J. Fish. Sci. Technol. 11(4), 205-208, 2008

JOURNAL OF
FISHERIES SCIENCE
AND TECHNOLOGY

Rapid detection and Quantification of Fish Killing Dinoflagellate *Cochlodinium polykrikoides* (Dinophyceae) in Environmental Samples Using Real-time PCR

Tae-Gyu Park, Yang-Soon Kang, Mi-Kyung Seo, Chang-Hoon Kim[1] and Young-Tae Park*

Marine Ecology Research Division, National Fisheries Research and Development Institute, Busan 619-705, Korea
[1]Department of Aquaculture, Pukyong National University, Busan 608-737, Korea

The mixotrophic dinoflagellate *Cochlodinium polykrikoides* was reported to be linked to major fish kills in Korea and Japan since the 1990s. Rapid and sensitive detection of microalgae has been problematic because morphological identification of dinoflagellates requires light microscopic and scanning electron microscopic observations that are time consuming and laborious compared to real-time PCR. To address this issue, a real-time PCR probe targeting the ITS2 rRNA gene was used for rapid detection and quantification of *C. polykrikoides*. PCR inhibitors in water column samples were removed by dilution of template DNA for elimination of false-negative reactions. A strong association between cell quantification using real-time PCR and microscopic counts suggests that the real-time PCR assay is an alternative method for cell estimation of *C. polykrikoides* in environment samples.

Key words: *Cochlodinium polykrikoides*, Harmful algal blooms, Real-time PCR, Red tide

Introduction

Harmful algae are defined as toxic or nontoxic species that can cause mass mortalities of aquatic organisms, and/or can cause human illness and have environmental impacts (Smayda, 1997). The outbreaks of harmful algal blooms (HABs) and toxicity episodes have been increasing globally over the last two decades, resulting in considerable economic losses and public health problems (Hallegraeff, 2003). Massive *Cochlodinium polykrikoides*-related fish kills have been recorded with losses of US $60 million and US $11 million in 1995 and 2007, respectively (NFRDI, 2007). Traditional methods to identify dinoflagellates require morphological analyses by light microscopy and scanning electron microscopy (SEM) on cultured strains, which are unlikely to offer accurate species identification when applied to field samples (Hallegraeff, 2003; Taylor, 2004). To overcome this difficulty, real-time PCR was used for detection and quantification of *C. polykrikoides* in environment samples. Real-time PCR method is highly sensitive becauseof the amplifycation of short fragments and the use of fluorescent probes. Since this method eliminates the requirement for post-amplification processing steps and is achieved through the generation of a fluorescent signal in the early stages of PCR, it takes 1-1.5 h to complete the assay (Mumford et al., 2000; Schena and Ippolito, 2003). Real-time PCR, incurporating fluorogenic 5′ nuclease (TaqMan) chemistry, has been used for rapid and sensitive detection of a number of dinoflagellates in environmental samples (Lin et al., 2006; Park et al., 2007). In this study, abundances of *C. polykrikoides* in the coast of Yeosu where recurring harmful algal blooms occur were estimated using species-specific real-time PCR (Park et al., 2008) and the results were compared to microscopic counts.

*Corresponding author: ytpark@nfrdi.go.kr

Materials and Methods

Cultures

C. polykrikoides cultures were obtained from NFRDI. The cultures were maintained in f/2 medium (Guillard and Ryther, 1962) at 24°C, with cool white fluorescent lamps on a 12:12-h light:dark cycle with a

light intensity of 100 μmol photons $m^{-1}s^{-1}$.

Environmental samples

Surface water samples of 250 mL were collected at three stations located on the southern coasts of Korea (Fig. 1), at 2-week intervals from June to September 2007 for investigation of temporal occurrences of *C. polykrikoides* and *G. impudicum* by real-time PCR, The water sample was filtered onto a 1.2 μm pore-size, 25 mm diameter glass microfibre GF/C filter (Whatman, Ltd. Maidstone, England). The filter was placed in a 2 mL microcentrifuge tube, and stored at -70°C until DNA extraction.

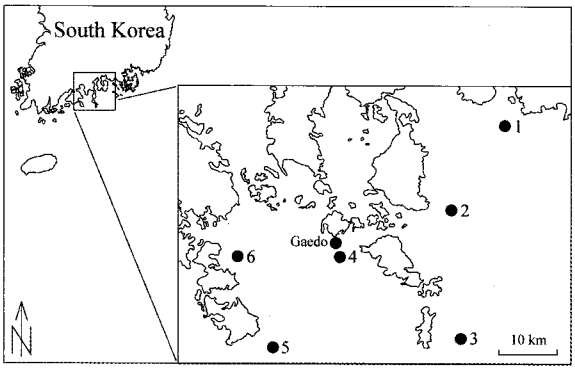

Fig. 1. Sampling locations for collection of surface water samples.

DNA extraction

Surface water samples were suspended in 900 μL of TE buffer (pH 8.0) and ground using wooden applicator sticks. The sample was then boiled at 100°C for 5 min. After adding 900 μL of phenol : chloroform : isoamyl alcohol (25 : 24 : 1), the sample was mixed thoroughly and centrifuged at 14,000 rpm for 10 min. The supernatant was transferred to a new tube, and 40 μL of 3 M sodium acetate (pH 5.2) and 900 μL of 100% of ethanol (-20°C) were added, followed by incubation of the sample at -20°C for 30 min. After centrifugation at 14,000 rpm for 20 min at 4°C, the DNA was rinsed twice with 70% of ethanol. The samples dried and dissolved in 100 μL of TE buffer.

Real-time PCR conditions

Real-time PCR was performed with 1 μL of template DNA, primers and a probe at final concentrations of 0.2 and 0.15 μM, 5 μL of platinum quantitative PCR supermix-UDG (Invitrogen, Eugene, Oregon, USA) and PCR grade water to a final volume of 10 μL. The thermal cycling conditions consisted of 2 min at 50°C and 2 min at 95°C following by 45 cycles of 10 s at 95°C and 45 s at 60°C. Fluorescence data were collected at the end of each cycle, and determination of the cycle threshold line was carried out automatically by the real-time PCR instrument (Rotor Gene 6000, Corbett Research, Sydney, Australia). All samples were analyzed in triplicate wells.

Standard curve

Ten-fold serial dilutions of the DNA extracts were used to construct the standard curve (triplicate measurements by real-time PCR). The cell number of the target species in environmental samples was calculated as C_T values, and was measured by comparison with the standard curve. To evaluate the accuracy of the standard curves, known concentrations of *C. polykrikoides* were spiked with sterile-filtered field samples (0.2 μm membrane filter; ADVANTEC MFS, Inc., California). Followed by DNA extraction, 10-fold diluted DNA extracts were amplified by the real-time PCR instrument. The calculated cell numbers by the standard curve were then compared to the cell numbers estimated by light microscopy.

Microscopic counts

Cell numbers of *C. polykrikoides* in environmental samples were estimated by light microscopy using a hemocytometer (Blau Brand, Germany).

Results and Discussion

The measured C_T values from standard curve correlated well with the values calculated from the log of the starting cell number (R^2 values of 0.99). The assay detection limit was less than one cell of *C. polykrikoides* in a reaction. PCR inhibitors in surface water samples were effectively removed by 10-fold dilution of template DNA. False-negative and - positive reactions were eliminated by running positive and negative controls with field samples in each reaction. The results of real-time PCR assays with DNA extracted from environmental samples showed a wide range of *C. polykrikoides* cell densities (Figs.

Table 1. Primers and probes for *Cochlodinium polykrikoides*-specific real-time PCR assays

Primers and probe	Code	Sequence (5'→3')	Reference
Forward	CPITSF	CGGCAACCTTTGTCAAACA	
Reverse	CPITSR2	GGTTTGCTGATCTAACTTCATGTCT	Park et al., 2008
Probe	CPITSP	FAM-CAACCGTGATACCCGCTAGCTTTGC-TAMRA	

2, 3). The cell numbers ranged from 1 cells mL^{-1} to 12,040 cells mL^{-1}. The cell numbers peaked at 12,040 cells mL^{-1} in 10th August. Cell abundances determined via real-time PCR were compared to abundances determined by microscopic counts (Fig. 3). Quantification of *C. polykrikoides* abundance by the two methods was in close agreement with a correlation coefficient (R^2) of 0.890 (Fig. 3).

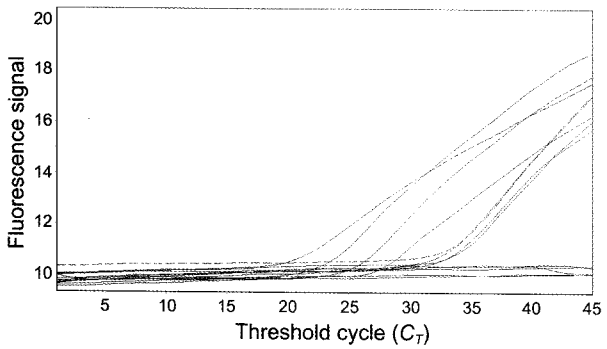

Fig. 2. Positive detection for *Cochlodinium polykrikoides* from water samples collected in August from the coast of Yeosu using species-specific real-time PCR. Samples were analyzed in triplicate wells.

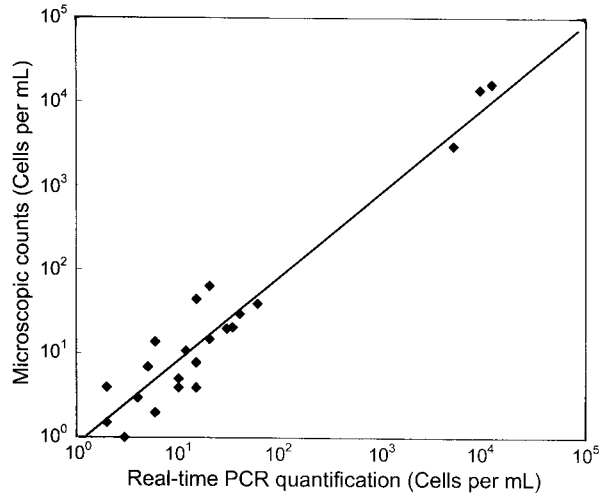

Fig. 3. Cell abundances of *Cochlodinium polykrikoides* in the coast of Yeosu from June to September 2007 estimated by TaqMan based real-time PCR and microscopic counts. The solid line indicates a 1:1 relationship between the two methods. A correlation coefficient (R^2) was 0.890.

C. polykrikoides can have a significant impact on marine animals and fisheries industry because of the formation of massive blooms. This dinoflagellate superficially looks similar to *Gymnodinium impudicum*, *Gymnodinium catenatum*, and other *Cochlodinium* species, and discrimination requires the identification of ultrastructural characters by light microscopy or scanning electron microscopy (SEM), and genetic chracterization by rDNA sequence analyses on cultured cells (Iwataki et al., 2008; Matsuoka et al., 2008). The culture dependent identification is time consuming and laborious. For rapid and sensitive detection of the organism in environmental samples, a real-time PCR protocol was used in the present study. The TaqMan probe system is based on the use of sequence-based fluorogenic probes labeled with a reporter dye at the 5' end and a quencher at the 3' end (Holland et al., 1991). This approach reduces time and labor because post-PCR gel electrophoresis is excluded and it has high sample throughput by allowing automated detection. The real-time PCR assay has been used successfully to detect and quantify dinoflagellates in environment samples (Lin et al., 2006; Park et al., 2007). In the present study, the maximum cell concentration reached 12,040 cells mL^{-1} in the coast of Yeosu in August, while its abundance was low in most months. The low cell abundances in most months are not likely due to non-specific reaction or false-negative detection because assay specificity was previously confirmed by testing against related organisms and sequence analysis of PCR amplicons obtained from field samples. The results of real-time PCR for detection of *C. polykrikoides* showed comparable results to those of microscopic counts. The strong association between two methods suggests the real-time PCR assay provides sensitive and specific detection of *C. polykrikoides* in field samples. Since first description of *Cochlodinium* genus in 1896 (Schütt, 1896), more than 40 species have been reported in this genus. In recent years, new *Cochlodinium* species, *C. fulvescens*, has been described from Japanese waters based on morphological and genetic characters. *C. fulvescens* morphologically distinguished from *C. polykrikoides* based on cell size, shape of chloroplasts, and the position of narrow sulcus situated in the cell surface (Iwataki et al., 2007). These ultrastructual characters are not readily distinguished from those of related organisms by traditional identification methods such as light microscopy. The real-time PCR protocol developed in this study may provide a sensitive and rapid identification tool for field-based studies such as geographic distribution and seasonal variations in *C. polykrikoides* abundances.

Acknowledgements

We thank Ga-Young Ha of NFRDI for technical support on real-time PCR, and NFRDI' personnel for providing water samples. We are also grateful to

Young-Sil Kang of NFRDI for valuable advice and suggestions. This work is funded by a grant from the National Fisheries Research and Development Institute (RP-2008-ME-078).

References

Guillard, R.R.L. and J.H. Ryther. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* cleve. Can. J. Microbiol., 8, 229-239.

Hallegraeff, G.M. 2003. Harmful algal blooms: a global overview. In G.M. Hallegraeff, Anderson, D.M. and Cembella, A.D. (Eds) Manual on Harmful Marine Microalgae. IOC Manuals and Guides, UNESCO, New York, 25-50.

Holland, P.M., R.D. Abramson, R. Watson and D. Gelfand. 1991. Detection of specific polymerase chain reaction products by utilizing the 5'-3' exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Acad. Sci. USA, 88, 7276-7280.

Iwataki, M., H. Kawami and K. Matsuoka. 2007. *Cochlodinium fulvescens* sp. nov. (Gymnodiniales, Dinophyceae), a new chain-forming unarmored dinoflagellate from Asian coasts. Phycol. Res., 55, 231-239.

Iwataki, M., H. Kawami, K. Mizushima, C.M. Mikulski, G.J. Doucette, Jr. J.R. Relox, A. Anton, Y. Fukuyo and K. Matsuoka. 2008. Phylogenetic relationships in the harmful dinoflagellate *Cochlodinium polykrikoides* (Gymnodiniales, Dinophyceae) inferred from LSU rDNA sequences. Harmful Algae, 7, 271-277.

Lin, S., H. Zhang, Y. Hou, L. Miranda and D. Bhattacharya. 2006. Development of a dinoflagellate-oriented PCR primer set leads to detection of picoplanktonic dinoflagellates from Long Island Sound. Appl. Environ. Microbiol., 72, 5626-5630.

NFRDI. 2007. Data on red tides. Retrived from http://www.nfrdi.re.kr/redtideInfo in 2007.

Matsuoka, K., M. Iwataki and H. Kawami. 2008. Morphology and taxonomy of chain-forming species of the genus *Cochlodinium* (Dinophyceae). Harmful Algae, 7, 261-270.

Mumford, R.A., K. Walsh, I. Barker and N. Boonham. 2000. Detection of *potato mop top virus* and *tobacco rattle virus* using a multiplex real-time fluorescent reverse-transcription polymerase chain reaction assay. Phytopathology, 90, 448-453.

Park, T.G., M.F. de Salas, C.J.S. Bolch and G.M. Hallegraeff. 2007. Development of a real-time PCR probe for quantification of the heterotrophic dinoflagellate *Cryptoperidiniopsis brodyi* (Dinophyceae) in environmental samples. Appl. Environ. Microbiol., 73, 2552-2560.

Park, T.G., G.H. Park, Y.T. Park, Y.S. Kang, H.M. Bae, C.H. Kim, H.J. Jeong and Y. Lee. 2008. Identification of the dinoflagellate community during *Cochlodinium polykrikoides* (Dinophyceae) blooms using amplified rDNA melting curve analysis and real-time PCR probes. Harmful Algae (In press).

Schena, L. and A. Ippolito. 2003. Rapid and sensitive detection of *Rosellinia necatrix* in roots and soils by real time Scorpion-PCR. J. Plant Pathol., 85, 15-25.

Schűtt, F. 1896. 'Peridiniales' in Engler and Prantl, 'Die natűrlichen Pflanzenfamillien'. Leipzig, Engelmann, I, Abt. B, 43 Figs.

Smayda, T.J. 1997. What is a bloom? A commentary. Limnol. Oceanogr., 42, 1132-1136.

Taylor, F.J.R. 2004. Illumination or confusion? Dinoflagellate molecular phylogenetic data viewed from a primarily morphological standpoint. Phycol. Res., 52, 308-324.

(Received October 2008, Accepted December 2008)