Detection of Mycobacteriosis in guppy, *Poecilia reticulata*, by loop-mediated isothermal amplification method

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Abstract

Mycobacterium spp. were isolated from diseased guppies *Poecilia reticulata* from Nakorn Pathom province, Thailand, during a period of severe disease loss. Two mycobacterial isolates, Thai 3 and Thai 4, were closely related to *M. gordonae* by phylogenetic analyses. A loop-mediated isothermal amplification (LAMP) technique was designed for *M. gordonae* identification in guppies. The 16S rRNA gene of the *M. gordonae* of Thai 4 was used to construct specific primers for LAMP. The LAMP reaction condition was tested and optimized at 65 °C for 60 min. The sensitivity of the LAMP was about 2.5 x 10^2 CFU. The LAMP method was applied to examine those guppies challenged with *M. gordonae* and healthy guppies from farms. The LAMP test detected *M. gordonae* in 66.6 % of the experimentally inoculated fish whereas 11.1 % was detected by microbiological culture and both of the techniques detected 37.8 % of diseased fish among the healthy fish. The results suggest that the LAMP test is a rapid and reliable method for the detection of *M. gordonae* infected guppies.

Introduction

Mycobacteriosis is a systemic, chronic, progressive infectious disease found in a wide range of marine and fresh water fish. Many freshwater ornamental fish such as bettas, neon tetra and guppies are susceptible species (Conroy & Conroy, 1999; Decostere et al., 2004). The disease is caused by non-tuberculosis mycobacteria including *Mycobacterium marinum*, *M. fortuitum*, *M. chelonea* (Frerichs, 1993; Adams et al., 1996; Puttinaowarat et al., 2000; Beran et al., 2006) and *M. gordonae* (Lescenko et al., 2003). The mycobacteria in this group produce small granuloma in the internal organs of chronically infected fish. The fish show no clinical signs in the early stages of infection but later they become listless, anorexic, emaciated, dyspnoeaic, exophthalmic and develop skin ulcerations (Frerichs, 1993; Beran et al., 2006). However these symptoms are common clinical signs of many fish diseases therefore it is difficult to diagnose Mycobacteriosis from these signs. Although microbiological techniques can be used to identify *Mycobacterium* they are time-consuming and not really appropriate for use in the rapid diagnosis, especially, of small

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sized fish where it is difficult to isolate the causative bacteria from their relatively small internal organs; therefore many methods use homogenized whole fish (Puttinaowarat et al., 2000; Puttinaowarat et al., 2002; Pate et al., 2005). The Loop-mediated isothermal amplification (LAMP) technique was first developed in 2000 (Notomi et al., 2000) and the test has been developed for the diagnosis of many disease agents for example *Edwardsiella tarda* in Japanese flounders *Paralichthys olivaceus* (Savan et al., 2004), *Edwardsiella ictaluri* in channel catfish *Ictalurus punctatus* (Yeh et al., 2005), *Nocardia seriolae* in yellowtails *Seriola quinqueradiata* (Itano et al., 2006), Iridovirus in red sea bream *Pagrus major* (Ohira et al., 2004), the herpes virus in common carp *Cyprinus carpio* (Gunimaladevi et al., 2004) and the infectious hematopoietic necrosis virus in rainbow trout *Oncorhynchus mykis*s (Gunimaladevi et al., 2005) and the spring viremia of carp virus in koi carp *Cyprinus carpio* L. (Shivappa et al., 2008). Although the LAMP test has been used for the detection of *M. tuberculosis* Complex, *M. avium*, and *M. intracellulare* in human sputum samples (Iwamoto et al., 2003) it has never been used on *M. gordonae* in guppies. The LAMP test has become widely used because of the simplicity, rapidity and sensitivity of the reaction that use only ordinary laboratory equipment. The aim of the present study was to develop a LAMP test for the rapid detection of *Mycobacterium* in guppies from farms.

Materials and methods

Bacterial samples and the extraction of DNA

Clinical isolates of *M. gordonae* were isolated from the livers and kidneys of diseased guppy which were collected from Mycobacteriosis suspected outbreak farms. The clinical diagnosis, microbiology and histopathology were studied. Thai 3 and Thai 4, two isolates of *Mycobacterium* sp. were successfully identified and 16S rRNA was characterized. *M. gordonae* was reported as the infected species (Areechon et al., 2001; Sakai et al., 2005; Ponpornpisit et al., 2005). The purified isolated Thai 4 were kept in -70 °C and re-grown in Ogawa-egg medium (Nissui, Japan) for 14 days at 27 °C prior to use. DNA of the bacteria was extracted using an isoplant DNA kit according to the manufacture's instructions (Nippon Gene, Tokyo, Japan).

Primer construction and optimization of LAMP conditions

Design of primers

Based on the sequence of 16S rRNA of *M. gordonae* (Sakai et al., 2005), a set of LAMP primers was designed using Primer Explorer V3 software (http://primerexplorer.jp/lamp3.0.0/ index.html). The set consisted of four primers : two inner (FIP: 5′-GAGCGACGACAGC CATGCATTTTCG GCAGAGATGTCGGTTC-3′, BIP: 5′-GTTGGGTTAAGTCCCGCAACGAT TTTGGCAGTC TCTCACGAGT CC-3′) and two outer (F3: 5′-GGGTTTGACATGCACAGGAC-3′, B3: 5′-ACCTT CCTCCGAGTTGACC-3′) primers (Figure 1). The DNA template from Thai 4 was amplified and used as a positive referent strain.

Determination of conditions

The LAMP was carried out in a total 25 µl reaction volume containing 2 µl (40 pmol) each of 16S-FIP and -BIP, 1.0 µl (5 pmol) and of 16S-F3 and -B3, 12.5 µl of 2X reaction mixture (40 mM Tris-HCl, 20 mM KCl, 16 mM

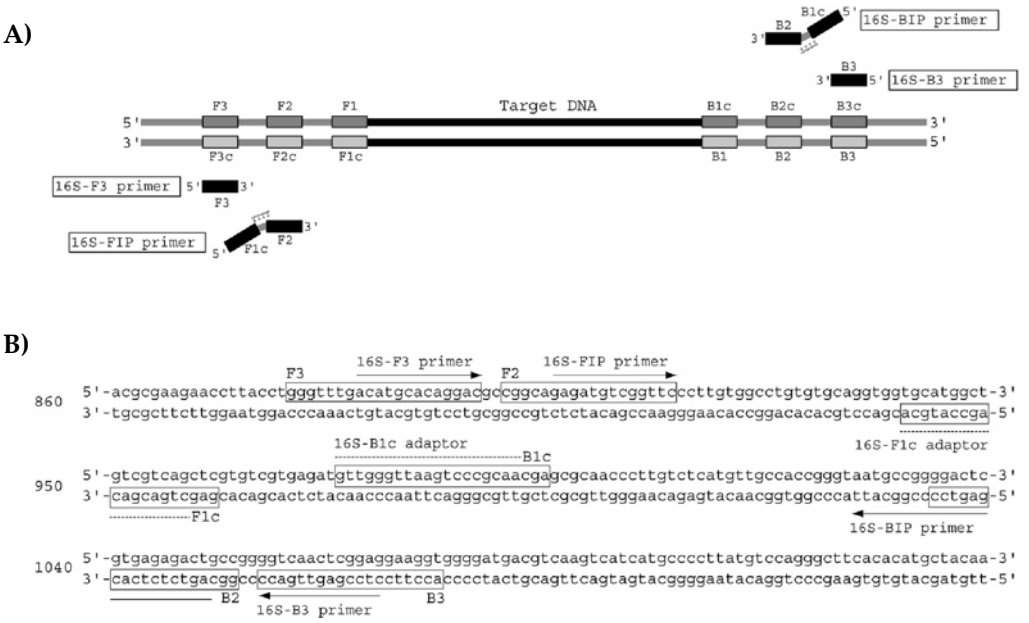

Figure 1. (A) Schematic diagram of two-inner (FIP, BIP) and -outer (F3, B3) primers for LAMP. This diagram was adopted from Eiken Chemical Co. Ltd. (B) Nucleotide sequence of 16S rRNA gene of *Mycobacterium* sp. (accession no. AB178775) used for the two-inner (FIP, BIP) and two-outer (F3, B3) primers. DNA sequences used for primer design are shown by boxes and arrows.

$MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2 % Tween-20, 1.6 M betaine and 2.8 mM dNTPs each), 1.0 µl of target DNA, 4.5 µl of distilled water and 1.0 µl (8U) of *Bst* DNA polymerase (Eiken chemicals Co. Ltd.Japan). The reaction temperature was optimized at 60, 63 and 65°C and LAMP was carried out for 15, 30, 45, 60 minutes. The amplified products were viewed on a 2 % agarose gel and documented using a gel doc system to determine the optimal conditions.

Detection limit of LAMP

Sensitivity of LAMP was tested on mycobacterial suspensions. The suspension was prepared by vortex 1 gram of purified Thai 4 with 10 ml of 0.85 % normal saline. A 0.1 ml of the bacterial suspension dropped for plate and bacterial colony was counted and 0.1 ml of the suspension was centrifuged and extracted DNA. A 10-fold serial dilution of Thai 4 DNA extracted from the suspensions adjusted to 2.5×10^8 CFU ml^{-1} (CFU, colony forming units) by the normal saline was used as template for LAMP following the predetermined conditions described above. The products from all preparation dilution were electrophoresed on 2% agarose gel.

Detection of M. gordonae from bacterial challenge and field samples

Parallel testing by bacterial culture and LAMP test were performed from bacterial challenge and field samples. The bacterial challenging group was prepared using 9 healthy guppies inoculated with M. gordonae, isolated Thai 4. The laboratory bred guppies were intraperitoneal injected with 2.76 x 10^7 CFU

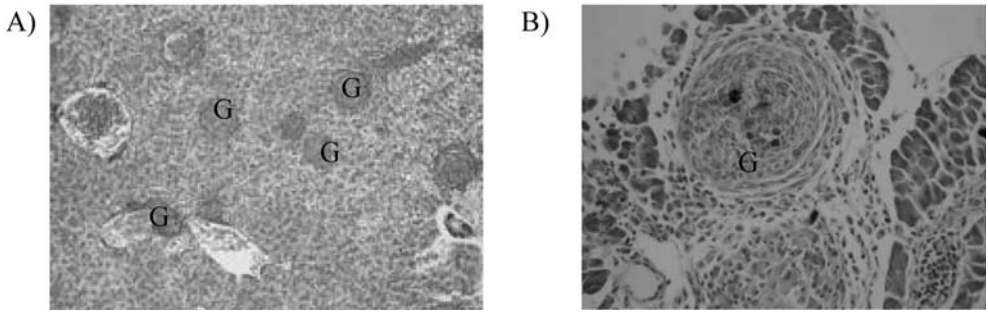

Figure 2. (A) Multifocal granulomas (G) in the liver of diseased guppy (H&E,100X). (B) Granuloma consisted of a central macrophage aggregation and surrounding lymphocytes and fibroblasts. (H&E,400X).

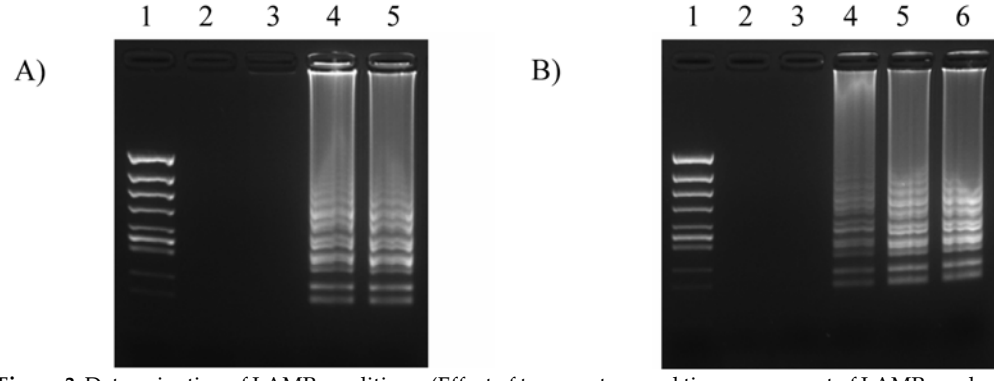

Figure 3. Determination of LAMP conditions. (Effect of temperature and time on amount of LAMP product.) (A) temperature; lane 1: molecular size marker (φ/X174/*Hinc* digest), lane2: negative control (template: D.W.), lane 3-5: LAMP carried out at 60, 63 and 65 °C, respectively. (B) time; lane 1: molecular marker (φ/X174/*Hinc* digest), lane2: negative control (template: D.W.), lane 3-6: LAMP carried out for 15, 30, 45 and 60 min, respectively. All the products were electrophoresed on a 2 % agarose gel and stained with ethidium bromide.

of the bacteria. The challenged guppies were reared in a 10 L glass aquarium, supplied daily with pellet feed and had no water change during the 20 days observation. Field samples that were collected from 3 fish farms in Nakorn Pathom province consisted of 37 male and female guppies without skin lesions or abnormal clinical signs. The fish from the challenge and the farms were euthanized with 2-phenoxy ethanol and then the livers and kidneys of the fish were dissected and divided. One piece was pretreated in 2 % NaOH for 10 min and then inoculated directly onto 1% Ogawa-egg medium. The other piece was subjected for DNA extraction using isoplant DNA kit and used for the LAMP test.

Results

The diseased guppies showed multifocal granulomas distributed in the visceral organs of all the fish. The granulomas consisted of central macrophage aggregations and surrounding lymphocytes and fibroblasts (Figure 2). Rod-shaped acid-fast bacteria were detectable in the macrophage aggregation. The bacterial inoculation from moribund fish liver took 14 days to show purified yellowish colonies on Ogawa-egg medium. The

LAMP test	Bacterial culture		Total %
	Positive (%)	Negative (%)	
Positive (%)	0 (0)	6 (75)	6 (66.7)
Negative (%)	1 (100)	2 (25)	3 (33.3)
Total	1 (100)	8 (100)	9 (100)

Table 1. Result of LAMP test and bacterial culture from challenging samples.

LAMP test	Bacterial culture		Total %
	Positive (%)	Negative (%)	
Positive (%)	9 (64.3)	5 (21.7)	14 (37.8)
Negative (%)	5 (35.7)	18 (78.3)	23 (62.2)
Total	14 (100)	23 (100)	37 (100)

Table 2. Result of LAMP test and bacterial culture from field samples.

developmental of a LAMP test at three different reaction temperatures and three different reaction times gave the result that at 60 °C the amplified product could be detected at a reaction time of 30, 45 and 60 minutes and at a 30 minutes reaction time, the amplified product could be detected at 63 and 65 °C (Figure 3). For the detection limit, the LAMP test could detected bacterial DNA from 2.5×10^2 CFU.

The challenging samples, 9 guppies infected with Thai 4, showed no clinical signs at 20 days and yielded one positive guppy (11.1%) by bacterial culture in contrast with 6 positive guppies (66.6%) from the LAMP test (Table 1). Both of the tests detected 14 positive fish (37.8%) from 37 apparently healthy guppies from the field samples (Table 2). The overall results of *Mycobacterium* infection in guppy was confirmed by bacterial culture and LAMP tests in 32.6 % and 43.5 % of cases, respectively.

Discussion

In this study, 43.5 % of *M. gordonae* from subclinical guppies were successfully detected by the LAMP test which is higher than that from the bacterial culture (32.6%). However the sensitivity, specificity, accuracy and positive predictive value were very low in the challenging group. The reasons for the lack of those were the small numbers of the sample in the testing group and might have been because the infection time was too short consequent on insufficient *Mycobacterium* organisms in the fish tissues (Fegan, 2000). Nevertheless the kappa value indicates fair to moderate agreement between the two tests the low detection rate was still a problem in the present study.

The limitations of the two tests were that the LAMP test is specific only *M. gordonae* and the bacterial culture requires a large number of viable organisms in the sample which are difficult to obtain from early infection, young or small sized fish. However, the LAMP technique can be an alternative in outbreak areas for rapid disease diagnosis because the bacterial culture method and acid-fast staining, the best currently available methods for *Mycobacterium* identification in fish, normally take up to 2 weeks to obtain result. Moreover, this method is inconvenient for the rapid detection of mycobacteriosis from guppies on farms because mycobacteria usually localize and induce pin point granuloma in the visceral organs of the fish, therefore tissue for bacterial cultures sometimes may not be from the actual infection site and sampling requires a skilful technician.

Thus other methods for *Mycobacterium* detection including histology, immunology and polymerase chain reaction (PCR) (Robert et al., 2001; Puttinaowarat et al., 2002; Sakai et al., 2005; Pate et al., 2005) have been used. The histological technique can detect only chronic *Mycobacterium* infection while immunological and PCR require sophisticated instruments.

DNA probe (Boddinghaus et al., 1990) and reverse cross blot PCR (Puttinaowarat et al., 2000) also have been successfully described, the capability of the LAMP to use simpler instruments makes the technique more appropriate on an ordinary laboratory scale. The benefit of LAMP in this study was to detect *M. gordonae* within 2 hours compared to bacterial culture technique that needs up to 14 days. Due to the limitation of available techniques, screening methods by a simple and rapid test is very important.

In conclusion, we recommend the LAMP test for screening *M. gordonae* in intensive culture farms, to confirm with other diagnostic methods as well as clinical correlation for the final diagnostic result.

Acknowledgement

This work was fully supported by the Japan Society for the Promotion of Science (JSPS) under JSPS-NRCT joint project.

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