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MOLECULAR DIAGNOSIS OF CYLINDROSPERMOPSIN-PRODUCING CYANOBACTERIA IN AMIRKELAYEH LAGOON

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ABSTRACT

Cyanobacteria commonly were found in fresh water and brackish. During the bloom, they produce toxins that threaten the health of water. One of the most important Cyanobacteria toxins is Cylindrospermopsin being a kind of alkaloid poison that consists the Cyclic Hepatotoxinpeptide. Toxicity from Cylindrospermopsin is associated with the death in humans and the domestic animals. The purpose of this study is quick identification of Toxigenic Cyanobacteria International AmirKelayeh lagoon by PCR Technique.

A total of 20 water samples were collected from 20 different stations from International AmirKelayeh lagoon. DNA samples were extracted by modified DNG method. PCR test was performed using Cyanobacteria diagnosis universal primers (CYA781R, CYA359F). The test has been used detection of specific primer of Cylindrospermopsin gene. It also checked for specificity and limited of detection. Amplicon was cloned in PTZ57R plasmid and providing positive control by the T/A cloned method.

In this study PCR test were amplified by using of cyanobacteria diagnosis specific primers (CYA781R, CYA359F) and Cylindrospermopsin diagnosis specific primers (cynsulfF, cylnamR). Clone was confirmed by PCR test. In specificity survey, primers test were formed only by positive control band and any bands weren't observed with other microorganism. Limited of detection were confirmed in range of 100 copies of genome. Five percent of Toxigenic Cyanobacteria was observed in one station from the 20 sample stations.

PCR teqnique used in this study by using of universal and specific primers is more accurate and faster method for detecting the cyanobacteria and Cylindrospermopsin-producing cyanobacteria in waters reservoir.

Keywords: Amirkelayeh Lagoon; Cyanobacteria; Cylindrospermopsin; Diagnosis; PCR

INTRODUCTION

Cyanobacteria are commonly known as blue-green algae which consist group of photosynthetic bacteria that inhabit a wide range of terrestrial and aquatic environment. They also have shown incredible morphological diversity. Many species of bloom-forming cyanobacteria are capable to produce biologically active secondary metabolites that are highly toxic to humans and animals(1). Cyanobacteria bloom produce dangerous toxin in local waters which is causing problems on global scale. It is known that some freshwater cyanobacteria like Microsistis, Anabaena, Osillatoria and Planktothrix are included a range of toxic cyanobacteria that are create taste and smell by secondary metabolites(2).Toxicology of cyanotoxins are included four categories as follows: Hpatotoxins, Neurotoxins, dermatoxin, and General synthesis inhibitor toxins. Neurotoxins are Alkaloid toxins and usually have molecular weight of less than 1000. All the non-sulfated alkaloid toxins in freshwater are neurotoxins, while the sulfated alkaloids from Cylindrospermopsin (CYN) is Neurotoxic (3) and have carcinogenic effects (4). Toxicity of CYN is inhibited glutathione and protein synthesis (5). CYN is detected in 1979 for the first time, when 148 people were hospitalized in Palm Island with symptoms of liver inflammation (6).Onset of disease was related with *Cylindrospermopsin rociborskii* in drinking water source. First reported in the lake,located in java, Indonesia. CYN producing cyanobacteria types are as follows: *Aphanizomenono valisporum, Cylindrosxermoxsis racixorskii, Aphanizomenonflox-aquae, Umezakianatans, Rhaphdiopsiscurvata, Anabaenabergii, Anabaenalapponica, Lygnbyawollei* (7). There are several methods to detect cyanobacteria toxins. Each has advantages and disadvantages. Molecular methods such as PCR have higher accuracy, sensitivity and speed than traditional methods such as culture. (8). The purpose of this study is quick identification of toxigenic cyanobacteria international Amirkelayeh lagoon in Gilan province with the method of molecular PCR technique.

METHODOLGY

Sample preparation: 20 water samples at 20 stations in different parts of the Amirkelayeh lagoon were collected in sterile containers and transferred to the Iranian Gene Fanavar institute laboratory at 4 °C and lack of light 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, 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 (1200 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 (1200 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 (1200 rpm for 15 minutes), decanted, mixed with 500 µl of 70% ethanol, agitated, and centrifuged (1200 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).Polymerase chain reaction was performed by using the universal primers detection of cyanobacteria (9,10)and specific primer of Cylindrospermopsin gene. (11)

Optimization of PCR test: Optimization of PCR test was performed by optimization of components density which used in PCR reaction and also by designing and selecting appropriate thermal application in thermocycler device (Table 1). PCR test was optimized by using DNA extraction from standard strain *Cilindrospermopsis rosiborskii.* By appropriate thermal application, PCR test was performed at time for cyanobacteria detection primers (CYA359F, CYA781R) and specific primers (cynsulf 5´-ACTTCTCTCCTTTCCCTATC-3´) and cylnamR (5´-GAGTGAAAATGCGTAGAACTTG-3´). Amplicon (template) DNA standard stain was evaluated beside the negative control samples and size markers on an agarose gel 1/5%.specific band from the amplicon that was 578bp in length was determined in PCR electrophoresis. PCR´s product electrophoresis: To detect target sequence amplification, agarose gel electrophoresis and stained with cyber green dye was used in different stages. Electrophoresis was performed for 45 minute at voltage V100.PCR`s product was moved during gel, after that it was determined by means of ultraviolet trans illuminator.

Table1.Thermal profile of Cylindrospermopsin

First Denaturation	**94°C**	**3min**	
Denaturation	**94°C**	**10 Sec**	**35 Cycle**
Annealing	**57°C**	**20 Sec**	
Extension	**72°C**	**60 Sec**	
Final Extension	**°C72**	**7Min**	

Determine limit of detection (LOD): To determine the limit of detection optimized PCR test and the minimum number of required cyanobacteria, serial dilution of DNA was prepared with specific number in order to do the PCR reaction on them. Serial dilution was prepared from extracted DNA *Cilindrospermopsis rosiborskii* with CYN toxic. The way of **preparation of serial dilution:**

-90µl sterile distilled water was added in 7 micro tubes

-10 µl *Cilindrospermopsis rosiborskii* DNA was added to the first micro tube

-With micro tube vortex, the required dilution was created evenly

-Dilution was continued to 10^7 dilution

-Considering DNA density in first tube, LOD of optimized PCR test was determined by these bacteria.

Specificity of optimized PCR test: Another common toxin DNA were extracted from toxic cyanobacteria *Anabaena circinalis, Microcyctis aeruginosa, microsistin, estafilococosoreuse, fosariomsolani* and then optimum PCR test were evaluated them with positive and negative control sample. This test shows the specificity of PCR test, this means that except *Cilindrospermopsis rosiborskii*, none of the samples shouldn't form a band with specific primers designed CYN.

Cloning: To cloning of the PCR products, the T/A cloning (fermentase) kit and PT57ZR plasmid.

RESULTS

PCR test optimization: PCR (Polymerase chain reaction) assay using DNA (Deoxyribo nucleic acid) extracted from standard strain AWT205, *Cilindrospermopsis rosiborskii* were optimized by using of primers for detection of cyanobacteria CYA359F, CYA781R and specific primers CylnamR and CynsulfF was conducted for one time according appropriate thermal application. In PCR product electrophoresis, specific band of slightly amplicon was visible that it had 578bp length. (fig1)

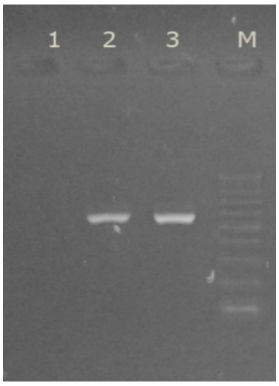

Fig 1: Cylindrospermopsin optimized test. M- DNA Ladder Mix 1- Negative control 2-3- PCR optimized test (positive control 587 bp)

Determine the sensitivity of PCR for the detection of cyanobacteria produce cilindorspermopsin: The limit of detection PCR test showed 100 cyanobacteria (100 copies of genome) (fig2)

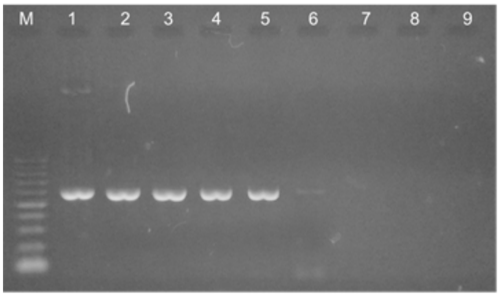

Fig 2: Determine the sensitivity (LOD) toxin gene Clindrospermopcin: M: size marker (100 bp DNA Ladder Mix) 1.Positive Control (578 bp) 2.10^{-1} 3.10^{-2} 4.10^{-3} 5 .10^{-4} 6.10^{-5} 7. 10^{-6} 8. 10^{-7} 9. Negative Control

Determine the specificity of PCR for detection of cyanobacteria and cylindrospermopsin:

To determine feature of optimized PCR test, primers used for cyanobacteria DNA proliferation and CYN producing gene were evaluated. The PCR test is been able to detect cyanobacteria and producing-CYN, however DNA of other species weren't amplified by primers test. (fig3)

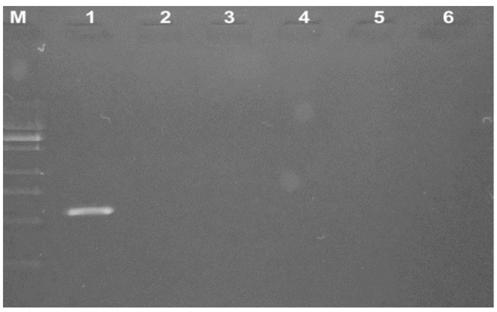

Fig 3: PCR specificity testM: size marker (100 bp DNA Ladder 1- positive Control 2-*Microcyctis aeruginosa 3-fishera spp* 4- *Anabaena circinalis* 5-*Microsistis spp.* 6-negative control

Cloning of PCR product: Plasmid with alkaline phosphatase was loaded from Lb-broth medium (with extracted clonies) and agarose gel 1.5% and electrophoresed. Since one of the target components 487bp and the other 578bp were interred the initial plasmid, the recombinant plasmid was heavier than PTZ57R.it is also has shown slower mobility and on the agarose gel was perched higher than no insert plasmid. After observing 487p and 587bp on the gel, it was found that cloning is done correctly and plasmid well extracted and gene is proliferate. Result of PCR test on water samples on the Amirkelayeh lagoon: The presence of CYN toxigenic cyanobacteria in 1 sample of 20 cases was recorded. (fig4)

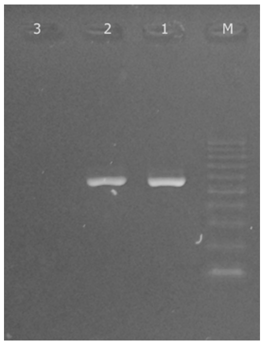

Fig4-PCR tests on water samples taken from Amirkelayeh lagoonM: size marker (100 bp DNA Ladder.1-possitive control.2-PCR test on samples.3-negative control

DISCUSSION

Cyanobacteria are the oldest photoautotroph organisms that usually Live in the seas, salt water and freshwater like planktonic. Also they can be tolerated Wide range of salinity and temperature. Toxins produced by some cyanobacteria and Increase in drinking water is developing acute and chronic effects that can Threaten organisms and aquatic life and other entities that use the contaminated water (12). Also human activities cause algae blooms that reduce oxygen and water and finally, cause increased mortality of organisms and acute and chronic diseases. Blooms of toxic cyanobacteria were considered in the first reports about the death of fish. The first evidence of this kind of report has been observed in Australia in 1878(13). Hepatomegaly Outbreak, for 148 indigenous inhabitants of Australia was reported in 1979. (14,15) All the patients were not at risk of disease. From 148 people reported, were 138 children (On average, 4 to 8 years that were 41% men and 59% women) and 10 of them adults (Age and gender were not reported). The symptoms were reported Fever, headache, vomiting, bloody diarrhea and kidney damage. The results showed that possible cause of the disease is from *Cylindrospermopsis raciborskii*. *Cylindrospermopsis rosiborskii* toxin cause damage to the liver and kidneys and other organs that the toxin was identified as CYN (15,16). Then in 2001 the prevalence of liver failure were reported in one of the clinics in Brazil. After a week of dialysis, patients were headache, blurred vision, nausea and vomiting at last half they died. Investigation of patients in clinic showed that the presence of CYN-producing cyanobacteria in water was caused liver failure **(17).** Many methods has introduced to detect CYN toxin. For example wood et al. in 2007 by chromatography method was examined Cyanobacteria toxicity in the Lake in New Zealand. Analysis on 16s rRNA (Ribo nucleic acid) gene showed that with Seasonal changes always *Cylindrospermopsis raciborskii* species CYN toxigenic is in the lake. This method is sensitive, but it is too slow. Also in this study used *Cylindrospermopsis raciborskii* species with PCR test which was highly sensitive with less time and fast (17)**.** In 2007 Rasmussen et al. were used Real-time-PCR method for the detection of a particular gene version *Cylindrospermopsis raciborskii* and CYN toxigenic cyanobacteria and the results were positive. This study was reported that Real-time-PCR is fast and sensitive method for the detection of CYN toxigenic cyanobacteria and genes involved in the production of this toxin are aoaA and aoaB and aoaC(18). In this study used as well as same Species and PCR assay was cheaper and more efficient than Real time PCR test and the results were positive only 1 From 20 samples.Kariana et al. in 2005 were succeed isolated CYN toxin *from Aphanizomenonflos-aquae* species of

Germany Lake. In this way, if the energy of the ions change it will reduce separation power and precision (19). This method is generally used reference laboratory and it is necessary to prepare the devices too expensive (Hundreds of dollars) and has created strong dependence, requires great expertise but PCR method is cheaper and done in less time. Amanda J in 2013, in an article checked out CYN toxigenic in tow urine and serum samples by using of LC / MS / MS and Elisa (Enzyme Linked immunosorbent assay) test. The results showed that the LC / MS / MS (Liquid chromatography- mass spectrometry) is an effective way to identify and quantify CYN toxins. And limit of detection for both serum and urine samples respectively were determined 0.5 and 0.25mg / ml. But Elisa method was inappropriate for serum sample and false positive for urine. Despite the positive features of the above methods. Every methodhas advantages and disadvantages which has limited their use in laboratories. For example, an ELISA is screening test and false positives and it is necessary to produce monoclonal antibodies for each of the toxins. It is also can evaluate certain pesticides LC / MS techniques require great expertise (20). While the water samples in this study were collected Amir Kalaye lagoon and DNA samples were extracted by DNG modified method. The advantages of this kit are high purity DNA extraction in the shortest time. In this way, by using of specific primers and PCR optimized test was detected CYN toxigenic cyanobacteria**.** Liu et al. in2011 by using of LC / UV method Identified CYN toxins in algae food supplements. On average 70 to 90% of them had been without CYN toxin and limit of detection was 16 mg / g. This way Announced expensive and accurate to detect toxins in food supplements (21). In this study, DNA was extracted from were used that PCR method is accurate way to detect CYN toxins in water samples.MikolajKokocinski and et al. In 2011 had examined species of cyanobacteria that produce CYN toxins and mentioned toxins Checked out cyrJ target gene by PCR assay. That was able to isolate this poison from the lake and found the original manufacturer's *Aphanizomenongracile* (11). Target gene of this study is sulfotransferases and a major manufacturer was not specified and only CYN toxin was diagnosed. Elena in 2013, with real time PCR test Identified CYN toxin in a paper and the results were positive. The limit of detection was 10^2 and 10^5 copies of the gene in average in each performance. This method can be used with speed and high quality to detect the CYN-producing cyanobacteria to monitor(22)and limit of detection in this study with PCR method was 10^2 gene copies in each performance. Lagoons as animal's environment and water resources used by humans of ecology are particular importance in the other field of aquatic ecology. In the meantime, the development of industrial activities and encroach human on the natural areas has been changed in the balance of biological cycles. Cyanobacteria as water microorganisms across the globe with their role as primary producers is created role of harmful to the environment and serve humans. Which some of these micro-organisms produce toxins that can be cited. One of the most toxic of these microorganisms is cylindrospermopsin. In this study, the ability of the PCR method for the identification of CYN-produce cyanobacteria is evaluated in international Amirkelayeh lagoon and show that CYN-produce cyanobacteria has identified In the area of water. And the presence of this toxin in the water supply Particular lagoons that are environments of different animal species and can obtained information of flowering time or otherwise. Also the advantage of molecular methods, especially PCR test ata lower cost than other methods of identification is searched fast and exclusive.

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