PCR- Based Test for the Early Warning of Both Cylindrospermopsin and Saxitoxin in Iraqi Freshwater

Ibrahim J. Abed
Department of Biology, College of Science, University of Baghdad, Baghdad-Iraq.
E-mail: ibrahimabed95@yahoo.com.

Abstract

Cylindrospermopsins (CYN) and saxitoxins (STXs) are neurotoxins produced by a variety species of cyanobacteria. CYN is recognized as one of the most globally important of the freshwater algal toxins. STXs are mostly associated with marine dinoflagellates (eukaryotes) and several species of freshwater cyanobacteria. The aim of this study was to provide a rapid tool to detect cylindrosprmopsin and saxitoxin biosynthesis genes in freshwater and bloom. Microscopic identification of the studied samples demonstrated that the prevalent cyanobacteria in samples of freshwater and blooms were *Lyngbya*, *Oscillatoria*, *Aphanizomenon, Chroococcus, Microcystis***,** *Cylindrospermopsis* and *Anabaena*. The molecular analysis revealed that *aoa*C gene was not detected in the freshwater samples at the three studied sites while *sxt*A was detected at one only. Also, the *aaa*C and *sxt*A genes were detected in the bloom at the three studied sites. In conclusion, The PCR technique applied in this paper was found to be useful and rapid test, particularly when the number of the target organism is very low in the freshwater sample.

Keywords: Cylindrospermopsins, saxitoxins, Iraqi freshwater, PCR.

Introduction

Cylindrospermopsins (CYN) and saxitoxins (STXs) are neurotoxins produced by a variety species of cyanobacteria. CYN is a wide spread, stable alkaloid and its toxicity is diverse. This toxin is rapidly being recognized as one of the most globally important of the freshwater algal toxins. The ever-expanding distribution of CYN producer into temperate regions is heightening concern that this toxin will represent serious environmental and health hazards (1). STX are among the most potent natural toxins known and its 57 analogs are a broad group of natural neurotoxic alkaloids, commonly known as paralytic shellfish toxins that caused the paralytic shellfish poisoning, and are mostly associated with marine dinoflagellates (eukaryotes). Several species of freshwater cyanobacteria produce STX including *Anabaena circinalis*, *Aphanizomenon* spp., *Lyngbya wollei* and *Cylindrospermopsis raciborskii*. (2,3,4,5). The lethal oral dose of STX in humans is between 1 to 4 mg depending upon the age and physical condition of the patient. There has been only one reported case of human poisoning through consumption of PST contaminated fish (6). Guidelines for water quality and treatment have suggested that the health alert level expressed in terms of saxitoxin-equivalent concentrations in water for consumption is 3 μg/L (7).

Polymerase chain reaction (PCR) test targeting cyanotoxins biosynthesis genes provide a rapid and sensitive means for detecting potentially toxic populations of cyanobacterial in water supplies. Several studies have been developed PCR techniques including multiplex PCR and real-time PCR for detection of CYN in cyanobacterial isolatesand in mixed population of cyanobacteria (8,9). Marbun et al., (10) were tested quantitative polymerase chain reaction (qPCR) to minimize the harmful effects of cylindrospermopsin in freshwater.

In a study by Al-Tebrineh., (11), a specific qPCR method based on SYBR green was developed to quantify saxitoxin- producing *Anabaena circinalis* cyanobacteria which are major bloom-forming fresh water cyanobacteria.

Therefore, the aim of this study was to provide a rapid tool for detecting cylindrosprmopsin and saxitoxin biosynthesis genes in both freshwater and blooms samples

Materials and Methods

Environmental samples

The study area included three sites located in Tigris River within Baghdad city; the first

was located at North of Baghdad in Sader Al-Qanat area (site 1), and the second at the middle part in Al-Jadiryia area (site 2), whereas the third site located at South part in Al-Zafrania area (site 3). During 2014, on summer months, freshwater and bloom samples were collected from the sites above-mentioned. Freshwater samples were centrifuged and concentrated into one ml and prepared to the DNA extraction.

Microscopic Identification

Each sample was observed under the microscope. Cell shape and size were observed, measured by micrometry. Identification of specimens was carried out using the taxonomic publication (12).

DNA extraction

Genomic DNA was extracted from approximately 200 mg wet weight from the environmental bloom sample and 1ml of concentrated freshwater samples (freshwater sample was centrifuged and concentrated into one ml and prepared to the DNA extraction) were extracted using lysis buffer (13). The cells were combined with 600µl lysis buffer (800 mM ammonium acetate; 20 mM EDTA; 1% SDS; 100 mM Tris–HCl, pH 7.4). The mixture was vortex-mixed and incubated at 65ºC for 2 h, and the extracts were cooled for 10 min on ice. Cell debris was removed by centrifugation at 12,000g for 10 min. DNA was precipitated by the addition of 1 volume of isopropanol and 1/10 volume of 4 M ammonium acetate for 15 min at 4ºC. The precipitated DNA was pelleted by centrifugation at 12,000g for 10 min and washed with 70% ethanol. The extracted DNA was resuspended in 100 µ of sterile water.

Primers selection

The first set of primers Ckc-F: AATGATCGAAAACAGCAGTCGG and Ckc-R: TAGAACAATCATCCCACAACCT was used to detect the cylidrospemopsin by targeting cylidrospemopsin biosynthesis *aoa*C gene (Baron-Sola et al., 2012) while the second set of primers sxtA-F: GATGACGGAGTATTTGAAGC and sxtA-R: CTGCATCTTCTGG ACGGTAA was used to detect the saxitoxin by targeting the saxitoxin biosynthesis *sxt*A gene (11).

PCR amplification

PCR mixture was set up in a total volume of 20µl included 5µl of PCR premix (Accupower, Bionear), 1µl of each primer and 2µl of template DNA were added then the rest volume was completed with sterile D.W. Negative control contained all material except that distilled water was used instead of template DNA. PCR reaction tubes were placed into thermocycler PCR instrument. The reactions were carried out using master cycler gradient PCR (Eppendroff, Germany). PCR conditions consisted of an initial denaturation at 94 ºC for 7 min to cylindrospermopsin and 5 min to saxitoxin; 40cycles of denaturation at 94 ºC for 30 s to cylindrospermopsin and 1 min to saxitoxin, annealing for 1min. at 54 ºC to cylindrospermopsin and 40 s at 63 C˚ to saxitoxin, extension for 1 min at 72 ºC and final extension for 10min at 72 ºC. PCR product was separated in 1.5% agarose gel electrophoresis stained with ethidium bromide and visualized on UV transilluminator.

Results and Discussion

Microscopic identification

The microscopic results revealed that all samples which collected in July and August of freshwater and blooms from Tigris River were dominated by genera of cyanobacteria Table (1). The studied samples in site (3) which located southern Baghdad city compared with the other sites revealed an increase in the identified dominated genera of potential cyanobacteria (*Anabaena, Lyngbya, Aphanizomenon* and *Cylindrospermopsis*) to produce cylindrospermopsin and saxitoxins in both types of samples (freshwater and blooms). Monitoring of freshwater supplies is very necessary by surveillance of toxic algal blooms in the river which provides the raw water to the drinking water treatment plants.

Molecular Analysis

Two sets of primers (*Ckc*-F/R- *sxtA*-F/R) were used to detect *aoa*C and *sxtA* genes directly in both samples of the freshwater and the bloom and there is no need to consume time by isolating

cyanobacteria in these samples to know which of them is produced for these cyanotoxins. The Ckc-F/R set primer was demonstrated to be specific to cylindrospermopsin producing cyanobacteria (8).

Table (1)
Dominated genera of cyanobacteria in the studied samples.

Kind of sample	*Site 1*	*Site 2*	*Site 3*
Freshwater	*Oscillatoria, Microcystis Lyngbya.*	*Oscillatoria, Microcystis Lyngbya.*	*Oscillatoria, Chroococcus, Lyngbya, Anabaena, Microcystis Nostoc.*
Bloom	*Oscillatoria, Anabaena, Aphanizomenon, Microcystis Lyngbya.*	*Oscillatoria, Anabaena, Cylindrospermopsis, Microcystis Lyngbya.*	*Oscillatoria, Anabaena, Aphanizomenon, Chroococcus, Cylindrospermopsis, Microcystis, Lyngbya.*

The PCR analysis in this study revealed that *aoa*C not detected in the freshwater samples for all sites Fig.(1). In bloom samples which genera *Oscillatoria, Anabaena, Aphanizomenon, Chroococcus, Cylindrospermopsis, Microcystis* and *Lyngbya* were prevalent, the *aoa*C gene was detected at the three studied sites. The poisoning cases by cyanobacterial toxins in freshwater bodies have almost been caused by Cylindrospermopsin and microcystin (14).

Results also showed that *aoa*C was not detected in freshwater in the studied sites Table (2), Fig.(1), that might be related to the low concentration of potential Cylindrospermopsin-producers in the freshwater samples. While in the bloom samples the concentration of potential cyanobacteria producing Cylindrospermopsin was high, this point explains the positive result in the studied sites.

Many studies detected the cylindrospermopsin producers in freshwater by PCR methods. Although bio-molecular detection methods have become popular because of its specificity and speed, only very few studies have focused on the quick monitoring of cylindrospermopsin producers in freshwaters by PCR techniques. Marbun *et al*., (10) have showed the applicability of the qPCR method for rapid on-site detection of *C. raciborskii* in reservoirs. In addition, the results also suggest that cylindrospermopsin is an important cyanotoxin in the reservoirs in Kinmen Island. Rasmussen *et al*., (9) have developed and tested a real-time PCR assay to detect and quantify genes specific to *Cylindrospermopsis sp*. and cylindrospermopsin-producing cyanobacteria.

The *sxtA*-F/R set primer developed by (11) which was demonstrated to be specific to saxitoxin-producing *Anabaena circinalis*, based on conventional PCR. The primers *sxtA*-F/R also amplified the *sxtA* gene from other saxitoxin-producing cyanobacteria.

The results in this study showed that *sxtA* was detected in freshwater at site 3 only Fig.(2), Table (2) this might be related to the prevalence of *Anabaeana circinalis*, *Lyngbya* and *Aphanizomenon sp.* in this sample, which are potential to produce the saxitoxin, While *sxtA* gene was observed at all sites in the bloom sample.

The positive results in the bloom samples at most studied sites might be belong to the heavy growth and high diversity of cyanobacteria in these blooms.

Al-Tebrineh *et al*., (11), showed a specific qPCR method based on SYBR green chemistry was developed to quantify saxitoxin- producing Anabaena circinalis, a major bloom-forming freshwater cyanobacterium the analysis of bloom samples in this study revealed that sxtA was not detected in three samples which did not give amplification in PCR or qPCR for either the cyanobacterial 16S rRNA or sxtA genes, most probably due to PCR inhibitors in the

extracted DNA or because the low density for growth of cyanobacteria.

During the last few decades, the increase in frequency of cyanobacterial blooms in freshwater bodies has led water management authorities to pay more attention to the risks associated with toxic cyanobacteria. The diagnosis of toxic occurrences still focuses mainly on microcystins, although reports of neurotoxins are becoming more frequent (15).

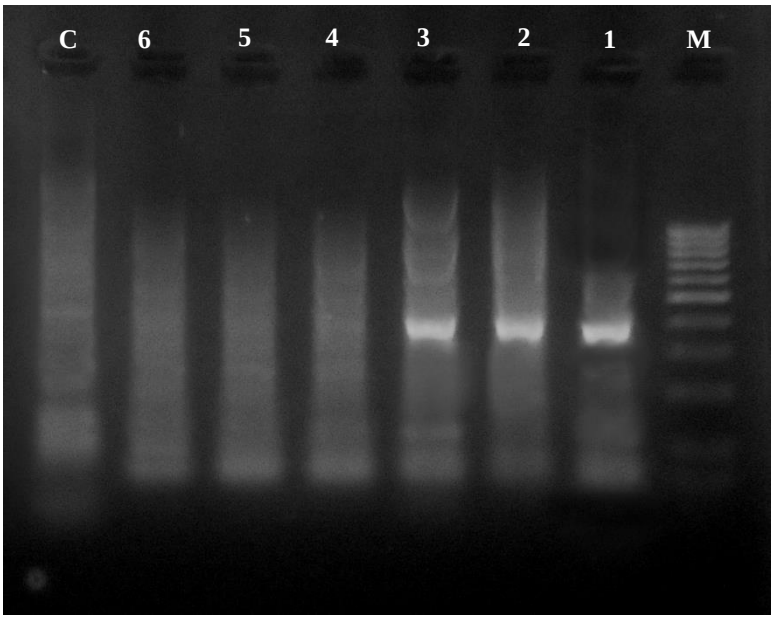

Fig.(1) PCR amplification of the aoaC gene (325bp) in freshwater and bloom samples. Lanes 1-3: bloom samples, lanes 4-5: freshwater samples. C: negative control. M:100bp DNA marker.

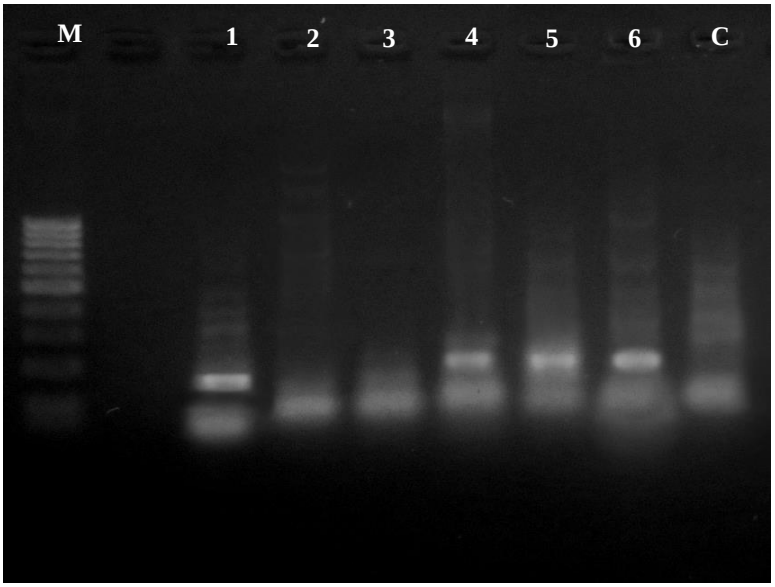

Fig.(2) PCR amplification of the sxtA gene (125bp) in freshwater and bloom samples. Lanes 1:site 3 freshwater samples, 2-3: other freshwater samples, lanes 4-5: bloom samples. C: negative control. M:100bp DNA marker.

During the last few decades, the increase in frequency of cyanobacterial blooms in freshwater bodies has led water management authorities to pay more attention to the risks associated with toxic cyanobacteria. The diagnosis of toxic occurrences still focuses mainly on microcystins, although reports of neurotoxins are becoming more frequent (15).

Table (2)
Amplification state of cylindrospermopsin and saxitoxin in in freshwater and bloom samples.

Kind of sample	*Site 1*	*Site 2*	*Site 3*
Cylindrospermopsin			
Freshwater	-	-	-
bloom	+	+	+
Saxitoxin			
Freshwater	-	-	+
bloom	-	+	+

(-): no PCR product in conventional PCR.
(+): PCR product in conventional PCR.

Conclusion

We were able to estimate the toxigenicity of cyanobacterial blooms based on the detection of *sxtA* and *aoaC* genes by PCR assay. This method is useful for managers in terms of being able to monitor the formation and progression of STX and CYN –producing cyanobacterial blooms in Iraqi fresh water - Tigris River. Also, The PCR technique applied in this study was found to be rapid test, particularly when the number of the target organisms is very low in the freshwater sample.

Reference

[1] Wormer L., Cires S., Carrasco D., Quesada., "Cylindrospermopsin is not degraded by co-occurring natural bacterial communities during a 40-day study", Harmful Algae, 7, 206–213, 2008.

[2] Anderson D.M., Cembella A.D., Hallegraeff G.M., "Physiological Ecology of Harmful Algal Blooms". 1st ed., Springer: Berlin, Germany, p. 662, 1998.

[3] Anderson D.M, Glibert P.M., Burkholder J.M., "Harmful algal blooms and eutrophication": Nutrient sources, composition, and consequences", Estuaries, 25, 704–726, 2002.

[4] Sellner K.G., Doucette G.J., Kirkpatrick G.J., "Harmful algal blooms: Causes, impacts and detection", J. Ind. Microbiol. Biotechnol., 30, 383–406, 2003.

[5] Zingone A., Enevoldsen H.O., "The diversity of harmful algal blooms: A challenge for science and management". Ocean Coast. Manage, (43) 725–748, 2000.

[6] NHMRC. "Australian Drinking Water Guidelines". 596 National Health and Medical Research Council and the Agricultural Resource Management Council of Australia and New Zealand, 2001.

[7] Fitzgerald D. J., Burch M. D., "Development of health alerts for cyanobacteria and related toxins in drinking water in South Australia. Environ", Toxicol, 14,203-9,1999.

[8] Baron-Sola A., Ouahid Y., delCampo F.F., "Detection of Potentially Producing Cylindrospermopsin and Microcystin Strains in Mixed Populations of Cyanobacteria by Simultaneous Amplification of Cylindrospermopsin and Microcystin Gene Regions" , Ecotoxicology and Environmental Safety, 75,102-108, 2012.

[9] Rasmussen J. P., Giglio S., Monis P. T., Campbell R. J., Saint C. P., "Development and field testing of a real-time PCR assayfor cylindrospermopsin-producing cyanobacteria." J Appl Microbiol, 104, 1503–1515, 2008.

[10] Marbun Y. R., Yen H., Lin T., Lin H., Michinaka A.,"Rapid on-site monitoring of cylindrospermopsin-producers in reservoirs using quantitative PCR", Sustain. Environ. Res, 22(3), 143-151, 2012.

[11] Al-Tebrineh J., Mihali T.K., Pomati F., Neilan B.A., "Quantitative PCR detection of saxitoxin-producing cyanobacteria and Anabaena circinalis in environmental water blooms", Appl. Environ. Microbiol. 4-10, 2010.

[12] Desikachary T. V. "Cyanophyta. Indian Council of Agricultural Research". New Delhi, pp 686, 1959.

[13] Neilan B.A, Burns B.P, Relman D., Lowe D., "Molecular identification of cyanobacteria associated with stromatolites from distinct geographical locations", Astrobiol, 2, 271–280, 2002.

[14] Falconer I.R. "Cyanobacterial Toxins of Drinking Water Supplies: Cylindrospermopsins and Microcystins". CRC Press, New York, pp 279, 2005.

[15] Ledreux A., Thomazeau S., Catherine A., Duval C., Ye pre mian, C. Marie, A., Bernard C., "Evidence for saxitoxins production by the cyanobacterium Aphanizomenon gracile in a French recreational water body", Harmful Algae, 10, 88–97, 2010.

الخلاصة

سموم السلندروسبيرموبسين والساكسيتوكسين هي سموم عصبية تُنتجها انواع مختلفة من الطحالب الخضر المزرقة. السلندروسبيرموبسين يصنف كأحد السموم المهمة على نطاق العالم والمنتج من الطحالب الخضر المزرقة المستوطنة في المياه العذبة. سموم الساكسيتوكسين هي على الاغلب يكون افرازها مرتبط بالسوطيات الدوارة البحرية (حقيقية النواة) وبعض الطحالب الخضر المزرقة التي تستوطن المياه العذبة. هدفت هذه الدراسة الى تقديم طريقة سريعة للكشف عن الجينات المصنعة لسموم السلندروسبيرموبسين والساكسيتوكسين في المياه العذبة والازدهار الطحلبي. اظهر التشخيص المجهري للعينات التي دُرست ان الطحالب الخضر المزرقة السائدة في كلتا العينتين (المياه العذبة والازدهار الطحلبي) كانت *Lyngbya*, *Oscillatoria*, *Aphanizomenon*, *Chroococcus*, *Microcystis*, *Cylindrospermopsis* و *Anabaena*. اظهر التحليل الجزيئي ان الجين *aoa*C لم يشُخص في المياه العذبة في المواقع الثلاثة التي دُر ست, بينما الجين *sxt*A شخُص في موقع واحد فقط، كذلك شخصت الجينات *aoa*C و *sxt*A في الازدهار الطحلبي في المواقع الثلاثة التي دُرست. تعتبر تقنية تفاعل سلسلة البلمرة المطبق في هذه الدراسة كفحص مفيد وسريع, ولا سيما عندما يكون تركيز الكائن المستهدف منخفض جدا في عينة المياه العذبة.