RESEARCH LETTER

Loop-mediated isothermal amplification method for rapid detection of the toxic dinoflagellate *Alexandrium*, which causes algal blooms and poisoning of shellfish

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Abstract

The marine dinoflagellate genus *Alexandrium* includes a number of species that produce potent neurotoxins responsible for paralytic shellfish poisoning, which in humans may cause muscular paralysis, neurological symptoms and, in extreme cases, death. Because of the genetic diversity of different genera and species, molecular tools may help to detect the presence of target microorganisms in marine field samples. Here we employed a loop-mediated isothermal amplification (LAMP) method for the rapid and simple detection of toxic *Alexandrium* species. A set of four primers were designed based upon the conserved region of the 5.8S rRNA gene of members of the genus *Alexandrium*. Using this detection system, toxic *Alexandrium* genes were amplified and visualized as a ladder-like pattern of bands on agarose gels under isothermal condition within 60 min. The LAMP amplicons were also directly visualized by eye in the reaction tube by the addition of SYBR Green I. This LAMP assay was 10-fold more sensitive than a conventional PCR method with a detection limit of 5 cells per tube when targeting DNA from *Alexandrium minutum*. The LAMP assay reported here indicates the potential usefulness of the technique as a valuable simple, rapid alternative procedure for the detection of target toxic *Alexandrium* species during coastal water monitoring.

Introduction

Toxic dinoflagellates of the genus *Alexandrium* are the primary organisms responsible for harmful algal blooms (HABs) (Du *et al*., 2002; Usup *et al*., 2002). Moreover, for some reasons, such HABs appear to be increasing in frequency, intensity and distribution (John *et al*., 2003). HABs are now recognized worldwide as having serious implications for seafood safety, and environmental and economic concerns. In addition to the formation of red tides, some species of dinoflagellates produce a range of toxins that are poisonous to organisms higher in the food chain (Taroncher-oldenburg & Anderson, 2000). Marine dinoflagellates of the genus *Alexandrium* include a number of species responsible for paralytic shellfish poisoning (Judge *et al*., 1993). Paralytic shellfish toxins (PSTs), produced by *Alexandria*, are potent neurotoxins that can be concentrated by filter-feeding shellfish (Gallacher *et al*., 1997). Consumption of PST-contaminated shellfish may cause muscular paralysis, neurological symptoms and, in extreme cases, death (Anderson, 1997; Pierce & Kirkpatrick, 2001). HAB events have been reported from the South China Sea, where Hong Kong and other coastal cities have suffered considerable economic losses from frequent occurrences of HABs, some of which have been highly toxic (Anderson *et al*., 1996; Wang *et al*., 2003).

It is important to monitor coastal waters for the presence of toxin-producing *Alexandrium* from the source. Monitoring coastal waters for the presence of HAB species is essential in assessing the potential for bloom formation. Traditionally, this type of monitoring involves morphological identification and bioluminescence capacity, mating compatibility and enumeration of target species by light or electron microscopy in addition to toxicity tests of shellfish (Anderson *et al*., 1994). However, these identification methods can be difficult for long-term monitoring and the characteristic morphological features are often difficult to determine because they can be influenced by environmental factors and culture conditions (Taylor & Fukuyo, 1998).

Because of the genetic diversity of different genera and species, molecular methods may be useful for the detection of target microorganisms in marine field samples (Leaners *et al.*, 1991; Medlin *et al.*, 1998; LaJeunesse, 2001; Galluzzi *et al.*, 2004). Because of their rapidity, PCR-based methods, molecular probes, restriction fragment length polymorphism (RFLP), and immunological techniques using polyclonal and monoclonal antibodies have been widely studied for the detection of toxic *Alexandrium* species (Nagasaki *et al.*, 1991; Judge *et al.*, 1993; Penna & Magnani, 1999; Bowers *et al.*, 2000; Coyne *et al.*, 2001; Godhe *et al.*, 2001; Galluzzi *et al.*, 2004; John & Medlin 2005). Although these techniques have significantly increased the ability to detect toxic *Alexandrium* species, their requirement for a high-precision instrument for amplification is complicated and costly. Immunological techniques require the identification of a phenotypic epitope, which may be influenced by the environment (Hosoi-Tanabe & Sako, 2005). This has prevented their widespread use in field laboratories, for example, as a routine diagnostic tool. Therefore, recent studies of *Alexandrium* species have focused on the search for better methods of identification.

The invention of loop-mediated isothermal amplification (LAMP) has opened up a new method for molecular detection and identification (Notomi *et al.*, 2000). The principle of LAMP is autocycling strand displacement DNA synthesis in the presence of *Bst* DNA polymerase with high strand displacement activity under isothermal conditions between 60 and 65 °C within 60 min. The detailed amplification mechanism has been described elsewhere (Notomi *et al.*, 2000; Mori *et al.*, 2001; Enosawa *et al.*, 2003; Parida *et al.*, 2004). The reaction relies on recognition of the DNA target by six independent regions, making this kind of assay highly specific. The LAMP assay is rapid and the amplification efficiency is equivalent to that of PCR-based methods (Nagamine *et al.*, 2002; Poon *et al.*, 2004). More importantly, the approach is less costly, and all reactions can be developed in an isothermal environment. The potential applications of LAMP methodology have been demonstrated in recent years (Maruyama *et al.*, 2003; Maeda *et al.*, 2005; Ohtsuka *et al.*, 2005). Here we demonstrate the feasibility of using the LAMP technique to detect toxic *Alexandrium* species.

Materials and methods

Algal cultures

Eight algal strains used in this study were isolated from the south coast of China. The six *Alexandrium* strains, *Alexandrium minutum* AMTW02, *Alexandrium andersoni* ADC02, *Alexandrium catenella* Balech 1985 ACDH03, *Alexandrium tamarense* ATMJ01, *A. catenella* L65 and *A. tamarense* L66, are toxic and their identification was confirmed by the Institute of Hydrobiology of Jinan University, China. *Prorocentrum donghaiense* PD01 and *Karenia mikimotoi* KM01 were used to determine the specificity of LAMP detection. All strains were maintained in f/2 medium (Guillard & Ryther, 1962) at 20 ± 1 °C. Cool-white fluorescent bulbs provided light on a standard 12/12-h light–dark cycle. Between 8 and 15 days after inoculation, when cultures were in the exponential phase of growth, algal cell density was accurately determined by enumeration using a 0.1-mL Plankton count box. Algal cells were collected by centrifugation at 10 000 ***g*** for 5 min at 4 °C.

DNA preparation from cultures

DNA was extracted using a DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. *Alexandrium minutum* DNA was isolated by two methods. The first used a DNeasy Plant Mini Kit (QIAGEN). In the second method, *A. minutum* pellets containing 5×10^4 or 1×10^5 cells were incubated at 95 °C for 10 min and quickly placed on ice for 5 min, then centrifuged at 12 000 ***g*** for 1 min at 4 °C. The supernatants containing DNA were used in the following tests.

LAMP primer design

rRNA gene sequences have been successfully employed for the detection of various toxic dinoflagellates in seawater samples, because these sequences are highly conserved (Scholin *et al.*, 1995; Hershkovitz & Lewis, 1996; Medina *et al.*, 2001; Moon-van der Staay *et al.*, 2001; Galluzzi *et al.*, 2004; Bolch & de Salas, 2007). The primers used in this study were designed by an alignment of all available ITS1–5.8S–ITS2 rRNA gene sequences for the genus *Alexandrium*. Sequences were downloaded from GenBank or obtained from the literature. The alignment was constructed by using CLUSTALW. The alignment included sequences of several strains of *A. minutum*, *A. tamarense*, *A. catenella* and *A. andersoni*. The 5.8S region is very conserved among these species. A set of four primers was designed to target six conserved sequences of the 5.8S region. In order to confirm the sequence specificity, we used the Basic Local Alignment Search Tool (BLAST) to search the GenBank and DDBJ databases for all published sequences identical to the primers. The primers were selected based on the criteria described by Notomi *et al.* (2000). In addition to the general criteria of primer design, such as 40–60 mol% G+C content but avoiding terminal dimer formation, 3′ hairpins, and self-complementarity, special care was taken to adjust the melting temperatures (T_m) of the primers in such a way that the T_m values were in the following order: F1C and

Table 1. DNA oligonucleotide primer sequences used for LAMP

Primer	Primer type	Length	Sequence (5′ → 3′)*
FIP	Forward inner (5′F1C-TTTT-F2-3′)	48 nt F1C, 25 nt; F2, 19 nt	ACMTTCTTCCAACAGCATCTCTTAC *TTTT*GCTGTATCGTATCGTCCTG
BIP	Backward inner (5′B1C-TTTT-B2-3′)	48 nt B1C, 24 nt; B2, 20 nt	TCGAACAGAACAAGGTTGATTACC *TTTT*GGAYATTCAGATCATTCGCG
F3	Forward outer	22 nt	CACCRGATACCAACCTCACAGG
B3[†]	Backward outer	23 nt	CAAGCAHACCTTCAAGMATATCC

*M, A or C; Y, T or C; H, A or C or T.

[†] See Galluzzi *et al*. (2004) for sequence details.

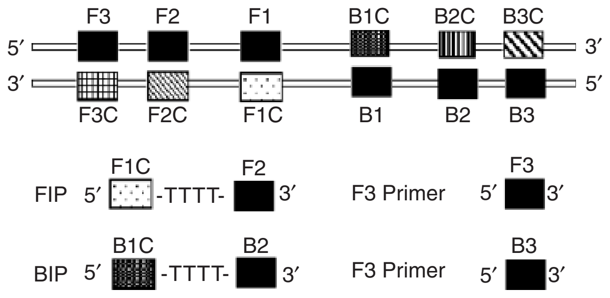

Fig. 1. Schematic representation of primers used in this study. The LAMP inner (FIP and BIP) and outer (F3 and B3) primer pairs are shown.

BIC > F2 and B2 > F3 and B3. The inner primers are described as forward inner primer (FIP) and backward inner primer (BIP). The forward inner primer consisted of the complementary sequence of F1C (25 nt), a T-T-T-T linker and F2 (19 nt). The backward inner primer consisted of B1C (24 nt), a T-T-T-T linker and the complementary sequence of B2 (20 nt). The outer primers were F3 and B3, which located outside of the F2 and B2 regions, respectively. The primer sequences and locations are indicated in Table 1 and Fig. 1. The primers were synthesized commercially by Invitrogen biotech (Guangzhou, China).

LAMP reaction

The LAMP was carried out in a total reaction mixture of 25 μL containing 1.6 μM (each) of the primers FIP and BIP, 0.2 μM (each) of the primers F3 and B3, 1.6 mM of dNTPs, 6 mM $MgSO_4$, 1 M betaine (Sigma), 1 × thermopol buffer (New England Biolabs), 1 μL (8 U) *Bst* DNA polymerase (New England Biolabs), and the specified amounts of target genomic DNA, which were incubated at 65 °C for 60 min. Positive and negative controls were included in each run, and all precautions to prevent cross-contamination were observed.

Monitoring of amplification by LAMP assay

Following incubation at 65 °C for 60 min, a 5-μL sample of the LAMP assay products was separated by electrophoresis on 2% agarose gels, stained with ethidium bromide and visualized on a UV transilluminator. In order to facilitate the field application of the LAMP assay, monitoring of amplification by the LAMP assay was also checked by eye. Following amplification, the tubes were inspected through observation of a colour change following addition of 1 μL (1 : 100) of SYBR Green I dye to the tube. In the case of a positive amplification, the original orange colour of the dye changes to green, which can be judged by eye under natural light.

PCR reaction

In order to compare the sensitivity of the LAMP assay, PCR was performed with the two outer primers F3 and B3. The amplification was carried out in a total reaction volume of 50 with 5 μL of the buffer solution (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, pH 8.3), 5 μL (10 pmol μL^{-1}) of a pair of appropriate primers, 4 μL dNTPs mixture (2.5 mM of each dNTP) and 0.25 μL (5 U uL^{-1}) *Taq* DNA polymerase were mixed. The thermal profile for PCR was 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and a final extension cycle at 72 °C for 7 min. The amplified products were then analysed through a 1.5% agarose gel by electrophoresis in Tris-borate buffer, and the target bands were visualized by staining with ethidium bromide.

Specificity of the LAMP assay

To evaluate the specificity of the LAMP, five cells of *A. minutum* were employed in the LAMP reaction at 65 °C for 60 min in the absence or presence of 100 ng of non-*Alexandrium* DNA. The LAMP assay was also used to amplify the DNAs of different species of toxic *Alexandrium* and other non-*Alexandrium* cultures (10 ng per reaction, respectively). The DNAs of all strains were obtained via a simple boiling method. The products were separated by 2% agarose gels electrophoresis.

Results

A successful LAMP reaction with specific primers produced many bands of different sizes. The LAMP assay used here was standardized with the toxic *A. minutum*. When the sample tube did not contain target DNA, no amplification was seen. The LAMP yields extremely large amounts of DNA, and this enabled inspection by eye (Mori *et al.*, 2001). The LAMP reaction mixture, which contained amplified fragments, turned green after the addition of SYBR Green I, whereas a solution with no amplicons retained the original orange colour of SYBR Green I. Inspection by eye with SYBR Green I demonstrated equivalent sensitivity to agarose gel electrophoresis under natural light (Fig. 2b). Inspection by eye was simple and rapid. Therefore, this method facilitates the application of LAMP, especially in field laboratory settings.

Sensitivity of the LAMP and PCR assays for the detection of *A. minutum*

To ascertain the detection limit of the LAMP assay for the detection of *A. minutum*, serial 10-fold dilutions of the extracted DNA were used and compared with the results of conventional PCR. A serial dilution of *A. minutum* cells was also used to evaluate the detection limits of LAMP and PCR. The detection limits of the LAMP assay and PCR for purified DNA were found to be 1 and 10 pg per tube, respectively (Fig. 2a). The comparative sensitivity of LAMP and PCR indicated that LAMP was 10-fold more sensitive than PCR. Amplification by LAMP revealed a ladder-like pattern, whereas the PCR showed a 176-bp amplicon.

The detection limits of the LAMP assay and PCR for *A. minutum* cells were found to be 5 and 50 cells per tube, respectively (Fig. 2b); LAMP was again 10-fold more sensitive than PCR. The LAMP products turned green after the addition of SYBR Green I. Thus, the sensitivity of the LAMP methods can simply be judged by eye, and the results were in agreement with detection via electrophoresis. The boiling method shortened the time for DNA extraction and facilitated the usefulness of the LAMP assay for rapid detection of *Alexandrium* species, especially for offshore operations.

Specificity of the LAMP reaction for *Alexandrium*

Five cells of *A. minutum* were amplified in the LAMP reaction at 65 °C for 60 min in the absence or presence of 100 ng of non-*Alexandrium* DNA. The products were

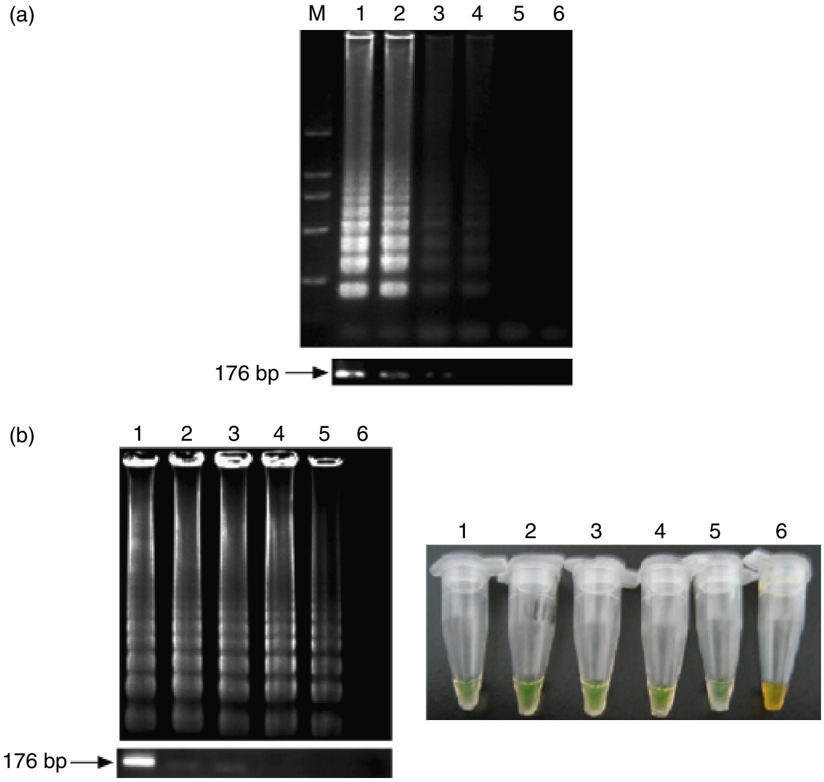

Fig. 2. Comparative sensitivities of visual inspection and electrophoretic analyses of LAMP and PCR for the detection of *Alexandrium minutum*. (a) The number above each lane represents the dilution of the purified *A. minutum* DNA: lane M, 2-kb ladder used as a size marker; lanes 1-6, DNA of *A. minutum* at 1, 100, 10, 1 pg per tube, 100 and 10 fg per tube, respectively. The lower figures are electrophoretic data from the PCR analysis. PCR shows a 176-bp amplification product. (b) Lane M, 2-kb ladder used as a size marker; lanes 1–6, dilution of the *A. minutum* cells (DNA extracted by boiling) at 500, 100, 50, 10, 5 and 1 cells/tube, respectively. The right figure is the visual inspection of the LAMP products following the addition of SYBR Green I. The lower figure is the sensitivity of PCR for the detection of *A. minutum* cells as observed by agarose gel analysis.

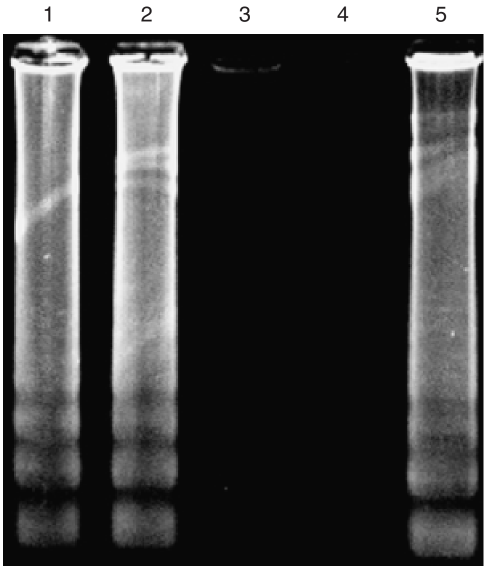

Fig. 3. Specificity of the LAMP reaction for detection of *Alexandrium* (standardized with the toxic *Alexandrium minutum*): lane 1, 5 cells of *A. minutum* were amplified in the presence of 100 ng of *Prorocentrum donghaiense* 01 DNA; lane 2, 5 cells of *A. minutum* were amplified in the presence of 100 ng of *Karenia mikimotoi* 01 DNA; lane 3, LAMP reaction in the presence of 100 ng of *P. donghaiense* 01 DNA; lane 4, LAMP reaction in the presence of 100 ng of *K. mikimotoi* 01 DNA; lane 5, positive control (five cells of *A. minutum*).

separated by agarose gel electrophoresis, as shown in Fig. 3. We can clearly see that the LAMP reaction was not influenced by the presence of large amounts of non-*Alexandrium* genomic DNA. Notomi *et al.* (2000) reported that the presence of 100 ng of human genomic DNA in a LAMP reaction mixture to detect six copies of hepatitis B virus target did not adversely affect the amplification efficiency and produced insignificant background. Our results were consistent with their results.

The specificity of the LAMP was also established by checking the reactivity with other algal strains, as discussed in the 'Materials and methods'. Significant amplification of DNAs isolated from the toxic *Alexandrium* species was observed after 60 min of incubation. Reaction products were detected only when DNA of cells of members of the genus *Alexandrium* was present, giving rise to a typical ladder-like pattern. In contrast, the DNAs of non-*Alexandrium* strains were not amplified even after 90 min of incubation (Fig. 4).

Discussion

In recent years, it has been shown that the geographical range of toxic *Alexandrium* species has been increasing (Anderson *et al.*, 1996; Lilly *et al.*, 2002; Choong & Yoshihiko 2005). The best approach to minimize the risk to humans should involve the continuous monitoring of activity of toxic *Alexandrium* species to track their presence and provide advance warning of a risk of a large-scale harmful bloom.

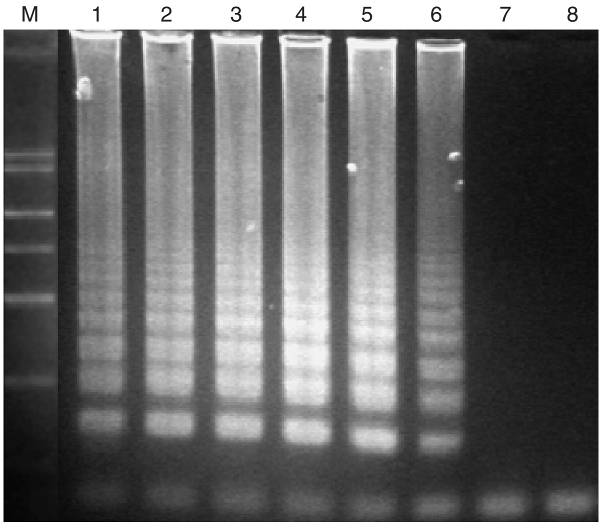

Fig. 4. Electrophoretic analysis of LAMP amplified products. Lane M, 2-kb ladder used as a size marker; lanes 1–8, LAMP carried out in the presence of genomic DNA (10 ng per tube) from *Alexandrium minutum* AMTW02, *Alexandrium andersoni* ADC02, *Alexandrium catenella* Balech 1985 ACDH03, *Alexandrium tamarense* ATMJ01, *A. catenella* L65, *A. tamarense* L66, *Prorocentrum donghaiense* PD01 and *Karenia mikimotoi* KM01, respectively.

To our knowledge, this is the first report of the application of the LAMP assay technique for the rapid and specific detection of toxic *Alexandrium* species. Compared with conventional PCR, the LAMP assay reported here is advantageous owing to its simple operation, rapid reaction and ease of detection. The LAMP assay is a simple detection tool in which the reaction is carried out in a single tube by mixing the thermopol buffer, primers and *Bst* DNA polymerase, and incubation of the mixture at 65 °C for 1 h. There is no need for a thermal cycler because there is no heat denaturation step of the template DNAs with this method. The only equipment needed for the LAMP reaction is a regular laboratory water bath or a heating block that can provide a constant temperature of 65 °C. Although there is no need for a thermal cycler, some of the double-stranded DNA seems to become single-stranded at high temperatures in the presence of high concentrations of betaine, a reagent that facilitates DNA strand separation because it stabilizes DNA (Nagamine *et al.*, 2001).

It is known that PCR inhibitors in samples reduce the sensitivity of PCR when attempting to detect a target gene (Wilson, 1997; Horisaka *et al.*, 2004). However, the LAMP method is able to detect 1 pg of the target gene even in the presence of 100 ng of other bacterial genomic DNAs (Notomi *et al.*, 2000). The sensitivity of LAMP was less affected by various components of the clinical samples than was PCR; therefore, DNA purification from samples could be omitted

(Kaneko *et al.*, 2007). As such, the sensitivity level of the LAMP method will allow detection of *Alexandrium* species not only at bloom concentrations but also in field samples containing only a small number of cells, which will be extremely useful for long-term monitoring programmes.

DNA extraction by the boiling technique prior to the LAMP test and visualization of reaction products using SYBR Green I DNA stain were employed to reduce the time required to perform the electrophoretic test and to simplify the procedure. We believe that the inexpensive running costs of the method make this technology very applicable to monitoring harmful algae in developing countries.

Although the present study provides only preliminary results, it does suggest that the LAMP assay will prove to be useful for the rapid monitoring of toxic and harmful *Alexandrium* algae.

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