Journal of Microbiological Methods 89 (2012) 179–184

Contents lists available at SciVerse ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Development of a visual loop-mediated isothermal amplification method for rapid detection of the bacterial pathogen *Pseudomonas putida* of the large yellow croaker (*Pseudosciaena crocea*)

Zhijuan Mao [a,*], Yangyu Qiu [b], Lei Zheng [a], Jigang Chen [a], Jifang Yang [a]

[a] *Biological and Environmental College, Zhejiang Wanli University, Ningbo 315100, China*
[b] *Fishery College, Huazhong Agricultural University, Wuhan, Hubei Province 430070, China*

ARTICLE INFO

Article history:
Received 16 February 2012
Received in revised form 15 March 2012
Accepted 16 March 2012
Available online 25 March 2012

Keywords:
Large yellow croaker (*Pseudosciaena crocea*)
Pseudomonas putida
Loop-mediated isothermal amplification (LAMP)
Visualization
rpoN

ABSTRACT

In recent years, the large yellow croaker (*Pseudosciaena crocea*), an important marine fish farmed in the coastal areas of Zhejiang province, east China, has become severely endangered as a result of the bacterial pathogen *Pseudomonas putida*. This paper reports the development of a visual loop-mediated isothermal amplification (LAMP) assay for rapid detection of the pathogen. Four primers, F3, B3, FIP and BIP, were designed on the basis of DNA sequence of the *rpoN* gene of *P. putida*. After optimization of the reaction conditions, the detection limit of LAMP assay was 4.8 cfu per reaction, 10-fold higher than that of conventional PCR. The assay showed high specificity to discriminate all *P. putida* isolates from nine other Gram-negative bacteria. The assay also successfully detected the pathogen DNA in the tissues of infected fish. For visual LAMP without cross-contamination, SYBR Green I was embedded in a microcrystalline wax capsule and preset in the reaction tubes; after the reaction the wax was melted at 85 °C to release the dye and allow intercalation with the amplicons. The simple, highly sensitive, highly specific and cost-effective characteristics of visual LAMP may encourage its application in the rapid diagnosis of this pathogen.

1. Introduction

Pseudomonas putida has been reported to be an important fish pathogen that has endangered the aquaculture of rainbow trout (Altinok et al., 2006), European eel (Fan, 2001), oyster toadfish (Smolowitz et al., 1998) and large yellow croaker (*Pseudosciaena crocea*) (Shen et al., 2008). The large yellow croaker, an important economic valuable marine fish, is farmed in coastal areas of Zhejiang province, China. In this species the infection shows no obvious symptoms on the body surface but white nodules develop in the spleen and kidney of diseased individuals; the disease induces mass mortality and results in severe economic loss. Rapid field diagnosis of the pathogen in the early stages of infection may help to prevent disease outbreaks.

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid-based amplification method for rapid detection of pathogens; it has high sensitivity and specificity, yet it is cost-effective (Notomi et al., 2000). The principle of LAMP is based on autocycling strand displacement DNA synthesis in the presence of exonuclease-negative Bst DNA polymerase, with four specific primers that recognize six distinct sequences on the DNA template, under isothermal conditions, within 1 h (Notomi et al., 2000). The LAMP assay has been developed for the detection of many microbial pathogens that affect aquaculture, such as viruses (Gunimaladevi et al., 2005; Mekata et al., 2009; Shivappa et al., 2008; Chen et al., 2011), bacteria (Savan et al., 2004; Yeh et al., 2005; Yamazaki et al., 2008; Saleh et al., 2008; Surasilp et al., 2011) and protozoan parasites (El-Matbouli and Soliman, 2005; Sakai et al., 2009).

Three methods are used commonly for detection of the results of LAMP assay: turbidity detection of LAMP results, agarose gel electrophoresis, and visual detection of the amplicons using a fluorescent intercalating dye, such as SYBR green, calcein or hydroxy naphthol blue (HNB) (Mori et al., 2004; Parida et al., 2008; Goto et al., 2009). Visual LAMP detection with fluorescent dye is thought to be the most straightforward, because the color change can be observed with the naked eye under natural or ultraviolet (UV) light, and this technique can therefore be used for rapid detection in most laboratories. In practice, gel electrophoresis and most methods of visual detection of LAMP results require opening of the tubes after the reaction, which leads to a high risk of cross-contamination (Tomita et al., 2008). Given the high sensitivity of the method, the amplicons disseminated with the aerosol may result in contamination of the workplace and produce false positive results in subsequent detection steps (Njiru et al., 2008). Visualization of the reaction in closed tubes to which the fluorescent dye has been added before the reaction may provide an ideal solution. In general, SYBR green is the dye used

* Corresponding author. Tel.: +86 574 88223093; fax: +86 574 88223016.
E-mail address: mzjjane@hotmail.com (Z. Mao).

0167-7012/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2012.03.011

most widely in visual LAMP (Parida et al., 2008). However, it inhibits amplification, so pre-addition of the dye to the reaction mix would not work (Goto et al., 2009). Recently, Tao et al. (2011) solved the problem by embedding SYBR green I in high melting point microcrystalline wax, and presetting the capsule in the reaction tubes. The wax melted after the reaction and released the dye to combine with DNA, thus the color change could be observed easily with the naked eye under natural or UV light (Tao et al., 2011).

In this study, we developed a visual LAMP method to detect the bacterial pathogen *P. putida* of the large yellow croaker. We designed the primers, optimized the reaction conditions, and visualized the LAMP assay with SYBR green I embedded in a microcrystalline wax capsule. The sensitivity and specificity of the primers in the LAMP assay were determined, and the method was applied to the detection of the pathogen in infected fish.

2. Materials and methods

2.1. Bacterial strains and DNA preparation

The bacteria used in this study, and their sources, are listed in Table 1. The bacteria were cultured in Luria broth (LB) at 28 °C for 18 h. Bacterial genomic DNA was prepared by lysis of the bacterial pellet in 100 μL of lysis buffer (20 mM Tris–HCl, pH 8.0, 2 mM EDTA, pH 8.0 and 1.2% Triton X-100) that was boiled for 10 min (Iwamoto et al., 2003).

2.2. LAMP primer design

The gene that encodes *RpoN*, the alternative sigma factor δ^{54} of *P. putida*, was selected as the target gene. A set of four primers was designed according to our published data on the *rpoN* coding sequence (GenBank ID: JN041208.1) of *P. putida* NB2011 (a pathogenic isolate obtained from an infected large yellow croaker collected by our staff during an outbreak in 2011) using Primer Explorer ver. 4 (http://primerexplorer.jp/elamp4.0.0/index.html). A forward inner primer (FIP), a backward inner primer (BIP) and two outer primers (F3 and B3) were used for the LAMP method. The sequences of the primers and their locations are shown in Table 2.

Table 1
Bacterial strains used in this study.

Bacterial isolates	Origin	Source
P. aeruginosa 1.1129	EI[a], China	CGMCC[b]
P. fluorescens 1.1823	EI, China	CGMCC
P. stutzeri 1.1803	EI, China	CGMCC
E. coli ATCC44752	Denmark	CMCC[c]
V. harveyi ATCC33868	EI, USA	CGMCC
V. parahaemolyticus ATCC33845	EI, USA	CGMCC
Listonella anguillarum	China	IHB[d]
V. alginolyticus 1.1607	EI, USA	CGMCC
Aeromonas hydrophila 1.1816	EI, China	CGMCC
P. putida 1.645	EI, China	CGMCC
P. putida 1.643	EI, China	CGMCC
P. putida 1.593	EI, China	CGMCC
P. putida 1.2309	EI, China	CGMCC
P. putida 1.1839	EI, China	CGMCC
P. putida PT01	*P. crocea*, China	ZWU[e]
P. putida PT02	*P. crocea*, China	ZWU
P. putida PT03	*P. crocea*, China	ZWU

The strains *Pseudomonas putida* PT01, PT02 and PT03 were isolated, identified and preserved in our laboratory.
[a] Environmental isolates.
[b] China General Microbiological Culture Collections Center.
[c] National Center for Medical Culture Collections.
[d] Institute of Hydrobiology, Chinese Academy of Sciences
[e] Zhejiang Wanli University, China.

2.3. Determination of LAMP reaction conditions

The LAMP assay was carried out in a total volume of 25 μL containing 0.8 μM of each of the inner primers (FIP and BIP), 0.2 μM of each of the outer primers (F3 and B3), 1.0 mM dNTP, 8 mM $MgSO_4$, 0.8 M Betaine (Sigma), 8 U of Bst DNA polymerase (New England Biolabs, MA, USA) and 1 μL of supplied buffer and DNA template. The reaction temperature was 60–65 °C, the concentration of Mg^{2+} was 2–18 mM; the amplification time was optimized at 30, 45 and 60 min, and terminated at 85 °C for 10 min. The LAMP products were analyzed by 2% agarose gel electrophoresis.

2.4. Visualization of the LAMP assay with SYBR green I encapsulated in microcrystalline wax

The microcrystalline wax–dye capsule with SYBR green I was prepared according to the method described by Tao et al. (2011) with some modifications. Briefly, a sterile paper slot, 50 mm × 5 mm × 7 mm, was prepared, and melted microcrystalline wax (melting point = 80 °C; IGI, Toronto, Canada) was pushed into the slot with a 5 mL sterile syringe, to form a long strip with a thickness of 2 mm. After the wax had cooled and solidified, 1 μL of SYBR green I solution (10×) was added to the surface of the wax on each 5 mm strip. The dye was absorbed by the wax. Subsequently, the melted wax was added to the top of the dye solution to form the top layer of wax (also with a thickness of about 2 mm). When the top of layer wax had cooled and solidified, the paper slot was removed, and the wax slot was cut with a sterile blade and shaped to form capsules of about 5 mm × 5 mm × 4 mm that would remain in the neck of 0.5 mL PCR tubes. Such capsules could be prepared in batches, kept away from light and stored at −20 °C until use.

After the addition of the 25 μL reaction system to the tubes, a capsule was preset in the neck of the tube, and the termination of the reaction was carried out by keeping the tube at 85 °C for 10 min, which allowed the wax capsule to melt to release the SYBR green I. This combined with the amplicons to allow visual detection under natural light or a UV lamp.

2.5. Polymerase chain reaction

The target gene of the PCR was also the *rpoN* gene of *P. putida*. Specific primers were designed using to the same data (GenBank ID: JN041208.1) to produce the following: primer p1, 5′CTGAACCAGGAAGCCATCCC3′, p2, 5′CCTTGTGCCTCCAGTAAACCA3′. The DNA extracted from bacterial samples was used as the template for PCR amplification. The reaction was carried out with primers p1 and p2 for 30 cycles, each of which consisted of denaturation at 94 °C for 30 s, annealing at 57.2 °C for 30 s and extension at 72 °C for 30 s. The length of the expected PCR amplicon was 485 bp.

2.6. Determination of sensitivity of LAMP and PCR with pure culture

The sensitivity of the LAMP assay for *P. putida* in pure culture was determined as described previously (Surasilp et al., 2011) with some modifications. Briefly, serial 10-fold dilutions (10^8 cfu mL^{-1} to extinction) of a mid-log phase culture of *P. putida* grown in LB were prepared in phosphate buffered saline (PBS) and quantified using the standard plating method. For preparation of DNA from pure cultures, 100 μL of each dilution was centrifuged at 18,000 × *g* for 5 min, after which the pellet was suspended in 50 μL of 25 mM NaOH and heated subsequently to 95 °C for 5 min. After neutralization with 4 μL of 1 M Tris–HCl buffer (pH 7.5), the suspension was centrifuged at 18,000 × *g* for 5 min. For the LAMP and PCR assay, 1 μL of each supernatant was used as the template. Sensitivity tests were repeated in triplicate and the lowest limits of detection (cfu/reaction) were reported.