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# IDENTIFICATION OF *CYANOBACTERIA* PRODUCING MICROCYSTIN IN AMIRKELAYE LAGOON BY PCR

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## ABSTRACT

**Introduction**: Microcystin is one of the important hepatotoxin that is produced by *cyanobacteria*. Accumulation of microcystin in aquatics that are creates arisk ofpoisoningfor humansis debatableissues and of course the level of consumption and severity of toxic blooms are communicated in the area.The purpose in this study is identifying the molecular microcystin toxin producing gene in *cyanobacteria* of Amirkelayeh lagoon in Gilan province.

**Methods**: In this study, two primers first: Universal (CYA106F, 23S30R) only to identify the *cyanobacteria* .second: (mcyA-Cd1F mcyA-Cd1R) for microcystin toxin were used. PCR test for both primers optimized and were determined for limit of detection and specificity. In order to confirm the PCR product amplicon was cloned. A total of 20water samples were collected from

20 stationslocated inAmirkelayeh lagoon. DNA of samples were extracted by DNG modified method andthe presence of*cyanobacteria* and microcystin-producing *cyanobacteria*were analyzedby PCR method.

**Result and discussion**: optimized PCR product were amplified for universal primers (487 bp) and specific primers of microcystin toxin (297 bp), The amplicon were also observed by gelelectrophoresis. Specificity test was 100% for selected primers with DNA of other microorganisms. The presence of *cyanobacteria* was checked in all stations by PCR assay. One sample was positive for the presence of microcystin-producing *cyanobacteria* in Amirkelayeh lagoon.**Conclusion**: PCR method can be diagnosis of microcystin toxin-producing *cyanobacteria* in water resources.

**Keyword: PCR, *Cyanobacteria*, Microcystin, Amirkelayeh lagoon**

## INTRODUCTION

*Cyanobacteria*, are blue-green algae are photosynthetic prokaryotes that live in the warm, sweet and eutrophic environment.*Cyanobacteria* bloom in local waters with produce dangerous toxins known as Cyanotoxin,Causes problems on a global scale(1).Microcystin is an important *Cyanobacteria* toxin. Variety of microcystin quality was observed in most Anabaena speciesand also Microcystis.Typically, some speciesare microcystin types such as*Nostoc, Chroococcus, Planktothrix, Anabaena and Microcystis*(2). Micricystin, encoded by mcy gene and so was synthesizedby microcystinsynthesase(2),(3)The Amirkelayelagoonis an important ecosystemThis part of the nature inGilan has several attractions that has important role in the life of other aquatic and non-aquatic species in area, As well as, along with the ecological effects, economic and tourism are important (4). Many species of *cyanobacteria*, in estuaries, rivers, freshwater lakes, oceans and drinking water storage, create significant problems (5).

So, to protect consumers, from poisoning and exposure to *cyanobacteria* toxin, it is necessary that water resources was assessed for the presence of dangerous toxins from to quantitatively and qualitatively and prevention the possible risks arising from their use (2), (6).Are also required.

Nowadays, ,using varietymethods for identification of *Cyanobacteria* producing microcystin havetheir advantages and disadvantages.Some of these methods was noted ELISA (ELISA), protein phosphatase inhibition assay (PPIA),Identifying toxin

absorption in the solid phase (SPATT),High performance liquid chromatography (HPLC), And liquid chromatography mass spectrometry (LC / MS)(7-9)

Molecularmethods are such as, PCR (Polymerase Chain Reaction), Can be used as a tool for the identification of toxins *cyanobacteria* encoding genes (10-12) For example, in 2009, by Valerio an et al. were detected several mcy target gene in micricystin(13). In 2010, Oliveira and her colleagues were examined *Cyanobacteria* blooms in northeastern Brazil. Cyanibacteria were detected in 11 districts and 7 water resource. In all cases PCR technique was used to identify microcystin encoding gene (2). In 2011, Pedro and partners identifiedthe predominant genotype of *Cyanobacteria*producing microcystin by PCR in three water zon of Mozambique and they also introduced Microcystis as dominant genotype (14).

PCR method, isas raised a sensitive, accurate, and efficient technique to identify *Cyanobacteria* producing toxins such as microcystin.Therefore,the purpose of this study, wasIdentification of *Cyanobacteria* producing microcystin in Amirkelaye lagoon by PCR method.

**METHODS**

***Sampling:*** A total of 20 water samples from 20 stations, collectedfrom different parts of the Amirkelayeh lagoon and were transferred in sterile containers,in terms of lack of light and temperature in 4 ° C to laboratory. Then, each sample was centrifuged (12000 rpm for 5 minutes), and after removal of supernatant, remaining pellet was mix with 100 µl of deionized water.

***DNA extraction:*** DNA extraction was performed by using DNG-Plus Kits (Sinaclon DN8117C), according to the manufacturer′s instructions. In details, for each sample, 100 µl of sample-containing deionized water was mixed with 400 µl of DNG solution in 1.5 ml micro-tubes, heated on heater block for 4 hours, agitating every 30 minutes. Then, the mixture was centrifuged (12000 rpm for 5 minutes) and its supernatant was transferred into a new 1.5-ml micro-tube and was mixed with 500 µl Chloroform solution, agitated and centrifuged (12000 rpm for 10 minutes). The resultant supernatant was mixed with 300 µl cold ethanol in a new 1.5-ml micro-tube and mixture was lightly inverted ten times, and then, was centrifuged (12000 rpm for 15 minutes), decanted, mixed with 500 µl of 70% ethanol, agitated, and centrifuged (12000 rpm for 5 minutes) and decanted for second time. Finally, each micro-tube was dried on heater block for removal of alcohol,

and was mixed with 100 µl of deionized water and incubated for on heater block (65 °C degree for 5 minutes).

***Optimization of PCR test:***PCR tests, were optimized by used the primers to identify *cyanobacteria* (CYA359F ,CYA781R) and specific primers for mycrocystin encoding gene (mcy-cd1F,mcy-cd1R)(15-17)and using standard DNA *microcystis aeruginosa* strains PCC7806( Table 1).

**Table 1: Sequences of used primers**

| Primers | Sequences | PCR product size |
|---|---|---|
| **CYA359F**<br>**CYA781R** | **5'-GGGGAATYTTCCGCAATGGG-3'**<br>**5'-GACTACWGGGGTATCTAATCCCWTT-3'** | 487**bp** |
| **mcyA-Cd1R**<br>**mcyA-Cd1F** | **5'-AAAAGTGTTTTATTAGCGGCTCAT-3'**<br>**5'AAAATTAAAAGCCGTATCAAA-3'** | 297**bp** |

The temperature profile for *Cyanobacteria* and microcystin in order to following step:

First denaturation; 94°C/95°C for 3/5 min, Denaturation: 94°C/95°C for 20 sec/1 min ,Annealing:50°C/56°C for 20/40 sec, Extention: 72°C/72°C for 40/60 sec and final Extention 72°C/72°C for 5/7 min.

***Mixture PCR reaction:***ThePCRmixturewas prepared: Double disterile water (DDW): 15µl, 10x buffer (Thrmoscientific): 2.5µl, $Mgcl_2$ (Thrmoscientific): 0.75µl (1.5 mMol), dNTP (Thrmoscientific): 0.5µl (0.2mMol), forward primer: 0.5µl (0.2mMol), reverse primer: 0.5µl (0.2mMol), Taq DNA polymerase (Thrmoscientific): 0.3µl (1.5 Unit), template DNA: 5µl and the final volume of a test was 25 µl.

***Limit of detection (LOD) and specificity tests:***To determine of LOD,optimized PCR assay was applied on serial dilution of positive control DNA,with significant number of genome.Specificity of PCR tests for detection of *cyanobacteria*,common bacteria DNA such as*Staphylococcus spp* ، *Streptococcus spp* ،*legionella pneumophila* ، *E.coli* ،*Pseudomonas aeruginosa*,Mouse *DNA and* human *DNA*were extracted and Optimized PCR test were evaluated on them with positive and negative control samples.As well asspecificity of microcystin primers was determined with microorganisms: *Fisherella, Synechococcus, Pleurocapsa, Cylindriospermopsis,Aphanothece, Schizothrix, Microcystiswesenbergii*.

***Cloning:***The cloned PCR product into the vector may be suitable for a variety of purposes including use as a template for PCR positive control and be used for further research,.After purification, the PCR product was cloned with using T/A cloning kit (Thrmoscientific K1213) and pTZ57R vector. The resulting plasmids were extracted with the Plasmid Mini Extraction Kit

(Bioneer K-3030-1) Corporation. Then plasmidscontaining the PCR product was confirmed.

***PCR Test in Samples:***Optimized PCR test to detection of*cyanobacteria* and microcystin were applied on collected samples.

**RESULTS**

PCR assay for detection of *Cyanobacteria* and microcystin, was optimized by using specific primers and thermal profile and product (487bp) for*cyanobacteria* (Figure 1a) and product (297 bp) for microcystin (Fig. 1 b), was observed on the gel.

Limit of detection of PCR test to *cyanobacteria*, 100 copies and intended for microcystin10 copies of genome was determined. This results, wereidentifiedinFigure (2a) and (2b).

To determine Specificity of optimized PCR tests, used primers were evaluated (for replication Cyanobacteria DNA and gene producing microcystin). Specificity of both tests showed no amplification with any other organism that shown, Specificity of primers is very high. (Fig 3A, B).

Of 20 collected samples, one sample was positive for the presence of microcystin-producing cyanobacteria in Amirkelayeh lagoon and the presence of cyanobacteria was confirmed in all stations by Specificprimers (Fig 4A, B).

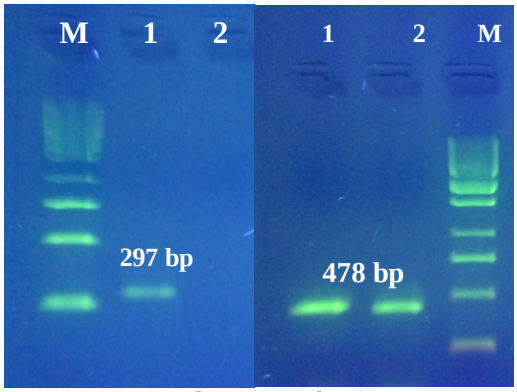


**Figure 1: The optimized PCR tests**

**A. Optimized PCR test to detection of microcystin. M:Size marker 1 Kb DNA Ladder (Thermoscientific) 1. Positive control (*MicrocystisaeruginosaPTCC7806 DNA)* 2. Negative control.**

**B. Optimized PCR test for Detection of *Cyanobacteria*. M:Size marker 1 Kb DNA Ladder (Thermoscientific) 1. Positive Control (*MicrocystisaeruginosaPTCC7806DNA) 2. Positive Control (AnabaenacircinalisAWQC131C DNA)***

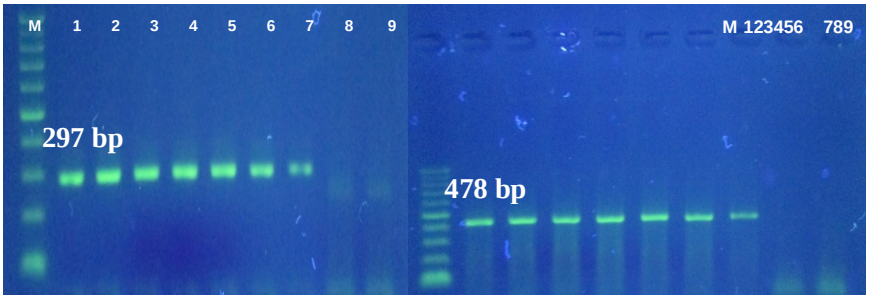


**Figure 2: The results of LOD tests**

**A. Result of LOD of microcystin. M:Size marker 100bp DNA Ladder (Thermoscientific) 1. : Positive control sample. The DNA of *Cyanobacteria*Microcystis aeruginosa PTCC7806. 2. $10^6$ DNA of *Cyanobacteria*Microcystis aeruginosa PTCC7806 3. $10^5$. 4. $10^4$. 5. 1000 6. 100 7.10 8. 1 9. Negative Control.**

**B. Result of LOD of *Cyanobacteria*. M:Size marker 100bp DNA Ladder (Thermoscientific) 1. : Positive control PCR product DNA of *Cyanobacteria*Cylindrospermopsisraciborskii AWT205 (478 bp) 2: DNA of $10^7$*Cyanobacterium*Cylindrospermopsisraciborskii and line 2-8. $10^6$-1 DNA of *Cyanobacterium*Cylindrospermopsisraciborski**

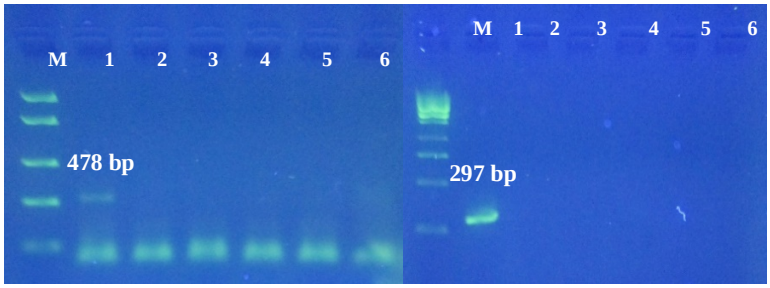


**Figure 3: The results of specificity tests**

**A. Result of *Cyanobacteria* SpecificityM:Size marker 1 Kb DNA Ladder (Thermofusariuscientific) 1. Positive Control. 2-5 DNA of *Staphylococcus aureus, Hepatitis B, Fusarium solani, Saccharomyces cerevisiae* 6. Negative Control**

**B. Result of microcystin Specificity. M:Size marker 1 Kb DNA Ladder (Thermofusariuscientific) 1. Positive Control.2-5 DNA of *Cylindrospermopsisraciborskii* AWT205,*Nostoc spp.*,*Anabena spp.*, *Fischerellaspp*.6. Negative Control**

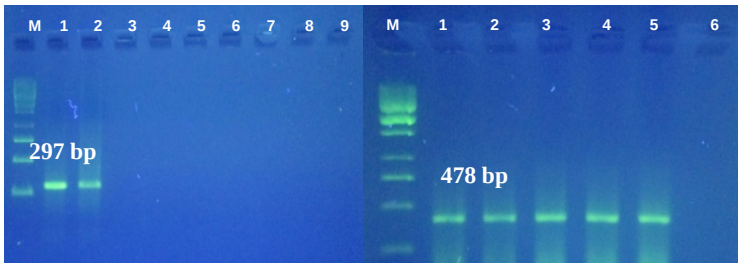


**Figure 4: results of Optimized PCR tests on samples**

**A. Optimized PCR test for detection microcystin in samplesM:Size marker 1 Kb DNA Ladder (Thermofusariuscientific) 1. Positive Control 2. Positive Sample 3-8. Negative samples 9. Negative Control**

**B. Optimized PCR test for detection *Cyanobacteria* in samples M:Size marker 1 Kb DNA Ladder (Thermofusariuscientific) 1. Positive Control. 2-5 Positive Samples 6. Negative Control**

**DISCUSSION**

The results of this study show thatthrough of 20 collected samples**,** all samples containing *cyanobacteria* and one sample (stations 8) includes *Cyanobacteria* producing microcystin.

Determining the presence of *cyanobacteria* and their toxins in the water, is a fundamental problem in the world.*Cyanobacteria* bloom createS vital issues in eutrophic waters.For example,It is estimated that in Alberta River or other rivers in Canada, 96% of bloom occurs in summer, istoxic and dangerous (14).Recent concerns about the proliferation of *cyanobacteria* and their potential toxicity,Led to identification of large number of contaminated areas with known Cyanotoxins.Including: Microcystin (MCs), Anatoxin (ANTX), Saxitoxin (STXs) and Cylindrospermopsin (CYN) (18).

Previous studies in the Caspian Sea,Reported*cyanobacteria*Bloom(Nodularia spumigena). And finally in 2009 it bloom, swept the southern coast of the seaand began early studies about *Nodulariaspumigena*species(19).

Microcystin as a poison that most research has been done on it,is a toxin with complex structure and 65 isoformand each of them have their own toxicity that Following the use of polluted water sources can be dangerous for humans (20). For example, Teixera in 1993, reported thatmakes more than 88% of child deaths in the city of Bahia (Brazil), were Anabaena species and Microcystin(21).

Several methods have been proposed to identify Microcystin, But each of these methods have their limitations (7-9, 22)Therefore, molecular techniques such as PCR, is Very important to identification of genes coding for*cyanobacteria* and their toxins (10-12)Biosynthesis genes of Microcystin,causesto identify the specific molecular target (23).This sequence around the world were used,In order to design and build primer based on PCR and And to identify the gene producing toxin (24-28).

Roland, by using primers to detection of *Cyanobacteria*PCβF&PCxR and primers to identify microcystin, *mcyA-cd1F* و *mcyA-cd1R,* that were used in this study,examined Toxicity and development of *cyanobacteria* in water resources Itaipu in Brazil.They found genetic similaritybetween samples of water resources Itaipu and *Microcystis species*.

Oliveira et al, in 2010were molecular identified*cyanobacteria* toxic on 7 water samples,In North East of Brazil.In the meantime,they found that all samples containing *Cyanobacteria* producing

microcystin by using two sets of primers to detect microcystin, *mcyB&mcyB*-A and primers to detection of*Cyanobacteria* PC-F&PC-R.*Mcy-B*primer had negative answered in Botafogo sample,While byusing mcB-A primers the same sample was positive (2).

Valerio et al in 2009, to reduce the number of false-positive identification of strains producing microcystin by ELISA technique, paided to identification of Three genomic areas mcyA ، mcyA-B&mcyB at the same time by Multiplex-PCR technique. So In this study, was used fromspecific primers toArea A for detection of *Cyanobacteri*a producing toxin.They determined that PCR can be a reliable technique for environmental samples (13).

Also, Pedro and his colleagues in 2010. By Using the same primers were identified the predominant genotypes *Cyanobacteri*a producing microcystin in the lake Mozambique.They determined that 33 percent of the 13 water samples containing *Cyanobacteri*a producing microcystin.However,in this study by Using the same primers,15% of water samples were positive to presence of *cyanobacteria* producing microcystin(14).

**CONCLUSION**

In this paper, PCR method was evaluated to detection of *Cyanobacteri*a producing microcystin in Amirkelayeh lagoon and show that can be identified *Cyanobacteri*a producing microcystin in this important water body and was informed presence of this toxin in the water resource that Habitat for many animal species at the time of blooming and none of it. And can be searching about advantage of molecular methods, specially PCR and their lower cost than other identification methods, fast and specificity.

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