Toxic cyanobacteria in reservoirs in northeastern Brazil: detection using a molecular method

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(With 2 figures)

Abstract

Cyanobacterial blooms are a frequent occurrence in northeastern Brazil and constitute a serious public health problem. Using the polymerase chain reaction (PCR) method, eleven environmental samples with cyanobacteria from seven reservoirs were used to determine the presence of the gene involved in microcystin biosynthesis (*mcy*B). Two sets of oligonucleotide primers were designed from the sequencing of Brazilian populations of microcystin producing cyanobacteria (*mcy*B-F/R and *mcy*B-F/R-A). The presence of the *mcy*B gene involved in microcystin biosynthesis was found in all samples, indicating the potential of this gene for producing the toxin. The PCR method proved sensitive and appropriate for the detection of potential producers of microcystins in environmental samples. Its ability to reveal potentially toxic cyanobacteria demonstrates that it can be a valuable tool in the monitoring of blooms.

Keywords: northeast Brazil, *mcy*B, microcystin, molecular markers, environmental monitoring.

Cianobactérias tóxicas em reservatórios do nordeste do Brasil: detecção utilizando método molecular

Resumo

Florações de cianobactérias são frequentes no nordeste do Brasil, constituindo um sério problema de Saúde Pública. Através da utilização da técnica de PCR, onze amostras ambientais com cianobactérias provenientes de sete reservatórios foram utilizadas para verificar a presença de um dos genes envolvido na biossíntese de microcistina (*mcy*B). Para isso foram utilizados dois conjuntos de oligonucleotídeos primers (*mcy*B-F/R e *mcy*B-F/R-A) desenhados a partir de sequências de populações brasileiras de *Microcystis* produtoras de microcistina. A presença do gene *mcy*B foi verificada em todas as amostras analisadas, indicando a capacidade de produzir esta toxina. O método de PCR apresentou-se sensível e apropriado para a detecção de potenciais produtores de microcistinas em amostras ambientais. Sua habilidade em detectar genótipos indicadores de produção de microcistina nos reservatórios investigados sugere que este método pode auxiliar no monitoramento de florações.

Palavras-chave: nordeste do Brasil, *mcy*B, microcistina, marcadores moleculares, monitoramento ambiental.

1. Introduction

Cyanobacterial blooms in public water supply reservoirs have become a matter of ever increasing concern throughout the world due to their potential for producing toxins. Water scarce semiarid northeast reservoirs, coupled with cultural eutrophication, have given rise to a recurring bloom formation of toxin-producing cyanobacterial species (Bouvy et al., 2000; Chellappa et al., 2000; 2008a, b; Costa et al., 2006; Panosso et al., 2007). The infrequent rainfall pattern, its heterogeneous distribution, punctuated by prolonged and intense dry periods, have all caused drought polygon in water storage in reservoirs of this region (Almeida et al., 2009). The occurrence of cyanobacterial blooms is therefore due to the combination of diverse factors and are favoured by high temperatures, high light intensities and run-off from agro-fertilizers originating from inadequate agricultural procedures around these bodies of water.

Microcystin is a cyanotoxin associated with a number of cases of intoxication in animals and humans. The worst case involving a human population occurred in the city of Caruaru in northeastern Brazil, in which patients at a hospital had been exposed to microcystins (Jochimsen et al., 1998). Brazilian legislation imposes the monitoring of bodies of water for public supply. This monitoring should encompass the density of cyanobacteria (20×10^3 cell.ml^{-1})

and maximum acceptable level of microcystin of 1 μg.L^{-1} in drinking water (Regulation MS N.518/2004).

Microcystin production depends on the existence of genes that codify microcystin synthetase. Microcystin is a hepatotoxin produced by a non-ribosomal pathway through a polyketide synthase/non-ribosomal peptide synthetase (PKS/NRPS) complex known as microcystin synthetase, which is responsible for the incorporation of amino acids in the peptide chain (Kleinkauf and von Döhren, 1996; Dittmann et al., 1997). The *mcy* gene cluster is responsible for toxin production, coding for microcystin synthetase (Dittmann et al., 1997; Tillett et al., 2000). Some cyanobacteria are unable to produce toxins, but all those that have genes associated to microcystin biosynthesis should be viewed as potential toxin producers (Ouellette et al., 2006).

Kurmayer et al. (2002, 2004) found that a few strains of cyanobacteria in nature have no microcystin production, but contain all the genes for microcystin synthetase. The authors speculate that these strains had lost the ability to synthesise microcystin due to gene inactivation and that another small peptide could functionally substitute this toxin.

A large number of authors have used microcystin synthetase genes (*mcy*) as molecular markers for microcystin-producing cyanobacteria (Baker et al., 2002; Nonneman and Zimba, 2002; Bittencourt-Oliveira, 2003; Hisbergues et al., 2003), for, in other studies, the presence of microcystin has been correlated, almost without exception, to the presence of *mcy* genes (Kurmayer and Kutzenberger, 2003; Via-Ordorika et al., 2004; Dittmann and Börner, 2005). There are no morphological differences between toxic and non-toxic cyanobacteria. Thus, the early detection of toxic cyanobacteria in water resources is advisable in order to avoid contamination and public health problems. Predicting toxic blooms is quite important in view of both their increasing occurrence in large water supply systems and the high cost of the current technology used for their removal.

The aim of the present study was to evaluate the existence of microcystin-producing genotypes using the molecular marker for the *mcy*B gene in public water supply reservoirs.

2. Material and Methods

Eleven environmental samples were collected with a 25 μm mesh plankton net from seven public water supply reservoirs in northeastern Brazil (Figure 1, Table 1). The cyanobacterial bloom-forming was determined from morphological characteristics used for the identification of taxa.

DNA was extracted from fresh cells of the environmental samples following the procedure described by Rogers and Bendich (1985). Polymerase chain reaction (PCR) was performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) using pure Taq Ready-To-Go PCR Beads kits (Amersham, Piscataway, NJ, USA) with 20 μM of each oligonucleotide (*mcy*B-F/R and *mcy*B-F/R-A). For PCR amplification, the following cycling parameters were used: 94 ºC for 2 minutes, 35 cycles at 94 ºC for 10 seconds; 50 ºC for 20 seconds, and 72 ºC for 1 minute, followed by a final extension at 72 ºC for 5 minute. The oligonucleotide primers were designed using sequences from Brazilian populations of microcystin-producing *Microcystis*. All primers were synthesised by IDT (Medley, FL, USA).

As a positive control, amplification reactions with oligonucleotide primers were used for the phycocyanin operon (PC) (Neilan et al., 1995) following the procedure described by Baker et al. (2001). Amplification products were visualised by electrophoresis on 0.7% agarose gels stained with ethidium bromide (0.2 μg.mL^{-1}) in 1X TBE running buffer and recorded using the Electrophoresis Documentation and Analysis System 290 (EDAS 290)

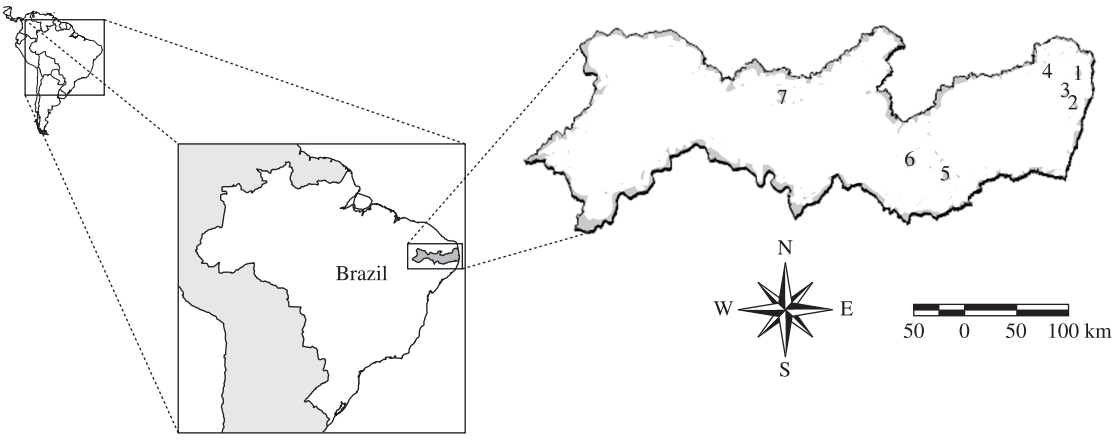

Figure 1. Map of study areas; Reservoirs: 1. Botafogo; 2. Duas Unas; 3.Tapacurá; 4. Carpina; 5. Mundaú; 6. Arcoverde; 7. Jazigo; State of Pernambuco, northeastern Brazil.

(Kodak, Melville, NY, USA). In all reactions sets, a control treatment (without DNA) was included (data not shown). All tests were performed in duplicate. The reproducibility band was tested with DNA from different extractions using the same methodology and conditions. Fragments obtained from the primers *mcy*B-F/R and *mcy*B-F/R-A resulted in sizes with approximately 570 and 315 bp, respectively.

3. Results

Potential microcystin-producing cyanobacteria were identified in the seven reservoirs investigated (Table 2). The prevailing species were *Microcystis aeruginosa* (Kützing) Kützing, *M. novacekii* (Komárek) Compère, *M. panniformis* Komárek et al., *Anabaena constricta* (Szafer) Geitler and *Planktothrix agardhii* (Gomont) Anagnostidis & Komárek.

The *mcy*B gene involved in microcystin biosynthesis was found in all samples. The oligonucleotide primer *mcy*B-F/R-A revealed a higher number of unexpected bands (Table 2 and Figure 2).

4. Discussion

Microcystis aeruginosa, *M. novacekii*, *M. panniformis*, *Planktothrix agardhii* and species from the genus *Anabaena* are frequent in bodies of water in Brazil. However, not all these organisms or populations necessarily have a potential for microcystin production. The presence of microcystin synthetase genes does not necessarily indicate the production of microcystins, but it does indicate the potential for their production. Recent studies have demonstrated that some strains have no capacity for toxin production because they do not have the entire *mcy* gene cluster (Christiansen et al., 2008).

The evaluation of the genetic potential for microcystin production can be determined by investigating the presence of *mcy* genes, for microscopic analysis does not allow the distinction of microcystin-producing from non-microcystin-producing organisms. Studies using ELISA (Bittencourt-Oliveira, 2003; Hisbergues et al., 2003; Vaitomaa et al., 2003), MALDI-TOF MS (Kurmayer et al., 2002; Saker et al., 2005), HPLC (Nonneman and Zimba, 2002; Ouahid et al.,

Table 1. Sampling locations and dates used.

Reservoirs	Latitude	Longitude	Sampling date
Arcoverde	8° 33' 33" S	36° 59' 07" W	Mar. 1, 2005
Arcoverde	8° 33' 33" S	36° 59' 07" W	Aug. 23, 2006
Botafogo	7° 53' 02" S	35° 03' 32" W	Oct. 18, 2006
Duas Unas	8° 05' 31" S	35° 02' 19" W	Oct. 17, 2006
Carpina	7° 53' 51" S	35° 20' 13" W	Feb. 13, 2006
Carpina	7° 53' 51" S	35° 20' 13" W	Aug. 26, 2006
Carpina	7° 53' 51" S	35° 20' 13" W	Sep. 18, 2006
Jazigo	7° 59' 58" S	38° 14' 31" W	Mar. 3, 2005
Mundaú	8° 57' 17" S	36° 29' 55" W	Sep. 19, 2006
Tapacurá	8° 02' 14" S	35° 09' 46" W	Aug. 26, 2004
Tapacurá	8° 02' 14" S	35° 09' 46" W	Oct. 16, 2006

Table 2. Presence (+) or absence (-) of amplified fragments using the oligonucleotide primers designed for Brazilian populations of cyanobacteria (*mcy*B-F/R, *mcy*B-F/R-A). (*) Non-specific fragments.

Reservoirs	Most frequent cyanobacteria	PC	*mcy* B-F/R	*mcy* B-F/R-A
Arcoverde	*Cylindrospermopsis raciborskii* (Woloszynska) Seenaya & Subba Raju	+	+	+
Arcoverde	*Planktothrix agardhii* (Gomont) Anagnostidis & Komárek	+	+	+*
Botafogo	*Microcystis aeruginosa* (Kützing) Kützing	+	–	+*
Duas Unas	*Anabaena constricta* (Szafer) Geitler	+	+	+*
Carpina	*Cylindrospermopsis raciborskii* (Woloszynska) Seenaya & Subba Raju	+	+	+
Carpina	*Microcystis aeruginosa* (Kützing) Kützing, *M. panniformis* Komárek et al.	+	+	+
Carpina	*Microcystis aeruginosa* (Kützing) Kützing, *M. panniformis* Komárek et al.	+	+	+
Jazigo	*Geitlerinema amphibium* (Ag. ex Gom.) Anagnostidis	+	+	+*
Mundaú	*Microcystis aeruginosa* (Kützing) Kützing	+	+*	+*
Tapacurá	*Cylindrospermopsis raciborskii* (Woloszynska) Seenaya & Subba Raju	+	+	+
Tapacurá	*Microcystis aeruginosa* (Kützing) Kützing	+	+	+

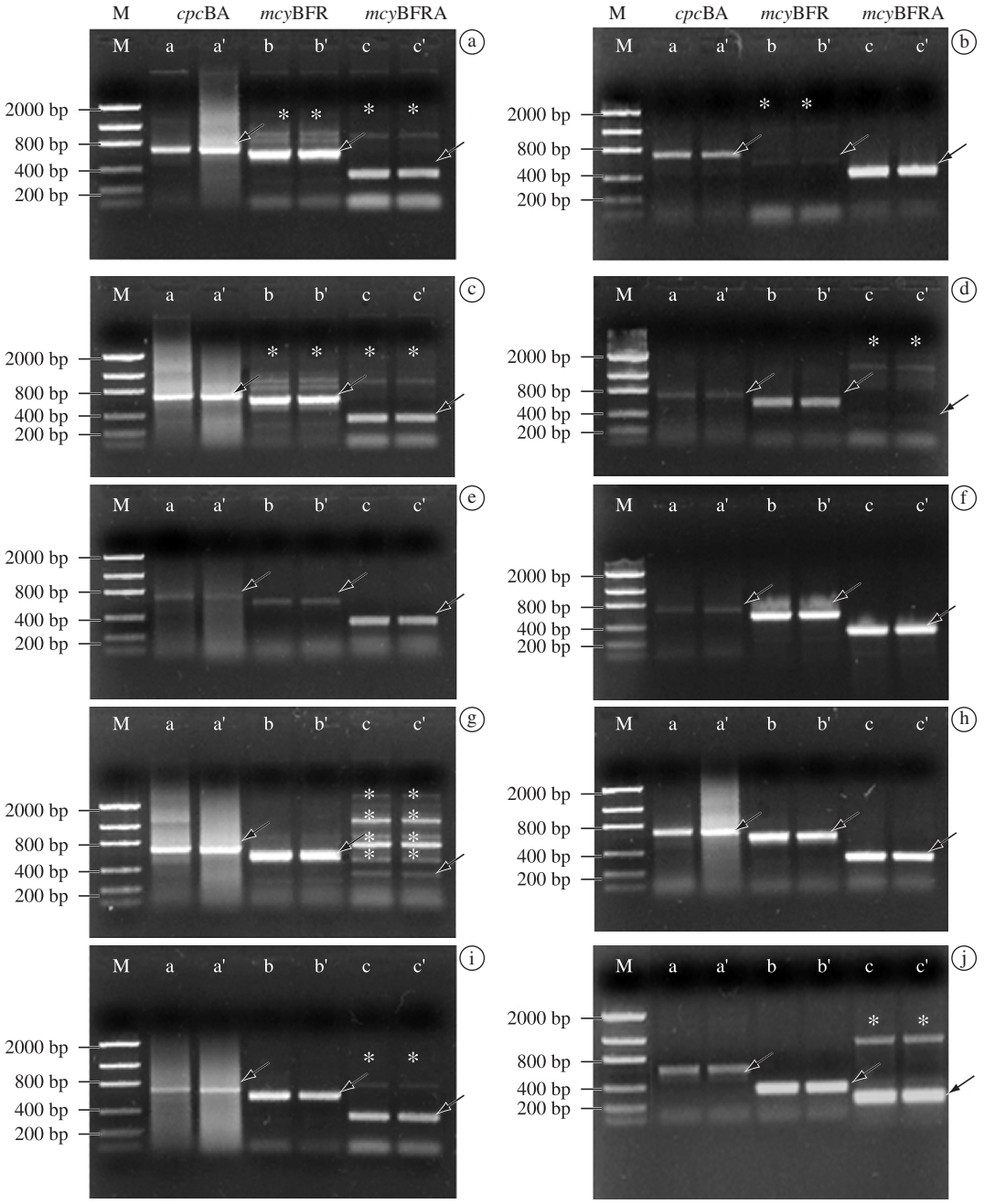

Figure 2. Amplification products (with replicas) revealed in electrophoresis with 0.7% agarose gels; Lanes M are DNA molecular mass standards (Low DNA); Lanes a-a': *cpc*BA; Lanes b-b': *mcy*B-F/R; Lanes c-c': *mcy*B-F/R-A; Expected fragment (arrow); non-specific fragment (asterisk); a) Arcoverde Aug 23, 2006; b) Mar 1, 2005; c) Mundaú Sep 19, 2006; d) Jazigo Mar 3, 2005; e) Tapacurá Oct 16, 2006; f) Tapacurá Aug 26, 2004; g) Botafogo Oct 18, 2006; h) Carpina Aug 26, 2006; i) Duas Unas Oct 17, 2006; and j) Carpina Feb 13, 2006.

2005) and bioassays with mice (Baker et al., 2002; Pan et al., 2002) have confirmed the production of microcystin in cyanobacteria with *mcy* genes. However, recent studies report the presence of amplified fragments in samples in which microcystins were not detected through high-performance liquid chromatography (HPLC) (Saker et al., 2007; Ouahid et al., 2005; Mankiewicz-Boczek et al., 2006). There are a number of interpretations for this, such as microcystin-producing genotypes that are not expressed (Kurmayer and Kutzenberger, 2003; Bittencourt-Oliveira, 2003), or the toxin is present but at concentrations below the HPLC detection limit.

The occurrence of these inactive microcystin genotypes is rare and poorly understood, but this should not be a significant limitation to the applicability of molecular detection. In previous studies, the presence of microcystin has been correlated, almost without exception, to the presence of *mcy* genes (Kurmayer and Kutzenberger, 2003; Via-Ordorika et al., 2004; Dittmann and Börner, 2005).

The presence of non-specific bands in the samples using *mcy*B-F/R-A did not impede the correct reading of the results obtained, since the size of the expected marker was previously known. Among the oligonucleotide primers tested, *mcy*B-F/R proved to be more specific than *mcy*B-F/R-A, as it did not exhibit non-specific bands.

Molecular analysis confirms the need for the monitoring of water quality in public water supply reservoirs in Brazil due to the occurrence of genotypes indicating toxicity. The PCR method was sensitive and appropriate for the detection of potential microcystin-producing cyanobacteria in environmental samples. The method could be used either alone or in conjunction with other techniques, such as the screening of samples to be sent for quantitative toxicological analysis using HPLC, thereby reducing monitoring cost and time. The usefulness of this molecular method in the investigation of potentially toxic cyanobacteria in reservoirs suggests that PCR could be a valuable tool in the monitoring of blooms.

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