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

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Abstract
Cylindrospermopsins (CYN) and saxitoxins (STXs) are neurotoxins produced by a variety 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 cylindrospermopsin 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 aoaC gene was not detected in the freshwater samples at the three studied sites while sxtA was detected at one only. Also, the aaoC and sxtA 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 of cyanobacteria. CYN is a widely 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 isolates and 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 cylindrospermopsin 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
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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 µl of sterile water.

Primers selection
The first set of primers Ckc-F: AATGATCGAAAAACGCAGTCGG and Ckc-R: TAGAACAATCATCCCACAACCT was used to detect the cylindrospermopsin by targeting the cylindrospermopsin biosynthesis aoaC gene (Baron-Sola et al., 2012) while the second set of primers sxtA-F: GATGACGGAGTATTTGAAGC and sxtA-R: CTGCATCTTTCTGG ACGGTAA was used to detect the saxitoxin by targeting the saxitoxin biosynthesis sxtA 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 aoaC 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.

<table>
<thead>
<tr>
<th>Kind of sample</th>
<th>Site 1</th>
<th>Site 2</th>
<th>Site 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshwater</td>
<td>Oscillatoria, Microcystis Lyngbya</td>
<td>Oscillatoria, Microcystis Lyngbya</td>
<td>Oscillatoria, Chroococcus, Lyngbya, Anabaena, Microcystis Nostoc.</td>
</tr>
</tbody>
</table>

The PCR analysis in this study revealed that aoaC 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 aoaC gene was detected at the three studied sites. The poisoning cases by cyanobacterial toxins in freshwater bodies have almost been caused by Cylindrospermopsis and microcystin (14).

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

Many studies detected the cylindrospermopsis 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 cylindrospermopsis 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 cylindrospermopsis 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 cylindrospermopsis-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 Anabaena 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.](image)

![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.](image)

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

<table>
<thead>
<tr>
<th>Kind of sample</th>
<th>Site 1</th>
<th>Site 2</th>
<th>Site 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cylindrospermopsin</td>
<td>Freshwater</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>bloom</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saxitoxin</td>
<td>Freshwater</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>bloom</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(-): 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
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الخلاصة
سموم السلندروسبيرموبسين والساكسيتوكسين هي سموم عصبية تنتجها أنواع 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 الأغلب يكون افرادها مرتبطة بالسوطيات الدورة البحرية (الحمضية النواة) وبعض الطحالب الخضر المزرقة التي تستوطن المياه العذبة. هدفت هذه الدراسة إلى تقديم طريقة سريعة للكشف عن الجينات المصنعة لسموم السلندروسبيرموبسين والساكسيتوكسين في المياه العذبة والزهور الطحلبي. أظهر التشخيص المجهري للعينات التي درست أن الطحالب الخضر المزرقة السائدة في كلتا العينتين (المياه العذبة والازدهار الطحلبي) كانت Lyngbya, Oscillatoria, Aphanizomenon, Chroococcus, Microcystis, Cylindrospermopsis Anabaena و Cylindropermopsis لم يشُخص في المياه العذبة في المواقع الثلاثة التي درست أظهَر التحليل الجزئي IA sxtx كما في الزهور الطحلبي في المواقع الثلاثة التي درست. 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.