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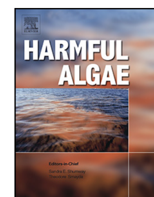

The development of loop-mediated isothermal amplification combined with lateral flow dipstick for detection of *Karlodinium veneficum*

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ABSTRACT

The aim of this study was to develop a loop-mediated isothermal amplification (LAMP) combined with a chromatographic lateral flow dipstick (LFD) assay to rapidly and specifically detect the *Karlodinium veneficum* ITS gene. Four groups of LAMP primers were specially designed to target the *K. veneficum* ITS gene. The LAMP-LFD detection limit was 7.4 pg/μL (approximately 6.5 cells/mL) of *K. veneficum* genomic DNA and was 10 times more sensitive than standard PCR. The LAMP-LFD method exhibited high specificity and accurately identified *K. veneficum* algal isolates, but not other algal isolates. To test the assay's accuracy, samples from positive results were further analyzed by sequencing and phylogenetic analysis, all of which were identified as *K. veneficum*. Over all, the LAMP-LFD assay established in this paper can be used as a reliable and simple method to detect the *K. veneficum*.

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1. Introduction

The *Karlodinium veneficum* is a common widespread dinoflagellate (Place et al., 2011) that forms blooms (Deeds et al., 2002; Kempton et al., 2002; Hallegraeff, 2002; Hall et al., 2008) and releases toxins into both aquaculture and natural systems (Adolf et al., 2007; Van Wagoner et al., 2008; Zhou et al., 2008a; Mooney et al., 2009). These toxic blooms can cause fish, shellfish, bird, and mammal mortalities (Sellner et al., 2003; Place et al., 2008). Since *Gymnodinium veneficum* Ballantine (syn. *K. veneficum*) was first described by Ballantine (Ballantine 1956), this species was also called *Gyrodinium estuariale* Hulbert, *Gymnodinium galatheanum* Braarud, *Gymnodinium micrum* and *Karlodinium micrum* (Leadbeater & Dodge) J. Larsen (Bergholtz et al., 2005). The blooms of *K. Veneficum* were first reported in South Africa (Braarud, 1957), then in North America (Li et al., 2000; Terlizzi et al., 2000), Europe (Bjornland and Tangen, 1979), and Australia (Ajani et al., 2001). Recently, *K. veneficum* blooms have been reported in the coastal waters off Hong Kong, but have not coincided with fish kills (Lam and Liu, 2005; Zhu et al., 2010). In June 2007, there were a large number of *K. veneficum* outbreaks in the shrimp ponds of Xiang Shan Bay (Zhou et al., 2011). However, *K. veneficum* is difficult to identify by standard epifluorescence microscopy because it is small and similar to many other dinoflagellates, e.g.,*Cryptoperidiniopsis brodyi*; *Pfiesteria piscicida*; *Pseudopfiesteria shumwayae* (Steidinger et al., 2006; Zhang et al., 2007a; Park et al., 2007a; Dai et al., 2014). To overcome this difficulty, several alternative detection methods based on molecular assays for harmful algae (Scorzetti et al., 2009; Smith et al., 2014; Nézan et al., 2014; Nagai et al., 2015) and especially *K. Veneficum* have been developed in the last decade. The ITS-ferredoxin dual-gene real-time PCR and the SYTO9 and TaqMan format real-time PCR probes were developed for rapid detection and quantification of *K. veneficum* (Zhang et al., 2008; Park et al., 2009). However, these molecular methods still have some disadvantages, such as expensive reagents, time consuming, expensive equipment, complicated procedure assays, and technical personnel to run them, which restrict the application of PCR in point-of-care testing (POCT). (Supplementary Table 1)

Loop-mediated isothermal amplification (LAMP) is a method that amplifies DNA under isothermal conditions and can be performed in one hour with high specificity (Notomi et al., 2000). The LAMP reaction products are usually detected by agarose gel electrophoresis (AGE), followed by either ethidium bromide (EB) staining or adding calcein (fluorescent detection reagent). There are several reports on using LAMP methods for detection of harmful algaes (Wang et al., 2008; Zhang et al., 2009; Nagai and

Abbreviations: LAMP, loop mediated isothermal amplification; LFD, lateral flow dipstick; ITS, internal transcribed spacer; PCR, polymerase chain reaction; KV, *karlodinium veneficum*; AGE, agarose gel electrophoresis; EB, ethidium bromide; PSU, practical salinity units; H7N9, avian influenza virus; FITC, fluorescein isothiocyanate; FIP, forward inner primer; BIP, backward inner primer; NJ, neighbour-joining; POCT, point-of-care testing.

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Itakura, 2012). To reduce the total processing time, avoid toxic substances, and improve amplification efficiency, LAMP combined with a lateral flow dipstick (LFD) has been developed to detect various organisms, e.g., Taura syndrome virus, white spot syndrome virus, infectious myonecrosis virus, infectious spleen and kidney necrosis virus, infectious hypodermal and hematopoietic necrosis virus, H7N9, Roundup Ready soybean, and *Ulva prolifera* (Kiatpathomchai et al., 2008; Jaroenram et al., 2009; Puthawibool et al., 2009; Ding et al., 2010; Arunrut et al., 2011; Ge et al., 2013; Wang et al., 2013; Chen et al., 2015). This method only takes 1–5 min compared with 35–50 min for AGE and does not require specialized detection equipment. In this study, the specificity, sensitivity, and convenience of the LAMP-LFD assay in both pure cultures and field water samples were established. Additionally, this method has potential value to make this technique more usable in remote locations.

2. Materials and methods

2.1. Algal species and culturing

The eight algal species used in this study were provided by Ying Jiang from the Key Laboratory of Applied Marine Biotechnology (Ningbo University). The following algal species were cultured for control experiments, *Karlodinium veneficum*, *Alexandrium tamarense*, *Prorocentrum donghaiense*, *Heterosigma akashiwo*, *Prorocentrum micans*, *Skeletonema costatum*, *Prorocentrum minimum*, and *Karenia mikimotoi.* (Table 1). A pure *K. veneficum* strain was cultured in f/2 medium (Guillard, 1975). All of the cultures were grown to either the exponential or stationary phase under the same culture conditions before being used in the experiments. The algal culture conditions were: f/2 seawater media (salinity: 23–25 practical salinity units (PSU)), 22 °C, 40 mmol $m^{-2} s^{-1}$ PAR (light: dark = 12:12), unless otherwise noted.

2.2. Field sample collection

Seventeen water samples were collected from the East China Sea (Table 2, Fig. 1). Water samples (1 L) for each station were collected by polyethylene terephthalate (PET) bottles and were then added to 3% acidic Lugol's solution to stabilize them. Upon arrival at the University of Ningbo, 1-L water samples were stored in a cold room (4 °C) and then were poured through a 50-μm mesh to remove large tissue and plankton. Finally, water samples were filtered with 0.45 μm acetate cellulose membrane (Millipore, USA). The membranes were kept frozen at −20 °C until DNA extraction from the membranes. (usually within 1–2 weeks but no longer than 2 months) for the LAMP-LFD and PCR assays as described below.

Table 2
Geographic location of field samples.

Sampling stations	Location	
	North Latitude	East Latitude
NB-68	29°03′48″	121°44′24″
NB-90	28°50′00″	121°50′00″
NB-104	29°40′00″	122°00′00″
NB-160	29°08′13″	122°13′13″
NB-168	30°04′01″	122°19′31″
NB-171	30°35′16″	122°24′54″
NB-173	29°42′07″	122°29′07″
NB-175	30°30′00″	122°30′00″
NB-179	30°44′39″	122°33′37″
NB-180	30°19′54″	122°33′44″
NB-181	30°40′20″	122°34′02″
NB-185	30°55′46″	122°41′51″
NB-188	30°40′00″	122°48′00″
NB-191	30°02′44″	122°59′44″
NB-192	30°24′00″	123°02′00″
NB-194	30°57′30″	123°11′47″
NB-195	30°24′00″	123°13′41″

2.3. DNA extraction and quality assurance

Algal cells in the exponential growth phase or stationary phase were collected by centrifugation at 12,000 × *g* for 5 min, genomic DNA was then extracted from the eight algal species using a Takara MiniBEST Universal Genomic DNA Extraction Kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. Briefly, 0.25 g of algal cells were lysed with a lysis buffer with RNaseA (10 mg/mL) and Proteinase K (20 mg/mL) for 30 min at 56 °C. The genomic DNA was eluted with 50 μL of elution buffer and stored at −20 °C until the PCR and LAMP-LFD assays. For the field samples, the membranes were shredded into fragments using sterile scissors and put into 2.0-mL Eppendorf collection tubes. DNA was extracted following the same procedure as with the pure culture samples. Finally, DNA quality was verified by spectrophotometer (Thermo Scientific NanoDrop 2000C, America). The extracted DNA was stored at −20 °C until use.

2.4. Design and synthesis of the primers and probe

Loop-mediated isothermal amplification (LAMP) was based on autocycling strand displacement DNA synthesis in the presence of Bst DNA polymerase that can amplify a few copies of DNA to 10^9

Table 1
Algal species for LAMP-LFD assay.

Algal species	Algal isolates	Source
Alexandrium tamarense (Accession number: JX524268.1)	NMBjah048	Hong Kong
Prorocentrum donghaiense[a]	NMBjah045	Dong tou
Heterosigma akashiwo[a]	NMBRah03-2	South tajima
Prorocentrum micans (Accession number: AB456035.1)	NMBjah041	Qingdao Marine research institute
Skeletonema costatum (Accession number: KF998561.1)	NMBguh004-2	Xia men
Prorocentrum minimum[a]	NMBjah049	Yu mountain
Karenia mikimotoi (Accession number: JN595877.1)	NMBjah052	Dong tou
Karlodinium veneficum (Accession number: DQ459434)	NMBjah047	Dong tou

[a] Sequences information in Additional file 1.

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