J. Fish. Sci. Technol. 11(4), 209-211, 2008

JOURNAL OF
FISHERIES SCIENCE
AND TECHNOLOGY

Detection of Heterotrophic Dinoflagellate *Pfiesteria piscicida* (Dinophyceae) in Surface Water Samples Using Real-time PCR

Tae-Gyu Park, Yang-Soon Kang, Mi-Kyung Seo and Young-Tae Park*

Marine Ecology Research Division, National Fisheries Research and Development Institute, Busan 619-705, Korea

Heterotrophic dinoflagellate *Pfiesteria piscicida* (Dinophyceae) has been claimed to produce potent ichthyotoxins that cause disorientation and eventually death of fish and other marine animals. A real-time PCR probe targeting for SSU rRNA gene was used for detection of *P. piscicida* in Chinhae Bay, Korea. PCR inhibitors were successfully removed by dilution of template DNA. Positive detections were shown from surface water samples indicating the presence of *P. piscicida* in Chinhae Bay.

Key words: Harmful algal blooms, *Pfiesteria piscicida*, real-time PCR, red tide

Introduction

The estuarine dinoflagellate *Pfiesteria piscicida* Steidinger et Burkholder has received considerable attention since they were identified as a causative agent of fish kills. PCR-based assays are a powerful tool to detect or quantify harmful dinoflagellates in environmental water samples (Rublee et al., 2005). Real-time PCR offers high sensitivity, specificity, and quantification of PCR products. The advantage of the real-time PCR method over other PCR-based quantification methods is that real-time PCR allows the simultaneous analysis of 96 samples (depending on instrument used) in a short time and reduces the risk of contamination because this method eliminates the requirement for post-amplification processing steps (Schena et al., 2002; Schena and Ippolito, 2003). Furthermore, real-time PCR monitors growth of the amplification product in each cycle, allowing quantification of starting DNA in the early stages of PCR [i.e. linear section of the log (starting DNA) versus DNA fluorescence curve] (Mumford et al., 2000). The fluorescent TaqMan® systems are based on the use of oligonucleotide probes labeled with a donor fluorophore and an acceptor dye (quencher) (Holland et al., 1991; Gibson et al., 1996; Heid et al., 1996). This system requires the design of primers (forward and reverse) and a probe that hybridizes the PCR product defined by the primers. The probe is labeled with a reporter dye at the 5' end and a quencher at the 3' end. DNA polymerase cleaves the annealed probe with its 5' nuclease activity, and the reporter fluorescence is separated from the quencher. The fluorescence detected during each PCR cycle results in an increase in fluorescence emission. In the present study, occurrences of *P. piscicida* in Chinhae Bay were investigated using a TaqMan-based real-time PCR probe.

*Corresponding author: ytpark@nfrdi.go.kr

Materials and Methods

Cultures and water sample collection

Two strains of *P. piscicida* (CCMP1975 and CCMP1974) were used for a control of real-time PCR. They were maintained in f/2 medium of 15 salinity at 20°C with a 12:12h light:dark cycle of 100 μmol photons·m^{-2}·s^{-1} light. The prey, *Rhodomonas salina* was added every 2-3 days. Surface waster samples were collected from 12 stations in Chinhae Bay in June 2007 (Fig. 1).

Conditions for real-time PCR

Species-specific real-time PCR was carried out using the Rotor-Gene RG-3000A (Corbett research). For real-time PCR using a TaqMan probe (Table 1), 2 μL of temperate DNA was added to each tube with 10 μL of platinum quantitative PCR supermix-UDG (Invitrogen, Australia Pty Ltd), forward and reverse primers each at a final concentration of 0.2 μM, fluorogenic probe at a final concentration of 0.15 μM, and PCR grade water to a final volume of 20 μL. The

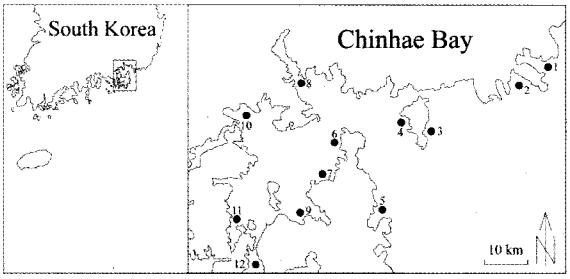

Fig. 1. Sampling locations for detection of *Pfiesteria piscicida*.

real-time PCR protocol consisted of holding samples at 50°C for 2min, followed by denaturation at 95°C for 2 min and then by 45 cycles of 95°C for 10s and 60°C for 45s. Fluorescence data were collected at the end of each cycle, and positive (*P. piscicida*) and negative (no template DNA) controls were included in every assay. The amplicon size of the target DNA and nonspecific products were inspected by gel electrophoresis using 2% agarose gels. For cell quantification, laboratory-cultured *P. piscicida* were collected and cell numbers were estimated by light microscopy using a hemocytometer (Blau Brand, Germany) before harvesting the cells (18,250 cells). DNA was extracted, and 10-fold serial dilutions of the DNA extracts were used to construct the standard curve (triplicate measurements by real-time PCR). The cell number of *P. piscicida* in environmental samples was calculated as C_T values, and was measured by comparison with the standard curve. Serial DNA dilutions of field water samples (non-dilution, 10-fold, and 100-fold dilutions) spiked with *Cryptoperidiniopsis brodyi* DNA (CBWA12; 1.5 ng μL^{-1}) were amplified using real-time PCR, and their C_T values were calculated and compared. Ten-fold dilutions of template DNA effectively removed PCR inhibitors from water samples.

Results and Discussion

A strong linear correlation between log (cell number) and C_T value with correlation coefficients (R^2) of 0.990 for the real-time PCR assay. Detection limit was of less than 1 cell per reaction. Positive detections for *P. piscicida* were shown from water samples (Stations 5 and 7) collected from Chinhae Bay and their abundances were 3 and 8 cells L^{-1}, respectively (Figs. 2, 3). When these water samples were observed under light microscopy, *P. piscicida* or look-alike species were found from Stations 5 and 7 at below quantification level. To prevent false-positive or false-negative reaction, each reaction was run with positive and negative controls. Assay specificity was previously tested against related organisms and this assay has been used for detection of *P. piscicida* in environmental samples (Bowers et al., 2000). For example, *P. piscicida* cysts were successfully detected in sediments from the U.S. using this assay suggesting a strong positive association between distribution of *P. piscicida* cysts and its presence in past water samples. This assay was also used for detection of *P. piscicida* in ballast water from Indonesia and Antarctic water samples indicating that it is a cosmopolitan species (Park et al., 2007a). The *P. piscicida*-specific real-time PCR assay based on SSU rDNA has three *P. piscicida*-specific regions for primers and a probe instead of one (Bowers et al., 2000). Therefore, the real-time PCR assay would provide higher specificity than the conventional PCR when the assays are applied to environmental water samples that may include unknown DNA sequences and genetic variants of related species. NTS regions of rDNA have also been employed for quantifying *P. pisicicda* DNA in standard and quantitative PCR-based format (Saito et al., 2002). NTS regions are more divergent than ITS, and may have unique and suitable regions for developing species-specific primers. However, extensive sequence analyses are required for NTS regions of other related dino-flagellates and they need to be compared with *P. pisicicida* DNA for additional confirmation of the assay specificity. In summary, real-time PCR offered

Table 1. Primers and TaqMan probes for species-specific real-time PCR assays

Dinoflagellate	Forward Reverse Probe	Code	Sequence (5'→3')	Reference
Pfiesteria piscicida	Forward	107	CAGTTAGATTGTCTTTGGTGGTCAA	Bowers et al., 2000
	Reverse	320	TGATAGGTCAGAAAGTGATATGGTA	
	Probe	Probe	FAM-CATGCACCAAAGCCCGACTTCTCG-TAMRA	
Cryptoperidiniopsis brodyi	Forward	CBITSF	TTGACACGTTGAAGTGAWGGA	Park et al., 2007b
	Reverse	CBITSR	ACAGCCAATGAAAGAGTKATGACAA	
	Probe	CBITSP	FAM- CATCTCATCGCTCGCCGTCGAT-TAMRA	

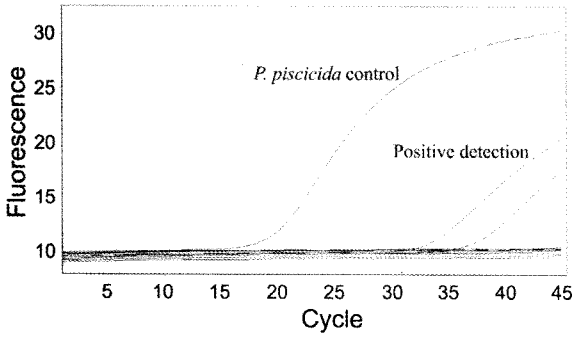

Fig. 2. Positive detection of *Pfiesteria piscicida* in water samples from Chinhae Bay (collected in June 2007) using real-time PCR.

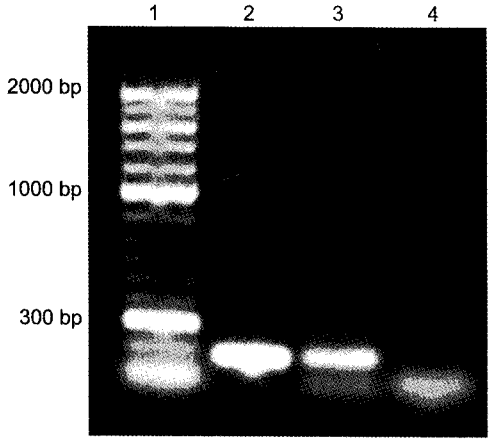

Fig. 3. Agarose gel analysis showing positive *P. piscicida* PCR products. Lanes: 1, 2-kb ladder molecular size marker; 2, *P. piscicida* positive control; 3, DNA extract from a water sample of Chinhae Bay; 4, no-template control.

highly sensitive and specific detection of *P. piscicida* in water column samples. The detection of *P. piscicida* by species-specific real-time PCR indicates the existence of *P. piscicida* and potential fish kills by this species in Chinhae Bay.

Acknowledgements

We thank Ga-Young Ha of NFRDI for technical support on real-time PCR. 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-077).

References

Bowers, H.A., T. Tengs, H.B. Glasgow, J.M. Burkholder, P.A. Rublee and D.W. Oldach. 2000. Development of real-time PCR assays for rapid detection of *Pfiesteria piscicida* and related dinoflagellates. Appl. Environ. Microbiol., 66, 4641-4648.

Gibson, U.E.M., C.A. Heid and P.M. Williams. 1996. A novel method for real-time quantitative RT-PCR. Genome Res., 6, 995-1001.

Heid, C.A., J. Stevens, K.J. Livak and P.M. Williams. 1996. Real-time quantitative PCR. Genome Res., 6, 986-994.

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

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., E.M. Bell, P. Imojen, P.A. Rublee, C.J.S. Bolch and G.M. Hallegraeff. 2007a. Detection of a novel ecotype of *Pfiesteria piscicida* (Dinophyceae) in an Antarctic saline lake by real-time PCR. Polar Biol., 30, 843-848.

Park, T.G., M.F. de Salas, C.J.S. Bolch and G.M. Hallegraeff. 2007b. 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.

Rublee, P.A., D.L. Reminfton, E.F. Schaefer and M.M. Marshall. 2005. Detection of the dinozoans *Pfiesteria piscicida* and *P. shumwayae*: A review of detection methods and geographic distribution. J. Eukaryot. Microbiol., 52, 83-89.

Saito, K., T. Drgon, J.A.F. Robledo, D.N. Krupatkina and G.R. Vasta. 2002. Characterization of the rRNA Locus of *Pfiesteria piscicida* and development of standard and quantitative PCR-based detection assays targeted to the nontranscribed spacer. Appl. Environ. Micro-biol., 68, 5394-5407.

Schena, L., F. Nigro and A. Ippolito. 2002. Identification and detection of *Rosellina necatrix* by conventional and real-time Scorpion-PCR. Eur. J. Plant Pathol., 108, 355-366.

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.

(Received October 2008, Accepted December 2008)