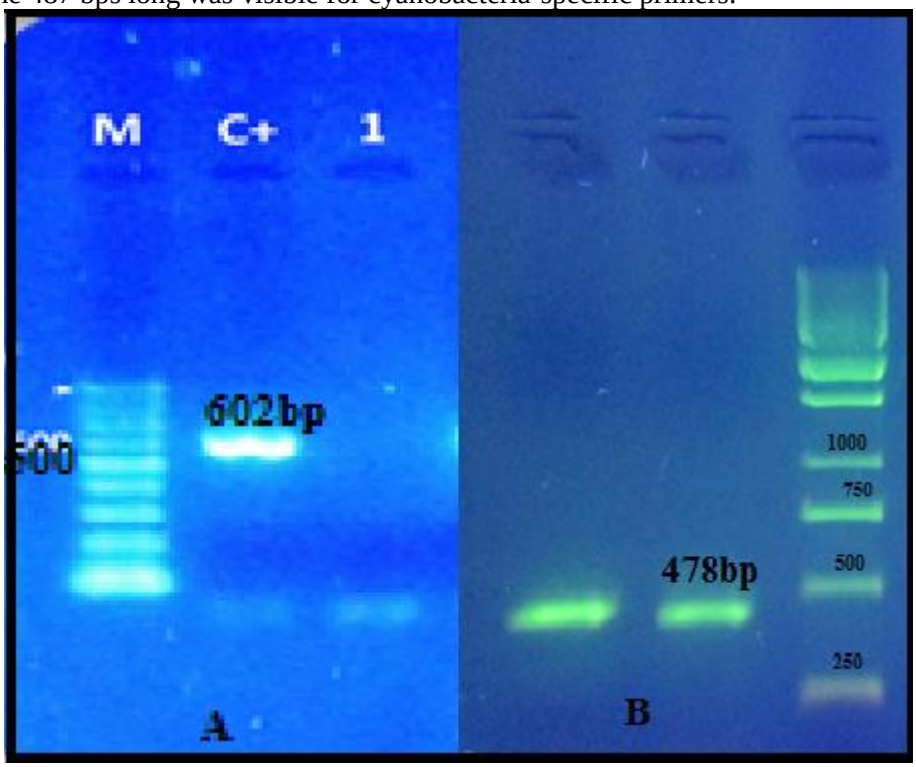

Figure 2. Optimized PCR assay for detection of (A) Saxitoxin-coding gene, and (B) cyanobacteria (*Microcystis aeruginosa* PCC7806, *Anabaena circinalis* AWQC131C)

Results

The specificity of primers toward cyanobacteria and saxitoxin detection was examined. The results showed that our proposed primers met saxitoxin more significantly than the others. Figure 3-A Indicates the strong specificity of the primers, which means they may be used for detecting cyanobacteria and their toxin-producing gene (figure 3-B).

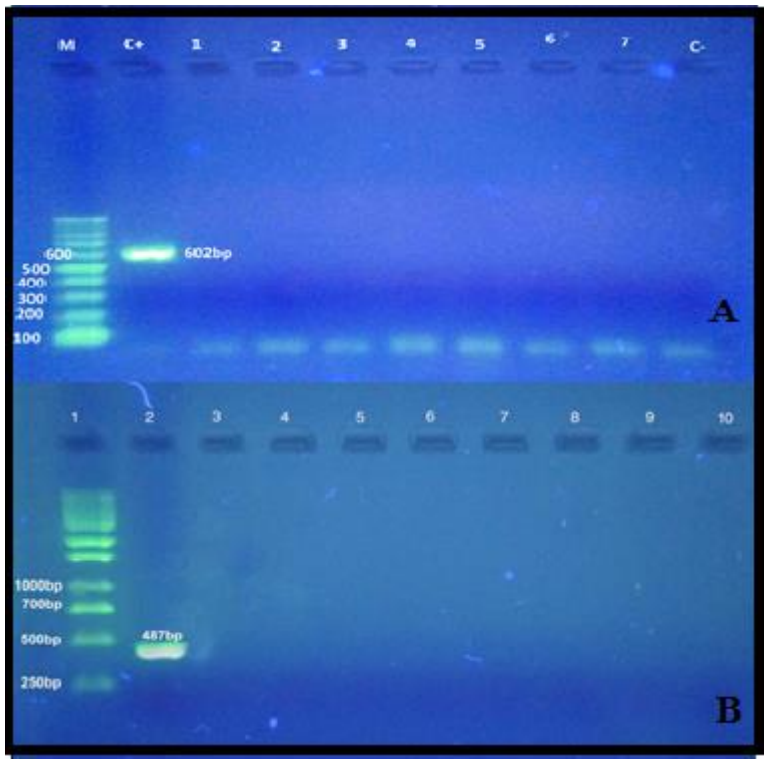

Figure 3. A- Specificity of PCR for detection of Saxitoxin; ; M)1Kb DNA Ladder Fermentas size marker, C+)Positive control, 1)Nostoc, 2)Anabeana, 3)Osilatora, 4)Ecoli, 5)Staphilococous aereus, 6)Legionella, 7)CMV, and C-)Negative Control.

B- Specificity of PCR for detection of Cyanobacteria; 1)1Kb DNA Ladder Fermentas size marker, 2) Positive control, 3)*Staphylococcus spp*., 4)*Staphylococcus aureus*, 5)*Legionella Pneumophila*, 6)Ecoli, 7)*Pseudomonas aeruginosa*, 8)Human DNA, 9)Mice DNA, and 10)Negative Control

As shown in Figure 4, the PCR sensitivity for the detection of saxitoxin was calculated, and the minimum number of bacteria used to detect the gene was 10 with different dilutions.
The Hormozgan stations used cyanobacteria to produce saxitoxin. According to this, 9 to 20 superficial samples of water are infected with saxitoxin.

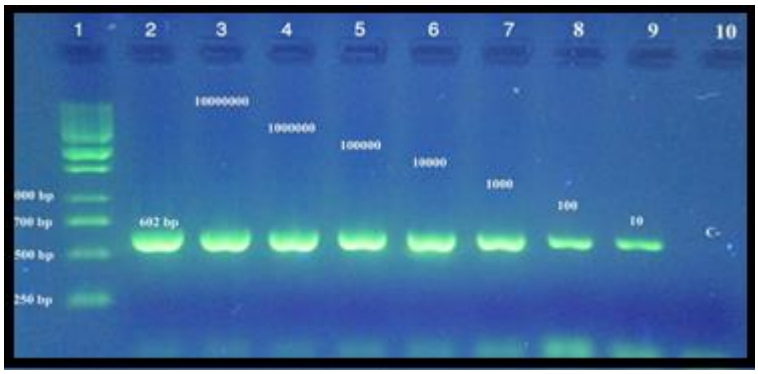

Figure 4. Sensitivity of PCR for detection of Saxitoxin; 1) 1Kb DNA Ladder Fermentas size marker, 2) Positive control, 3) 10000000 Genome, 4) 1000000 Genome, 5) 100000 Genome, 6) 10000 Genome, 7) 1000 Genome, 8) 100 Genome, 9) 10 Genome, and 10) Negative control.

The station´s survey showed that: it can conclude that the stations located in the estuaries (Tiab, Darsarokh, Jalabi) contain cyanobacteria producing saxitoxin. (Figure 5A,B).

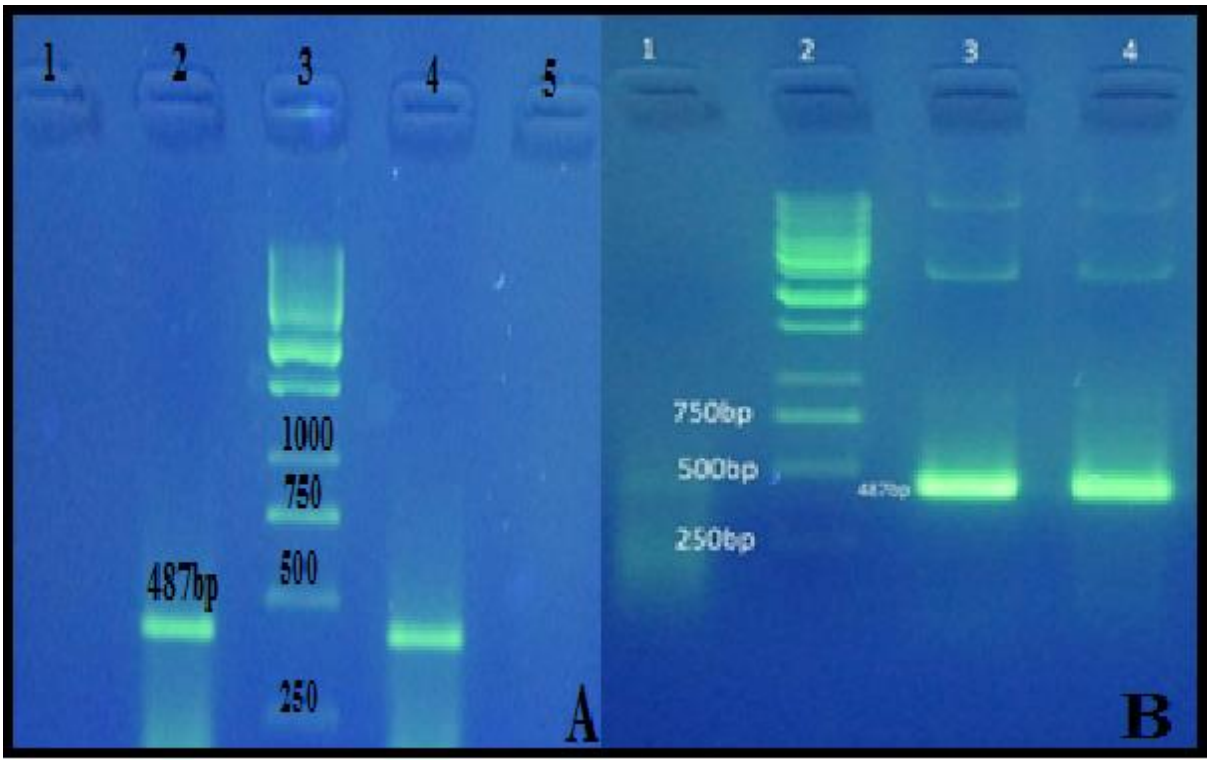

Figure 5. A- Confirmation of Cyanobacteria cloning product by PCR 1,5) Negative Control, 3) 1Kb DNA Ladder Fermentas size marker, 2,4) Insert fragment of 487 bp result proliferation.

Discussion

The increase of cyanobacteria causes the death of many marine organisms, such as fish and mollusks, and ultimately causes water pollution and the end of humans. Blooming cyanobacteria can produce biologically active secondary metabolites, which are highly toxic to humans and other organisms. From a toxicological point of view, cyanotoxins can be categorized into four main categories: neurotoxin, hepatotoxin, cytotoxin, and dermatotoxin[17]. Anabaena and Microcystis spp caused a deadly outbreak of toxic cyanobacteria in drinking water and caused the death of 88 children out of 2000 cases of abdominal edema in forty-four days[18]. Environmental factors are known to be practical factors in the deadly bloom form, including temperature, PH, light quantity, and nutrient concentration[17]. Around the world, hepatotoxin from freshwater cyanobacterial blooms are more abundant than neurotoxin blooms[19]. Several studies show that Dinoflagellates do not produce saxitoxin, but co-culture is produced by symbiotic bacteria, [5]. Saxitoxin (STX) is one of the neurotoxins produced in red shrimp by several species of freshwater cyanobacteria and dinoflagellate, *Anabaena circinalis, Aphanizomenon spp, Lyngbya wollei, Cylindrospermopsis raciborskii*(18). Saxitoxin is a neurotoxin that affects the environment and human health.

Cyanobacteria-producing toxins have been seen in different countries; for example, *Anabaena flos-aquae* was introduced in Canada as a producer of neurotoxins and anatoxin A [15]. Homoanatoxin A was found only in the Anabaena flos-aquae species in America and Scotland and the Anabaena lemermanii species in Denmark [21, 22]. The blooming of Aphanizomenon flos-aquae in the United States has long been considered the only major producer of saxitoxin among cyanobacteria. The species of Lynbya wollei in North America and Cylindrospermopsi raciborskii species in Brazil were introduced as saxitoxin producers. The cause of saxitoxin in rivers in Australia is anabaena circinalis[22, 23]. The cyanobacterium Nodularia spumigena frequently produces nodularin in salt water, such as the Baltic Sea or salty lakes and estuaries in Australia and New Zealand[24, 25].

In this study, the presence or absence of Saxitoxin-producing cyanobacteria was measured at 20 stations in the Hormozgan region in the autumn of 2012 using the molecular PCR method. In addition, cyanobacteria were identified from 9 sites by microscopic techniques that were in estuaries. There are harmful cyanobacteria in the Iranian regions. Oscillateria, Lyngbya, Microcoleus, Aphanotheca, Anabaena, Plectonema, and Phormidium were observed in the mangrove ecosystem of the Tiab region[20]. Microcystis species, mainly *Microcystis aeruginosa*, have repeatedly caused the growth and production of liver toxins worldwide (20). Initially, Cylindrospermopsines are produced in Cylindrospermom raciborski, Anabaena, Umezakia, Aphanizomenon, and Radhidiopsis[24, 26]. The biodiversity of phytoplankton in the Persian Gulf stated that the predominance

of the cyanophyte group, especially the specially Oscillatoria thiebautii, in Hormozgan (due to higher temperature, lower salinity, higher phosphate, and little feeding of grazers from this algae) the amount of diversity will decrease[27].In the study on the phytoplankton biodiversity in the Persian Gulf of Iran, five species of blue-green algae were identified, including *Merismopedia sp. Anabaena, Phormidium sp, Oscillatoria thiebautii , Oscillatoria spp[28]*.In this study, the presence or absence of Saxitoxin-producing cyanobacteria was measured at 20 stations in the Hormozgan region in the autumn of 2012 using the molecular PCR method. In addition, cyanobacteria were identified from 9 sites using microscopic techniques in estuaries.

Conclusion

Methods based on chemical structure were used to determining the number of STX. These methods are as follows[5]: Using the molecular method, they studied cyanobacteria blooms in the aquatic area of Itaipu, located in Brazil. The DNA extraction in their research was long and lasted for two days, and they used different materials to purify the DNA, while in the present research, the DNA extraction was done in only four solutions which were used for DNA extraction[29]. The result of this study was conducted on the cyanobacteria of the Hormozgan-Persian Gulf using the molecular PCR method. The presence of cyanobacteria producing saxitoxin in 9 out of 20 stations (4-6-7-10-11-12-13-16-17), or 45% of stations where cyanobacteria have saxitoxin. The production of position and the presence of cyanobacteria saxitoxin-producing is not related to all the stations. This study shows the stations located in the estuaries (Tiab, Darsarokh, Jalabi) contain saxitoxin-producing cyanobacteria. In the estuary areas, there is a lot of urban and industrial sewage, which increases harmful cyanobacteria, and with the increase of cyanobacteria, fish die, fishing, economic problems, and pollution increase. Contamination of crustaceans and fish has caused human poisoning, so we must avoid sewage from entering the estuary to avoid the rise in cyanobacteria and toxin production.

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Author contributions
Mozhgan Emtyazjoo conceived the study. Parisa Sahebi and Athena Sheibani Nia performed the experimental work and prepared the manuscript. All authors were involved in data analysis and interpretation. Mozhgan Emtyazjoo read and approved the final version.

Ethical approval
This research does not have an applicable subject.

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Data Availability
All data generated or analyzed during this study are included in this published article.

Conflict of Interest
The authors declare no competing interests.

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