

PCR-based detection of microcystin-producing cyanobacterial blooms from Central India

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Microcystin synthetase-gene-specific primers were used to identify hepatotoxic microcystin producing genotypes in six *Microcystis* spp.-dominant water blooms. Four blooms gave positive PCR reaction. They produced microcystin-RR and -LR amounting to 0.037 to 0.095% of the dry mass.

Keywords: Cyanobacteria, Microcystin synthetase genes, Microcystin variants, *Microcystis*-dominant blooms, PCR amplification

Occurrence of cyanobacterial blooms and associated animal and human poisoning has been documented from over sixty five countries¹, including India², Sri Lanka³ and Bangladesh⁴. Several bloom forming planktonic cyanobacteria produce hepatotoxic microcystins (MCs)⁵⁻⁹. MCs are synthesized nonribosomally by a peptide/polyketide synthetase complex encoded by the microcystin synthetase (*mcyA-J*) gene cluster, consisting of highly conserved sequences¹⁰. These genes are chosen for PCR amplification to deduce MC production, because the microscopic and molecular phylogenetic methods using 16S rRNA or other housekeeping genes can not differentiate between MC-producing and -non-producing strains¹¹. Choice of primers specific to *mcyD* and *E* genes, which could recognize a number of genera present in natural population of cyanobacteria^{12,13}, has several advantages in toxigenicity typing. Recently, the aminotransferase domain of *mcyE* amplified using HEP primers in natural samples of *Microcystis*, *Anabaena*, *Nodularia*, *Nostoc*, *Planktothrix* and *Phormidium* revealed that PCR amplification and hepatotoxin production was correlated by 100%⁸.

In India the warm water temperature promotes dense *Microcystis* growth in most eutrophic water bodies almost throughout the year. In an epidemiological study, the patients with a history of

bathing in *Microcystis*-infested ponds have been reported to develop acute rhinosporidiosis; a disease caused by “pathogenic” strains of *Microcystis*¹⁴. However, the possible cause of such pathogenicity, whether due to MCs or some other toxin was not determined. *Arthrospira*, *Anabaena*, *Phormidium* and *Oscillatoria* species are the other common cyanobacteria present in Indian freshwaters, and risk of them being potentially toxic can not be ruled out¹.

The bloom samples collected in Central India during 2001-03 exhibited predominance of *M. aeruginosa*, with only 20% of the population producing MCs². Most likely the other blooms also had below detectable levels of MC. To ascertain this, in this communication, HEP primers were used to diagnose MC producing cyanobacteria in the bloom samples and production of toxin was detected using RP-HPLC and MALDI-TOF MS. Current methods of DNA isolation from freshly collected environmental samples use an irritant, phenol, some expensive reagents like potassium methylxanthogenate, proteinase K, cetyltrimethylammonium bromide etc. and gel materials¹⁵. Several steps which involve above material have either been omitted or modified making the protocols simple, cost effective, rapid and safe. Another advantage is that the freeze-dried blooms collected at different times of a year or locations can be stored so that DNA can be simultaneously extracted, thus minimizing the error component.

Materials and Methods

Bloom samples from ponds and lakes of Bhopal (Shahpura lake), Jabalpur (Sadar lake), Sihora,

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Gosalpur, Kundam and Seoni towns of Madhya Pradesh were collected by skimming across the water surface using a 25 μm plankton net during March-April, 2006. These samples were kept cool (10°C) and brought to the laboratory. The *Microcystis* species present in the blooms were identified following Via-Ordorika *et al.*¹⁶. *Anabaena fertilissima* was identified, courtesy Dr. Radha Prasanna, Center for Conservation & Utilization of Blue Green Algae, IARI, New Delhi. The frequency (i.e. number of individual colonies or filaments/100 colonies, referred as “f”), of each species was determined using a haemocytometer³. At least three enumerations were done in minimum three water samples collected simultaneously from a surface area of 8 m², and the average values are presented. The phytoplankton samples were concentrated by placing the material in plastic cylinders and the buoyant cyanobacterial scum collected from the surface. *Synechococcus elongatus* PCC7942 was grown in BG-11 medium¹⁷. *Microcystis aeruginosa* PCC7806 was provided in freeze-dried form by Prof. J. Weckesser, Albert-Ludwigs Universität, Freiburg i. Br, Germany. The samples collected from nature as well as the harvested cultures were freeze-dried and kept at -20°C until further use.

Total genomic DNA was extracted from 25 mg dry matter following the methods described previously^{8,18}. In method I the cells were combined with 3 ml of extraction buffer containing 45 mg lysozyme replacing potassium methylxanthogenate. After this incubation an additional step of RNase (500 μg) treatment for 30 min at 37°C was included. In method II the cells were homogenized in NET buffer, and incubated with 1% SDS for 1 hr followed by RNase as above. Proteinase K and phenol-chloroform-isoamyl alcohol treatments and sonication steps were skipped.

PCR amplification was done according to Jungblut and Neilan⁸.

Preparation and RP-HPLC resolution of methanolic extracts of dried bloom cells, quantification of MCs, and MALDI-TOF MS of the whole cells were carried out as given earlier².

Fine chemicals, gel materials and primers were purchased from Sigma (U.S.A.) and Bangalore Genei (India) and other chemicals from Qualigens (India). Microcystin standards were procured from Calbiochem (Germany).

Results

In early summer of 2006 profuse blooms appeared in several water bodies situated in and around Bhopal, Jabalpur and Seoni towns. While *M. aeruginosa* (f = 30-80%; colony size, 200-400 μm) and *M. viridis* (f = 20-85%; size, 50-300 μm) were the predominant cyanobacteria present in most of the water bodies, *M. botrys* (f = 10-30%; size, 50-150 μm), *M. panniformis* (f = 5-20%; size, 150-350 μm) and *M. ichthyoblabe* (f = 5-15%; size, 200-400 μm) constituted minor proportions of the phytoplankton population. Occasionally, filaments of *Anabaena fertilissima* (f = 5-15%; filament size, 200-400 μm) were also detected along with *Microcystis* colonies.

Method I yielded high quality DNA (A_{260}/A_{280} within 1.56-1.65 and distinct bands in agarose gel; Fig. 1 A and C – lanes -2, 5 and 6) for the blooms of Sihora, Gosalpur and Seoni, whereas method II was suitable for the other blooms and *M. aeruginosa* PCC7806 and *S. elongatus* PCC7942 cultures (Fig. 1 B and C – lanes -1, 3, 4, 7 and 8). Possibly polysaccharide (mucilage) and humic acid composition varied considerably in the dried bloom samples, and therefore no single treatment could

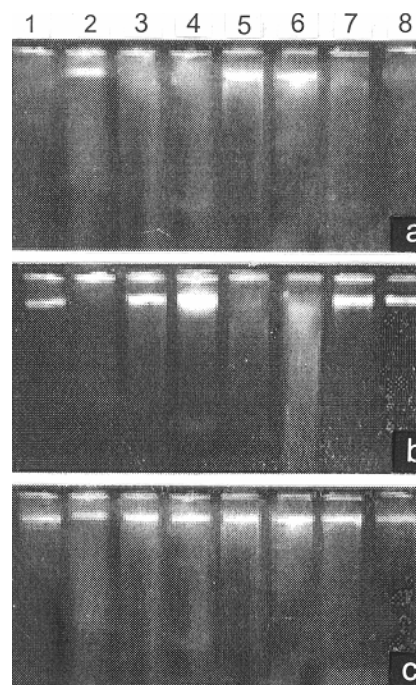


Fig. 1—Agarose gel electrophoresis of DNA prepared by (a)- method I and (b)-method II: lane 1- *M. aeruginosa* PCC7806; lane 8: *S. elongatus* PCC7942, lane 2-7- blooms collected from the water bodies of Sihora, Jabalpur, Bhopal, Gosalpur, Seoni and Kundam respectively. (c)-Repeat extraction by method I (lanes 2, 5 and 6) and method II (lanes 1, 3, 4, 7 and 8).

optimally remove the contaminants and yet kept the DNA intact. The presence of *mcy* genes was examined in *M. aeruginosa* PCC7806, known to produce MCs¹⁹, and also in bloom samples. The HEP PCR reactions resulted in amplification of 472-bp fragment for *M. aeruginosa* PCC7806, and four out of six bloom samples (Fig. 2). *S. elongatus* PCC7942, which was used as negative control did not give amplification product.

The presence of MCs in the bloom samples was examined using RP-HPLC and MALDI-TOF MS. The presence of MC-RR and -LR at 5.7 and 14.8 min was confirmed by comparing the peaks with the standards (Fig. 3). A majority of the blooms produced MC-RR variant at concentration range of 0.45-0.95 mg/g dry weight, and only one also co-produced -LR (0.37 mg/g dry weight). In accordance with the HPLC results, the observed prominent parent ions in MS analysis, according to the database¹⁶, were characteristic of MC-RR and -LR variants (Fig. 4). The remaining fragments did not correspond to any

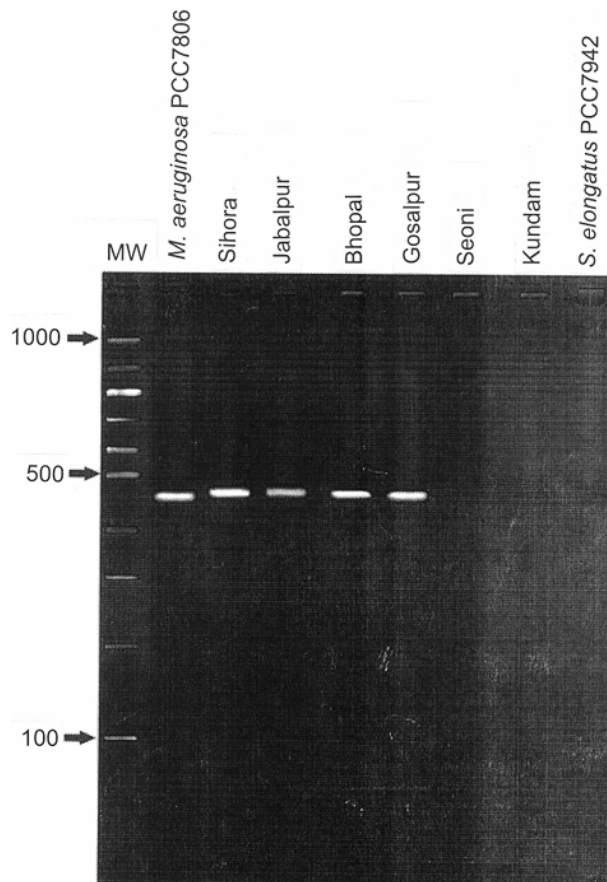


Fig. 2—PCR amplification of the *mcyE* with DNA extracted from cyanobacterial cultures and blooms. MW=100 bp DNA ladder.

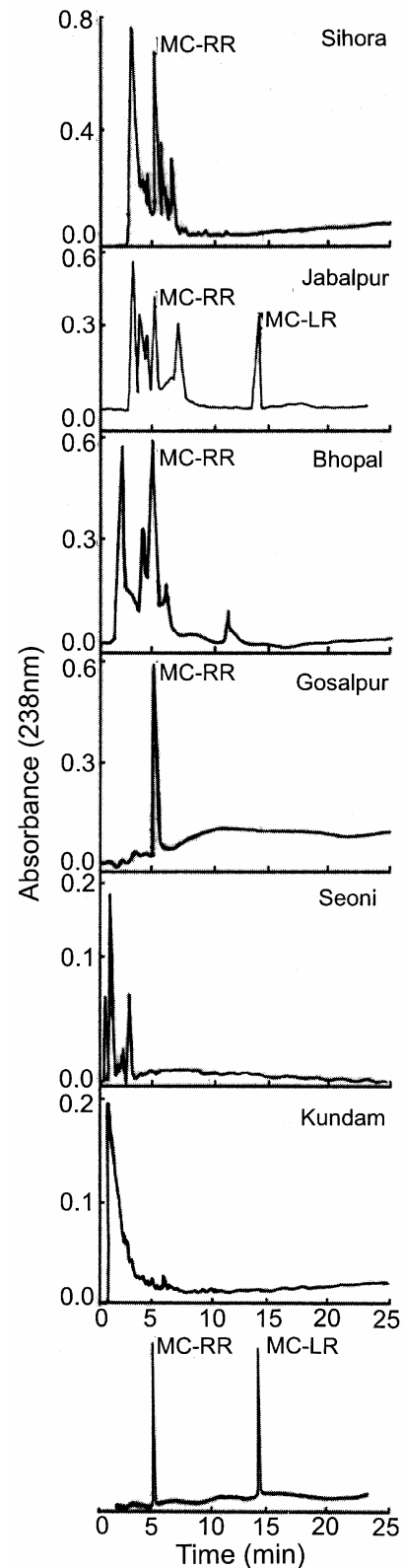


Fig. 3—RP-HPLC elution profile after methanolic and solid phase extraction of 2 mg bloom samples from different water bodies and standard MC-LR and -RR (20 ng each).

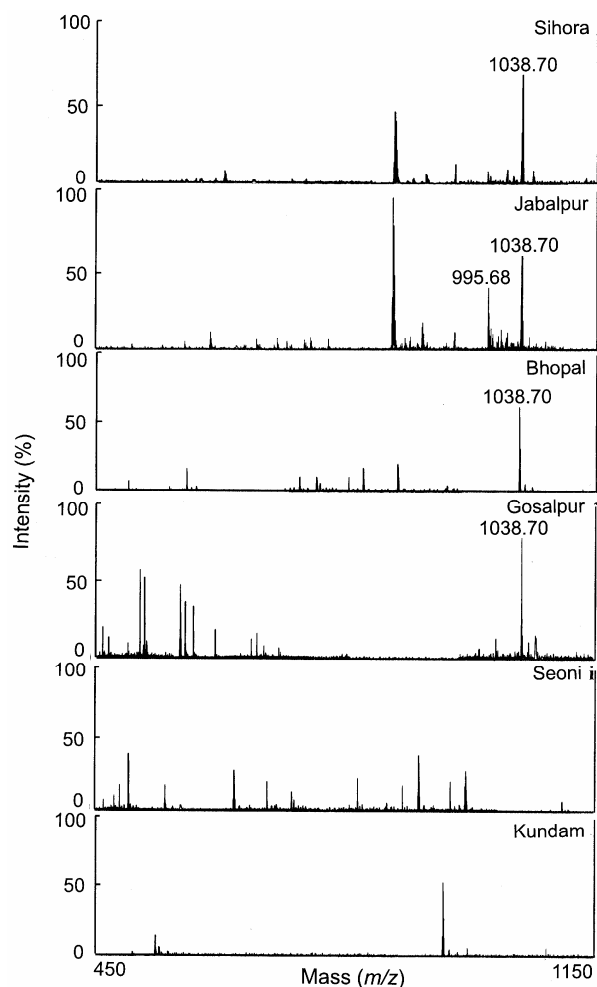


Fig. 4—MALDI-TOF MS of bloom samples from different water bodies. The m/z 995.68 = microcystin-LR and m/z 1038.70 = microcystin-RR.

cyanobacterial toxin. Presence of toxins was found in >65% of the blooms and this attribute corresponded to the positive PCR signals.

Discussion

It was observed that *M. viridis* and *M. aeruginosa* dominated the phytoplankton population in the water bodies at Bhopal, Jabalpur and Sihora, and they harbored *mcy* genes and produced predominantly MC-RR variant. Conversely, MC-non-producers and *mcy*-negative strains of the two species also occurred in some blooms (at Seoni and Kundam). Such unusual distribution of *mcy*⁺ and *mcy*⁻ genotypes together with corresponding ability to synthesize MC without a species bias agrees with the findings of Rantala *et al.*¹², which suggests that an ability to synthesize MC is an ancient feature but eventually natural mutations

including insertions and deletions have caused inactivation of *mcy* genes in non-toxic strains.

Production of MC-RR by phytoplankton blooms of Bangladesh⁴, Chile²⁰ and Kenya⁷ has also been reported. From these results it can be speculated that MC-RR genotypes are more successful to establish under tropical conditions, whereas MC-LR and -YR variants are more prevalent in the temperate European waters¹⁶ and perhaps the strains producing them belong to different genotypes. The -RR variants are less toxic as compared to -LR²¹, and thus the difference in abundance of MC variants in the water bodies of Central India could explain why there is no apparent animal or human poisoning associated with the MC-containing blooms in this region²⁻⁴.

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References

- 1 Codd G A, Azevedo S M F O, Bagchi S N, Burch M D, Carmichael W W, Harding W R, Kaya K & Utkilen H C, CYANONET, A global network for cyanobacterial bloom and toxin risk management - Initial Situation Assessment and Recommendations, *Int Hydrol Progr-VI Tech Doc Hydrol 76* (UNESCO, Paris) 2005, 1.
- 2 Agrawal M K, Ghosh S K, Bagchi D, Weckesser J, Erhard M & Bagchi S N, Occurrence of microcystin-containing toxic water blooms in Central India, *J Microbiol Biotechnol* 16 (2006) 212.
- 3 Jayatissa L P, Silva E I L, McElhiney J & Lawton L A, Occurrence of toxigenic cyanobacterial blooms in fresh waters of Sri Lanka, *Syst Appl Microbiol* 29 (2006) 156.
- 4 Welker M, Khan S, Haque M Md, Islam S, Khan N H, Chorus I & Fastner J, Microcystins (cyanobacterial toxins) in surface waters of rural Bangladesh: pilot study, *J Water Health* 3 (2005) 325.
- 5 Vieira J M D S, Azevedo M T D P, Azevedo S M D O, Honda R Y & Correa B, Microcystin production by *Radiocystis fernandoi* (Chroococcales, cyanobacteria) isolated from a drinking water reservoir in the city of Belem, P A, Brazilian Amazonia region, *Toxicon* 42 (2003) 709.
- 6 Oksanen I, Jokela J, Fewer D P, Wahlsten M, Rikkinen J & Sivonen K, Discovery of rare and highly toxic microcystins from lichen-associated cyanobacterium *Nostoc* sp. strain IO-102-I, *Appl Environ Microbiol* 70 (2004) 5756.
- 7 Ballot A, Krienitz L, Kotut K, Wiegand C & Pflugmacher S, Cyanobacteria and cyanobacterial toxins in the alkaline crater lakes Sonachi and Simbi, Kenya, *Harmful algae* 4 (2005) 139.
- 8 Jungblut A-D & Neilan B A, Molecular identification and evolution of the cyclic peptide hepatotoxins, microcystin and

- nodularin, synthetase genes in three orders of cyanobacteria, *Arch Microbiol* 185 (2006) 107.
- 9 Izaguirre G, Jungblut A-D & Neilan B A, Benthic cyanobacteria (Oscillatoriaceae) that produce microcystin-LR, isolated from four reservoirs in southern California, *Water Res* 41 (2007) 492.
 - 10 Dittmann E & Börner T, Genetic contributions to the risk assessment of microcystin in the environment, *Toxicol Appl Pharmacol* 203 (2005) 192.
 - 11 Tillett D, Parker D L & Neilan B A, Detection of toxigenicity by a probe for the microcystin synthetase A gene (*mcy A*) of the cyanobacterial genus *Microcystis*: Comparison of toxicities with 16S rRNA and phycocyanin operon (Phycocyanin Intergenic Spacer) phylogenies, *Appl Environ Microbiol* 67 (2001) 2810.
 - 12 Rantala A, Fewer D P, Hisbergues M, Rouhiainen L, Vaitomaa J, Börner T & Sivonen K, Phylogenetic evidence for the early evolution of microcystin synthesis, *Proc Natl Acad Sci (USA)* 101 (2004) 568.
 - 13 Mankiewicz-Boczek J, Izydorczyk K, Duda Z R, Jurczak T, Stefaniak K & Kokocinski M, Detection and monitoring toxigenicity of cyanobacteria by application of molecular methods, *Environ Toxicol* 21 (2006) 380.
 - 14 Dhaukhandi D B, Ahluwalia K B, Poduval P & Ravi A K, Comparative RAPD analysis of causative agent of rhinosporidiosis and *Microcystis* isolated from pond waters, *Curr Sci* 86 (2004) 386.
 - 15 Srivastava A K, Ara A, Bhargava P, Mishra Y, Rai S P & Rai L C, A rapid and cost-effective method of genomic DNA isolation from cyanobacterial culture, mat and soil suitable for genomic fingerprinting and community analysis, *J Appl Phycol* 19 (2007) 373.
 - 16 Via-Ordorika L, Fastner J, Kurmayer R, Hisbergues M, Dittmann E, Komarek J, Erhard M & Chorus I, Distribution of microcystin-producing and non-microcystin-producing *Microcystis* sp. in European freshwater bodies: detection of microcystins and microcystin genes in individual colonies, *Syst Appl Microbiol* 27 (2004) 592.
 - 17 Rippka R, Deruelles J, Waterbury J B, Herdman M & Stanier R Y, Generic assignments, strain histories and properties of pure cultures of cyanobacteria, *J Gen Microbiol* 111 (1979) 161.
 - 18 Kondo R, Yoshida T, Yuki Y & Hiroishi S, DNA-DNA reassociation among a bloom-forming cyanobacterial genus, *Microcystis*, *Int J Syst Evol Microbiol* 50 (2000) 767.
 - 19 Cho J E, Bang S W & Han M S, Development of oligonucleotide primers for the detection of harmful *Microcystis* in water, *Bull Environ Contam Toxicol* 72 (2004) 655.
 - 20 Neumann U, Campos V, Cantarero S, Urrutia H, Heinze R, Weckesser J & Erhard M, Co-occurrence of non-toxic (cyanopeptolin) and toxic (microcystin) peptides in a bloom of *Microcystis* sp. from a Chilean lake, *Syst Appl Microbiol* 23 (2000) 191.
 - 21 Gupta N, Pant S C, Vijayraghavan R & Rao P V L, Comparative toxicity evaluation of cyanobacterial cyclic peptide toxin microcystin variants (LR, RR, YR) in mice, *Toxicol* 188 (2003) 285.