Molecular detection of some toxogenic cyanobacteria in Tigris River in Baghdad–Iraq

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Abstract
Cyanobacteria and their pollution are being increasingly commonly reported worldwide that cause a serious hazard to environmental and human health. Cyanotoxin was the most algal toxin reported to be produced by several orders of cyanobacteria. This study aimed to provide a technique to detect cylindrospermopsin and saxitoxin biosynthesis genes in the river. In November, December 2019, and January 2020. Cyanobacteria were isolated from freshwater of Tigris River and identified by compound microscope also conventional PCR. Five isolates of cyanobacteria that successfully amplified a gene fragment from the phycocyanin were found in all cyanobacteria (Microcystis flosaquae, Microcystis sp, anabaena circinalis, nostoc commune and westiellopsis prolifica) and all isolates successfully amplified aoaC gene to detecting the cylindrospermopsin and the saxitoxin. Our results concluded that PCR assay can be used for early detection of cylindrospermopsin and the saxitoxin producing cyanobacteria in river water that useful to stations responsible for the preparation of drinking water to public.

Background
Cyanobacteria (or blue-green algae) are a unique alga; they are the only algae that are bacteria and the only bacteria that can photosynthesize by consuming carbon dioxide and producing oxygen. Cyanobacterial harmful algal blooms (CyanoHABs) are globally on the increase in both frequency and intensity as a result of eutrophication and climate change. CyanoHABs, are now a problem of global environmental concern and efforts are being taken to prevent, predict, minimizes, and suppress their occurrences. HAB-forming cyanobacteria are generally considered to be inedible or at least poor food for zooplankton, mainly because of three common properties: size, toxicity and poor nutritional quality.

Cyanobacteria also possess significant potential for lipid production, which can be used as biofuel feedstock. Water pollution has been a recurrent environmental issue and global challenge. This is particularly due to the increasing rate of anthropogenic activities, such as population expansion, industrialisation, and agricultural practices making the issues of water quantity, quality, and availability a concern in many parts of the world.

Algae are involved in water pollution in a number of important ways. Due to the enrichment of inorganic phosphorous and nitrogen is responsible for the growth of algae in water bodies. Cyanobacteria are geographically widespread in freshwater, marine and terrestrial habitats and can cause serious threats to drinking water supplies using surface water as source.

Cyanotoxins
Cyanobacteria are producing a wide variety of bioactive compounds, including some toxins, also known as cyanotoxins, such as microcystins, anatoxins, and saxitoxins, etc.

Bioactive compounds produced by cyanobacteria have been described: non-ribosomal peptides, alkaloids, lipopeptides, esters, amino acids and polyketides. Cyanotoxins are usually divided into groups according to the effect that they provoke. Hepatotoxins cause liver damage, cytotoxins are responsible for cell dysfunction and damage, neurotoxins cause damage to nervous tissue, whereas dermatotoxins are linked with allergic reactions. Toxicity can only occur when toxins are transported into cells or interact with specific receptors or channels present on cells membrane.
Cylindrospermopsin (CYN) is a biologically active alkaloid found in several cyanotoxin genera [17]. It is a general cytotoxin that blocks protein synthesis, the first clinical symptoms being kidney and liver failure.

Saxitoxins: The toxin can be produced by dinoflagellates as well as by cyanobacteria. The toxin then accumulates in filter-feeding shellfish such as mussels, clams, oysters, and scallops over time [18, 19].

The neurotoxicity of saxitoxins (STXs) relies on the blocking of nerve axon membrane sodium ion channels [20]. STXs are also known to block calcium channels [7]. STXs also affect the cells of the heart muscle by prolonging potassium channel gating, which can lead to alterations in the influx of ions into the cell [21].

**The genetic basis of microcystin production**

MCs are synthesized by the thiotemplate mechanism. The MCs (mcy) gene cluster contains peptide synthetases, polyketide synthases and tailoring enzymes [22]. Over 80 structural analogues of MC have been isolated and characterized to date that differ in the type of amino acids incorporated into the MC or by modifications to the peptide backbone [23]. This group of toxins is encoded by the MC synthetase (mcy) gene cluster, which spans 55 kb of DNA [24]. The cluster of mcy genes contains 10 genes, namely mcy A to mcy J, which have been fully sequenced and characterized in many cyanobacterial species [25].

The carboxyl group of the D-glutamate side chain is present in all known variants of MCs [26]. The mcyE gene is responsible for the activation and condensation of D-glutamate with Adda moiety, which can be used as a specific target for cyanobacteria that produce MCs [22].

**Materials and methods**

**Collection of samples**

Surface water samples were collected weekly between November 2019 to January 2020 from one sampling station at Tigris river of Baghdad city. (Samples were collected in sterile bottles) and stored for later analysis. Cyanobacterial culturing by Tow Culture Medium were used in these study BG-11 culture medium and Chu-10media. The cyanobacteria in liquid medium were directly spreader over the entire surface of the solid medium resulting in separation at many places. 0.1 ml of diluted cyanobacterial mixture was inoculated onto the surface of agar and spreader [27]. The cultures were incubated at 28 ± 2 °C under continuous illumination of 54–67 50 μE/m²/s light intensity. Microscopic examination were performed to ensure the culture were unialgal.

**Genomic DNA extraction and PCR amplification**

The DNA extractions were carried out using Easy Pure® Genomic DNA Kit following the Gram-negative bacterial protocol as per manufacturer’s instructions. Extracted DNA was quantified and stored at − 20 °C until further use. Each sample was analyzed using three different primer sets, The sequences of the oligonucleotide primers used for PCR are listed in (Table 1) with final concentrations 25 µl. All the PCRs were carried out 5 µl of the extracted DNA were mixed with PCR mixture that composed of 12.5 µl of Easy Taq PCR Super Mix, 1 µl from each of primers (Forward and Reverse/10poml) and 5.5 µl of nuclease free water was added to complete volume to 25 µl. PCR reaction tubes were mixed by vortex and finally placed into thermocycler PCR instrument, the PCR conditions for these primers were involved as follows: an initial denaturation for 5 min at 95 °C; 36 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 62 °C (PCβ-PCα primer set) and for 1 min at 56 °C (Ckc and sxtA primer set), extension for 1 min at 72 °C and final extension for 1 min at 72 °C. After the reaction, 5 µl of amplified DNA was separated on 1% agarose gels (Promega, USA), stained with ethidium bromide and visualized on a UV transilluminator, the size of amplified products were compared with DNA ladder to determine the exact size of these products.

| Target | Primer | Sequence (5′-3′) | C | bp | Reference |
|--------|--------|------------------|---|----|-----------|
| cpcB-IGC-cpcA region to detect the presence of cyanobacterial | PCβF | (GGCTGCTTTTGTTACGCAGCA) | 62 | 650 | [15] |
| | PCαR | (CCGTAACCCAGCAACTAA) | 62 | | |
| aoaC gene to detect the cylidrospemopsin | Ckc-F | AATGATCGAAAACAGCAATGCG | 56 | 325 | [16] |
| | Ckc-R | TAGAACAATCTCCCCACAACCT | 56 | | |
| sxtA gene to detect the saxitoxin | sxtA-F | GATGACGGAGTATTGAAGC | 56 | 125 | [16] |
| | sxtAR | CTGCATCTTTCTGGACGGTAA | 56 | | |
Results and discussion

Isolation and identification of algae

Isolation and identification of algae from water samples, five isolates of cyanobacteria were obtained from the Tigris River included, *Microcystis flosaquae*, *Microcystis sp*, *Anabaena circinalis*, *Nostoc commune* and *Westiellopsis prolifica* Which belonged to four cyanobacterial orders: Chroococcales, Stigonematales and Nostocales as well as one isolate of chlorophyceae included *Chlorella vulgaris* where used as negative control test.

Extraction of DNA algae

Genomic DNA was successfully extracted from samples (algae) by Easy Pure® Genomic DNA Kit, DNA bands were confirmed and analyzed by gel electrophoresis as in (Fig. 1).

Detection of cyanobacteria by PCR test

A phycocyanin operon gene fragment containing the IGS (cpcBA-IGC) from cyanobacteria was amplified. A distinct amplicon patterns was produced from all of the DNA extracts with a size of 650 bp when analyzed in gel electrophoresis (Fig. 2) confirming the existence of cyanobacterial DNA from isolates collected from fresh water of the Tigris River in Baghdad. While the line of a green alga *Chlorella vulgaris* does not possess Pycocyanin operon, gave no PCR product suggested the highly specificity of used primers. The results confirming the presence of cyanobacterial DNA from isolates that take from Tigris River in Baghdad city and successfully amplified of fragment by used PCβ-PCα primes set for cyanobacterial detection and reported the same results [28].

Detection of cylidrospemopsin by PCR assay

In this analysis, traditional PCR was used to recognize cyanobacteria that produce cylidrospemopsin and have aminotransferase enzyme, the (CKc-F/R) primers successfully amplified the 325 bp fragments of the aoaC gene from all microcystin-producing cyanobacterial isolates Except for Chlorella vulgaris. (Fig. 3). The specificity of 325 bp
primers appeared to be highly specific for isolates producing cylidrospemopsin, since there was no DNA amplified from chlorophyta used in this study. The results agreed with the study of [30].

PCR methods were used in several experiments to find the cylidrospemopsin producers in freshwater. While biomolecular detection methods have gained popularity due to their specificity and speed, only a few studies have focused on using PCR techniques to quickly track cylidrospemopsin producers in river water. Marbun et al. [31] have shown that the qPCR approach can be used to detect C. raciborskii in reservoirs quickly and on-site. Furthermore, the findings indicate that cylidrospemopsin is a major cyanotoxin in Kinmen Island’s reservoirs.

Detection of saxitoxin by PCR assay

The sxtA-F/R set primers were used to detect the sxtA gene, which is unique to cyanobacteria that produce saxitoxin and amplified 125 bp. The results showed that the sxtA gene was found in all samples isolated from the Tigris River except C. Since no DNA from chlorophyta was amplified (Fig. 4). The findings were in line, its stratification with those of many other studies. [32, 33].

The sxtA-F/R set primer developed by [34] was shown to be specific to saxitoxin-producing Anabaena circinalis based on conventional PCR. The sxtA/R primers also amplified the sxtA gene from other cyanobacteria that produce saxitoxin. Saxitoxins (STXs) cause neurotoxicity by blocking sodium ion channels in nerve axon membranes; STXs are also known to block calcium channels. STXs also affect heart muscle cells by prolonging potassium channel gating, which can result in changes in ion influx into the cell. [20, 22]. To generate fresh portable water, most existing drinking water treatment plants use traditional treatment methods such as coagulation-flocculation, sedimentation, sand filtration, and disinfection [26].

The increased occurrence of cyanobacterial blooms in freshwater bodies over the last few decades has prompted water management authorities to pay more attention to the threats posed by toxic cyanobacteria. While reports of neurotoxins are becoming more common, the diagnosis of toxic occurrences still focuses primarily on microcystins [35].

Conclusions

The ability to estimate the toxigenicity of cyanobacterial in river water based on the detection of sxtA and aoaC genes by PCR assay is based on the findings presented in this work. Managers would benefit from this technique because it allows them to monitor the development and progression of cylidrospemopsin and saxitoxin in Iraqi river water. The simultaneous identification of cyanotoxin-producing genes and potentially cyanotoxin-producing species demonstrates the utility of the PCR technique as a monitoring tool, particularly when the number of target organisms in the freshwater sample is low.

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Declaration

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical approval The Ethics Committee of the Mustansiriyah University approved and oversaw this study.

Research involving human participants No human sample was used.

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