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Development and evaluation of a real-time fluorogenic loop-mediated isothermal amplification assay integrated on a microfluidic disc chip (on-chip LAMP) for rapid and simultaneous detection of ten pathogenic bacteria in aquatic animals

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ABSTRACT

Rapid, low-cost, and user-friendly strategies are urgently needed for early disease diagnosis and timely treatment, particularly for on-site screening of pathogens in aquaculture. In this study, we successfully developed a real-time fluorogenic loop-mediated isothermal amplification assay integrated on a microfluidic disc chip (on-chip LAMP), which was capable of simultaneously detecting 10 pathogenic bacteria in aquatic animals, i.e., *Nocardia seriolae*, *Pseudomonas putida*, *Streptococcus iniae*, *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio fluvialis*, *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio rotiferianus*, and *Vibrio vulnificus*. The assay provided a nearly-automated approach, with only a single pipetting step per chip for sample dispensing. This technique could achieve limits of detection (LOD) ranging from 0.40 to 6.42 pg per 1.414 μL reaction in less than 30 min. The robust reproducibility was demonstrated by a little variation among duplications for each bacterium with the coefficient of variation (CV) for time to positive (Tp) value less than 0.10. The clinical sensitivity and specificity of this on-chip LAMP assay in detecting field samples were 96.2% and 93.8% by comparison with conventional microbiological methods. Compared with other well-known techniques, on-chip LAMP assay provides low sample and reagent consumption, ease-of-use, accelerated analysis, multiple bacteria and on-site detection, and high reproducibility, indicating that such a technique would be applicable for on-site detection and routine monitoring of multiple pathogens in aquaculture.

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1. Introduction

Aquaculture is an economically important global activity, with up to 197 countries participating in export of fishery products. Disease emergence has become one of the most important risk factors to the expansion of aquaculture, and particularly for intensive fishery farming. Bacterial diseases caused by a wide range of conditional pathogens, such as *Vibrio* spp. (Austin, 2010; Frans et al., 2011), *Streptococcus* spp. (Agnew and Barnes, 2007), *Aeromonas* spp. (Pridgeon and Klesius, 2011), *Nocardia* spp. (Labrie et al., 2008), and *Pseudomonas* spp. (Altinok et al., 2006), result in considerable economic losses in aquaculture worldwide. Likewise, some members from *Vibrio* spp. and *Streptococcus* spp. are also among the most important causal agents in terms of public health.

Rapid, early, and accurate identification of pathogens is critical for successful disease control and timely treatment in aquaculture. Until recently, methods for detecting microbes were divided into four major categories, i.e., conventional microbiological methods, immunoassay-based methods, nucleic acid amplification-based methods, and DNA microarray-based methods. Conventional microbiological methods are reliable and accurate for identification of pathogenic bacteria, making important contributions to the development of aquaculture (Frans et al., 2008). However, culture conditions (e.g., medium, temperature, time, salinity, and oxygen, among others) for these bacteria are frequently fastidious and varied, and microbiological procedures are time-consuming in general. Immunoassay-based methods, on the other hand, are rapid and precise, without the need for isolation of bacteria, and have been used for detecting pathogenic bacteria in aquaculture (Bisha et al., 2012). Nevertheless, the availability of pure

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antigens and subsequent production of specific antibodies limit the application of such techniques (Churchill et al., 2006; Schneid et al., 2006). Nucleic acid amplification-based methods are preferred for their direct detection of microbial pathogens in environmental and clinical samples, without the requirement of culturing (Jadhav et al., 2012; Jones et al., 2012). In addition to the widely used methods based on the classical polymerase chain reaction (PCR), several isothermal nucleic acid amplification techniques, such as loop-mediated isothermal amplification (LAMP), have been developed and applied in the diagnosis of pathogens for rapid, sensitive, and specific detection (Kim and Easley, 2011). LAMP needs no thermal cycler and is more tolerant to inhibitory components from a crude sample compared to PCR (Notomi et al., 2000; Kaneko et al., 2007). The LAMP method has successfully been employed for detection of various aquatic pathogens, including bacteria, viruses, and protozoa, and also shows promise for clinical applications (Biswas and Sakai, 2014; Ding et al., 2010; Picón-Camacho et al., 2013). Although LAMP is convenient for the detection of a single pathogen, use of the technique for a panel of specific pathogens depends on a significant number of parallel tests, which are both time-consuming and expensive (Frans et al., 2008).

DNA microarray is a technology developed for gene analysis, and is a promising approach for simultaneous detection of multiple pathogens (Yoo and Lee, 2008). DNA microarray assays utilize similar-sized products amplified by PCR before hybridization, thereby reducing PCR template bias. Due to their high-throughput capacity and accuracy, DNA microarray has successfully been employed for identification of some aquatic pathogens (Cao et al., 2011; Chang et al., 2012; Lee et al., 2010; Lievens et al., 2011; Shi et al., 2012; Warsen et al., 2004). However, this method is almost exclusively performed in centralized laboratories using high-end instrumentation and skilled personnel and thus is not well suited for on-site pathogen screening.

Microfluidics emerged in the beginning of the 1980s and is now used in the development of techniques such as DNA chips, lab-on-a-chip technologies, and micro-thermal technologies (Bjerketorp et al., 2008; Lei, 2012; Sakamoto et al., 2007). Recently, microfluidic chips integrate with electrodes or heating elements and provide a promising "sample in, answer out" gene analysis platform for disease diagnostics (Asiello and Baeumner, 2011; Foudeh et al., 2012; Saleh-Lakha and Trevors, 2010; Zhang et al., 2006). Many improvements apparently led to by the microfluidic chips including decreased time of DNA amplification, less consumption of samples and reagents, increased portability, compactness, and potential for automation of analyses were well reviewed (Ahmad and Hashsham, 2012; Asiello and Baeumner, 2011; Zhang et al., 2006). Because there is no need for the additional thermal cycling steps as in miniaturized PCR devices, the isothermal microfluidic chips make the reaction take place in separate reaction microchambers and simplified the reaction system (Chang et al., 2013a; Craw and Balachandran, 2012). When specific primers for LAMP are dispensed in each separated microchamber or reaction well, the chips are fabricated by methods like soft-lithography, hot embossing, laser micromachining, and micro-injection molding (Chang et al., 2013a; Craw and Balachandran, 2012). The durable materials, such as polydimethylsiloxane (PDMS), permit the use of disposable chips at low cost, which effectively prevents the contamination. Most recently, attempts have been made to develop microfluidic chips integrated with LAMP for the detection of some aquatic pathogens (Wang et al., 2011; Chang et al., 2013b). Wang et al. (2011) combined one-step, reverse-transcription LAMP (RT-LAMP) on a microfluidic chip to detect the grouper *Nervous necrosis virus* (NNV). This group further designed another integrated microfluidic chip for simultaneous detection of four microbial pathogens in ornamental fish (i.e., *Streptococcus agalactiae*, *Aeromonas hydrophila*, *Koi herpes virus*, and *Iridovirus*) (Chang et al., 2013b). Similarly, Ahmad et al. (2011) developed a microfluidic chip to perform LAMP for the detection of six waterborne pathogens, i.e., *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Legionella pneumophila*, *Salmonella enterica*, *Vibrio cholerae*, and *Cryptosporidium parvum*.

The aim of this study was to develop a LAMP integrated microfluidic chip (on-chip LAMP) for multiplex detection of 10 pathogenic bacteria in aquaculture, i.e., *Nocardia seriolae*, *Pseudomonas putida*, *Streptococcus iniae*, *Vibrio alginolyticus*, *Vibrio anguillarum*, *Vibrio fluvialis*, *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio rotiferianus*, and *Vibrio vulnificus*. In addition, assays were performed to evaluate the sensitivity, specificity, and reproducibility of on-chip LAMP. The performance of this method was determined by screening blinded tissue samples from diseased aquatic animals, and comparing these with conventional microbiological methods and PCR assays.

2. Materials and methods

2.1. Bacterial isolates and culture conditions

A total of 49 bacterial isolates from aquatic animals or products, or water environments were used in the study (Supplementary Table 1). Standard biochemical tests were performed to identify the bacteria, and 16S rDNA was amplified for bacterial validation as previously described (Shi et al., 2012).

Vibrio spp. was cultured overnight at 30 °C using thiosulfate citrate bile salt sucrose agar (TCBS agar) or alkaline peptone water. *N. seriolae* was grown in Luria–Bertani broth at 28 °C for 5 days. *S. iniae* was grown on trypticase soy agar or in broth (TSA or TSB) at 30 °C for 24 h. Other bacteria were cultured overnight at 37 °C using brain heart infusion broth (BHI) or TSB. Specific mediums were purchased from Difco Laboratories, Detroit, USA.

2.2. Primer design

LAMP primers targeting a bacterial virulence or house-keeping genes were selected from established methods described in the literature or newly designed for this study (Table 1). Briefly, one or more sets of LAMP primers (FIP, BIP, F3, B3, LF, LB) were designed for each of 6 bacteria, i.e., *P. putida*, *S. iniae*, *V. fluvialis*, *V. harveyi*, *V. rotiferianus*, and *V. vulnificus*, using Primer Explorer ver. 4 (http://primerexplorer.jp/elamp4.0.0/index.html). Primer specificity was checked using a basic local alignment search tool (Primer-BLAST) against the nucleotide sequences from all organisms in the non-redundant GenBank database. The performance of the primer sets, e.g., the specificity test and real-time amplification plot, was tested using the optimized LAMP conditions, after which the primer sets were selected by sensitivity analysis using a 10-fold serial dilution set of genomic DNA (gDNA) of the abovementioned 6 bacteria (Table 1). For *V. anguillarum* and *V. parahaemolyticus*, all primers were chosen from the literature and used in LAMP (Dong et al., 2011; Yamazaki et al., 2008). For *N. seriolae*, the primers F3, B3, FIP, and BIP were chosen from the literature (Itano et al., 2006), but LF and LB primers were newly designed (Table 1). For *V. alginolyticus*, the primers F3, B3, FIP, and BIP were chosen from the literature (Ding et al., 2009), but LF primer was newly designed (Table 1). All oligonucleotide primers were custom synthesized by Invitrogen (Shanghai, China).

2.3. Animal sample collection

Seventy-nine samples of fish, crab and shrimp were collected along the coast of Zhejiang Province in the China foreland such as Xiangshan Bay, or purchased from markets in Ningbo, China between 2008 and 2013. Each sample exhibited one or more clinical signs of disease as follows: erratic swimming behavior, ulcerated or festered skin, rotted fins, erosion and darkened coloration to the skin and fins, swollen or protruding hemorrhaged anus, hemorrhage to gill, intestine, body cavity, spleen, and muscle, exophthalmia, and corneal opacity, among others. The liver and kidney (fish), gut and gill (crab), or muscle (shrimp) were collected from each animal, and used to assess the clinical sensitivity

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