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Easy detection of multiple *Alexandrium* species using DNA chromatography chip

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ABSTRACT

In this study, the Kaneka DNA chromatography chip (KDCC) for the *Alexandrium* species was successfully developed for simultaneous detection of five *Alexandrium* species. This method utilizes a DNA–DNA hybridization technology. In the PCR process, specifically designed tagged-primers are used, i.e. a forward primer consisting of a tag domain, which can conjugate with gold nanocolloids on the chip, and a primer domain, which can anneal/amplify the target sequence. However, the reverse primer consists of a tag domain, which can hybridize to the solid-phased capture probe on the chip, and a primer domain, which can anneal/amplify the target sequence. As a result, a red line that originates from gold nanocolloids appears as a positive signal on the chip, and the amplicon is detected visually by the naked eye. This technique is simple, because it is possible to visually detect the target species soon after (<5 min) the application of 2 μL of PCR amplicon and 65 μL of development buffer to the sample pad of the chip. Further, this technique is relatively inexpensive and does not require expensive laboratory equipment, such as real-time Q-PCR machines or DNA microarray detectors, but a thermal cycler. Regarding the detection limit of KDCC for the five *Alexandrium* species, it varied among species and it was <0.1–10 pg and equivalent to 5–500 copies of rRNA genes, indicating that the technique is sensitive enough for practical use to detect several cells of the target species from 1 L of seawater. The detection sensitivity of KDCC was also evaluated with two different techniques, i.e. a multiplex-PCR and a digital DNA hybridization by digital DNA chip analyzer (DDCA), using natural plankton assemblages. There was no significant difference in the detection sensitivity among the three techniques, suggesting KDCC can be readily used to monitor the HAB species.

1. Introduction

The genus *Alexandrium* includes 28 described species (Balech, 1995; MacKenzie and Todd, 2002; Moestrup et al., 2002; Montresor et al., 2004), 8 of which are known to cause paralytic shellfish poisoning; the others are nontoxic species (Moestrup et al., 2002). Therefore, monitoring programs, especially important in aquaculture of oysters and in shellfish processing, have been established in many coastal regions. A clear understanding of the taxonomic diversity of harmful algal bloom-causing species, as well as their closely related species and their characteristics, constitutes a crucial prerequisite for the success of the monitoring programs (Scholin and Anderson, 1994; Montresor et al., 2004; Nagai et al., 2012; Medlin et al., 2013).

The monitoring of *Alexandrium* species in Japan has been hitherto conducted through microscopic studies of the plate morphology; for example, the arrangement of plates and the presence or absence of a ventral pore. However, as several *Alexandrium* species lack distinctive characteristics such as a horn, spine, or heavy ornamentation of the thecal plates, inadequate

Abbreviations: KDCC, Kaneka DNA chromatography chip; DDCA, digital DNA chip analyzer; PSP, paralytic shellfish poisoning; HAB, harmful algal bloom.

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information on which to base identification may lead to misidentification and result in an inaccurate estimation of the abundance of toxic species. Therefore, molecular diagnostic techniques such as PCR-RFLP (Scholin and Anderson, 1994), real-time PCR (Hosoi-Tanabe et al., 2004; Kamikawa et al., 2005, 2007), techniques using RNA target probes (Hosoi-Tanabe and Sako, 2005) and DNA target probes (Anderson et al., 2005; Ki and Han, 2006) for nuclear rRNA genes, DNA microarray technology (Ahn et al., 2006; Gescher et al., 2008; Medlin et al., 2013), loop-mediated isothermal amplification method (LAMP) (Nagai et al., 2010, 2012; Nagai and Itakura, 2012; Nagai, 2013), multiplex-PCR (Nagai, 2011) have been developed to accurately identify *Alexandrium* species.

Alexandrium catenella (Whedon & Kofoid) Balech, and *A. tamarense* (Lebour) Balech are dominant *Alexandrium* species causing paralytic shellfish poisoning (PSP). Appearances of the toxic tropical dinoflagellate species *A. tamiyavanichii* Balech have been confirmed in the Seto Inland Sea since the 1990s, the blooming of this species has often been observed not only the Seto Inland Sea but also around the Tsushima, Gotou, and Amami Islands in Japanese coastal waters (Nagai et al., 2003, 2011). *Alexandrium affine* (Inoue et Fukuyo) Balech is a coastal, bloom-forming and cosmopolitan dinoflagellate and typically forms chain of 2–8 cells long, but chains as long as 16 cells have been observed (Hallegraeff et al., 1991; Band-Schmidt et al., 2003). The vegetative cell and cyst of this species were first described in Japan by Fukuyo et al. (1985), after the summer blooms of 1974, 1975, and 1977. There is no report that Japanese populations produce PSP toxins. *Alexandrium fraterculus* (Balech) Balech is a long chain forming species (up to 64 cells per chain; MacKenzie et al., 2004) and is also distributed widely in coastal areas of western Japan and appears in seasons of high water temperature. This species does not fundamentally produce PSP toxins (Noguchi et al., 1985; Moestrup et al., 2002; MacKenzie et al., 2004; Omachi et al., 2007; Nagai et al., 2009b), but is an exception (Mendez, 1993). In Japan, the monitoring of species causing PSP has mainly been conducted by local government researchers who do not have easy access to expensive laboratory equipment such as real-time quantitative PCR machines (even though the prices are now more affordable than earlier). Therefore, it has become necessary to develop easy-to-use and cost-effective molecular techniques for identifying *Alexandrium* species, especially in areas where several *Alexandrium* species coexist, as Nagai (2011, 2013) has emphasized repeatedly.

Very recently, Kaneka Corporation, Japan has developed a novel molecular diagnostic method utilizing nucleic acid chromatography (Kaneka DNA chromatography chip), i.e., a captured DNA probe is solid-phased on a strip of paper (chip) (http://www.kaneka-labtest.com/defection/chromato.html). PCR amplification is performed using primer pairs added to two different tags (each for their 5′ ends) to visualize positive signals with colloidal gold and to capture target DNAs by solid-phased DNA probes. At present, it is possible to print six solid-phased probes per one chip, i.e., six species are detectable per chip at maximum, by combination with multiplex-PCR techniques. After PCR amplification, putting 2 μL of PCR amplicon on a chip with 65 μL of development buffer was sufficient enough to visualize the amplicons and detect the target species by naked eye within 5 min. This DNA chromatography chip technology will be widely applicable to many targets through the use of different sequence capture probes. This technology was applied to the species-specific detection of HAB species for the first time. The successful development of the multiple-PCR assay, that enabled the specific detection of five *Alexandrium* species, indicates that this method is one of the most convenient, rapid, sensitive, objective and cost-effective molecular tools for HAB monitoring.

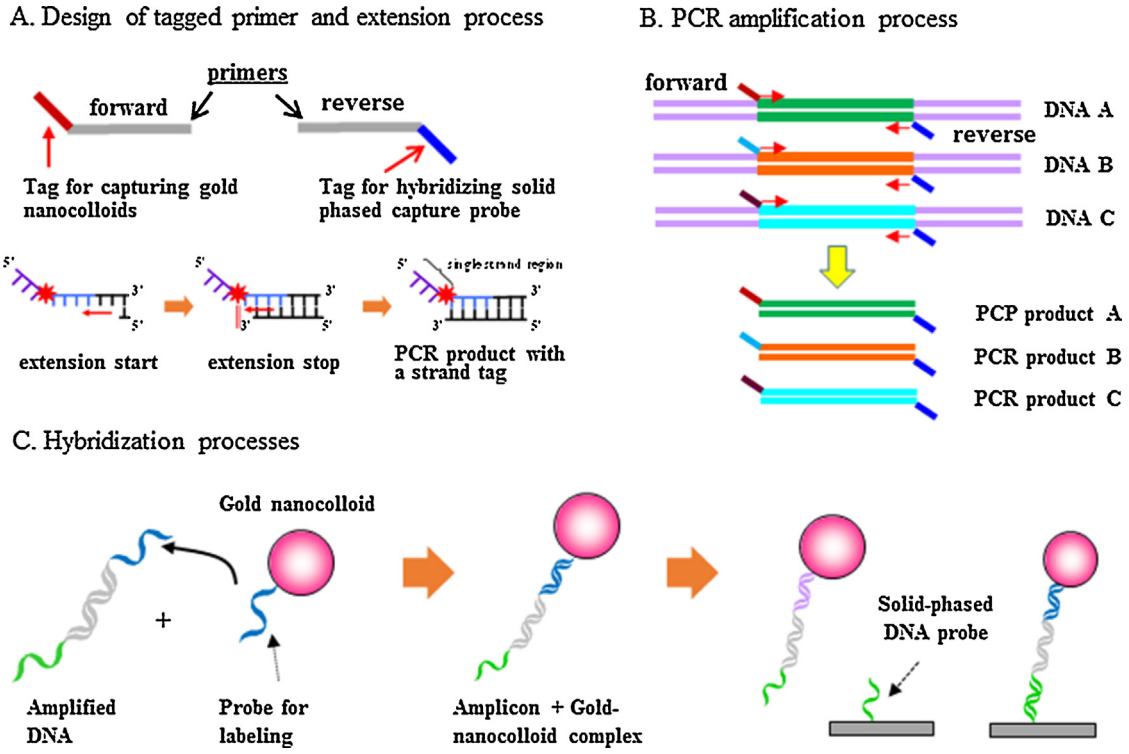

Fig. 1. *Principle of detection by the Kaneka DNA chromatography chip (KDCC).* (A) Design of tagged primer and extension process: a forward primer consists of a tag domain which can conjugate with gold nanocolloids of the chip, and a primer domain which can anneal/amplify the target sequence. The reverse primer consists of a tag domain, which can hybridize to the solid-phased DNA probe on the chip and a primer domain, which can anneal/amplify the target sequence. Both domains are connected by a spacer, which stops the extension of DNA polymerase. (B) PCR amplification process; using the tagged-primer sets, an amplicon is obtained, which has a tag to conjugate a gold nanocolloid at one end and a tag to capture a solid-phased probe at the other. (C) Hybridization process; the KDCC enables a double strand DNA, which has single-strand tags on both ends, to hybridize one tag to make a conjugate with a gold nanocolloid and the other tag to be captured by the solid-phased probe. It does not include the process of single strand DNAs for detection, therefore resulting in higher sensitivity of detection than conventional methods.