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Original Article

CYANOBACTERIA AND CYANOTOXINS IN WELL WATERS OF THE GIRANDURUKOTTE, CKDu ENDEMIC AREA IN SRI LANKA; DO THEY DRINK SAFE WATER?

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Cyanobacteria produce potent toxins which have been responsible for numerous livestock and human poisonings. Among cyanotoxins, microcystins, cylindrospermopsins and nodularins are known to cause acute and chronic illnesses. Concern about cyanotoxins in the dry zone water sources of Sri Lanka has grown due to a numerous negative health impacts, including the epidemic of Chronic Kidney Disease of unknown etiology (CKDu). The study aimed a region of high CKDu prevalence in Sri Lanka, Girandurukotte which consisted of CKDu and CKD patients and their well waters. Control samples were collected from other parts and all were analyzed in terms of potential cyanotoxin producers. A questionnaire analysis was carried out with 330 subjects (CKD n=33, CKDu n= 244 patients and healthy individuals n=53). Eleven factors showed significant difference ($p<0.05$) that could be related to the CKDu. Among them, well water source for drinking was ($p=0$) notable. Potential microcystin and cylindrospermopsin producing cyanobacteria were morphologically identified from 110 (CKD n=11, CKDu n=74 and Healthy individuals n=25) water samples. Compared to Girandurukotte patients' well water samples, cyanobacterial diversity was found to be less in healthy individuals' well waters. Among potential toxin producers, presence of *Phormidium* spp in CKDu patients' well waters were found to be significant ($P=0.004$) compared to other two populations. 50 CKDu and 15 CKD + healthy individuals' well water samples were assayed for partial *mycE* gene, cylindrospermopsin specific NRPS and PK genes and partial nodularin synthetase (*nda*) gene. Among these, presence of cylindrospermopsin producers ($p= 0.049$) and nodularin producing *Nodularia* species ($p= 0.0029$) were found to be significant.

Key words; CKDu, Cyanobacteria, Cyanotoxins, Cylindrospermopsin, Nodularin, Microcystins

1. Introduction

Cyanobacteria are diverse group of aquatic photosynthetic prokaryotes[1]. Under positive conditions they produce potent cyanotoxins which cause numerous livestock and human poisonings[2,3]. Among cyanotoxins, microcystins (MCs), cylindrospermopsins (CYNs) and nodularins (NODs) are the most common and persuasive cyanotoxins in fresh waters that cause acute and chronic illnesses[4,5]. Being a tropical country, Sri Lanka (7.0000° N, 81.0000° E) has a wide range of topographic and climatic variation which grants excellent ecological niches providing exceptional growth conditions for varied cyanobacteria. The occurrence of toxic cyanobacterial blooms have also been reported from different aquatic systems of the country[6]. Among them, toxic cyanobacteria in dry zone water sources need extensive attention due to the epidemic of Chronic Kidney Disease of unknown etiology (CKDu) prevailing in dry zone and other health impairments associated with water. The study aimed water samples both from CKDu and Chronic Kidney Disease (CKD) patients' well waters in Girandurukotte (High CKDu endemic area) (7°28'10"N, 81°0'54") in the dry zone of Sri Lanka and analyzed in terms of cyanobacteria and their potential toxin producing ability with respect to morphology and molecular methods.

2. Materials and Methods

2.1 Study population and sampling

Water samples from wells were collected from CKDu (n=74) and CKD (n=11) patients

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attending the kidney dialysis clinic, Girandurukotte, and from control subjects (n=25) from different geographic areas including Kandy, Galle, Ratnapura and Monaragala Districts in Sri Lanka.

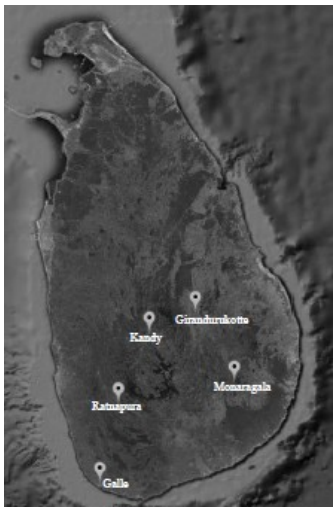

Fig 1 Sri Lankan map showing the water sample collected sites

2.2 Culturing and microscopic observations

Collected samples were centrifuged and inoculated into cyanobacterial specific liquid media (BG11, $BG11_0$ and MLA). Cultures were incubated and were observed under the compound light microscope (Olympus BH-2).

2.3 DNA extraction and amplification

75 water samples were selected for molecular studies and DNA extractions were carried out using Boom's method[7]. Toxic *M. aeruginosa* (PCC 7941, France) and toxic *C. raciborskii* (CCMP1973, USA) were used as standard cultures.

Polymerase Chain Reaction (PCR) was used to amplify 16S rRNA, partial *mcyE*, CYN specific AMT (Amidinotransferase) domain, NRPS/PK and PK genes and partial nodularin synthetase (*nda)* gene cluster in order to detect cyanobacteria, MC,
CYN and NOD producing *Nodularia* species respectively. The primers used, their respective sequences, target gene, annealing temperatures, expected DNA fragment size and related references[8,9,10,11] are shown in **Table 1**.

2.4 Data analysis

Associations between variables were tested using independent sample t-tests and Odds ratios. Ninety-five percent confidence intervals were calculated for the Odds ratios.

Table 1 PCR primers selected for molecular identifications

No	Primer	Sequence (5' – 3')	Target gene	Annealing Tem °C	Expected size bp	Reference
1	CYA 359F	GGGGAATCTTCCGCAATGGG	16SrRNA gene	60	450	Nübel et al.1997
2	CYA 781Rb	GACTACAGGGGTATCTAATCCC TTT				
3	CYA 781Ra	GACTACTGGGGTATCTAATCCC ATT				
10	HEPF	TTTGGGGTTAACTTTTTTGGGCA TAGTC	MC synthetase gene E	55	472	Jungblut and Neilan, 2006
11	HEPR	AATTCTTGAGGCTGTAAATCGG GTTT				
19	CatF1	AGATGGTGCTTATTTTGAAC	CYN specific amidinotransferase (aoaA) gene		881	
20	CatR1	TCTTCACAGATGACCTTCTT				
21	CpbF2	CACCATTGGCTATGTAGAAGCT	CYN specific NRPS/PKS complex (aoaB) gene	54	550	Baron-Sola et al. 2012
22	CpbR2	TATTGGCTGTGAAAGAGAGGTC				
23	CkcF3	AATGATCGAAAACAGCAGTCGG	CYN specific PKS (aoaC) gene		325	
24	CkcR3	TAGAACAATCATCCCACAACCT				
25	NPSF3	CTTATCGAGGAGGTCGTGAAG	Partial NOD synthetase (*nda*) gene cluster	54	1000-1200	Moffitt and Neilan, 2004
26	HLIPR	CAGAAAGTCAGTATTAGG				

3. Results and Discussion

3.1 Morphological identification

Each population's samples had cyanobacterial species belonging to all five orders. Comparing CKDu and CKD populations, the prevalence of species belonging to order Nostocales and order Stigonematales was less in CKD patients' compared to others. Additionally, of CKDu patients' waters, out of 74 samples, 10 (13.51%) did not have any cyanobacterial growth. Among three populations, identified MC and CYN producers and their percentages are shown in **Table 2**.

Table 2 Morphologically identified potential MC and CYN produced cyanobacteria in well waters collected from CKDu, CKD and healthy populations

Population	MC producers	CYN producers
CKDu patients (n=64)	*Anabaena* (11%) *Phormidium* (56%) *Chroococcidiopsis* (55%) *Arthrospira* (12.5%) *Anabaenopsis* (1.6%) *Nostoc* (8%) *Nodularia*(1.6%) *Calothrix*(3%) *Aphanocapsa* (9%) *Oscillatoria* (11%) *Hapalosiphon* (6%) *Microcystis* (6%) *Limnothrix* (3%)	*Anabaena* (9%), *Raphidiopsis (5%)* *Lyngbya* (11%)
CKD patients (n=11)	*Phormidium* (36%) *Anabaena* (9%) *Chroococcidiopsis* (18%) *Hapalosiphon* (18%) *Nostoc* (18%) *Microcystis* (9%) *Aphanocapsa (*9%)	*Anabaena* (9%) *Lyngbya* (9%)
Healthy individuals (n=25)	*Phormidium* (32%) *Chroococcidiopsis* (72%) *Synechococcus* (12%) *Arthrospira* (4%) *Calothrix* (8%) *Planktotrix* (4%) *Aphanothece* (4%)	*Lyngbya* (4%)

Among potential toxin producers, presence of *Phormidium* spp in CKDu patients' well waters were found to be significant (P=0.004) compared to other two populations. Further, presence of *Chroococcidiopsis* (55%) spp was also notable in each population. Apart from MC and CYN producers, some wells had *Nodularia* species which produce NOD.

3.2 Molecular identification

Amplification with cyanobacterial specific oligonucleotide primers confirmed the presence of either unicellular, non-heterocyst / heterocyst forming filamentous types of cyanobacterial communities in water samples collected from all subjects. Out of 85 well waters, 75 water samples (CKDu n=64, CKD n= 11) were positive for cyanobacteria. Further, of 25 well waters from healthy individuals, one was negative. Negative amplification total of 11 samples (CKDu/CKD n= 10 and control n= 1) showed linear relationship with the morphological identifications. Therefore, presence of cyanobacteria in CKDu patients' well waters were not significant ($p>0.05$) compared to rest.

To confirm the presence of MC, CYN and NOD producers in well water samples, 50 CKDu patients, seven CKD patients and 18 healthy individuals' well waters were tested. The amplification results obtained are summarized in **Table 3** and the gel profiles obtained for each primer are shown in **Fig. 2-4**

Table 3 PCR amplification results for the well water samples collected from CKDu, CKD and healthy populations

Subjects	Number of positive amplifications		
	HEP	CYN	NOD
CKDu patients (n=50)	3	12	26
CKD patients (n=07)	0	1	3
Healthy individuals (n=18)	1	0	1

From analysis, presence of CYN ($p= 0.049$) and NOD ($p= 0.0029$) in CKDu patients' well waters was statistically significant compared to other two. However, presence of MC in CKDu patients' well waters was not significant ($p>0.05$).

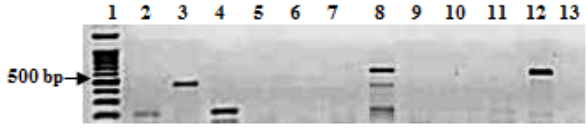

Fig. 2 Agarose gel profile (1.5%) obtained for DNA samples amplified with HEPF/ HEPR primer pair

Lane 1: DNA marker (100 bp); patients' well water samples, Lane 2: G/60; Lane 3: G/81; Lane 4: G/66; Lane 5: G/127; Lane 6: G/183-44; Lane 7:G/111; Lane 8: G/100-A; Lane 9: G/5-A; Lane 10: G/3-A; Lane 12: *M. aeruginosa* (PCC 7941) (positive control); Lane 13: water (negative control).

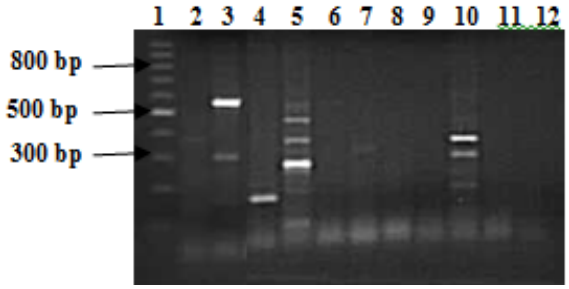

Fig. 3 Agarose gel profile (1.5%) obtained for DNA samples amplified with CatF1/CatR1, Cpb2/CpbR2 and CkcF3/CkcR3 primer pairs

Lane 1: DNA marker (100 bp); Lane 3: *C. raciborskii* (CCMP1973) (positive control); Patients' well water samples, Lane 4: G/183-44; Lane 5: G/5-A; Lane 6: G/66; Lane 7: G/127; Lane 8: G/81; Lane 9: G/3-A; Lane 10: G/100-A; Lane 11: G/148; Lane 12: water (negative control)

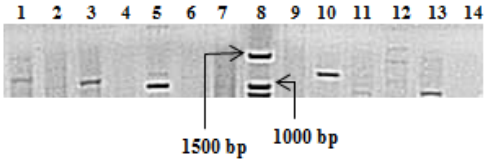

Fig. **4** Agarose gel profile (1.5%) obtained for DNA samples amplified with NPSF3/HLIPR primer pairs

Patients' well water samples, Lane 1: G/31; Lane2: G/14; Lane 3: G/34-112; Lane 4: G/56-A; Lane 5: G/109; Lane 6: G/113; Lane 7: G/116; Lane 8: DNA marker (100 bp); Lane 9: G/138; Lane 10: G/160; Lane 11: G/170; Lane 12: G/74-77; Lane 13: G/120; Healthy individuals' water sample, Lane 14: I/10

The quality of drinking water in relation to CKDu is an important potential root cause since shallow and deep wells are the main source of potable water in the affected region compared to the control population. Considering morphological data, presence of potential MC and CYN producers were abundant in patients' populations compared to control. MC is a potent hepatotoxin, causing damage to the liver function leading to liver injury and also a tumor promoter[12]. A number of human fatalities have been recorded on exposure to MC through ingestion of contaminated drinking water and through recreational contacts with contaminated water[13]. Among MC producers, *Phormidium* spp were significant (56%), and is a well-known MC[14] and a neurotoxin producer[14] and its health impacts to animals were well documented[14,15]. Further, presence of *Chroococcidiopsis* (55%) spp was also notable and also documented as a novel and potent cyanotoxin producer[16]. Therefore, *Phormidium* and *Chroococcidiopsis* spp pose a risk to human health as these waters are used extensively for drinking and recreational activities. NOD which is similar to MC in toxicity and mode of action and its toxicity is well documented among animals[17]. CYN is a potent hepatotoxin, cytotoxin and a neurotoxin, affects preliminary to kidney and liver function. It also causes damage to other organs and also is a potential carcinogen[18].

In addition, certain cyanobacterial species present in these water samples were also known to produce other cyanotoxins such as Anatoxin-a (*Anabaena, Oscillatoria, Aphanizomenon, Planktothrix, Phormidium*), Homoanatoxin-a (*Planktothrix, Phormidium*), Anatoxin-a(S) (*Anabaena*) and Saxitoxins (*Planktothrix, Anabaena, Lyngbya*) which are known to be hepatotoxins, and neurotoxins[14].

In molecular studies, although HEP primers showed the specific amplification for patients' samples, result was not significant. However, presence of CYN in CKDu patients' waters was significant with CatF1/CatR1, Cpb2/CpbR2 and CkcF3/CkcR3 primers and toxic *Raphidiopsis, Anabaena* and *Lyngbya* species would have contributed to the result. Further, presence of NOD was found to be strongly significant ($p = 0.0029$) in CKDu population compared to others. The specific amplification with NPSF3/HLIPR primers confirmed the presence of toxic *Nodularia* strains and thereby the possibility of water contamination with NOD. Further, it shows the importance of molecular detections

over morphology for accurate identification of cyanobacteria.

4. Conclusion

Well waters of Girandurukotte, Sri Lanka contained vast cyanobacterial diversity with potential MC, CYN and NOD producing species and the presence of cyanobacteria with toxin generating ability in these water bodies is an indication regarding the numerous health impacts faced by the people living in the dry zone of Sri Lanka. Consequently, more epidemiological studies are required to explore the relationship existing between CKDu and cyanotoxins in water.

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