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Semi-Quantitative PCR for Quantification of Hepatotoxic Cyanobacteria

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ABSTRACT

Blooms of microcystin-producing cyanobacteria are a problem worldwide. Microcystin is a liver hepatotoxin commonly found in bodies of water and is produced mainly by the genus *Microcystis*. The aim of the present study was to develop and assess a competitive PCR method for the quantification of toxic and non-toxic *Microcystis* cells using the *cpc*BA and *mcy*B genes, which are respectively involved in the formation of phycocyanin and biosynthesis of microcystin. For the acquisition of competitor DNA, amplification sequences were carried out of the "cell DNA equivalent" of microcystin-producing (BCCUSP18) and non-microcystin-producing (BCCUSP03) strains of *Microcystis* spp. using primers described in the literature as well as others designed for the present study. The method was successfully developed, as competitor DNA was constructed and co-amplified with the target DNA. Competitive PCR proved to be useful in quantifying toxic and non-toxic cells of *Microcystis* spp. strains, representing a helpful methodology tool to study isolated toxin-producing cyanobacteria.

Keywords: Cyanobacteria; *Microcystis*; Competitive PCR; *mcy*B; *cpc*BA

1. Introduction

Cyanobacterial blooms have become increasingly frequent in freshwater systems worldwide, causing countless adverse effects on public health and the environment. One of the greatest problems is that these organisms produce toxins with hepatotoxic and neurotoxic effects [1-3]. These toxins are released in water through cell lysis and remain dissolved for varying periods of time. Among the cyanobacteria capable of producing toxins is genus *Microcystis* Kützing *ex* Lemmermann, which produces several microcystin isoforms (hepatotoxins). Microcystin damages liver cells, especially through disruption of cytoskeleton components [3].

The lack of observable morphological differences between toxic and non-toxic colonies by optical microscope makes molecular detection of the potentially micro-cystin-producing genotype quite useful.

In Brazil, concerns over cyanobacteria and cyanotoxins increased following the incident in Caruaru, Pernambuco in 1996 [4]. Decree 518/2004 was the first legal mechanism to mandate the monitoring of cyanobacteria and cyanotoxins in public water supply reservoirs. That document sets 2.0×10^4 cyanobacteria cells per milliliter and 1.0 $\mu g \cdot L^{-1}$ of microcystins as the maximum levels allowed in crude samples and treated water, respectively [5], in tune with the recommendations set by the World Health Organization [6].

The quantification of cyanobacteria cells using Sedgwick-Rafter or sedimentation chambers [7] is made difficult by the dense aggregates that are formed, hindering cell distinction [8].

The monitoring of cyanobacteria in public supply reservoirs could be made easier by applying quicker methods able to overcome the difficulty in cell visualization in colonial individuals. This requires the development and employment of sensitive methods that can guarantee the detection and quantification of these cyanobacteria at low cost. In that task, highlight is given to molecular detection of the potentially microcystin-producing genotype. Using PCR assays, molecular detection aims to investigate the existence of *mcy* genes in the populations, which are responsible for coding of the enzyme complex (microcystin synthetase), along with polyketide synthase.

Competitive PCR is based on the co-amplification of the target gene and a competitor DNA of known concentration; both can be amplified by the same primer [9]. Assuming there is a copy of the studied gene in the ge-

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nome of the organism, once quantified the number of copies of the gene can be converted to the number of chromosomes, and consequently to the number of cells [10].

The objective of this study was to develop and investigate the applicability and efficiency of the competitive PCR method to quantify toxic and non-toxic *Microcystis* cells. To that end, nucleotide sequences named "competitor DNA" were obtained for phycocyanin (PC) genes and for the *mcy*B gene microcystin synthetase. The accuracy of the competitive PCR technique was later assessed against a cell count by optical microscope.

2. Materials and Methods

2.1. Strains and Environment Samples

The study used strains *Microcystis* sp. BCCUSP18 and BCCUSP03 from the "Brazilian Cyanobacteria Collection—University of São Paulo" (BCCUSP), microcystin-producing and non-producing, according to [11,12], respectively, under controlled lighting conditions (30 $\mu mol \cdot m^{-2} \cdot s^{-1}$), photoperiod (14:10 hours, light-dark) and temperature (21˚C ± 0.5˚C), BG-11 culture medium [13] modified according to [14].

2.2. Cell Quantification

The samples were quantified using a Fuchs Rosenthal hemocytometer with the aid of an Olympus binocular microscope. A minimum of 400 cells were counted in order to achieve an error of approximately 10% for a confidence level of 95% [15].

2.3. DNA Extraction

Single-mL aliquots of the culture and environment sample containing a quantified number of cells were used to extract the DNA according to the methodology of [16]. This made it possible to relate the volume of the extracted DNA solution to the corresponding cell number, which was expressed as the number of "cell DNA equivalent" (hereby referred to as CDE in this study).

2.4. Competitor DNA Construction

Competitor DNA was synthesized from consecutive amplifications, as performed by [17], as shown in **Figure 1**. The phycocyanin intergenic spacer (PC-IGS), considered a housekeeping gene in cyanobacteria, was amplified, as was the *myc*B gene involved in the biosynthesis of microcystin. Amplifications were carried out in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, USA) using the pureTaq Ready-To-Go PCR Beads kit (GE Healthcare, Piscataway, NJ, USA), 20 µM of each primer, 5 to 10 ng of DNA and ultrapure water (Mili-Q, Millipore, USA) to fill a 25 µL reaction. Primers and amplification conditions are shown in **Table 1**.

The amplification products were visualized by 1.2% agarose gel electrophoresis stained with ethidium bromide (2.0 $\mu g \cdot mL^{-1}$). The quantity and purity of competetor DNA were measured using a Nanodrop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA).

2.5. Competitive PCR

Trials were carried out in triplicate to evaluate the amplification efficiency of the different competitor DNA constructed. A total of four ten-fold dilutions of competitor *mcy*B (7.4×10^{-2} ng, 7.4×10^{-3} ng, 7.4×10^{-4} ng or 7.4×10^{-5} ng) and competitor *cpc*BA (6.6×10^{-3} ng, 6.6×10^{-4}

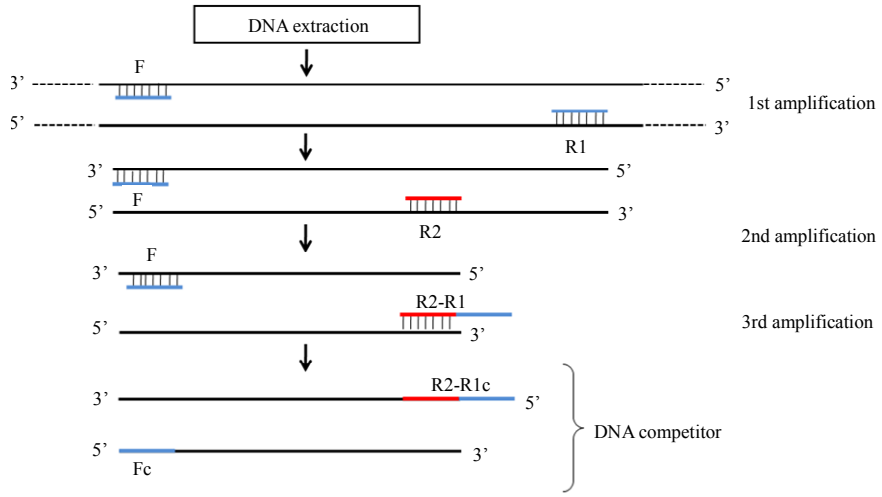

Figure 1. Phases of competitor DNA construction. F. forward primer obtained in Neilan *et al.* (1995); R1. Reverse primer obtained in Neilan *et al.* (1995); R2. Reverse primer designed in the present study; R2-R1. Reverse primer with combined R1 and R2 synthesis; Fc. Genomic region complementary to forward primer F; R1/R2c. Genomic region complementary to reverse primer R1.

Table 1. Primers and conditions of the reactions used to construct the competitor DNA.

Gene	*Primer*	Sequence (5' - 3')	Fragment (pb)	Source	PCR conditions
PC-IGS	*cpc*B-F	GGCTGCTTGTTTACGCGACA	685	[18]	1 (94˚C/4'); 30 (94˚C/10", 50˚C/20" and 72˚C/40"); 1 (72˚C/7')
	*cpc*A-R	CCAGTACCACCAGCAACTAA			
	*cpc*A-R2	AGGGGGGTTTTCATTGTTTGG	363	This study	
*mcy*B	*mcy*B-F	TTCAACGGGAAAACCCAAAG	570	[12]	1 (94˚C/2'); 35 (94˚C/10", 56˚C/20" and 72˚C/1'); 1 (72˚C/5')
	*mcy*B-R	CYAATATGTAAYTCTCCAG[a]			
	*mcy*B-R2	TTAACCAGTCTCGCAAGTC	233	This study	

[a]Y. C or T.

ng, 6.6×10^{-5} ng or 6.6×10^{-6} ng) were used, along with 2.0×10^4 CDE from toxic and non-toxic strains.

Amplifications were carried out in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, USA) using the pureTaq Ready-To-Go PCR Beads kit (Amersham, Piscataway, NJ), 20 μM of each primer, 2.0×10^4 CDE, competitor *mcy*B or competitor *cpc*BA and ultrapure water (Mili-Q, Millipore, USA) to fill a 25 μL reaction.

A control was performed for the target DNA (Ct), using only target DNA in the amplification, without competitor DNA. A control was also performed for the competitor DNA, without target DNA (Cc).

The assay was carried out to investigate the amount of competitor DNA at which the amplification of target DNA (2.0×10^4 cell DNA equivalents) and competitor DNA would be equivalent. The optical densities (OD) of the bands of amplified target and competitor DNA were measured using Kodak Digital Science 1D 3.6 software from the digital image of the 1.2% agarose gel stained with ethidium bromide (2.0 $\mu g \cdot mL^{-1}$); the obtained data were used to plot a line graph in Excel 2007 (Microsoft, USA).

2.6. Quantification by Competitive PCR

Competitor DNA and increasing quantities of CDE from DNA extracted from the different strains were amplified to obtain the standard curve.

The amplifications, reaction controls and visualization of amplified DNA in agarose gel were carried out as previously described.

The linear regression equation for each assay was obtained using SAS statistical software for Windows (SAS Institute, Inc.), interposing the $\log_{10}$ of the ratio between the ODs of the target and competitor DNA on the X-axis, and the $\log_{10}$ of the quantity of target DNA CDE on the Y-axis.

3. Results and Discussion

It was identified that 3.7×10^{-3} ng of competitor *mcy*B and 6.6×10^{-5} ng of competitor *cpc*BA co-amplified with DNA of BCCUSP18 cells were enough to quantify 2.0×10^4 CDE of toxic cells. For strain BCCUSP03 this value was 3.3×10^{-4} ng of competitor *cpc*BA. Visual observations in the digital image of agarose gel, as well as analysis of the obtained OD graphic data, confirmed the results (**Figure 2**).

From the assays of competitor c*pc*BA with cells from the different strains, 1.84×10^4 cells of *Microcystis* sp. were obtained. The quantification of toxic cells using co-amplification with competitor *mcy*B was 1.54×10^4 cells of *Microcystis* sp. BCCUSP18 (**Table 2**).

Cell counts and real-time PCR analysis in strains of microcystin- and nodularin-producing cyanobacteria using *mcy*E and *nda*F, respectively, suggest there are 1.3 copies of these genes per cell [19]. Those authors normalized the toxigenic potential for one copy of the genes per cell. Our results with genes *cpc*BA and *mcy*B, for strain BCCUSP18, are comparable to those of [19] as the *cpc*BA:*mcy*B ratio was equal to 0.94. The ratio between copy number of *cpc*BA and cell number in BCCUSP03 was 1.07.

As is known through genetic sequencing, *Microcystis* spp. has only one copy of the *mcy* operon [20] and two copies of gene PC [21]. As such, the results found herein express the number of copies of genes *mcy*B and *cpc*BA in the genome of this cyanobacterium. [22,23] found similar results in the quantification of *mcy*B and *cpc*BA by real-time PCR. Both concluded that the ratio between *mcy*B and *cpc*BA is 1:1.

Quantifying toxic cells, either by competitive PCR or real-time PCR, includes the possibility that intrinsic errors can affect the results [24]. Nevertheless, it is believed that the data obtained in this study allowed a standardization of the competitive PCR technique with high reproducibility, as represented by highly significant coefficients of explanation of the line (R^2) values; it is less onerous than real-time PCR and can be applied in experiments with isolated cultures of toxic and non-toxic cells.

4. Conclusion

In conclusion, it can be affirmed that the methodology developed to construct competitor DNA was successful, allowing the co-amplification of the target and competi-

Table 2. Quantitative analysis of strains and environment sample using competitive PCR. R^2. Coefficient of explanation of the line; Q. Quantification by competitive PCR; M. Mass of competitor DNA used in competitive PCR.

Samples	Repetitions	R^2	Line equation	Q (CDE)	M (ng)
		Competitor *cpc*BA			
BCCUSP18 and BCCUSP03	6	0.895	y = 4.265 + 5.6968x	1.84×10^4	-
		Competitor *mcy*B			
BCCUSP18	3	0.950	y = 4.187 + 4.607x	1.54×10^4	3.7×10^{-3}

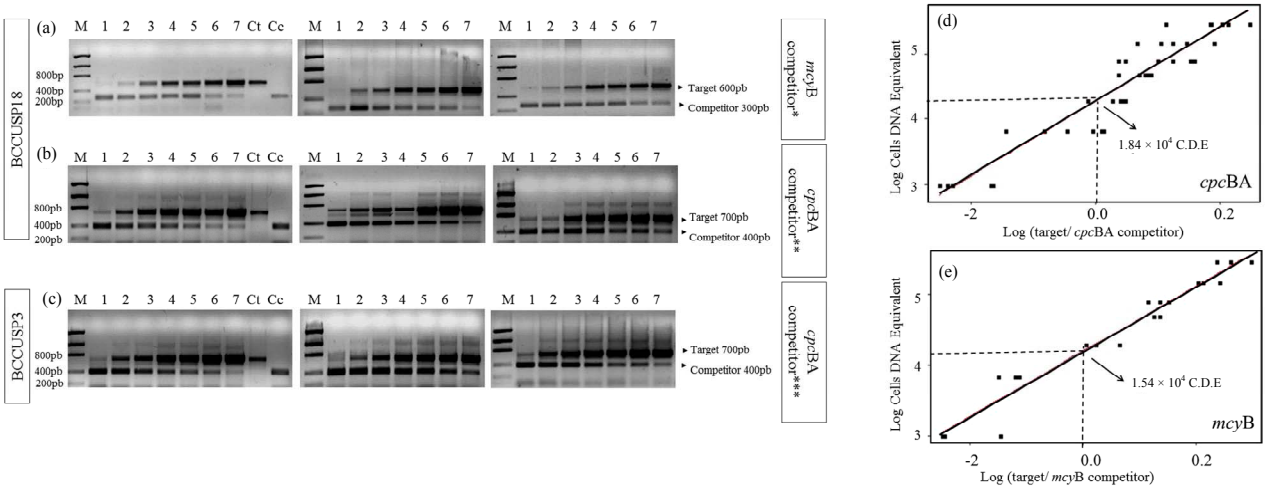

Figure 2. Agarose gels with competitive PCR assays for strains BCCUSP03 and BCCUSP18 and their respective graph analyses. (a) Competitor *mcy*B with BCCUSP18; (b) Competitor *cpc*BA with BCCUSP18; (c) Competitor *cpc*BA with BCCUSP3; M. Low DNA Mass LadderTM; 1. 1.0×10^3 CDE; 2. 7.0×10^3 CDE; 3. 2.0×10^4 CDE; 4. 5.0×10^4 CDE; 5. 8.0×10^4 CDE; 6. 1.5×10^5 CDE; and 7. 3.0×10^5 CDE. Ct. Target DNA controle; Cc. Competitor DNA control; (*). 3.7×10^{-3} ng, (**). 6.6×10^{-5} ng; (***). 3.3×10^{-4} ng; (d) Amplification with competitor *cpc*BA; (e) Amplification with competitor *mcy*B.

tor DNA with high reproducibility. Competitive PCR showed potential for use in scientific studies as a less onerous alternative to real-time PCR, and as an option for water source monitoring, abiding by the limits of Brazilian legislation.

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