Korean Journal of Life Science
Vol. 12. No. 3. 250~255, 2002

Application of Species-specific DNA Probe to Field Samples of *Alexandrium tamarense* (Lebour) Balech

Eun-Seob Cho[1], Gi-Young Kim, Hyung-Sik Park, Hak-Gyoon Kim[1], Sung-Ki Moon[2] and Jae Dong Lee*

Department of Microbiology, Pusan National University, Pusan 609-735, Korea
[1]Harmful Algal Research Division, National Fisheries Research and Development Institute, Pusan 619-900, Korea
[2]Department of Biology, College of Science, Kyungsung University, Pusan 608-736, Korea

Abstract

Fluorescent species-specific DNA probe (AT1) of toxic dinoflagellate *Alexandrium tamarense* was tested on several other species, on comparison of binding activity at different preservatives for fixation of the cells, at different culture age and estimation of cell density by light microscope or epifluorescent microscope using whole cell hybridization. Th AT1 probe specifically bound to *Alexandrium tamarense*, whereas it did not bind to other phytoplankton, in particular *Alexandrium catenella*, morphologically similar to *Alexandrium tamarense*, could not react to AT1 probe. When cells were fixed with all three preservatives, labeling cells of *Alexandrium tamarense* emitted strong fluorescent signal intensity. In addition, regardless culture days, binding activity with AT1 probe was strong. The cell densities estimated by epifluorescent microscope were than those estimated by light microscope. The enumeration and identifying of *Alexandrium tamarense* using DNA probe method will be contributed to a new biotoxin monitoring and prediction system in field.

Key words – DNA probe, *Alexandrium tamarense*, identification, PSP, toxication,

Introduction

Korea has rich shellfish resources and a vigorous shellfish aquaculture industry that was threatened from marine biotoxins of algal origin. Paralytic shellfish poison (PSP) contaminated mussels and oysters, caused by *Alexandrium tamarense* (Lebour) Balech, have affected exports to other countries with associated losses for shellfish harvesters. It is important to meet the needs of the shellfish industry by exploring a rapid and accurate way of identifying and counting a PSP-producer, *Alexandrium tamarense*. However, considerable time and taxonomic skills are required to identify and count a particular species in the field samples using light microscope. In order to avoid these risks, biochemical methods (lectin-binding assay, immunoassay and DNA probe) have been developed to detect targeted phytoplankton species in the last decade [1,2,7,16,]. Previously, we applied FITC-conjugated lectins to some Korean red tide microalgae including several species of *Alexandrium* [4,5]. However, little is investigated on the application of species-specific DNA probe to toxic *Alexandrium tamarense* in Chinhae Bay, where the shellfish biotoxin contamination occurs annually. In this study an attempt was made for a new biotoxin monitoring system using DNA

*To whom all correspondence should be addressed
Tel : 82-51-510-2269, Fax : 82-51-510-2269
E-mail : leejd@hyowon.cc.pusan.ac.kr

probe that is quick and accurate means of identifying toxic *Alexandrium tamarense* in the field samples from Chinhae Bay.

Materials and Methods

Cultures

Four strains of *Alexandrium tamarense* (AT-2, 6, 10, B) were taken from red tide waters in Chinhae Bay and the strains were isolated using capillary pipette under microscope. The isolation procedure was done at National Fisheries Research and Development Institute, Pusan. *Alexandrium catenella* (Whedon & Kofoid) Balech was obtained from Inje University, Kimhae, Korea. The organisms in this study were maintained in f/2-Si medium [9] under 12 h light/12 h dark cycle (100 mol s^{-1}) at 20℃ (Table 1).

Field samples

Dong-Woo Kang provided natural seawater collected from Chinhae Bay on May 2000.

Preservation

Preservation methods using 1%, 2% glutaraldehyde, 1%, 3% Lugol's idoine solution and 1%, 2% formaldehyde were evaluated for their effect on fluorescence intensity according to the procedures of Scholin *et al.* [13].

Fluorescent signal intensity

The visual fluorescent signal in each sample, from + (weak) to ++ (moderate) to +++ (very strong), with a (-) indicating a negative result.

Whole cell hybridization

Whole cell hybridization with species-specific probes was tested against *Alexandrium tamarense* from cultures and natural samples, using the filtration method [11,14]. This study used 4 kinds of probes: AT1 probe (5'-ATCGTACACAAACACAGCAC-3', specific probe for *Alexandrium tamarense*) [2], UniC probe (5'-GWATTACC-GCGGCKGCTG, positive control for all eukarytotes) [11, 13], UniR probe (5'-CAGCMGCCGCGGUAAUWC, negative control to test for non-specific retention of probe) [11, 13], and no probe (negative control to compare against UniR for autofluorescence versus non-specific retention of probe). Cells were fixed for 1 h, and following washing and probe addition, were hybridized as described by Scholin *et al.* [14] and Miller and Scholin [11]. Binding reactivity was examined under a Nikon Optiphot microscope with FITC optical filter set (excitation 450-490 nm; emission 529 nm).

Table 1. Isolates of harmful algal species used in DNA probe

Species	Location	Toxicity[1]
Alexandrium tamarense (AT-2)	Chinhae	+
A. tamarense (AT-6)	Chinhae	+
A. tamarense (AT-10)	Chinhae	+
A. tamarense (AT-B)	Chinhae	+
A. catenella[4]	Chinhae	+
Cochlodinium polykrikoides (CP-1)[2]	Tongyong	-
C. polykrikoides[3]	Kunsan	-
Eutreptiella gymnastica (EG-1)	Namhae	-
Gyrodinium impudicum (GI-1)	Tongyong	-
Gymnodinium sanguineum (GS-1)	Masan	-
Heterosigma akashiwo (HA-1)	Jindong	-
Prorocentrum micans (PM-1)	Masan	-
P. minimum (Pmini-1)	Tongyong	-
P. triestinum (PT-2)	Namhae	-
Scrippsiella trochoidea (ST-1)	Yousu	-

[1]Toxicity was analyzed by high-performance liquid chromatography with a fluromonitor detector (Ex. 330 nm, Em. 390 nm). Toxicity in four isolates of AT-2, AT-6, AT-10 and AT-B was 3.245, 5.716, 0.999 and 0.178 pg STX_{eq} $cell^{-1}$ in late exponential phase under the same culture conditions, respectively.
[2]Collected during the 1997.
[3]Collected during the 1999.
[4]Obtained from Inje University, Kimhae.

Effect of culture age

The effect of culture age on the fluorescence signal

intensity was determined by harvesting cells from batch cultures of *Alexandrium tamarense* (AT-2, 6, 10, B) in early to late exponential phase and at stationary phase.

Enumeration on field and mixed cultures samples

The usefulness of the fluorescence staining methods for counting field samples was determined by the staining and counting of the stored field samples and a sample from a mixed cultures (Table 1).

Results

Table 2 showed the reactivity of species-specific DNA probe (AT1 probe) for *Alexandrium catenella* and various dinoflagellates using whole cell hybridization. AT1 probe conjugated strongly several strains of *Alexandrium* in this study and had an appearance of ovoid in the periphery of cell (Fig. 1), however, showed no binding activity with other dinoflagellates. Glutaraldehyde, Lugol solution and formaldehyde for cell preservation in the field and laboratory used commonly fixatives. Under the epifluorescence microscope, the intensity of the fluorescent signal generated by fluorescently labeled cells showed higher fluorescent intensity regardless of different strains and methods (Table 3). The difference in fluorescence generated by cells fixed in these fixatives was minimal. Cells from early to late exponential growth (5-45 days) in batch culture showed the strongest binding activity consistently (Table 4). When cells were fixed with Lugol's idoine (3%) from field sample, AT1 probe showed a speciation in toxic *Alexandrium tamarense* that emitted strongly fluorescence intensity (Fig. 2). Tests on the field and mixed culture samples showed that it was easier to positively identify toxic *Alexandrium tamarense* and discriminate them from non-target species using epifluorescent microscope than using direct light microscope (Table 5).

Table 2. Binding activity of fluorescent DNA probe (NA1) at different isolates of harmful algal species using whole cell hybridization

Microalgae	Fluorescence intensity
Alexandrium tamarense (AT-2)	+++
A. tamarense (AT-6)	+++
A. tamarense (AT-10)	+++
A. tamarense (AT-B)	+++
A. catenella[3]	-
Cochlodinium polykrikoides (CP-1)[1]	-
C. polykrikoides[2]	-
Eutreptiella gymnastica (EG-1)	-
Gyrodinium impudicum (GI-1)	-
Gymnodinium sanguineum (GS-1)	-
Heterosigma akashiwo (HA-1)	-
Prorocentrum micans (PM-1)	-
P. minimum (Pmini-1)	-
P. triestinum (PT-2)	-
Scrippsiella trochoidea (ST-1)	-

[1]Collected during the 1997.
[2]Collected during the 1999. [3]Obtained from Inje University, Kimhae.
+++ (strong binding); ++ (moderate); + (weak); - (no binding)

Discussion

In Korea, toxin-producing dinoflagellate *A. tamarense* was observed from middle March to early July and distributed mostly in Chinhae Bay [10] that is a semi-

Fig. 1. Cultured cells hybridized with species-specific probe AT1.

Table 3. Comparison of different preservation on toxic *Alexandrium tamarense* (AT-2, 6, 10, B) using DNA probe assay in whole cell hybridization

Strains	Preservation (%)	Fluorescence intensity
AT-2	Glutaraldehyde (1%)	++
AT-2	Glutaraldehyde (2%)	+++
AT-2	Lugol's idoine solution (1%)	++
AT-2	Lugol's idoine solution (3%)	+++
AT-2	Formaldehyde (1%)	++
AT-2	Formaldehyde (2%)	+++
AT-6	Glutaraldehyde (1%)	++
AT-6	Glutaraldehyde (2%)	++
AT-6	Lugol's idoine solution (1%)	+++
AT-6	Lugol's idoine solution (3%)	+++
AT-6	Formaldehyde (1%)	++
AT-6	Formaldehyde (2%)	++
AT-10	Glutaraldehyde (1%)	+++
AT-10	Glutaraldehyde (2%)	+++
AT-10	Lugol's idoine solution (1%)	++
AT-10	Lugol's idoine solution (3%)	+++
AT-10	Formaldehyde (1%)	++
AT-10	Formaldehyde (2%)	++
AT-B	Glutaraldehyde (1%)	++
AT-B	Glutaraldehyde (2%)	++
AT-B	Lugol's idoine solution (1%)	+++
AT-B	Lugol's idoine solution (3%)	+++
AT-B	Formaldehyde (1%)	++
AT-B	Formaldehyde (2%)	++

+++ (strong binding); ++ (moderate); + (weak); - (no binding)

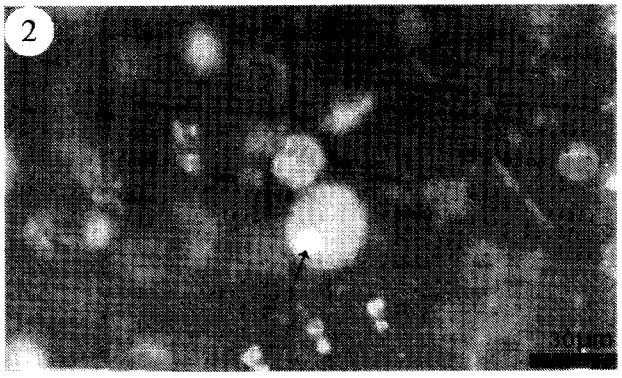

Fig. 2. Cells in field fixed with Lugol solution (3%) reacted with DNA probe. Arrow indicates the location of the label.

Table 4. DNA probe binding to toxic *Alexandrium tamarense* (AT-2) at different stages of growth

Culture days	Fluorescence intensity
5 days	+++
10 days	+++
20 days	+++
30 days	+++
45 days	+++

+++ (strong binding); ++ (moderate); + (weak); - (no binding)

Table 5. Comparison of cell density of field samples and mixed culture with *Alexandrium tamarense* by light microscope (LM) and epifluorescent microscope (EM)

Samples	LM (10^3 cells L^{-1})	EM (10^3 cells L^{-1})
Field		
Sujong	9	15
Wucheon	13	18
Dandong	5	9
Chilchundo	11	12
Mixed culture	17	20

enclosed bay with a restricting exchange of the waters. Since Chinhae Bay has been regarded as an important local shellfish cultivation region, the monitoring of *Alexandrium tamarense* has frequently taken place at many locations around the coast of Chinhae Bay. Lee [10] reported that four species of *Alexandrium* (*Alexandrium tamarense, Alexandrium affine* (Inoue and Fukuyo) Balech, *Alexandrium fraterculus* (Balech) Balech and *Alexandrium catenella*) have occurred in Chinhae Bay, with *Alexandrium tamarense* and *Alexandrium catenella* (PSP toxin producer), and *Alexandrium affine* and *Alexandrium fraterculus* (none toxin producer). The identification of genus *Alexandrium* has based largely on the morphological features of the thecal plates [8]. These features can be varied depending on environmental condition [12,15], however, four strains of *Alexandrium tamarense* (AT-2, AT-6, AT-10 and AT-B) used in our study indicated a strong binding activity with AT1 DNA probe (Table 2, Fig. 1). Even though AT1

probe is identical nucleic acids to cTAM-F1 [2], the reason of different intensity may be associated with modifications in the hybridization protocol. Four isolates in this study did not reveal considerable nucleotide variation in the internal transcribed spacers (ITS) regions, in particular complete identical sequence to Japanese *Alexandrium tamarense* OFX151-A [6]. It is thought that how to treat the cells are important to enhance the signal.

All three preservatives and at different concentrations used for fixing the *Alexandrium tamarense* cells were satisfactory in maintaining the general shape and structure of the cell, including consistently strong signal intensity (Table 3, Fig 2). However, cells treated with strong concentration (>3%) were associated with vulnerable morphological features, in particular decline of signal intensity (unpublished data). It is therefore that treatment of 1-2% in three preservatives may be desirable to identify and enumerate field samples of *Alexandrium tamarense*. In contrast to immunofluorescent detection targeted to cell surface, changes of binding reaction over times [3], fluorescent intensity of AT1 probe was not changed even in late growth phase (Table 4). As seen in Table 5, enumeration of *Alexandrium tamarense* cells from Sujong, Wucheon, Dandong and Chilchundo field samples were easy and great in estimating of *Alexandrium tamarense* cells using epifluorescent microscope than with light microscope. Since this method provides for more accurate *Alexandrium tamarense* discrimination and enumeration than that of traditional method, it will be established to new biotoxin monitoring and prediction program in Chinhae coastal waters.

Acknowledgements

We are indebted to Dr. C. K. Lee, for his contribution with cultures. We appreciate to Dr. J. G. Park, Inje University, Kimhae, for providing me a sample of *A. catenella*. This work was supported by a grant-in-aid from the Maritime Affairs and Fisheries Ministry of Korea.

References

1. Adachi, M., Y. Sako and Y. Ishida. 1993. Application of monoclonal antibodies to field samples of *Alexandrium* species. *Nippon Suisan Gakkaishi* **59**, 1171-1175.
2. Adachi, M., Y. Sako and Y. Ishida. 1996. Identification of the toxic dinoflagellates *Alexandrium catenella* and *A. tamarense* (Dinophyceae) using DNA probes and whole-cell hybridization. *J. Phycol.* **32**, 1049-1052.
3. Bates, S. S., C. Leger, B. A. Keafer and D. M. Anderson. 1993. Discrimination between domoic acid-producing and non-toxic forms of the diatom *Pseudonitzschia pungens* using immunofluorescence. *Mar. Ecol. Prog. Ser.* **100**, 185-195.
4. Cho, E. S., J. G. Park, H. G. Kim, C. H. Kim, L. L. Rhodes and C. S. Chung. 1999. The rapid differeniation of toxic *Alexandrium* and *Pseudonitzschia* species using fluorescent lectin probes. *J. Kor. Ocean. Soc.* **34**, 167-171.
5. Cho, E. S., G. M. Seo, S. G. Lee, H. G. Kim, S. J. Lee, L. L. Rhodes and Y. K. Hong. 1998. Application of FITC-conjugated lectin probes for the recognition and differentiation of some Korean coastal red tide microalgae. *J. Fish. Sci. Tech.* **1**, 250-254.
6. Cho, E. S., S. G. Lee, I. S. Kim and M. Chinain. 2001. Molecular phylogeny of the genus, *Alexandrium* (Dinophyceae), based on internal transcribed spacers ITS1 and ITS2, including 5.8S nuclear rDNA sequences. *Algae.* (accepted)
7. Costas, E., E. G. Chavarri, A. Aguilera, S. G. Gil, and V. L. Rodas. 1993. Use of lectins to recognize and differentiate unicellular algae. *Bot. Mar.* **36**, 1-4.
8. Fukuyo, Y. 1985. Morphology of *Protogonyaulax tamarensis* (Lebour) Taylor and *Protogonyaulax catenella* (Whedon and Kofoid) Taylor from Japanese coastal waters. *Bull. Mar. Sci.* **37**, 529-537.
9. Guillard, R. R. L. and J. H. Ryther. 1962. Studies of marine planktonic diatoms 1. *Cyclotela nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. *Can. J. Microbiol.* **8**, 229-239.
10. Lee, S. G. 1991. The taxonomy and distribution of marine toxic flagellates in Chinhae Bay. Ph. D. thesis.

University of Kyungsung, Pusan, 125 pp. (in Korean)
11. Miller, P. and C.A. Scholin. 1998. Identification and enumeration of cultured and wild *Pseudo-nitzschia* (Bacillariophyceae) using species-specific LSU rRNA-targeted fluorescent probes and filter-based whole cell hybridization. *J. Phycol.* **34**, 371-382.
12. Sako, Y., C. H. Kim, H. Ninomiya, M. Adachi and Y. Ishida. 1990. Isozyme and cross analysis of mating populations in the *Alexandrium catenella/tamarense* species complex. pp. 320-323. *In* Graneli, E., Sundstrom, B., Edler, L. and Anderson, D. M. (eds.) *Toxic Marine Phytoplankton*, Elsevier, New York.
13. Scholin, C. A., K. R. Buck, T. Britschgi, G. Cangelosi and E. P. Chavez. 1996. Identification of *Pseudonitzschia austalis* (Bacillariophyceae) using rRNA-targeted probes in whole cell and sandwich hybridization formats. *Phycologia* **35**, 190-197.
14. Scholin, C. A., P. Miller, K. R. Buck, E. P. Chavez, P. Harris, P. Haydock, J. Howard and G. Cangelosi. 1997. Detection and quantification of *Pseudo-nitzschia australis* in cultured and natural populations using LSU rRNA-targeted probes. *Lim. Oceano.* **42**, 1265-1272.
15. Seaby, D.A. 1996. Differentiation of *Trichoderma* taxa associated with mushroom production. *Plant Patho.* **45**, 905-912.
16. Vrieling, E. G., A. Draaijer, W. Vanzeijl, L. Peperzak, W. W. C. Gieskes and M. Veenhuis. 1993. The effect of labelling intensity, estimated by real-time confocal laser scanning microscopy, on flow cytometric appearance and identification of immunochemically labelled marine dinoflagellates. *J. Phycol.* **29**, 180-188.

(Received March 27, 2002; Accepted May 11, 2002)

초록 : 자연 시료로부터 *Alexandrium tamarense*을 위한 종 특이적 DNA탐침의 응용

조은섭[1] · 김기영 · 박형식 · 김학균[1] · 문성기[2] · 이재동*
(*부산대학교 미생물학과, [1]국립수산진흥원 적조생물과, [2]경성대학교 생물학과)

독성이 있는 특이한 편모충인 *Alexandrium tamarense*의 구별하기 위한 종특이적인 형광 DNA탐침 AT1이 서로 다른 3가지의 고정액에서, 그리고 배양기간의 차이와 whole cell hybridization을 사용하는 광학 현미경 또는 형광 현미경에 의한 세포밀도 측정에 있어서 biding activity를 비교하는 방법으로 여러 다른 종의 실험에 사용되어졌고, 그의 결과를 보고하는 바이다.

형광 DNA탐침 AT1은 특이적으로, *A. tamarense*에 결합했지만, 형태학적으로 유사한 다른 편모충에는 결합하지 않았다. 특히 형태적으로 *A. tamarense*에 유사한 *A. catenella*는 형광 DNA탐침 AT1에 결합하지 않았다.

*A. tamarense*은 다른 세가지 고정액을 처리하였을 때, 고정액과 상관없이 강한 형광신호를 발산하였다. 추가해서 말하면, 배양기간에 관계없이 형광 DNA탐침 AT1의 biding activity는 강했다. 광학 현미경에 의해서 측정되어지기 보다는 형광현미경에 의해서 세포밀도가 측정되었다. 형광 DNA탐침 AT1을 이용한 *A. tamarense*의 동정과 계산은 이 분야에서 새로운 생물독성 감시와 예측 시스템에 기여 할 것이다.