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Occurrence of Toxigenic *Microcystis* spp. in Major Water Bodies of North-East India

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ABSTRACT

Toxigenic cyanobacterial blooms in the water bodies represent a major ecological problem around the world. Some species produces a diverse range of toxins that have hepatotoxic, neurotoxic, cytotoxic and dermatoxic activity and hence have deleterious effect on humans, animals and fishes leading to death as well. Cultural eutrophication of water bodies leads to increased incidence of these harmful cyanobacterial blooms worldwide. North-East India being a biodiversity hotspot harbour many species of cyanobacteria. Few reports suggested presence of few toxigenic cyanobacteria in the water bodies of Assam, but no systematic studies have been undertaken to evaluate their toxicity. This work is being conducted to gather information on major toxigenic cyanobacteria, with special emphasis to Microcystin (a cyclic heptapeptides with high acute and chronic toxicities to humans and animals) producing strains. Water samples have been collected from few water bodies of North-East and enriched in specific media. The toxin Microcystin was detected using specific ELISA kit and positive results have been obtained. Further, 16s rDNA sequencing was employed for molecular identification of the strains.

Keywords: Cyanobacteria; Toxins; Microcystin; ELISA; 16s rDNA

1. INTRODUCTION

Cyanobacteria are ancient inhabitant of the earth and in fact an essential component of the aquatic ecosystem providing food for aquatic organisms. However, under certain circumstances, they bloom to reproduce exponentially, becoming harmful to other life forms and are called harmful algal bloom (HAB). Some produce cyanotoxins, which are detrimental to the aquatic ecosystem and possess a severe threat to the health of animals as well as human beings. These blooms are common during February to July as hydrological conditions are relatively stable during this period[1,2].

Harmful algal bloom is a global phenomenon caused by ecological and anthropogenic factors, and is increasingly affecting the aquatic ecosystem world over. Microalgae and blue green algae called cyanobacteria are two group of organisms that belong to the phytoplankton community and are responsible for HAB. High concentration of these cyanobacteria in the water bodies changes the colour of water to appear according to the pigments found in the species causing the bloom[3].

A range of structurally and functionally diverse cyanotoxins are produced by over 40 species of freshwater and marine cyanobacteria, some of which have been well characterised in terms of their toxicological effects as well as molecular mechanism of their production whereas some of them are less well-understood[4].

Toxicologist have classified these cyanotoxins with respect to their effect on the target organs of vertebrates. Neurotoxic exert their effect on the nervous system and, at high concentration can cause death by respiratory arrest, while hepatotoxins mainly Microcystin and nodularin targets and damages the hepatocyte cells of liver. Chronic exposure of hepatotoxins through drinking water even at low dose has also been associated with tumour promotion[5-10] and β-methylamino-L-alanine (BMAA), a neurotoxin is linked with the development of neurodegenerative diseases[11,12]. Cytotoxins, dermatoxins and lipopolysaccharide endotoxins are the other types of toxins produced by cyanobacteria.

Humans and other animals may be directly exposed to these toxins by using water contaminated by these toxins while drinking, bathing and swimming. Indirect exposure can occur *via* consumption of animal or plant products that have been exposed to cyanotoxins. These toxins can bio-accumulate in shell-fish and fishes and also in plants and vegetables, if contaminated water is used for their nurturing, leading to indirect exposure upon their consumption[13].

Well documented case of cyanotoxin poisoning have been reported worldwide for more than a century. Over this period, humans and a diverse range of both aquatic and terrestrial animals as well as insects have been reported to be affected by cyanotoxins[14-17]. Phytotoxicity of cultivated plants due to irrigation from water source containing toxigenic cyanobacteria has also been reported[18], which poses threat to human health due to bioaccumulation of these toxins in plant tissues. These toxins hampers growth and development of the plants as well[19,20].

North Eastern region of India is inflicted with insurgency and jungle warfare is one of the ways to tackle the situation.

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During jungle warfare or in search operations, a combatant carries a sizeable amount of food and water. In case of prolong operations or during emergency situations, they need to use natural source of water for drinking purpose or sometimes need to cross water bodies. If these water bodies are infected with toxigenic cyanobacteria, it will lead to casualties as well as other health complications as the current portable field water filter is not capable of removal of cyanobacterial toxins.

No reports on systematic survey of toxigenic cyanobacteria available in the water bodies of North East India could be found. Therefore, it was felt necessary to explore presence of cyanotoxin producers in this region of India.

2. MATERIALS AND METHODS

2.1 Sample collection

Water samples were collected from major water bodies, Ramsar sites and old temple ponds dug by the kings in various location of North-East India. Physical parameters of water were documented at the site of collection.

2.2 Culture and isolation

The collected water samples were filtered through Whatman paper No. 1 to discard mud and twigs. 5 ml of the filtrate was added in 100 ml of $BG11_0$ media and incubated at 25 ± 2 °C with a photoperiodic cycle of 16 h light and 8 h of dark. Enriched cultures were subjected to serial dilution agar plate technique for isolation of single colonies. Single blue-green colonies were picked and reinoculated in liquid $BG11_0$ media and incubated. The isolates that appeared to be colonial in microscopy which is a characteristic of *Microcystis* spp. were further subjected to molecular analysis.

2.3 Genomic DNA Extraction and PCR

Biomass of unialgal cultures were harvested through centrifugation at 5000 rpm. Genomic DNA was extracted from the biomass following phenol-chloroform-isoamyl alcohol method[23] with slight modification. For PCR amplification of 16S rRNA gene, 16S1 (5'-GAGTTTGATCCTGGCTCA-3') was used as forward and 16S2 (5'-CGGCTACCTTGTTACGACTT-3') as reverse primer[24,29]. An initial denaturation of 5 min followed by denaturation at 94 °C for 45 s, annealing at 55 °C for 30 s and extension at 72 °C for 45 s for 35 cycles and a final extension for 5 min at 72 °C was employed in a G-Box thermal cycler. The PCR amplicons were electrophoretic ally separated in 1% agarose gel and visualised under a UV transilluminator. An amplicon of 1.5 kb was obtained in the PCR reaction.

2.4 DNA Sequencing and Bio Informatics Analysis

For removal of excess primer, PCR products were column purified (Qiagen PCR clean-up kit) and the forward and reverse strands were sequenced on an ABI 3500 *xl* Genetic Analyser (Applied Biosystems Inc. Foster City, CA). Manual editing of the sequences was done using BioEdit software (*ver*. 7.2). NCBI BLAST algorithm was used to search for homologous sequences in the GenBank database. The sequences homologous to the query were retrieved and aligned using ClustalW program. Phylogenetic analysis was carried out using MEGA X software package and 500 bootstrap replicates was used to construct the phylogenetic tree using Neighbor-Joining method.

2.5 ELISA for Detection of Microcystin

Microcystin was detected in ELISA using a commercial Kit (Microcystins-ADDA ELISA, Microtiter plate, Abraxis kit). Microcystin was extracted from the sample using a previously described method[25]. The test was carried out in triplicate for each sample using manufacturer's protocols and read in a multiplate reader at 450 nm wavelength. The concentration of toxin in the test sample was deduced from the standard curve, which further validated our results.

3. RESULTS

3.1 Culture isolation and microscopic observation:

Light microscopy proves to be an essential tool to differentiate colonial *Microcystis* spp. as shown in Fig. 1 from other species. $BG11_0$ media supported proper growth of this cyanobacteria as shown in Fig. 2.

3.2 PCR and Sequence Analysis

16S rDNA PCR analysis yielded 1.5 kb amplicon as shown in Fig. 3 which was further sequenced and used for bioinformatics analysis. Online blast search of the sequence showed similarity to *Microcystis aeruginosa* (97 %) and to some other species of cyanobacteria. Phylogenetic tree revealed that the strain isolated from this study was of *Microcystis aeruginosa*. The

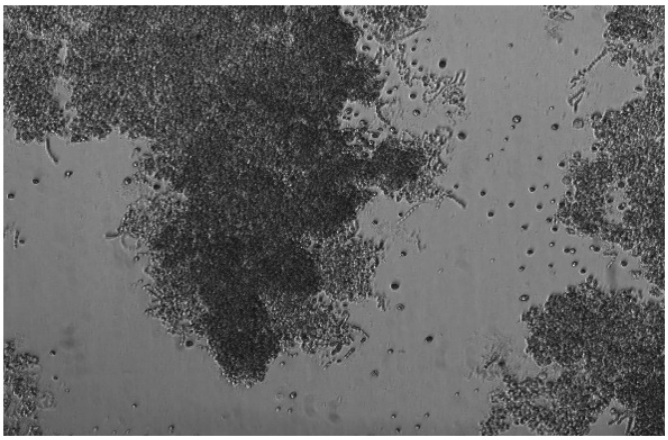

Figure 1. Light microscopic picture of colonial *Microcystis* spp. (40X magnification, Carl Zeiss, Germany).

Figure 2. Culture of cyanobacteria in $BG11_0$ media.

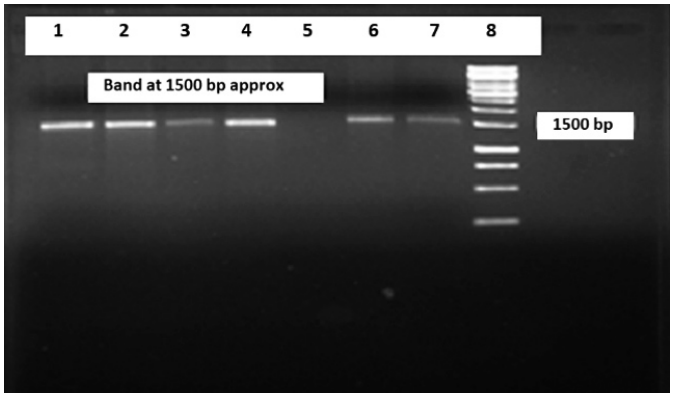

Figure 3. Electrophoretic separation of 16s rDNA PCR amplicon showing positive band at 1.5 Kb approx. in 1% agarose gel. Fermentas 1 kb plus DNA ladder is used in lane 8.

neighbour joining tree constructed using Tamura 3 parameters distance correction method has been depicted in Fig. 4.

3.3 Enzyme-linked Immunosorbent Assay for Determination of Microcystins in Water Samples

The test is an indirect competitive ELISA for the detection of Microcystins. It is based on the recognition of Microcystins by specific antibodies. The evaluation of the ELISA was performed using commercial ELISA evaluation programs such as 4-Parameter. The result has been depicted in Table 1.

4. DISCUSSION

Toxigenic cyanobacterial blooms producing toxin have deleterious effect on the health of animal and human[4]. Cultural eutrophication of water bodies has increased occurrence and intensity of cyanobacterial blooms. Cyanobacterial blooms and associated animal and human poisoning have been documented from over sixty-five countries, including India, Sri Lanka and Bangladesh. Cyanobacterial blooms are a potential health hazard due to the ability of some species to produce toxins that are harmful to other living organisms. Several bloom forming planktonic cyanobacteria produce hepatotoxic Microcystins (MC) which are synthesised non-ribosomally by a peptide/polyketide synthatase complex encoded by the Microcystin synthetase (*mcy*A-J) gene cluster, consisting of highly conserved sequences[27]. *Microcystis aeruginosa* is a species of toxin producing cyanobacteria and is

Table 1. Results of ELISA using Microcyatins-ADDA ELISA, Microtiter plate, Abraxis kit

Place of collection	Average concentration (ppb)	SD
Padumpukhuri (Tezpur, Assam)	3.0770	0.060811
Ganesh Ghat (Tezpur, Assam)	2.4635	0.068589
Deeporbeel (Guwahati, Assam)	2.588	0.062225
Sonari Pukhuri (Sivasagar, Assam)	2.448	0.098995
Sundubi Lake (Mirza, Assam)	1.497	0.004243
Dekhowmukhbeel (Dekhowmukh, Assam)	3.2095	0.113844
Dekhowmukh college Pond (Dekhowmukh, Assam)	2.677	0.060811
Lakhi Narayan pond (Tipura)	2.7115	0.109602
Rajbari Pond (Tipura)	2.9	0.029698
Rudra Sagar (Tripura)	1.808	0.067882
Jaganath sagar (Tripura)	3.181	0.069296
Mahadev Sagar (Tripura)	2.92	0.056569
Kalyan Sagar (Tripura)	2.508	0.019799
Amar sagar (Tripura)	3.1555	0.078489
SilcoriBeel (Silchar, Assam)	2.798	0.079196
Baba Baram Mandir (Silchar, Assam)	2.9445	0.033234
Ramnagar Annua (Silchar, Assam)	2.837	0.059397
Narsing tala pond (Silchar, Assam)	3.808	0.067882

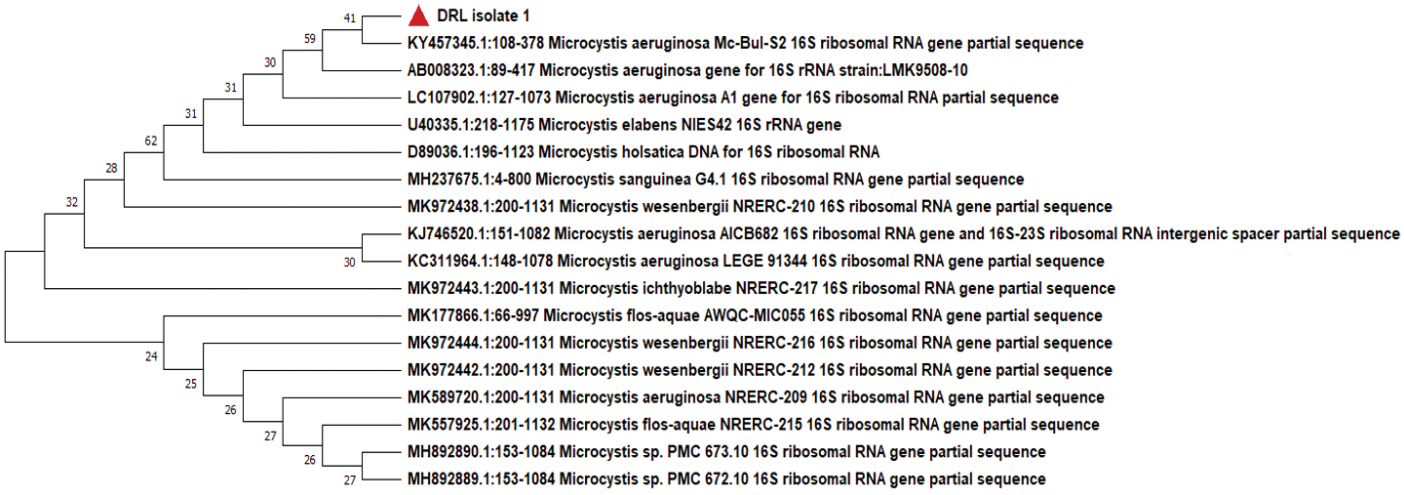

Figure 4. Phylogenetic tree constructed using neighbor joining method, showing relation of the isolated species with other homologous 16s rDNA sequences retrieved from GenBank.

responsible for production of a hepatotoxin *viz*. Microcystin. There are several cases of Microcystin toxicity throughout the world, affecting both humans and animals. A case report on acute cyanotoxin poisonings in animals and humans suggested that two-thirds of reported poisonings have occurred in the United States and Europe. However, there is a potential to spread worldwide[30]. There are also evidence of these toxins affecting military personal during special operations or during training. There were some cases of amyotrophic lateral sclerosis (ALS) among the army veterans that were deployed in the Gulf War during 1990-1991. The research group suggested that inhalation of 2,4-diaminobutyric acid (DAB), β-methylamino-L-alanine and other cyanotoxins in aerosolised form might contributed to the development of ALS and other neurodegenerative diseases[21].

Another study reported an outbreak of Microcystin poisoning among army recruits, who had accidently ingested water during canoe exercise from a reservoir where *Microcystis aeruginosa* was blooming. Two soldiers had detailed case reports with history of malaise, pleuritic and abdominal pain, sore throat, blistering around the mouth and dry cough. They also developed fever, abdominal tenderness and pneumonia. Testing of various pathogens such as influenza, adenovirus and other traditional disease-causing bacteria was negative. Another sixteen soldiers that were part of the same exercise also reported similar symptoms[22].

In India, marine cyanobacterial toxicity is being studied well and documented properly. However, there are very few reports of freshwater toxigenic cyanobacteria in India. A report suggested that severe fish mortality was caused due to toxic effect of Microcystin at Muttukkadu backwater situated at Southeast coast of India[31]. Chaturvedi[32], *et al.* had reported that spread of *Vibrio cholera* may be associated with harmful algal bloom. Research work on the field of toxin producing cyanobacteria is gaining momentum in India, but none could be traced from North-East India. Therefore, this research work was carried out to explore and detect presence of this toxin producer, particularly Microcystin through use of molecular markers and ELISA. Microscopic observation helped to differentiate the *Microcystis* spp. from other species of cyanobacteria but could not predict the toxigenic potential of the isolated strain. Animal bioassays method could be used to ascertain the toxigenicity but it may be subjected to ethical issue, labour and cost intensive as well as time consuming. Molecular detection methods like PCR and DNA sequencing of 16S rRNA have great potential in predicting an organism up to the species level[26,27]. In this study, we used a pair of primer targeting 16S rRNA gene to test its specificity for cyanobacterial detection. The selected primers successfully amplified a 1.5 base pair segment of DNA of the unialgal cyanobacterial strains. Sequence analysis of 16S rRNA gene could be used to identify some potentially toxic cyanobacterial genera. However, accurate diagnosis of bloom samples may be difficult, as both toxic and non-toxic strains are erratically distributed in an algal bloom[28]. ELISA have been successfully used by many researchers for detection of Microcystin and it also serves as a correlation with the molecular data. In this study we used Microcystins (Adda specific) ELISA kit by Abraxis, U.S.A which successfully detected the toxin in the tested sample. Though the kit is robust and specific to Microcystin, it is better to correlate the findings with other analytical techniques such as HPLC and LC-MS.

To the best of our knowledge, this is the first report of the presence of toxin producing *Microcystis* spp. in the water bodies of North-East India. The present study is significant in generating a database of toxigenic freshwater cyanobacteria of the North-eastern region and thereby to develop assays for detection of toxigenic cyanobacteria. These studies may also help medical and veterinary doctors and researchers for accurate diagnosis of causal agents other than medically established water-borne infectious agents for any suspicious casualty caused by consumption of water from natural sources. These will also help in monitoring of water quality in aquaculture as well as in detection of cyanotoxins during episodes of unnatural deaths of fishes and other aquatic animals. Besides, field detection kits may be developed for future use by our Armed Forces patrolling in deep jungles and difficult terrains of North East India which will ensure their safety while using water for drinking from natural sources under energency situtation.

5. CONCLUSIONS

This research work was conducted to gather information on major toxigenic cyanobacteria, with special emphasis on *Microcystis*. Results from this study suggested presence of these toxigenic cyanobacteria in some water reservoirs of North-Eastern region of India and there is a need to monitor the water quality used by humans and animals, so that, any negative impact on the health of the user can be averted as well as precautionary measure may be adopted. Moreover, Armed forces trained for jungle warfare need to be made aware of these threats for their personal safety during specific operations in the jungles of North-East India.

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