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Detection of *Alexandrium tamarensis* by Rapid PCR Analysis

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ABSTRACT

Alexandrium tamarensis is a toxigenic dinoflagellate found in coastal waters worldwide. A critical factor in alleviating the health and economic threats posed by this species is the development of a rapid and reliable method for detection. This study stream-lined a labor- and resource-intensive protocol for the isolation of A. tamarensis ribosomal DNA (rDNA). Subcultures of A. tamarensis were established in water samples from several coastal Atlantic sites. A commercial DNA isolation kit protocol for cultured cells was used for isolation of the dinoflagellate DNA. Samples were amplified by PCR using primers specific for a 700-bp sequence of A. tamarensis rDNA. It was determined that this method facilitated the detection of 10-4 ng/mL of A. tamarensis DNA. Furthermore, the kit enabled A. tamarensis to be isolated from the water sources with little signal degradation. This is a valuable technique for the rapid detection of A. tamarensis, even before cell numbers are large enough for morphological identification.

INTRODUCTION

Dinoflagellates constitute a significant portion of the primary productivity within the world's oceans, and toxic blooms or "tides" can pose serious health and economic risks (1,12). Alexandrium tamarensis, a thecate dinoflagellate found in warm coastal waters, produces saxitoxin, a potent neurotoxin responsible for paralytic shellfish poisoning (PSP) (5,6). Filter-feeding shellfish are capable of bioaccumulating the saxitoxin. Human consumption of the shellfish can result in serious health problems such as numbness of the mouth, lips and fingers, muscle weakness, memory loss and even respiratory paralysis (1,18). Harmful algal blooms can also initiate severe economic repercussions in the shellfish and tourism industries. Thus, the health and economic threats *A. tamarensis* poses have fostered the need for information regarding distribution, prevalence and methods for its detection.

Detection of *A. tamarensis* is arduous and requires light microscopy. The morphologies of the toxic and nontoxic strains of *Alexandrium* are similar, thus rendering light microscopy ineffective (3). A current detection and identification method capable of surpassing the confusion and controversy inherent with using morphological distinctions utilizes molecular biology techniques (15). One of the techniques emerging as an important tool in the identification of *Alexandrium* involves the analysis of ribosomal DNA (rDNA) genes.

There are several key factors for the emphasis on and use of rDNA (13). The ribosomal genes are present as multiple, tandomly repeated copies that significantly increase their detection as compared with a single-copy gene. In addition, the ribosomal genes are conserved in both structure and function yet are highly variable between species (8,9,16). These qualities contribute to the usefulness of rDNA as a means of delineating phylogenetic and taxonomic discrepancies between many organisms (16,17). Scholin and Anderson (16) have shown that the hypervariable regions of the large subunit ribosomal RNA (28S rRNA) can be used to distinguish A. tamarensis from other dinoflagellate species and strains. However, to fully utilize this unique characteristic, an expedient and efficient protocol for the isolation of the genomic DNA encoding this hypervariable region is essential. In the present investigation, we have found that the Gentra Systems' Puregene® DNA Isolation Kit Protocol for cultured cells was effective for isolation of the dinoflagellate DNA. The cellulose shell of the A. tamarensis did not interfere with the protocol, and neither proteinase K treatment nor the multiple phenol extractions used by Scholin and Anderson (16) were necessary for template purification. This study also confirmed the specificity and sensitivity of the 28S rDNA primers. Lastly, it was shown that this streamlined technique could be used to detect Alexandrium in its resting cyst form and detect its presence in several coastal Atlantic water samples.

MATERIALS AND METHODS

Culturing

A. tamarensis, Euglena gracilis and Amphidinium carterae were cultured in our laboratory in appropriate media for each genus. A. tamarensis cultures were obtained from Provasoli Guillard National Center for Culture of Marine Phytoplankton (CCMP) (West Boothbay Harbor, ME, USA) and were maintained in f/2 Si medium (2). The two other nontoxic genera were obtained from Carolina Biological Supply Company (Burlington, NC, USA). All cultures except E. gracilis were kept in a refrigerator at 10°-15°C and received 14 h of light and 10 h of dark. Euglena cultures were maintained at room temperature and were subjected to approximately 10 h of daylight. The number of cells present in a given culture was ap-



Figure 1. PCR amplification of a 700-bp fragment of the *A. tamarensis* rDNA. Lanes designated M contain the 1-kb ladder DNA molecular weight standard. Lanes 1–3 contain the amplified *A. tamarensis* rDNA 700-bp fragment. The 1-kb marker is indicated by the arrow.



Figure 2. PCR amplification of *A. tamarensis*, *E. gracilis* and *A. carterae* rDNA. (A) Lane 1 contains the 1000-bp amplified fragment of *E. gracilis*, and lane 2 is the 700-bp fragment of the *A. tamarensis*. (B) Lane 2 is the 700-bp fragment of the *A. tamarensis*, whereas lane 3 is the amplified product from *A. carterae*. In both A and B, the 0.5- and 1-kb markers are indicated with an arrow. The positive control *A. tamarensis* was cultured in water from Nantucket, MA, USA. No endogenous levels of *A. tamarensis* were present (lane 1).

proximated with a Sedgewick-Rafter counting chamber (Ben Meadows Company, Atlanta, GA, USA) and 30 random fields of view (20). *A. tamarensis* cultures were allowed to grow to a yield in excess of 500 cells per milliliter.

Inoculation of Water Samples

Water samples were obtained from Bar Harbor, ME, Provincetown, MA, Fort Pierce, FL and Nantucket, MA, USA. The sites were selected to represent a variety of media that could possibly sustain the dinoflagellate growth rather than on the basis of A. tamarensis blooms. Water samples were maintained in the same temperature and light/dark conditions as the A. tamarensis cultures. Twenty-five milliliters of each water sample were inoculated with 1 mL of A. tamarensis. Both uninoculated and inoculated water samples underwent DNA isolation and polymerase chain reaction (PCR) amplification according to the procedure below.

DNA Isolation

One milliliter of *A. tamarensis* culture was placed in a 1.5-mL microcentrifuge tube and centrifuged at 14 000× *g* for 5 s. This was repeated 3× to yield a larger, more visible pellet. The supernatant was removed, leaving approximately 20 μ L. The pelleted samples were subjected to the Puregene DNA Isolation Kit Protocol (Gentra Systems, Minneapolis, MN, USA) (4,7,10,11,14) for cultured cells.



Figure 3. Sensitivity of detection of A. tamarensis template. A. tamarensis was diluted serially from 2, 2×10^{-1} , 2×10^{-2} , 2×10^{-3} , 2×10^{-4} and 2×10^{-5} ng/µL, and PCR amplification performed. Lanes 1–6, respectively, show the results. PCR-amplified product was detected at a template concentration of 2×10^{-4} ng/µL, as seen in lane 5.

PCR Amplifications

PCR amplifications were performed by using GeneAmp® PCR System 2400 and GeneAmp PCR Core Reagents according to the manufacturer (Perkin-Elmer, Norwalk, CT, USA). A 700-bp segment of the rDNA unique to A. tamarensis was amplified with primers obtained from D. Kulis at the Woods Hole Oceanographic Institution (Woods Hole, MA, USA). The forward (D1R) and reverse (D2C) primers targeted nucleotide positions 25-45 and 733-714, respectively (16). The sequences of the primers are: D1R, 5'-ACCCGCTGAA-TTTAAGCATA-3' and D2C, 5'-CCT-TGGTCCGTGTTTCAAGA-3' (16). The final concentration of each primer was 0.1 μ M (19). The thermal cycler was programmed as follows: denaturing at 92°C for 1.5 min, cooling to 50°C for 30 s, annealing at 45°C for 1.5 min, warming to 72°C for 1.5 min and extension at 72°C for 2 min (19). This cycle was repeated 30 times. Amplified samples were then maintained at 4°C and subsequently analyzed on a 1% agarose gel in 1× TAE (0.089 M Tris-acetate, 0.089 M acetic acid, 0.02 M EDTA).

Detection of Cyst Vs. Vegetative Form of *A. tamarensis*

To determine if DNA isolation and PCR detection were limited by the life-



Figure 4. Detection of *A. tamarensis* DNA in the vegetative and cyst stage. The 700-bp amplified fragment of the *A. tamarensis* rDNA was detected from both the vegetative (lane 1) and cyst (lane 2) structures.

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cycle stage of the *A. tamarensis*, the resting cyst, or hypnozygote form, was induced by nutrient starvation over a period of approximately 3.5 months. DNA from the resting cysts and from *A. tamarensis* in its vegetative form then were isolated, PCR-amplified and subsequently analyzed on a 1% agarose gel.

RESULTS

A. tamarensis was grown in f/2 Si medium, and approximately 700–800 cells were subjected to the DNA Isolation Kit Protocol. PCR was performed, and the data appear in Figure 1. The results show that the Puregene procedure for cultured cells was capable of disrupting the dinoflagellate cellulose armor and purifying the genomic DNA. The primers recognized the DNA and amplified a distinct DNA fragment at the 700-bp position. This is congruous with the size of the rDNA target sequence and confirms the results of Scholin et al. (19).

To determine the specificity of the PCR product, DNA was purified from *E. gracilis* and *A. carterae* and tested for primer recognition. The primers recognized the *E. gracilis* and *A. carterae* sequences; however, the amplified products were 1000 (Figure 2A)



Figure 5. Multiple east coast water samples are capable of supporting *A. tamarensis* growth. Inoculated (lanes 2, 4 and 6) and uninoculated (lanes 1, 3 and 5) water samples from Provincetown, MA, Fort Pierce, FL, Bar Harbor, ME and Nantucket, MA, USA were tested for the presence of endogenous levels of *A. tamarensis* and for the ability to support their growth. No endogenous levels of *A. tamarensis* DNA were detected, yet all supported the growth, as evidenced by the 700-bp fragment after amplification in Provincetown (lanes 1 and 2), Fort Pierce (lanes 3 and 4) or Bar Harbor (lanes 5 and 6). Figure 2B shows data from Nantucket water samples.

- uct of *A. tamarensis*. The sensitivity of this assay was tested by serially diluting the *A. tamarensis* template. As Figure 3 shows, it was found that sequences could be detected at template concentrations of 2×10^{-4} ng/µL. Dinoflagellates form cysts in response to changing environmental conditions thus it was processed to test if

sponse to changing environmental conditions, thus it was necessary to test if this isolation procedure would effectively isolate DNA from the hypnozygote (cyst). *A. tamarensis* was grown under optimal conditions to promote the vegetative stage or was starved for 3 months to induce cyst formation. DNA was isolated from these cultures, and PCR was performed. Figure 4 shows the data. In both the vegetative stage (lane 1) and the cyst form (lane 2), the specific 700-bp fragment was amplified. Thus, it appears that both forms are easily detected by using this method.

and 600 bp (Figure 2B), respectively,

rather than the anticipated 700-bp prod-

Due to the likelihood that A. tamarensis blooms will expand into new areas, multiple water samples were inoculated and tested using this protocol. Water samples were obtained from Provincetown, Fort Pierce, Bar Harbor and Nantucket. Inoculated and uninoculated water samples were tested for the presence of endogenous levels of A. tamarensis and for the ability to support their growth. Figure 5 shows the data. No endogenous levels of A. tamarensis DNA were detected in Provincetown, Fort Pierce or Bar Harbor, yet all supported the growth, as evidenced by the 700-bp fragment after amplification. Water samples from Nantucket also sustained growth of A. tamarensis, and no A. tamarensis DNA was detected in uninoculated samples (Figure 2B, lanes 1 and 2).

DISCUSSION

The Puregene DNA Isolation Kit and PCR protocol for cultured cells proved to be effective for the isolation and subsequent amplification of a specific 700bp fragment of *A. tamarensis* rDNA. This study showed that template levels as low as 2×10^{-4} ng/µL were efficient-ly amplified. This finding suggests that relatively few cells could be detected within the water column. Such data could be used to more accurately track an algal bloom and develop predictive models. Furthermore, *A. tamarensis* was easily distinguished from the negative controls *E. gracilis* and *A. carterae*.

In conjunction with the specificity and sensitivity, the Puregene kit and PCR amplification (GeneAmp PCR Core Reagents) were found to be equally effective in the detection of the hypnozygote (resting cyst) form and the vegetative form of A. tamarensis. This suggests the possibility that A. tamarensis could be found and monitored in waters before a toxic bloom. Identification of A. tamarensis cysts by using this protocol would allow fisheries managers longer lead times to predict the occurrence of vegetative cells and sufficient time to use evasive actions when environmental conditions change.

This study describes a powerful tool for identifying the presence of a toxic dinoflagellate before cell numbers increase to bloom conditions. This protocol was found to be advantageous for several reasons. The kits allowed for rapid DNA isolation, and the use of salt precipitation eliminated the need for toxic organic extraction, which decreases sample yield. The procedure was found to have short incubation times, and, depending on the number of samples assayed, the entire process could be completed in as little as 2 h. Thus, the sensitivity and rapid nature of this protocol could be promoted for commercial use in the detection of A. tamarensis and for other toxic species.

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Enhancement of PCRs by Partial Restriction Digestion of Genomic Templates

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ABSTRACT

Genomic DNA preparations derived from mammalian cells can often exhibit poor template activity in PCR, particularly when carried out on target sequences present at low copy number. Using genomic DNA bearing SV40 sequences integrated into host chromosomal DNA at low copy number as a target, we show that template efficiency can be dramatically enhanced after treatment of the genomic template with restriction enzymes for varying periods of time. Also, our results indicate that, while template activity was enhanced by all of the restriction enzymes tested, optimal digestion time varied for each enzyme.

INTRODUCTION

The polymerase chain reaction (PCR) is a widely utilized technique for rapid and specific amplification of target DNA sequences. Despite the fact