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RESEARCH ARTICLE

PCR Detection of MycrocystinSynthetase E Gene in Toxogenic Cyanobacteria Isolated from Tigris River in Baghdad City

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Abstract

Cyanobacterial blooms and the associated cyanotoxins contamination are being increasingly reported worldwide that cause a serious hazard to environmental and human health. Mycrocystin was the most algal toxin and reported to be produce by several orders of cyanobacteria. Between 2010 and 2011, toxic cyanobacteria were isolated from fresh water of Tigris River and identified by light compound microscope as well as conventional PCR. The results revealed sixisolates of dominant toxic cyanobacteria which successfullyamplified a gene fragment from the phycocyanin shared by all cyanobacteria and *myc E* belonged to microcystin in all tested isolates. Our data concluded that PCR assay can be used for early detection of microcystin producing cyanobacteria in bloom and fresh water that useful to companies responsible for the surveillance of drinking water.

Introduction

Cyanobacterial blooms and the associated cyanotoxins contamination are being increasingly reported worldwide. Harmful algal blooms have been identified in fresh water, Estuarine, and marine system. In fresh water some cyanobacteriamay produce dermal toxins, neurotoxins and hepatotoxins which including nodularins and microcystins (Nanneman and Zimba, 2002) that accumulated in aquatic organisms and transferred to higher trophic levels, representing a health hazard to animals and humans (Chen et al., 2013; Pearson and Neilan, 2008). Among all the cyanotoxins, microcystins are the most frequently studied because of their wide distribution and high toxicity. Up to now, more than 80 different structural variants have been identified, among which microcystin-LR is the most common and potent variant, followed by microcystin-RR and microcystin-YR (Chen et al., 2013).

Good monitoring systems are needed to protect water users from these toxins. Excellent methods, such as ELISA and high performance liquid chromatography have been established for most cyanotoxins, but they frequently use laborious sample preparation protocols as well as expensive machinery and purified toxin standards that are often difficult to obtain. However, molecular detection techniques such as conventional PCR, quantitative real-time PCR and microarrays/DNA chips that are rapid, extremely sensitive and specific for detecting toxic cyanobacteria in water supplies (Pearson and Neilan, 2008; Sivonen , 2008).

The distribution of phycocyanin in the aquatic microorganism makes the study of phycocyanin genes ideal for the classification of freshwater cyanobacteria (Neilan et al., 1995). Phycocyanin operon contains genes coding for two bilin subunits and three linker polypeptides. The intergenic spacer (IGS) between the two bilin subunit genes, designated as b (*cpcB*) and a (*cpcA*) showed variations in their sequences which are capable of differentiating genotypes below the generic level make it useful for the identification of cyanobacteria via PCR (Wu et al., 2010).

Conventional PCR could be used to detect microcystin producers and several primers are available for these large gene clusters (55kb) (Rouhiainen et al. 2000; Fastner et al. 2001; Welker et al. 2004). *mcy*E primers were successfully used to get PCR product from all known microcystin and nodularin producers (Vaitomaa et al. (2003)

and Rantala*et al*. (2004)) while Jungblut and Neilan, (2006) chose the aminotransferase domain, which was located on the modules *mcy* E and *nda* F of the microcystin and nodulrinsynthetase enzyme respectively to detect all potential microcystin and nodularinprodusing cyanobacteria from laboratory cultures as well as in harmful algal blooms.

Tigris River is one of two main sources of drinking water for Iraq, serving population approximately seven million people settled in Baghdad city, this river usually affected by agricultural and industrial eutrification as well as the sewage effluent, which provide protected mesocosms of cyanobacteria growth and potential mycrocystin production. Therefore, this study was aimed to use PCR technique for identification of cyanobacteria and mycrocystin producing isolates from Tigris River for early detection of potentially toxic species that could be useful to companies responsible for the surveillance of drinking water.

Material and Methods

1. Samples collection

Water samples were collected from the higher superficial layer of Tigris River from a depth of 20-30 cm monthly between September 2010 and August 2011using 20µ mesh net. Samples were transported to the laboratory on ice. 10 ml of water sample was added to chu-10 culture medium and incubated at 28ºC for two weeks with continuous illumination of 50 µE/m²/s. one ml of growth inoculated on agar plates containing BG-11 and incubated in the same condition for one week to isolate unialgal (Stain, 1973). Regular microscopic examination were performed to ensure the culture were unialgal.

2. Extraction of DNA from cyanbacterial and chlorophyta isolates

Genomic DNA was extracted from the cyanobacterial isolates using the method described by Matehkolaei et al. (2012). Briefly, amount of fresh colony was placed in lysis buffer (200 mMTris-HCl, pH 7.5, 25 mM EDTA,0.5% w/v SDS, 250 mMNaCl), and crushed with a conical grinder. Samples were incubated for 20 min at 100°C and mixed with 150 µl of 3.0 M sodium acetate, kept at -20°C for 10 min and centrifuged at 12,000 g for 10 min. The supernatants were extracted once with phenol chloroform iso-amyl alcohol (25:24:1), and once with chloroform. DNA was precipitated with an equal volume of isopropanol, washed with 300 µl of 70% ethanol, dried and suspended in 50 µl of TE. The final solution was kept at -20 ºC until using as template for PCR. Genomic DNA was extracted from the chlorophyta isolates for specificity test using CTAB method (Doyle and Doyle, 1990).

3. Polymerase chain reaction

PCR was performed with two sets of primers. PCβF (GGCTGCTTGTTTACGCGACA) and PCαR (CCAGTACCACCAGCA ACTAA) (Saker et al., 2007) to amplify *cpcB*-IGC-*cpcA* region in phycocyanin operon while the HEPF (TTTGGGGTTAACTTTTTTGGG CATAGTC) and HEPR (AATTCTTGAGGCTGTAAATCGGGTTT) (Jungblut and Neilan, 2006) used to amplify *mcy*E gene of the microcystinsynthetase. All PCR mixture was set up in a total volume of 20µl included 5µl of PCR premix (Accupower, Bionear), 1picomole of each primer and 100 ng of template DNA then the rest volume was completed with sterile D.W. Negative control contains all material except that distal water was instead of template DNA. PCR protpcols involved an initial denaturation for 2 min at 95ºC; 35 cycles of denaturation for 90 sec at 95ºC, annealing for 30 sec at 52ºC (PCβ-PCα primer set) and for 90 sec at 95ºC(HEP primer set), extension for 1min at 72ºC and final extension for 8 min at 72ºC. 10µl of PCR product was separate in 1.5% agarose gel electrophoresis stained with ethidum bromide and visualized on a UV transilluminator, the size of amplified products were compared with the 100pb DNA ladder to determine the exact size of these products.

Results

1. Isolation and identification of algaefrom water samples

Sixisolates of dominant cyanobacteria were obtained from the Tigris River included *Microcystisaeruginosa*,*Microcystisflos-aquqae*,*Lyngbya* sp.,*Chroococcusturigidus*,*Oscillatorialimnetica*and *Nostoccarneum.* which belonged to four cyanobacterialorders: Oscillatoriales, Chroococales, Stigonematales and Nostocales as well as two isolates of chlorophyceae included *Scenedesmus*sp. and *Chlorella* sp. used for specificity test.

2. Detection of cyanobacteria by PCR assay

In the current study, the gene fragment of the phycocyanin operon 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. 1), confirming the presence of cyanobacterial DNA from isolates collected from fresh water of Tigris River in Baghdad. While lysates of a green algae *Scenedesmus*sp. and *Chlorella* sp. does not possess pycocyanin operon, gave no PCR product suggested the highly specificity of used primers.

3. Detection of Microcystin by PCR assay

In this study, conventional PCR used as a tool to identify potentially microcystin producing cyanobacteria possess aminotransferase enzyme. The HEP primers were successfully amplified the 472 bp fragments of *mcy* E gene from all microcystin-producing cyanobacterial isolates (Fig. 2). The specificity of HEP primers appeared to be highly specific for isolates producing microcystin since there was no DNA amplified from chlorophyta used in this study.

Fig. 1. Gel electrophoresis of amplified *cpcBA-IGC* (650bp) in cyanobacterial isolates. Agarose (1.5%), 5 V/cm for 2 hrs, stained with ethidum bromide and visualized on a UV transilluminator. M. 100 bp DNA ladder. Lane 1-6.*Microcystisaeruginosa, Microcystisflos-aquqae, Nostoccarneum ,Oscillatorialimnetica, Lyngbya* sp. *and Chroococcusturigidus*respectively. Lane 7-8.*Scenedesmus*sp. and *Chlorella* sp. Lane 9.Negative control.

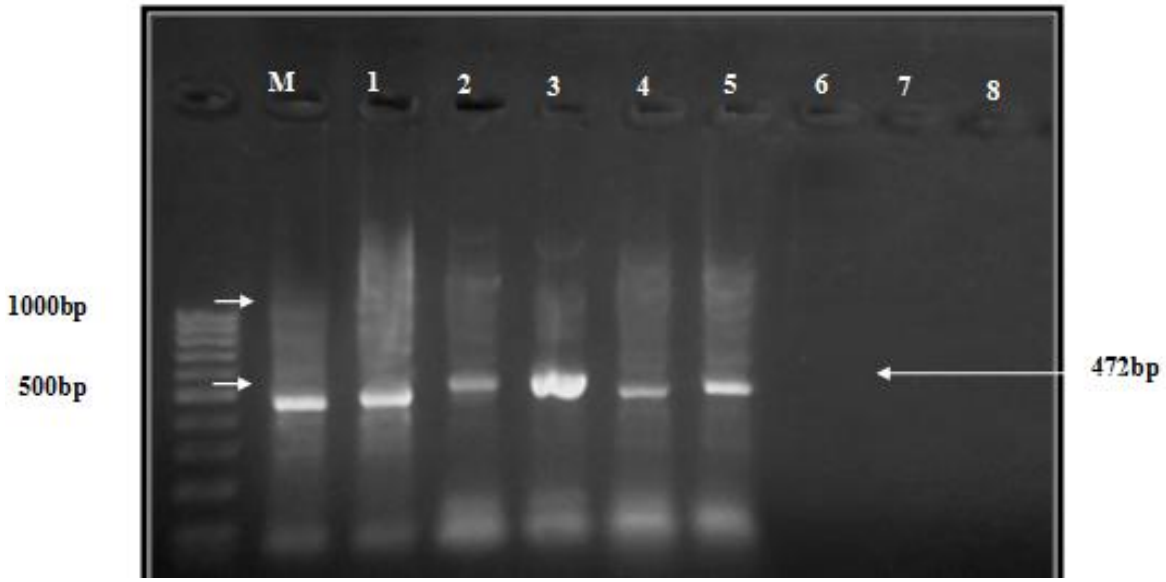
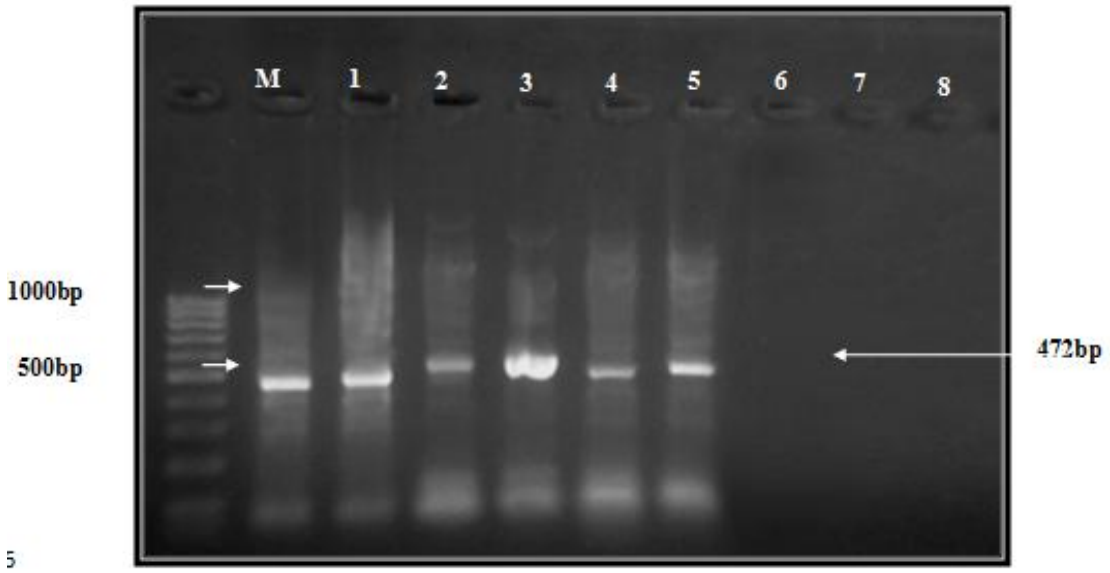


Fig. 2. Gel electrophoresis of amplified *mycE* (472bp) in cyanobacterial isolates. Agarose (1.5%), 5 V/cm for 2 hrs, stained with ethidum bromide and visualized on a UV transilluminator. M. 100 bp DNA ladder. Lane 1-6.*Microcystisaeruginosa, Microcystisflos-aquqae, Nostoc carneum, Oscillatorialimnetica, Lyngbya* sp. *and Chroococcusturigidus*respectively.Lane 7-8.*Scenedesmus*sp. and *Chlorella* sp. Lane 9.Negative control.



Discussion

The microscopic results revealed that all species of cyanobacteria that isolated from Tigris River related to toxic dominant genera which produce microcystins. This might be related to the capability of these species to highly competition to remain dominant utilizing all environmental conditions such as high temperature, optical density and abundance of nutrients (eutrification), all these factors allowed to form the blooming and can increase microcystin production rates (Kearns and Hunter, 2001).

Morphological identification is time consuming and it requires high expertise. In fact, morphological features used for the identification of species such as colonial form, mucilage patterns and cell arrangement in the colony is frequently variable and dependent on the environment (Otsuka et al., 2000). Furthermore, the co-occurrence of toxin producing and non-producing cells that are morphologically indistinguishable (Janse et al., 2005; Dittmann and Wiegand, 2006). Therefore, the development of a molecular method for the identification of cyanobacteria is essential for the rapid and accurate analysis members of cyanobacterial population (Nelian et al., 1995). Several investigators used PCβ-PCα primer set for cyanobacterial detection and showed the same results revealed in this study (Saker et al., 2007; Baron-Sola, et al., 2012). Except in Nelian et al. (1995) study, he was reported that phycocyanin gene fragments from *Nostoc commune* and *Nostocpunctiforme* were unable to be amplified using these primers while strains of all of the cyanobacterial genera were successfully amplified. In recent research, found that *Nostocpunctiforme*had short sequence and incomplete of *cpcBA*-IGC region resulting in high variability in these genes and cause heterogeneity of genus *Nostoc*(Teneva et al., 2012).

The detection of cyclic peptide hepatotoxin genes by using HEPF and HEPR primers was developed to identify potentially microcystin or nodularin-producing cyanobacterial blooms that possess the aminotransferase domain of either *mcy E* or *nda F*, involved in the production of microcystin or nodularin from four order of cyanobacteria included *Oscillatoriales, Chroococales, Stigonematales*and *Nostocales* (Jungblut and Neilan, 2006).

The aminotransferase domain was chosen as the target sequence because of its essential function in the synthesis of all microcystins as well as nodularins that catalyzes the addition of D- glutamate to Adda, an essential step in the synthesis of both microcystin and nodularin (Al-Tebrineh et al., 2011). Thus, can use these described primers to amplify a 472 bp PCR product from the aminotransferase domains of all tested hepatotoxic species and bloom samples. In adittion, these primers can be used for distinguished between toxic and non-toxic populations of cyanobacteria that coexist simultaneously in a single ecosystem and are indistinguishable by microscopy (Jungblut and Neilan, 2006).

Tigris River usually affected by agricultural and industrial eutrification as well as the sewage effluent, high turbidity, river discharge or by agricultural runoff which provide protected mesocosms of cyanobacteria growth and potential microcystins production. The results by Ranatal et al. (2006) and Bittencourt-Oliveira et al.(2011), suggested that eutrophication increased the co-occurrence of potentially microcystins producing cyanobacterial genera, raising the risk of toxic-bloom formation.

Conclusion

From these data revealed that using of PCR assay for detection and targeting toxin-producing cyanobacteria could replace laborious and time-consuming microscopic techniques for early detection of potentially toxic species that could be useful to companies responsible for the surveillance of drinking water allows these companies to implement appropriate measures for preventing the growth of these organisms, such as artificial destratification or the application of water cleansing procedures.

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