Molecular detection of cyanotoxins in water reservoirs in Anuradhapura District, Sri Lanka

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ABSTRACT

Most chemicals arising in drinking water are of health concern only after exposure of years, rather than months. A number of chemical contaminants have been shown to cause adverse health effects in humans due to prolonged exposure through drinking water. A number of human deaths have been reported through exposure to cyanobacterial toxins in drinking-water. Therefore, this research was undertaken to determine the value of using specific molecular techniques for the direct detection of toxin producing cyanobacteria in selected drinking water reservoirs in Sri Lanka. Environmental water samples were subjected to PCR to detect cyanobacteria, *Microcystis*, microcystin, *Cylindrospermopsis* and cylindrospermopsin targeting the specific 16S rRNA, *mcyE*, cylindrospermopsin synthetase (*rpoC1*) and cylindrospermopsin specific peptide synthase (PS) genes respectively. All tested were positive for unicellular and five were positive for filamentous cyanobacteria. In all three reservoirs, positive amplification was observed for *mcyE* genes indicating the presence of cyanobacterial strains with genetic potential to produce microcystins. A positive amplification was resulted in Nuwara wewa cultured sample for *rpoC1* gene confirming the presence of *Cylindrospermopsis raciborskii* in the reservoir. Further, an environmental sample from Kala wewa was positive for PS gene confirming the presence of toxic *Cylindrospermopsis* species and thereby cylindrospermopsin in that reservoir. According to the findings, all three lakes have a vast distribution of cyanobacterial species with toxic generating ability and it might be a major risk factor for the human health.

Keywords: Cyanobacteria; Cyanotoxins; PCR

INTRODUCTION

Cyanobacteria are aquatic and photosynthetic prokaryotes which in their mass occurrence produce harmful algal blooms (HAB) under optimal environmental conditions. Massive occurrence of HAB affects the water quality by changing the pH, transparency and biodiversity, and by production of odors and hazardous toxins (cyanotoxins) as their secondary metabolites. Therefore, their presence in freshwaters is of increasing concern worldwide. The incidence of wild and domestic animal poisoning and human health problems attributed to exposure to cyanobacterial toxins have been well documented. Toxic cyanobacteria are found in inland and coastal water environments. At least 46 species have been shown to cause toxic effects in vertebrates (Sivonen and Jones 1999). The most common cyanobacteria in fresh water are *Microcystis* spp., *Cylindrospermopsis raciborski*, *Planktothrix* (syn. *Oscillatoria*) *rubescens*, *Planktothrix* (syn. *Oscillatoria*) *agardhii*, *Gloeotrichia* spp., *Anabaena*, *Nostoc* spp., *Lyngbya* spp., *Aphanizomenon* spp., *Synechococcus* spp., some *Oscillatoria* spp., *Schizothrix* spp.,

Anabaenopsis and *Hapalosiphon*. Toxicity cannot be excluded for further species and genera. Among the potentially toxic cyanobacterial species, several chemically and toxicologically diverse compounds have been isolated, purified and characterized (Sivonen and Jones, 1999).

Health impairments are also seen from numerous anecdotal reports of irritations of the skin and/or mucous membranes and from documented cases of illness after exposure through drinking-water as well as accidental swallowing or aspiration of scum material.

Among the cyanotoxins, microcystins are the predominant toxins in freshwater lakes worldwide and have been implicated in several cases of animal and human intoxications (Kuiper-Goodman *et al.*, 1999). There are two aspects of chronic Microcystin, damage to the liver-progressive active liver injury (Falconer *et al.*, 1988) and the potential for promotion of tumour growth. A number of human deaths have been reported in exposure to cyanobacterial toxin; microcystin through renal dialysis and also microcystin and cylindrospermopsin implicated in drinking-water (Falconer *et al.*, 1983; Falconer, 2005; Hawkins *et al.*, 1985; Byth, 1980). Cylindrospermopsin is a general cytotoxin that blocks protein synthesis, the first clinical symptoms being kidney and liver failure. In contrast to the pure toxin, crude extracts of the organism also cause injury to the lungs, adrenals and intestine, indicating further, unknown toxins in the organism. Clinical symptoms may become manifest only several days after exposure, so it will often be difficult to determine a cause-effect relationship. Patients intoxicated with cylindrospermopsin via drinking- water in an incident in Australia escaped death only through skilled and intensive hospital care (Falconer, 2005).

Concern about cyanobacterial blooms in fresh waters in Sri Lanka has also grown in recent years due to a numerous reasons. These include: frequent recordings of cyanobacterial blooms, suspected fish kills by these blooms, problems created by blooms formed in aesthetic water bodies in the capital and other cities. In addition to these, a patchy distribution of thick cyanobacterial scum in irrigation water bodies in Sri Lanka is commonly observed, particularly in the dry season. Furthermore, waters in most of the irrigation reservoirs in the dry zone are also used for drinking without appropriate purification (Jayatissa *et al* 2005). Under these circumstances, investigations of cyanotoxins present in Sri Lanka water bodies' needs extensive attention. Therefore, this research was undertaken to determine the value of using specific molecular techniques for the direct detection of toxin producing cyanobacteria in selected drinking water reservoirs in Anuradhapura District, Sri Lanka

METHODOLOGY

Site Selection and Sample collection

Kala wewa, Tissa wewa and Nuwara wewa in the District of Anuradhapura were selected for water sample collection in June 2010. All the samples were collected in sterile brown glass containers and the collections were carried out from both on the surface and down the water column (~ 0.1 - 15m) to represent the whole water body. Temperature and pH were also recorded. Morphological observations were also made from each sample using compound light microscope (Olympus BH-2) (400–1,000×) by standard morphological methods.

Culturing of water samples

Collected water and soil samples were concentrated by centrifugation (3500 rpm, 10 min) and 500µL of the resulting pellet and 500µL from supernatant were inoculated into cyano specific BG11, $BG11_0$,

$BG11_0C$ and MLA media. Cultures were incubated at room temperature (27° C) with 12- 12 light dark cycling. Morphological observations were made from each sample using compound light microscope (Olympus BH-2) (400–1,000×) by standard morphological methods

DNA extraction and purification

500 µL of environmental water sample from each site (Table 1) along with 500 µL of standard *M. aeruginosa* culture (obtained from the Pasteur Culture Collection (PCC 7941), France.) were subjected for DNA extraction. In brief, above each sample were transferred to 500 µL of 1xTE buffer and three sequential heating (at 99 °C for 5 min) and freezing (at –5° C for 5 min) achieved lyses. Samples were centrifuged (12,000 rpm, 5 min) and to each resulting pellet, 40 µL of TES and 20 µL of lysozyme (10 mg /mL) was added, and incubated for overnight at 37°C. Cells were then treated with 10 µL of proteinase K (20 mg/mL) and 40 µL of TE/ SDS and incubated at 55°C for 2 h to lyse the organisms further. Subsequently, proteinase K was inactivated by heating the sample at 95°C for 10 min. Finally, nucleic acids were purified by Boom's method (Boom *et al.,* 1990) using silica particles and guanidium isothiocyanate

Detection of cyanobacteria, *Cylindrospermopsis*, *Microcystis*, microcystin and cylindrospermopsin generating genes using Polymerase Chain Reaction (PCR) amplification

DNA amplification was performed for the 16S rRNA gene to identify the presence of cyanobacteria using the modified protocols of Nübel *et al.,* (1997). Cyanobacterial specific primers, forward primer Cya 359F (5'-GGGGAATCTTCCGCAATGGG-3') along with the reverse primers Cya781Rb (5'-GACTACAGGGGTATCTAATCCCTTT-3') and Cya781Ra (5'-GACTACT GGGGTATCTAATCCCATT-3') and an equimolar mixture of reverse primers Cya781Ra + Cya 781Rb were used for 16S rRNA gene identification. The total 25 µL of reaction mixture contained 3 µL of 5 µM each primer, 2.5 µL of 1 µM each deoxynucleoside triphosphate, 5 µL of 5x PCR buffer, 2 µL of 25 mM $MgCl_2$, 1.25U of *Taq* DNA polymerase (Promega, Madison, Wisconsin, USA) and 5 µL of template DNA.

In order to detect the microcystin synthetase (*mcy*) gene cluster for the identification of toxic *Microcystis* strains in the water source, DNA amplification was performed for *mcy*E gene using the general microcystin synthetase gene E forward primer mcyE-F2 (5'-GAAATTTGTGTAGAAGGTGC-3') and the genus-specific reverse primer for *Microcystis* MicmcyER8 (5'-CAATGGGAGCATAACGA G-3') (Vaitomaa *et al.,* 2003) along with the other ingredients as mentioned above.

Detection of *Cylindrospermopsis raciborskii* were carried out according to the protocol of Kim *et al.,* 2000 using rpoC1 – 1 forward primer (3'GAGCTCCAAAACCATCCACTCAGG 5') and rpoC1 - T reverse primer (5'GGTACCAAACGGACAAATAGTTGG-3') which codes for *C. raciborskii* specific cylindrospermopsin synthetase gene. Further, for the detection of cylindrospermopsin, forward primer M13 (5'-GGCAAATTGTGATAGCCACGAGC-3') and reverse primer M14 (5'-GATGGAACATCGCTCACTGGTG -3') which codes for cylindrospermopsin specific peptide synthase (PS) gene were used according to the protocol of Schembri *et al.,* 2001 along with the other ingredients as mentioned above.

All the amplifications were carried out in Techine TC 3000 DNA Thermal Cycler. A single cycle of initial denaturation for 5 min at 94^0C followed by 35 incubation cycles each consisting of 1 min at 94^0C, 1 min at 60^0C and 59^0C for 16S rRNA gene and *mcy*E gene respectively and 1min at 72^0C

followed by 15 min extension at 72^{0}C. A touchdown PCR was carried out with a single cycle of initial denaturation for 5 min at 94^{0}C followed by 5 incubation cycles each consisting of 1 min at 94^{0}C, 1 min at 62^{0}C and 1 min at 72^{0}C and another 5 cycles at 60^{0}C and 30 cycles at 58^{0}C followed by 15 min extension at 72^{0}C for the amplification of cylindrospermopsin specific peptide synthase (PS) gene. Further, another touchdown PCR was carried out as above with 5 cycles at 61^{0}C, 5 cycles at 58^{0}C and 30 cycles at 56^{0}C for the amplification of cylindrospermopsin synthetase gene. Aliquots of the resulted amplified products were electrophoresed in 1.5% agarose gels containing 10 μgmL^{-1} ethidium bromide and documented through a Gel Documentation system (Syngene, UK)

RESULTS AND DISCUSSION

Site Selection and Sample collection

This study was performed to detect the cyanotoxins in three main reservoirs in Anuradhapura district. Those reservoirs are often use for drinking and recreational purposes, including bathing, fishing and various water related activities. Furthermore, they are the main water sources for their irrigation. According to the data, mean temperatures of 27.6^{0}C, 28^{0}C and 27^{0}C and mean pH of 8.5, 8.34 and 8.7 were found in Nuwara wewa, Tissa wewa and Kala wewa respectively. Under microscope, *Cylindrospermopsis* species were recorded as the dominant cyanobacterial species in the environmental water samples collected from all three reservoirs along with *M. aeruginosa*, *Anabaena*, *Chroococcus*, *Phormidium*, *Microcystis spp.*, *Oscillatoria*, *Limnothrix*, *Lyngbia*, *Calothrix*, *Anabaenopsis* and *Merismopedia* which were comparatively moderate to low in numbers. However, culture samples in BG11, $BG11_0$ and $BG11_0C$, no *Cylindrospermopsis* species were observed except *Phormidium*, *Microcystis*, *Limnothrix*, *Lyngbia* and *Merismopedia*. Moreover, the cultures in MLA medium which lacks the inorganic nitrogen, *Cylindrospermopsis* could be observed only in Nuwara wewa sample and also were biased to filamentous forms and with the minority of *Merismopedia*. Although *Cylindrospermopsis* is a filamentous heterocyst former, the reason for this contrast might be due to the culture media which used for culturing (BG11, $BG11_0$ and $BG11_0C$) did not facilitate the growth of *Cylindrospermopsis* species. Therefore, use of different culture media to represent all the communities of cyanobacteria is essential for further identification studies.

Molecular detection

The method employed for genomic DNA extraction resulted in high quality DNA in satisfactory amounts for amplification. According the study done by Boutte *et al.*, 2005, the primers cyanobacteria specific Cya 781 Ra and Rb were used separately to identify filamentous and unicellular cyanobacteria respectively and equimolar mixture of Cya 781Ra + Cya 781Rb to identify cyanobacterial community composition in those reservoirs. According to the gel profiles obtained, all DNA samples submitted to PCR reactions with cyanobacterial specific oligonucleotide primers of Cya 359F forward and Cya 781Rb reverse primer yielded the unique fragment of ~450bp (Table 1 and Figure 1) indicating the presence of unicellular cyanobacterial community in those reservoirs. However, the use of the forward primer Cya 359F and the reverse primer Cya 781Ra yielded ~450bp fragment only in AN 3 from Nuwara wewa and AK3 sample from Kala wewa (Table 1).Therefore this along with microscopic observations confirmed the presence of filamentous forms which could be either heterocyst formers or non-heterocyct formers. Furthermore, all the samples yielded ~450bp unique fragment with Cya 359F and the equimolar mixture of Cya 781 Ra + Cya 781Rb with different intensities (Table 1) signifying the presence of unicellular and filamentous cyanobacterial communities in those water bodies. The

reason for these different intensities might be due to the competition of reverse primers to the same template and thus results the less complete genetic fingerprint (Boutte *et al.*, 2005).

Further, all the samples gave positive amplification for the *mcy*E gene yielding the unique ~250 bp except AK1 sample (Table 1 and Figure 2). This indicates the presence of *Microcystis* strains in all three reservoirs that have the genetic potential to produce Microcystins.

Only AN3 DNA sample gave positive amplification with rpoC1 –1 forward and rpoC1 - T reverse primer confirming the presence of cylindrospermopsin synthetase gene and therefore the presence of *Cylindrospermopsis raciborskii* species. Although the expected size was 609bp, the only amplified product size was ~250bp (Table 3 and Figure 3). Eventhough, microscopic identifications indicated the presence of *Cylindrospermopsis* species, negative amplifications were might be due to the presence of other *Cylindrospermopsis* species other than *Cylindrospermopsis raciborskii. A* specific ~597bp amplification was resulted in AK3 environmental DNA sample with M13 forward and M14 reverse primers (Table 3 and Figure 4) indicating the presence of Cylindrospermopsin specific peptide synthase (PS) gene and therefore confirmed the presence of toxic producing *Cylindrospermopsis* species and also cylindrospermopsin.

Table 1. PCR amplification results

Sample No.	Sample code	PCR Amplification					
		Cya 359F with			Mcy E	M13/M14	rpoC1
		Ra	Rb	Ra+Rb			
1	Nuwara wewa 1 -ANI	-ve	+ve	+ve	+ve	-ve	-ve
2	Nuwara wewa 2 -AN2	-ve	+ve	+ve	+ve	-ve	-ve
3	Tissa Wewa 1 -AT1	-ve	+ve	+ve	+ve	-ve	-ve
4	Tissa wewa 2 -AT2	-ve	+ve	+ve	+ve	-ve	-ve
5	Kala Wewa 1 -AK1	-ve	+ve	+ve	-ve	-ve	-ve
6	Kala Wewa 2 -AK2	-ve	+ve	+ve	+ve	-ve	-ve
7	Kala Wewa 3 -AK3	+ve	+ve	+ve	+ve	+ve	-ve
8	Nuwara wewa 3 -AN3	+ve	+ve	+ve	+ve	-ve	+ve

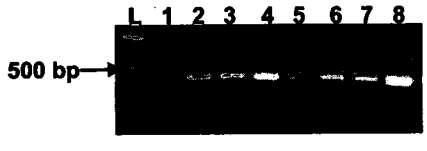

Figure 1. Agarose gel profile (1.5%) obtained for DNA samples amplified with cya359F and cya781Rb primers. Lane L- 1kb DNA maker, Lane 1- (-) ve control (water), Lane 2 – (+) ve control (*M.aureginosa*), Lanes 3-8 – AK1, AK3 AN1, AN2, AT1 and AT2 environmental samples.

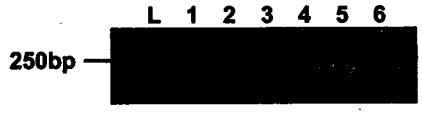

Figure 2. Agarose gel profile (1.5%) obtained for DNA samples amplified with mcyE-F2 and MicmcyER8 primer pair. Lane L- 1kb DNA maker, Lane 1- (-ve) control (water), Lane 2- (+ve) control, Lanes 3-6- AK1, AK3, AT1 and AN3 environmental samples respectively.

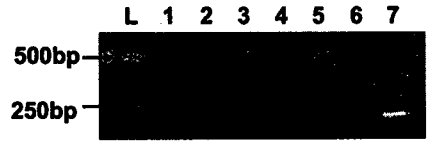

Figure 3. Agarose gel profile (1.5%) obtained for DNA samples amplified with rpoC1 –1 forward and rpoC1 - T primer pair. Lane L- 1kb DNA maker, Lanes 1-7 AK1, AK2, AK3, AT1, AT2, AN1, AN3 respectively

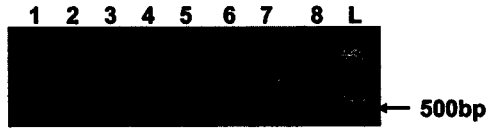

Figure 4. Agarose gel profile (1.5%) obtained for DNA samples amplified with M13 and M14 primer pair. Lane L- 1kb DNA maker, Lanes 1-8 AN1, AN2, AN3, AT1, AT2, AK1, AK3 and AK2 respectively

CONCLUSION

In conclusion, molecular detection data confirmed the presence of cyanobacteria in collected water samples and also the presence of cyanobacterial strains that have the genetic potential to produce microcystins and more importantly the presence of *Cylindrospermopsis* and also the presence of cylindrospermopsin producing *Cylindrospermopsis* species. This preliminary study was performed to detect the cyanotoxins in 3 main reservoirs in Anuradhapura district which are often used for drinking and recreational purposes, including bathing, fishing and various water related activities. Furthermore, they are the main water sources for irrigation. Concerns over the health risks that cyanotoxins pose to humans prompted the WHO to adopt a provisional guideline value of 1 μg/L for microcystin-LR in drinking water (WHO, 1998). Due to the lack of reliable analytical data, no guideline values have yet been set for concentrations of nodularin or cylindrospermopsin toxins in water. But in Sri Lanka, no guideline value has been set for the maximum permissible concentration for any cyanotoxin. However, the mere presence of toxin producing organisms does not mean that the water contains these toxins to the same extent, although there is an inherent danger of such toxins being released.

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