Simple and rapid detection of the toxic marine dinoflagellates *Alexandrium tamarens* and *A. catenella* with fluorescence *in situ* hybridization (FISH) using rRNA-targeted probes

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SUMMARY: Oligonucleotide probes targeting 28S ribosomal RNA for the toxic marine dinoflagellates *Alexandrium tamarens* (Lebor) Balech and *A. catenella* (Whedon and Kofoid) Taylor were designed for simple and rapid identification of these species. With fluorescence *in situ* hybridization (FISH) using these probes, each species could be detected and there were none non-specific stained cells. The efficiency of detectable cells remained constant through exponential and stationary phase, and more than 80% cells of both species could always be identified. Targeted cells in a field sample could be detected and discriminated within 1 hour by the labeling the two different probes with FITC and Rhodamine, respectively.

KEYWORDS: toxic dinoflagellate, *Alexandrium*, paralytic shellfish poisoning (PSP), rRNA, oligonucleotide probe, fluorescence *in situ* hybridization (FISH)

INTRODUCTION

Paralytic shellfish poisoning (PSP) caused by the marine toxic dinoflagellates *Alexandrium tamarens* (Lebor) Balech and *A. catenella* (Whedon and Kofoid) Taylor have increased in the last two decades in Japan and throughout the world.¹⁾ Filter-feeding shellfish accumulates the neurotoxins produced by these algae, making them toxic to humans. PSP is a serious problem to the public health and causes economic losses to the fisheries industry. Thus, there is a need for a rapid and precise monitoring of these toxic dinoflagellates.

However, identification of *Alexandrium* species is very difficult as only very small differences in plate pattern are used to distinguish these species.²⁻³⁾ Furthermore, monitoring programs need investigations of many samples, which is often time consuming.

Recently, new techniques that might solve these problems have been developed. One of these techniques is the molecular biological technique, fluorescence *in situ* hybridization method (FISH) using a fluorescent oligonucleotide probe.^{4,5)} In this method, DNA sequences of different species are compared, and probes are designed against a species-specific region of the gene or rRNA that can discriminate a certain species. The established probe is hybridized in the intact cells of a particular species directly and the labeled cells can be detected by epifluorescence microscopy or flow cytometry (Fig.1).⁶⁾ This technique has previously been used on bacteria and in population analysis.⁷⁾ On the other hand, there are relatively few studies of this method applied to phytoplanktons⁸⁾

We found a species-specific region in the 28S rRNA gene from the comparison of those sequences among

Fig. 1 Scheme of fluorescence *in situ* hybridization (FISH) method for unicellular microalgae. Fluorescent rRNA-targeted probes are hybridized to rRNA distributed in the whole cell. Cell stained with fluorescent probes can be detected using fluorescence microscopy.

various species of *Alexandrium* containing *A. tamarense* and *A. catenella*, and established Atm1-probe specific to *A. tamarense* and Act1-probe specific to *A. catenella*. Here we report a simple and rapid FISH method using these probes and the application of this method to natural samples.

MATERIALS AND METHODS

Culture and Natural sample

All used strains are shown in Table 1. All clonal strains of *Alexandrium* are grown in SWIIIm medium at 15°C under as condition described previously.⁹⁾ *Heterosigma akashiwo* and *Chattonella marina* were grown in SWIIIm at 20°C under the same conditions. Cultures were harvested in mid-exponential phase. Density was about 1×10^4 cells/ml. One ml culture was used to the hybridization.

The natural samples used in this study were collected from different stations of Tokuyama Bay in Japan in 1999 May and 2000 June. These samples were immediately sent to the laboratory at 4°C and put in the incubator at 15°C. When the density of the microalgae cells was low, the sample was concentrated before hybridization, and 1ml was used to the hybridization.

Fluorescent Oligonucleotide probes.

The oligonucleotide probes were designed against the sequence of D1/D2 region in 28S rRNA gene.¹⁰⁾ The Atm1 probe was complementary to the species-specific region in *A. tamarense* and was 5'-end labeled with tetramethyl rhodamine (Rhodamine). Act1 was specific to *A. catenella* and was 5'-end labeled with fluorescein 5-isothionate (FITC). These probes were stored at -20°C in the dark.

Hybridization.

Cells were fixed on ice with 5×PBS (685mM NaCl, 13.4mM KCl, 40.5mM Na₂HPO₄·12H₂O and 7.35mM KH₂PO₄, pH 7.5) containing 4% paraformaldehyde for 5min. After filtration, the samples were dehydrated in acetone. Then, cells were incubated at 30°C for 5min with 40% formamide hybridization buffer containing the fluorescent probes which had been incubated at 100°C for 3min and chilled on ice just before hybridization. After hybridization, samples were washed twice with 5×SSC (83mM NaCl, 83mM sodium citrate, pH 7.0) at 40°C, 5min in each change.

Microscopic observation was made using an ECLIPSE E800 (Nikon) with filter for FITC (excitation 450–490nm, dichroic beamsplitter 505nm, emission 515–555nm) and

Rhodamine (excitation 540/25nm, dichroic beamsplitter 565nm, emission 605/55nm). The digital images were captured using a chilled 3CCD color camera (Hamamatsu Photonics K. K.).

RESULTS AND DISCUSSION

We applied the two probes, Atm1 and Act1, to different strains of *A. tamarense* and *A. catenella* using the described protocol. Targeted rRNAs are distributed in the whole cell (Fig. 1), giving the cells a strong green fluorescence. The sensitivity of the two probes was excellent, making enumeration using epifluorescence microscopy easy (Fig. 2).

(a) *A. tamarense* cell

(b) *A. catenella* cell

Fig. 2 Micrographs of clonal *A. tamarense* HAT9 (a) and *A. catenella* TN7 (b) hybridized with Atm1 (A) and Act1 (B). (A) and (B) light microscopy. (A') Rhodamine labeled and (B') FITC labeled

Table1 List of strains and specificity of Atm1 and Act1 probes to several algae by FISH. +, labeled cell; -, non-labeled cell.

Species	Strains	Geographic origin	PSP Toxicity ^{A)}	Reactivity ^{B)}	
				Atm1	Act1
<i>A. tamarense</i>	OF151	Ofunato Bay, in Japan	Yes	+	-
<i>A. tamarense</i>	HAT9	Hiroshima Bay, in Japan	Yes	+	-
<i>A. tamarense</i>	SHO1	Kure Bay, in Japan	Yes	+	-
<i>A. catenella</i>	TN7	Tanabe Bay, in Japan	Yes	-	+
<i>A. catenella</i>	AM59	Amakusa, in Japan	Yes	-	+
<i>A. pseudogonyaulax</i>		Harima-Nada, in Japan	No	-	-
<i>A. affine</i>		Harima-Nada, in Japan	No	-	-
<i>Heterosigma akashiwo</i>	OS11	Osaka Bay, in Japan	No	-	-
<i>Chattonella marina</i>	NIES121	Kagoshima Bay in Japan	No	-	-

A); PSP toxicity was determined by high-performance liquid chromatography - fluorometric analysis⁸⁾.

B); Reactivity was observed under the epifluorescent microscope.

Fig. 3 Micrographs of natural sample from Tokuyama Bay hybridized with both of Atm1 and Act1. (a') was observed with Rhodamine band pass filter and (b') was observed with FITC bandpass filter. (a) and (b) was observed using light microscopy. Notice the intense labeling with the Act1 probe in (b').

When each of the Atm1 and Act1 probes was applied to both species, Atm1 was specific to *A. tamarense* (Fig.2a) and Act1 specific to *A. catenella* (Fig.2b). Moreover each probe reacted with other strains of each species respectively, but didn't react with *A. pseudogonyaulax* and *A. affine*, *Heterosigma akashiwo* and *Chattonella marina* (Table1). This high specificity

suggested that Atm1 and Act1 might be useful for analyzing natural seawater samples containing the cells of *A. tamarense* and *A. catenella*.

In flow cytometry, it has been found that the fluorescent intensity of the labeling decreases in stationary phase cells.¹¹⁾ However, we found that more than 80% of the targeted cells were labeled both in the exponential and

stationary phase using epifluorescence microscopy (data not shown).

Natural samples collected in 1999 May from Tokuyama Bay were hybridized with both of *Atm1* and *Act1*. Only cells that looked like *Alexandrium* species showed a green fluorescence (FITC = *Act1* probe) (Fig.3) indicating the presence of *A. catenella*. This was confirmed using light microscopy and morphological characteristic. These results indicated that this sample only contained *A. catenella*. *Heterosigma akashiwo* and *Prorocentrum dentatum* which were present in high number in the sample did not show any labeling. Therefore, we think that the FISH method may be useful for analyzing seawater samples for toxic species.

The two-color FISH method for *Alexandrium* sp. has not been reported before. In this study, the cells in seawater samples were detected within 1 hour, which is more rapid than the one-color FISH method (Sako unpublished). But because the red emission from Rhodamine resembled the autofluorescence of the algae, it was necessary to decrease the autofluorescence. Although ethanol has been used in previous reports, the acetone treatment was more efficient.

The percentage of labeled *Alexandrium* cells in natural samples of different stations and different seasons from Tokuyama Bay (two samples were collected in 1999 and one sample was collected in 2000) were always more than 80 %. Most of the non-detectable cells were thought to be due to the loss of cells by the pipetting and chemical treatments because these losses were also observed in cultured strains with a similar frequency (Sako unpublished). The present study shows that cells in natural samples contain enough rRNA for detection.

Although the FISH method for *Alexandrium* sp. in field samples has not been reported in any detail, this study indicated that it was possible to detect *Alexandrium* cells using FISH probes. Moreover the cells could be detected within 1 hour and the process was simple and without the need of special skills or machines.

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