Diagnosis toxogenic Saxitoxin Cyanobacteria by molecular method in Amir Kalayeh Lagoon–Iran

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Abstract

One of the important cyanobacteria toxins is Saxitoxin poison. It affects nervous and respiratory system. In the event of blooming, some of them produce toxins that threaten the health of water. This poison is one of the most neurotoxins, which feeds on the transmission of nerve signals and the best-known paralytic shellfish toxin (PST). The purpose of this study is quick identification of toxigenic cyanobacteria that produce saxitoxin in the International Amir Kelayeh lagoon using PCR Technique. Twenty water samples of different transect were collected fromAmir Kalayeh lagoon. The DNA was extracted by the modified DNG method kit. Polymerase chain reactions (PCR) test was optimized by the DNA toxogen standard strains *Anabaena circinalis* (AWQC131C) and then were evaluated for the specificity and sensitivity. Amplicon was cloned in PTZ57R plasmid for sequencing and provide positive control by the T/A cloning method. The PCR test was optimized and 602bp were produced by the standard strains. The Amplicon was cloned and the gene sequence was determined. In specificity survey, no production was produced with TOX cyanobacteria DNA. The sensitivity of the diagnostic method was able to detect 10 copies. The cyanobacteria which produce poison, was potentially observed in 6 out of 20 samples collected from different stations.

Keywords: Saxitoxin; Cyanobacteria; Amir Kalayeh Lagoon; Polymerase chain reactions.

1. Introduction

Cyanobacteria are photosynthetic prokaryotes, and most of them have a bluish pigment called phycocyanin, and so they are also known as blue-green algae (Hakanson *et al.* 2007, Pearson

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et al., 2010). These phytoplankton are usually found in fresh, brackish, and saline water (Hitzfeld *et al.*, 2000). When cyanobacteria bloom, they produce toxins that endanger the safety of drinking water and have adverse effects on living organisms. These toxins include: Microcystin, Nodularin, Saxitoxin, Anatoxin-a, Anatoxin-a(S) and Cylindrospermospin (Wiese *et al.*, 2010; Corbel *et al.*, 2014). The toxins are different in structure and affect the nerves. The existance of cyanobacteria and their toxins in drinking water reservoirs are due to lack of decent management of water resources and reservoirs. Mechanism of Action (MOA) of known cyanobacteria toxins are various and include Hepatotoxin, Neurotoxin, Dermatotoxin and Synthesis inhibitor toxins (Falconer, 1999; Ferrer *et al.*, 2015). Neurotoxins are the ones which influence the nerves and respiratory system. Three categories of neurotoxins which have been identified so far are as follow: (1) Anatoxin-a and Homo anatoxin-a, which has the same effects as Acetylcholine, (2) Anatoxin-a(S), which is an anti-cholinesterase, and (3) Saxitoxin which is known as a paralyzing poison for economical shellfish aquaculture (PSP). Neurotoxins are alkaloid toxins. Non-sulfated Alkaloid toxins produced by cyanobacteria in fresh water are considered as neurotoxins. The Alkaloid sulfate-induced of Cylindrospermospin will stop protein degradation in liver (Falconer, 1999; Hii *et al.*, 2016). Saxitoxin is the most known toxins which is sodium channel blocker that respiratory inhalation is really dangerous compared with other cyanotoxins. In fact, it is a neurotoxin that can lead to death immediately within a few minutes of inhaling the lethal dose (Madsen, 2001; Tsuchiya *et al.*, 2016). Saxitoxins such as: *Anabaena circinalis*,

Aphanizomenon flos-aquae, *Clindrospermopsis raciborskii,* and *Lyngbya wollei* have been found in cyanobacteria (Falconer, 1999).

Recently, the most concerns about monitoring the spread of cyanobacteria and their potential toxic led to the identification a number of contaminated areas with cyanotoxins (Sivonen and Jones, 1999). In Australia the *A.circinalis* specie, was reported as the only toxin-producing cyanobacteria forming blooms in freshwater systems such as; dams, rivers, and water reservoirs which can sometimes leads into animal diseases and ultimately death (Fristachi *et al.*, 2008; Al-Tebrineh *et al.*, 2010). In France, in freshwater ecosystems, the presence of toxin-producing cyanobacteria cause concerns about the health risks to animals and humans. This has led to concentration and detecting Saxitoxin in water samples collected from the water body (Ledreux *et al.*, 2010; Cusick and Sayler, 2013). In some part of the water bodies in Brazil, Saxitoxin was identified by Clindrospermopsis raciborskii specie and the poison resistance to the hardness of water was investigated and showed potential highlights and high growth in water (Carneiro *et al.*, 2013). The mouse bioassay for paralytic shellfish poisoning (PSP) toxins has been used in the official Japanese method, and the toxicity were evaluated in and compared between Saxitoxin and derivatives of Saxitoxin in the mouse (Suzuki and Machii, 2014). There are several approaches for identifying cyanobacteria toxins, and each has its advantages and disadvantages. Molecular detection of cyanobacteria saxitoxin by using PCR method has not been done by the interior research. Detection by molecular methods in terms of speed and sensitivity are more important than culture methods (Thebault *et al.*, 1995; Astuya *et al.*, 2015).

One of the dignostic methods for cyanobacteria and their toxins is PCR method; which is also used in this research. Studies were performed for the detection of Saxitoxin in cyanobacteria and Dinoflagellates by using PCR method for toxin detection (Stüken *et al*., 2011). The PCR method was used for assessing and identifying Saxitoxin producer algal bloom in marine water, (Murray *et al*., 2011).

Amir Kalayeh lagoon is an important water body in Iran, which is located in north of Lahijan and near southwestern coast of the Caspian Sea (located at a distance of 1 Km from the Caspian Sea) with an area of 1230 Hectares. The lagoon is colonized by a pretty diverse wetland flora and fauna, the lagoon is fed by a number of underground springs and the drainage and it is an inhabitant for thousands of local and migratory birds. Lagoon has an average depth of 1.5 meters and the deepest part is 2 meters. This lagoon is ecologically affected by many microorganisms, especially toxin-producing cyanobacteria (Figure 1).

In this study, considering the strong presence of toxins produced by cyanobacteria in water bodies, the molecular detection of Saxitoxin producing cyanobacteria in the International Amir Kalayeh Lagoon is studied which is environmentally important.

2. Materials and Methods

2.1. Sample preparation

For sampling, 20 stations were selected from western, central, and eastern parts of the lagoon (Figure 2). Geographic latitude and longitude of these stations are given in Table 1. Surface water from each station was taken by using distinct two-liter sterile bottles, which then, were immediately put into ice-gel containing box, and were transferred to the laboratory under dark and 4°C temperature conditions. Afterwards, each sample was centrifuged at 12000 rpm for 5 minutes, the supernatant was removed, and the pellet was collected and mixed with 100 μl of deionized water. Then, the samples were kept at -22°C for 24 hours, and finally, DNA was extraced from samples by DNG extraction kit (Massahi *et al*., 2014).
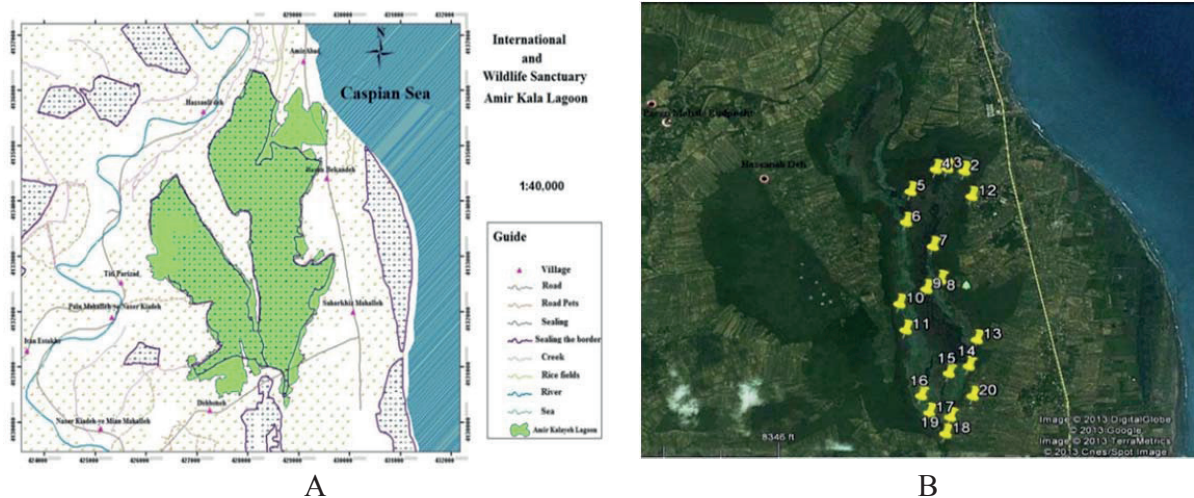

Figure 1. Amir Kalayeh Lagoon A) Geograghic map, B) Satellite imagery of stations

Table 1. Geographic latitudes and longitude of sampling station of Amir Kalayeh Lagoon

Sampling stations	Latitude	Longitude	Sampling stations	Latitude	Longitude
S_1	373 06.68	501 64.91	S_{11}	3733 37.1	501 07.23
S_2	373 21.92	501 80.11	S_{12}	373 92.42	501 89.45
S_3	373 25.11	501 58.62	S_{13}	373 25.72	501 96.76
S_4	373 23.13	501 32.67	S_{14}	373 95.19	501 86.69
S_5	373 98.12	501 12.39	S_{15}	373 86.72	501 61.61
S_6	373 62.21	501 06.91	S_{16}	373 60.8	501 27.01
S_7	37334.14	501 40.85	S_{17}	373 41.13	501 37.49
S_8	373 94.9	501 50.64	S_{18}	373 17.11	501 57.84
S_9	373 84.24	501 33.03	S_{19}	373 36.17	501 63.51
S_{10}	373 67.33	501 99.23	S_{20}	373 60.52	501 91.67

2.2. DNA extraction

DNG-Plus kits used for DNA extraction based on the protocol in the kit, which were performed by the following steps respectively.

▪ 100 µl of sample containing deionized water was mixed with 400 µl of DNG solution
▪ Heated for 4 hours and shake every 30 minutes
▪ Centrifuged the mixture at 12000 rpm for 5 minutes
▪ Supernatant was transferred into a new microtube and mixed with 500 µl Chloroform
▪ Resultant supernatant mixed with 300 µl cold ethanol and gently stir up for ten times.
▪ Micro-tube was centrifuged at 12000 rpm for 15 minutes
▪ Precipitate mixed with 500 µl of 70% ethanol for agitating and again centrifuged at 12000 rpm for 5 minutes
▪ Each microtube was dried on block heater for removal of alcohol
▪ Subsequently, it was mixed with 100 µl of deionized water and incubated on heater at 65 °C for 5 minutes (Masahi *et al*., 2014)

2.3. PCR and Electrophoresis

Optimization of PCR test for each primer pair was achieved using standard DNA strain of Anabaena circinalis (code number: AWQC131C) and general primers (CYA359F, CYA781R) for cyanobacteria detection (Sueoka *et al*., 1997; Namikoshi *et al*., 2003), and the specific primers (sxtAF, sxtAR) for Saxitoxin-coding gene (Ledreux *et al*., 2010). Furthermore, the specificity and sensitivity of the molecular detection test was evaluated. The optimized PCR was applied for detecting the Saxitoxin-producing gene of cyanobacteria in

Amir Kalayeh Lagoon. Then, 10 μl of each PCR product and 2 μl of 6X loading buffer was loaded in to 1.5% agarose gel that was previously mixed with CYBR Green dye. The electrophoresis was run for 45 minutes at 70 V. Finally, electrophoresis gel was appeared under Ultraviolet transilluminator (ShahHosseiny and Tehrani, 2005). Cloning of the gene was carried out using following processes. The PCR product was purified and transfected into PTZ57R plasmid vector. It transformed into E.coli JM107 using T/A cloning kit, Fermentas Company.

For purification, 50 μl of PCR product was mixed with 5 μl sodium acetate (3 M) and 125 μl of cold absolute alcohol within an eppendorf tube and incubated in a freezer (-20 °C) for one hour. After that, it was centrifuged at 12000 rpm for 15 minutes and decanted. Thereafter, it was mixed with 200 μl of cold 70% Alcohol, and it gently inverted ten times. The mixture was prepared for again centrifugation in 12000 rpm for 10 minutes. After removal of supernatant, the pellet was desiccated within 30-40 minutes at 37 °C. It was dissolved in 20 μl distilled water.

Cloning process involves three main steps; Ligation, Transformation, and Clone Selection. After cloning stages, the plasmid was extracted from recombinant bacteria (ShahHosseiny and Tehrani, 2005).

3. Results and Discussion

3.1. Optimization of PCR

PCR assay using DNA extracted from standard strains *Anabaena circinalis* AWQC131C optimized by listed primers (Table 2) for detecting the cyanobacteria CYA106F, 23S30R, and by specific primers stxA-F and stxA-R for detecting the Saxitoxin (Toxogene). Heat schedule optimized using Gradient PCR, which was 65 °C as an optimum annealing temperature. Optimization of forward and reverse primers′ concentration assay, performed between 0.1-1 μM, showed that the maximum amplification of DNA happened in 0.4 μM concentration of the primers. In addition, the optimum concentrations for $MgCl_2$ and dNTPs were determined as 1.5 mM and 0.5 mM, respectively. As shown in Figure 2-A, electrophoresis of PCR product was appeared in a specific band of the intended Amplicon at 602 bp in length which was slightly higher than 500 bp size marker for Saxitoxin. As seen in Figure 2-B, the 487 bp in length were visible for cyanobacteria-specific primers.

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

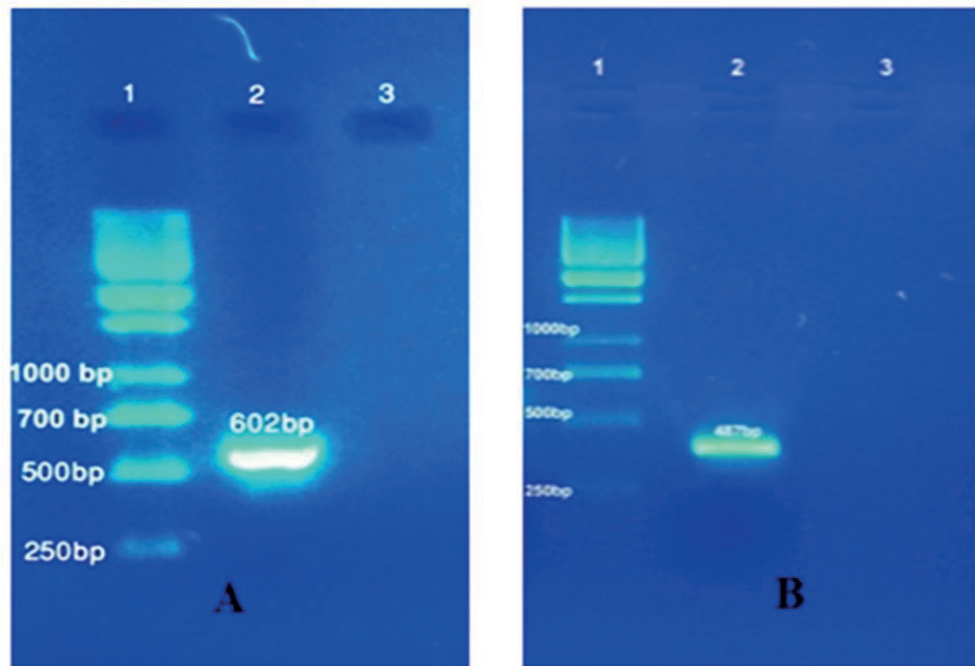| Gene	Primer sequences	Product Size of PCR (bp)
CYA359F	5'-GGGGAATYTTCCGCAATGGG-3'	487
CYA781R	5'-GACTACWGGGGTATCTAATCCCWTT-3'	
sxtAF	5'-AGG-TCT-TGA-CTT-GCA-TCC-AA-3'	602
sxtAR	5'-AAC-CGG-CGA-CAT-AGA-TGA-TA-3'	

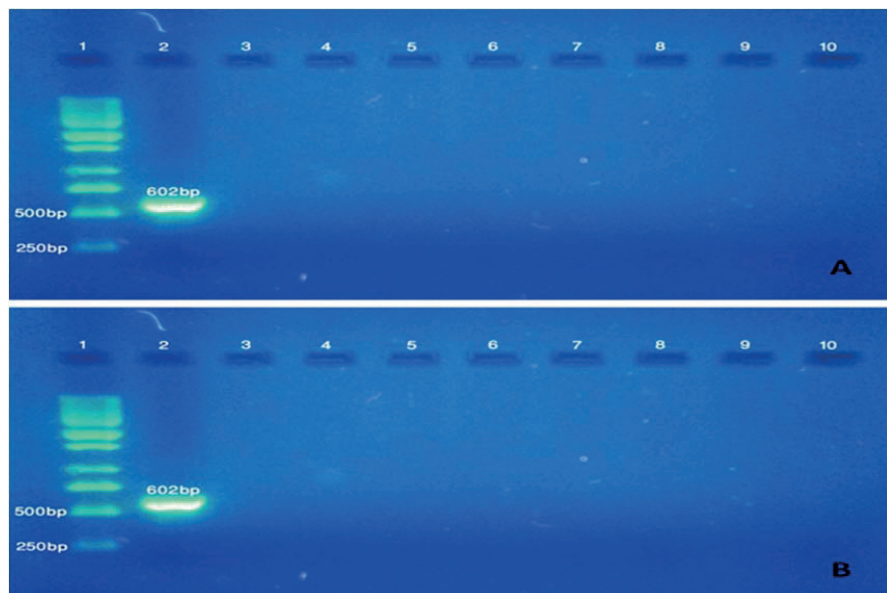Figure 2. Optimized PCR assay for detection of (A) Saxitoxin-coding gene, and (B) cyanobacteria

Figure 3. A- Specificity of PCR for detection of Saxitoxin; 1) 1Kb DNA Ladder Fermentas size marker, 2) Positive control, 3) *Nostoc*, 4) *Anabeana*, 5) *Osilatoria*, 6) *E-coli*, 7) *Staphilococcus aereus*, 8) *Legionella*, 9) Cyto megalo virus, and 10) 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) E-coli,7) *Pseudomonas aeruginosa*, 8) Human DNA, 9) Mice DNA, and 10) Negative Control

3.2. PCR specificity and sensitivity for detection of Saxitoxin and cyanobacteria

The specificity of primers toward cyanobacteria and Saxitoxin detection was examined. Figure 3-A, compares the specificity of primers for 1) 1Kb DNA Ladder Fermentas size marker, 2) Positive control, 3) *Nostoc*, 4) *Anabeana*, 5) *Osilatoria*, 6) *E-coli*, 7) *Staphilococcus aereus*, 8) *Legionella*, 9) Cyto megalo virus, 10) Negative Control. The PCR products were appeared at optimum conditions. The results revealed that our proposed primers responded significantly to Saxitoxin than the other.

The cyanobacteria determination was carried out by the optimized PCR. The specific primers were used to amplify DNA of cyanobacteria. No band was appeared for the other tested organisms *such as:* 1) 1Kb DNA Ladder Fermentas size marker,2) Positive control, 3) *Staphylococcus spp.*, 4) *Staphylococcus aureus*, 5) *Legionella Pneumophila*, 6) E-coli,7) *Pseudomonas aeruginosa*, 8) Human DNA, 9) Mice DNA, 10) Negative Control. This indicates a high specificity of the primers which means they can be safely used for detection of cyanobacteria

and their toxin-producing gene (Figure 3-B). As shown in Figure 4, the PCR Sensitivity for detection of Saxitoxin was calculated, the minimum number of bacteria used to detect the gene, was 10 with different dilution. This method represented that the selected primers had the potential to being used as a primer for detection of Saxitoxin.

3.3. Cloning of PCR products

Twenty hours after transformation, white and blue colonies were seen on LB-Agar plates containing X-Gal IPTG and Amp. The white colonies were containing recombinant plasmid but not the blue colonies. All colonies that were selected by PCR of a recombinant plasmid containing the target fragment were approved. The PCR reaction product indicates that the plasmid DNA obtained from four colonies was used as a template.

Plasmid was extracted by alkaline denaturation of LB-Broth culture medium containing colonies and was loaded on 1.5% Gel electrophoresis. Since the aimed fragments of 487bp and 602bp were inserted into the original plasmid, the recombinant plasmid
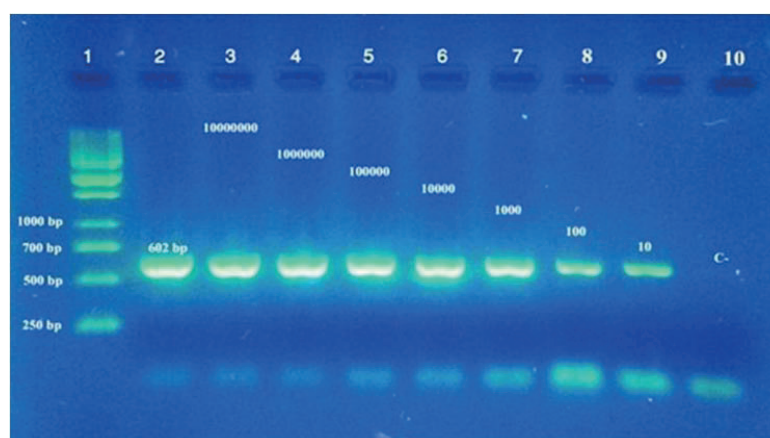

Figure 4. Sensitivity of PCR for detection of Saxitoxin; 1) 1Kb DNA Ladder Fermentas size marker, 2) Positive control, 3) 10000000 Genome, 4) 1000000 Genome, 5) 100000 Genome, 6) 10000 Genome, 7) 1000 Genome, 8) 100 Genome, 9) 10 Genome, and 10) Negative control.

was heavier than PTZ57R. Also observing the 602bp and 487bp bond on the gel determines that cloning was completed properly and the plasmid extraction and gene amplification were successfully finished.

3.4. PCR on water samples prepared from the Amir Kalayeh Lagoon

Optimized PCR assay was performed on the DNA extracted from the samples of Amir Kalayeh Lagoon. All the 20 DNA samples responded to these primers, and it was found that Cyanobacteria have been present in all stations. The samples were tested by PCR for the detection of Saxitoxin; the results showed that 6 out of 20 samples were positive with these primers (Figure 5).

3.5. Presences of Saxitoxin producing cyanobacteria in Amir Kalayeh Lagoon

Based on the Table 1, the stations of Amir Kalayeh Lagoon had cyanobacteria producing Saxitoxin. According to that, 6 out of 20 superficial samples of water were infected to Saxitoxin. The investigation of stations showed that: two samples from the eastern, one sample from central and three samples from the western areas of the lagoon were positive in terms of Saxitoxin producing cyanobacteria (Figure 6).

In a brief investigation, it was observed that the lagoon was surrounded by the farm fields in the northern parts and is located 1km far from the Caspian Sea. As seen in the aerial images of the lagoon (1-B), the samples taken from 20 stations. 16 out of 20 stations were from four directions and surrounding lagoon waters and other samples of 4 stations were taken from the central part of lagoon. Following the conducted experiments, it became clear that almost all of the sampling stations, the 6 stations contained Saxitoxin-producing cyanobacteria gene. The geographical locations of the 6 stations are as follow:

The northwestern part and the part of lagoon near cane fields are located close to the agricultural lands. The central part of the lagoon and the shallow part of the lagoon are located between
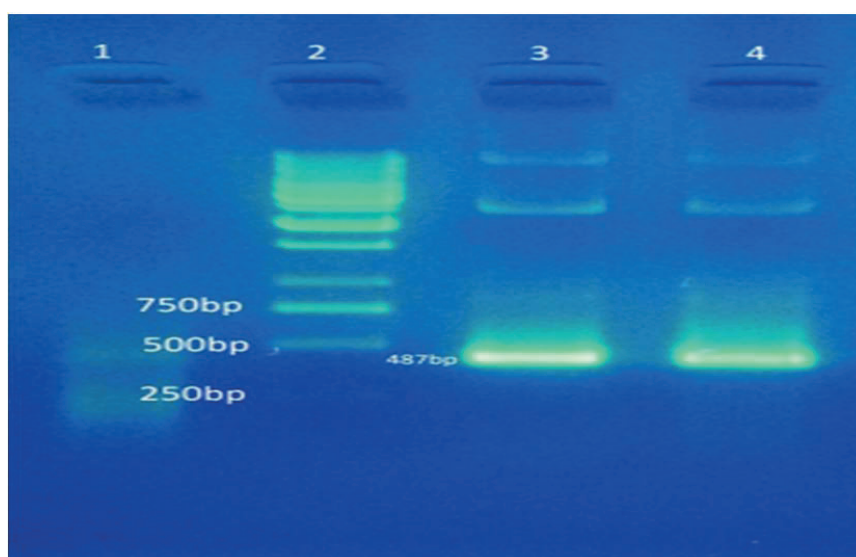

Figure 5. PCR on the plasmid containing the Insert; 1) Negative Control, 2) 1Kb DNA Ladder Fermentas size marker, 3 & 4) Insert fragment of 487 bp result proliferation.
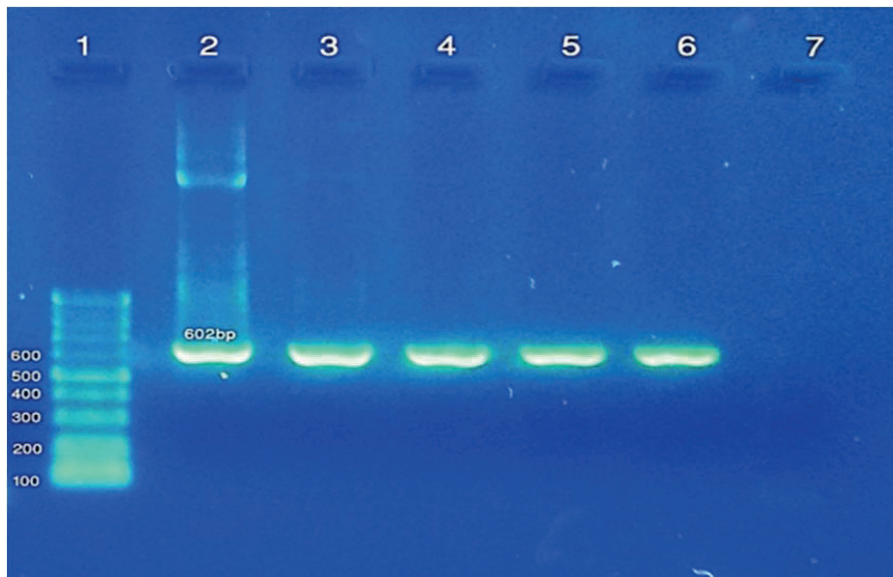
Figure 6. PCR on bacteria DNA containing Insert; 1) 1Kb DNA Ladder Fermentas size marker, 2 to 6) positive PCR product on colonies containing the Insert, and 7) Negative Control

the two north and west regions. Western district of the lagoon is close to the cane fields and the agricultural fields. There is an environmental monitoring station near the lagoon entrance and also a parking area for motor boats. In the southeastern part of the lagoon, a water pump channel is located for farming fields. The other station which was positive to Saxitoxin generator gene was associated with water entrance of the cane fields behind the lagoon and adjacent to farm lands in the southwestern parts.

According to an account from the stations containing toxigenic cyanobacteria, it can be stated that: 4 out of 6 stations (67%) were located near farm fields in which there are agricultural waste input flows. Based on current evidence two of the six stations were located on an area in which there was a stream of water from the depth to the surface. One of them is located in the entrance water pump channel, and the other one is located inside the environmental monitoring station which is used for patrol boats commuting. However, this problem might be due to the vacuum made by the suction of water pumps and the flow to the farm lands. Another reason is the overflow of water from the lagoon to the farm lands which is because of the difference between deep water and the water came from the surface. In addition, the gradient difference of distance happened which finally lead into existence of nutrients. As well, within the area of patrolling station and due to igniting the motors and their movement which makes flows of water, the nutrient may come up from the deep parts to the surface.

In our study, the PCR method was performed on cyanobacteria of international Amir Kalayeh Lagoon. The result showed that the 6 samples out of the 20 stations contained Saxitoxin producing cyanobacteria, which means that 30% of the stations contained toxin-producing cyanobacteria. Production and the presence of the toxigenic cyanobacteria are not related to other stations. The results revealed that the existence of such a toxin would be problematic for living organism of the lagoon ecosystem which is considered as one of the important lagoons in the region.

Conclusion

In this study, the ability of the PCR method in detecting Saxitoxin generator cyanobacteria of international Amir Kalayeh Lagoon was examined and proved to be effective for identifying Saxitoxin toxigenic cyanobacteria. The presence of this toxin can also be identified in other water bodies and the lagoons containing different living organisms' weather during blooming time or other circumstances.

Acknowledgements

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