Journal of Microbiological Methods 164 (2019) 105671

Contents lists available at ScienceDirect

Journal of Microbiological Methods

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

Establishment of loop-mediated isothermal amplification for rapid and convenient detection of *Mycobacterium marinum* complex

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ARTICLE INFO

Keywords:
Mycobacterium marinum
Mycobacterium ulcerans
Loop-mediated isothermal amplification
Digestion polymorphism

ABSTRACT

Mycobacterium marinum is a zoonotic pathogen that can cause dermatological infection mainly from contaminated water or fish. Some well-known genetically similar species and subspecies are *M. lifrandii* and *M. pseudoshottsii* from amphibians and fish in aquaculture, and *M. ulcerans*, a causative agent of a neglected tropical disease (NTD), Buruli ulcer. They are believed to survive in water as their major niche, which might be related to their source of infection, but detailed ecological surveillance of the species complex remains to be done. Herein, we present a new detection system for *M. marinum* complex based on isothermal DNA amplification that can be conducted conveniently with high sensitivity and specificity. The target was a chromosomal gene, *mrs*A, including a restriction polymorphism between *M. ulcerans* (except for the most ancestral subspecies, *M. ulcerans* subsp. *shinshuense*) and the other species. The system was able to detect less than 500 fg (approximately 70 copies) of genomic DNA of *M. marinum*, within 60 min, and caused no amplification from mycobacterial species other than *M. marinum* complex species. It was also verified that restriction of the amplified DNA fragments was able to discriminate *M. ulcerans* as expected. This easy, quick, and convenient system is expected to facilitate detection of *M. marinum* complex from various resources.

1. Introduction

Mycobacterium marinum is an aquatic pathogenic mycobacterial species, has been reportedly isolated from human cases as well as various fish species, and aquatic animals (Falkinham III, 1996; Haridy et al., 2014; Li et al., 2017; Waltzek et al., 2012). In human cases, it has been observed to cause occasional infection, mainly on skin (Aubry et al., 2002; Eberst et al., 2012), potentially spreading from an infected patient to secondary cases as in an earlier outbreak in New York City (Sia et al., 2016). Human infection has been associated with exposure of injured skin to contaminated water, causing granulomatous inflammation (Aubry et al., 2002; Gluckman, 1995). Phylogenetically, *M. marinum* is similar to *M. tuberculosis*, an important pathogen of human tuberculosis (Stamm and Brown, 2004). This aspect has enabled others to study this species as a model of tuberculosis to investigate its pathogenesis and host immune responses (Davis et al., 2002; Meijer et al., 2004; Ramakrishnan and Falkow, 1994; Stamm and Brown, 2004).

In addition to occasional infection of humans, *M. marinum* can be a threat to aquaculture industries and breeding of aquatic animals and ornamental fish (Bozzetta et al., 2010; dos Santos et al., 2002; Haridy et al., 2014; Li et al., 2017; Prearo et al., 2004). In the case of a fish farm, not only dead or moribund fish but also apparently healthy fish showed visible granulomas in their organs, which means that its outbreaks might damage farm productivity severely (dos Santos et al., 2002). The bacterial species can also be found through water environmental surveillance at sites such as lakes, ponds, and rivers (Tsai et al., 2007). Such observations underscore that this pathogen inhabits natural niches similarly to other mycobacterial species.

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https://doi.org/10.1016/j.mimet.2019.105671
Received 10 June 2019; Received in revised form 16 July 2019; Accepted 16 July 2019
Available online 18 July 2019
0167-7012/

Table 1
Primers for isothermal amplification specific to *Mycobacterim marinum* complex.

Primers	Sequence	Conc. (μM)
Mm-MrsA -FIP	CGCCGCCTGAACCGAAGGTATCAACGTCCAGGTCACCGA	1.6
Mm-MrsA -F3	GATGCAAACGTTGCCGC	0.2
Mm-MrsA -BIP	CCGAAACCGAGCTGGGTGACCGACCATCACCCGGATCA	1.6
Mm-MrsA -B3	TGGCCAACCGATGCGC	0.2

Evolution of *Mycobacterium ulcerans*, a highly virulent mycobacterium that can cause a neglected tropical disease (NTD) Buruli ulcer, is closely related to *M. marinum* (Stinear et al., 2007). Genomic comparison between the two species revealed that their identity as greater than 98% (Stinear et al., 2007), although the virulence of *M. ulcerans* is striking. Ancestral species isolated from other hosts, such as *M. liflandii* from frogs (Fremont-Rahl et al., 2011; Mve-Obiang et al., 2005; Suykerbuyk et al., 2007) and *M. pseudoshottsii* (Nakanaga et al., 2012; Rhodes et al., 2005) from cultured fish, are branched on the evolutionary process from *M. marinum* to *M. ulcerans* (Yip et al., 2007). These species have been regarded as a tight complex (*M. marinum* complex) because of their genomic similarity. *M. ulcerans* and these animal-related subspecies possess a large plasmid encoding pathogenic genes related to synthesis of mycolactone (Mve-Obiang et al., 2005; Stinear et al., 2007), a toxin causing effective suppression of host immunity (Adusumilli et al., 2005; Coutanceau et al., 2005). Reportedly, some clinical strains of *M. marinum* also possess mycolactone producing genes on their plasmids, which shows atypical necrotic lesion without granuloma (Li et al., 2017), partly because of immunosurppression by the toxin.

In contrast to deep concentration on establishment of the methods, used to date, for the molecular detection and diagnosis of *M. ulcerans*, genetic diagnosis systems for non-mycolactone *M. marinum* have been neglected. Efficient and inclusive methods to detect the entire *M. marinum* complex including mycolactone negative strains have not been established, although they can also cause fish or human skin infection. To detect *M. ulcerans* specifically, mycolactone-producing genes *mls*A and *mls*B have been targeted for PCR amplification (Fyfe et al., 2007; Mve-Obiang et al., 2005). In addition to the genes, two transposons (IS*2404* and IS*2606*) are useful as more sensitive targets for detection of *M. ulcerans* (Fyfe et al., 2007; Stinear et al., 1999), but they are less specific because of other mycobacterial species possessing them (Stinear et al., 1999). These targets are highly copied on a genome along with evolution to *M. ulcerans* (Fyfe et al., 2007), which increases sensitivity of the system. For detection of mycolactone-negative *M. marinum* complex, it is still necessary to find an appropriate target for molecular detection.

This report describes our study of an isothermal amplification assay for detection of *M. marinum* complex with high sensitivity and specificity, irrespective of mycolactone productivity. The amplified region of *M. ulcerans* is readily distinguishable from those of other species, based on a restriction site polymorphism within the amplified fragment. The ingenious amplification will provide us with a convenient tool for surveillance of *M. marinum* complex to scrutinize its contamination on fish industry production lines, aquariums, lesions such as granuloma, and water environments where humans can get access.

2. Material and methods

2.1. Design of primers for specific isothermal amplification of DNA fragments of the M. marinum complex

For this study, *mrs*A gene was set as the amplification target. The restriction enzyme sites in the gene where *M. ulcerans* is distinguishable were searched using the Restriction Mapper website (*http://www.restrictionmapper.org/*). A software program (LAMP Primer Explorer ver. 4; *http://primerexplorer.jp/e/v4_manual/index.html*) was used to design primers for amplification of DNA fragments including the restriction site.

2.2. Preparation of DNAs for sensitivity check

Template genome DNA was purified from *M. marinum* ATCC00927 colonies on Ogawa KY Media (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan) as described previously (Belisle and Sonnenberg, 1998). Purified DNA was quantified by Qubit 3.0 and was diluted with DNA-free water. The copy number (n) was calculated from the following equation with its approximate genome size (6.5 Mb) and molecular weight per nucleotide pair (660 Da).

$$\text{DNA weight (g)} = [(6.5\ \text{M} \times 660)/N_A] \times n \ (\text{therein}, N_A = \text{Avogadro constant}, 6.02 \times 10^{23})$$

2.3. Isothermal amplification

To amplify DNA fragments isothermally, we used Bst 2.0 DNA polymerase (New England Biolabs Inc., Ipswich, MA, USA). The condition followed its instruction. Briefly, for Bst 2.0 DNA polymerase, 0.32 U was used in a reaction (25 μL) with 2.5 μL of 10× Isothermal Amplification Buffer (New England Biolabs Inc.), 3.5 μL of 10 mM dNTPs, and 1.5 μL of 100 mM of $MgSO_4$. Primers concentrations are shown in Table 1. Reaction tubes were incubated at a constant temperature using a generic thermal cycler to amplify the targeted DNA fragment.

2.4. Detection of amplified DNA

Amplified DNA was checked using electrophoresis or direct visual inspection of fluorescence. Electrophoresis was done using 2.0% SeaKem LE Agarose gel (Lonza Rockland Inc., Rockland, ME, USA) in 1× TAE buffer, with submarine type system. For direct visual inspection, SYBR Green I (Thermo Fisher Scientific Inc., Waltham, MA, USA) was added to each reaction according to its users manual.

Sensitivity of the isothermal amplification was evaluated (Light Cycler 480; Roche, Ltd., Basel, Switzerland) using Isothermal Master Mix for Genie III (OptiGene, Ltd., Horsham, West Sussex, UK). The primer concentration and temperature were adjusted with the conditions described above. The detection format was set as SYBR Green I (465 nm as excitation, 410 nm as detection).

2.5. Restriction of DNA fragments

Amplified DNA was cut by a restriction enzyme (*Not* I; New England Biolabs Inc.) to identify whether the fragment was amplified from *M. ulcerans* or another species of the *M. marinum* complex. After being purified by phenol/chloroform purification and ethanol precipitation, amplified DNA fragments were incubated with 10 U of *Not* I at 37 °C for 6 h. After reaction, agarose gel (2.0%) electrophoresis was done in 1× TAE buffer, as described above.

Table 2
Specificity of isothermal amplification of *M. marinum* complex verified in this study.

Species[a]	Strain name	Source	Isothermal amplification	*Not* I digestion
Mycobacterium marinum	ATCC00927	Fish, Philadelphia	+	+
M. pseudoshottsii	JCM15466	Striped bass, USA	+	+
M. ulcerans	ATCC19423	Human, Australia	+	−
M. marinum	AOD100104	Grouper, Taiwan	+	+
M. marinum	AOD99182	Guppy, Taiwan	+	+
M. marinum	AOD99163	Africa Lampeye, Taiwan	+	+
M. marinum	AOD99066	Altum angelfish, Taiwan	+	+
M. marinum	AOD99066-W	Pool water of Altum angelfish, Taiwan	+	+
M. marinum	AOD100161	Paradise fish, Taiwan	+	+
M. marinum	AOD100011	Galaxy Rasbora, Taiwan	+	+
M. ulcerans	AOD96005	Bullfrog, Taiwan	+	−
Nocardia seriolae	BCRC 13745	n. d.	−	ND[b]
N. farcinica	BCRC 13722	n. d.	−	ND
N. salmonicida	BCRC 12441	n. d.	−	ND
Lactococcus garvieae	ATCC 43921	n. d.	−	ND
Streptococcus dysgalactiae	BCRC 12577	n. d.	−	ND
Streptococcus pyogenes	BCRC 10797	n. d.	−	ND
Enterococcus faecalis	BCRC 10789	n. d.	−	ND
Staphylococcus aureus	BCRC 10781	n. d.	−	ND
Chryseobacterium meningosepticum	BCRC 10677	n. d.	−	ND
Aeromonas hydrophila subsp. hydrophila	BCRC 13018	n. d.	−	ND
Edwardsiella tarda	BCRC 10670	n. d.	−	ND
Flavobacterium sp.	BCRC 10098	n. d.	−	ND
Vibrio parahaemolyticus	BCRC 12864	n. d.	−	ND

[a] Other Mycobacterial species are listed in Table S1.
[b] Not determined.

2.6. Preparation of DNAs for specificity check

All control mycobacterial strains were cultured on Ogawa media at respectively appropriate temperatures aerobically for sufficient culture periods: seven days for rapid growers and four weeks for slow growers (Table S1). After culturing, the colonies were suspended in 200 μL of TE and were heat-killed in boiling water for 20 min. Supernatants were used for DNA templates.

M. marinum and *M. ulcerans* strains isolated originally in this study (Table 2) were secondarily cultured on Lowenstein Jensen Slant (Creative Microbiologicals, Ltd., Taipei, Taiwan) at 25 °C for up to 7 days. Their DNAs were prepared following the methods developed by Telenti et al. (1993). They were verified by the 16S rRNA direct sequencing. PCR was conducted using forward primer 5F (TGGAGAGTT TCCTGGCTCAG) and reverse primer 1540R (AAGGAGGTGATCCARC-CGCA), according to the original method (Edwards et al., 1989).

Most of other bacteria, *Lactococcus garvieae*, *Streptococcus dysgalactiae*, *Streptococcus pyogenes, Enterococcus faecalis*, *Staphylococcus aureus*, *Chryseobacterium meningosepticum*, *Aeromonas hydrophila* subsp. *hydrophila, Edwardsiella tarda, Flavobacterium sp.*, *and Vibrio parahaemolyticus* (Table 2), were grown on brain and heart infusion broth (BHI) medium at 25 °C. Overnight cultures (3 mL) were isolated by phenol/chloroform extraction followed by precipitation with isopropanol. The DNA samples of *Nocardia* sp. were prepared using the same protocol as that used for mycobacterial strains (Telenti et al., 1993). These negative control species were mainly selected from various common fish bacterial pathogens, on the assumption that this system would be used to confirm clinical applications in aquaculture.

3. Results

3.1. Amplification of DNA fragment of M. marinum complex by isothermal condition

To establish an easy, sensitive, and specific detection system for *M. marinum* complex using isothermal DNA amplification, *mrs*A gene was selected as a candidate. This gene is useful to discriminate *M. ulcerans*, a severe etiological agent of humans, and other *M. marinum* complex by difference of the existence of the restriction site of *Not* I, except for *M. ulcerans* subsp. *shinshuense* (Fig. 1). To amplify DNA fragments of *mrs*A including the restriction site, four primers for isothermal amplification were designed (Table 1).

Using control DNA templates of *M. marinum* and *M. ulcerans*, amplification was observed sufficiently with a typical condition: at 60 °C or 65 °C for over 45 min (Fig. 2). When digested by *Not* I, ladder fragments from *M. marinum* of amplified DNA had disappeared but those from *M. ulcerans* were stable (Fig. 3). This result is consistent with mutation of the digestion site of *M. ulcerans*, as presented in Fig. 1.

3.2. Sensitivity and specificity

To estimate the sensitivity, serial dilution series of purified genome DNA of *M. marinum* were prepared and used for the isothermal amplification (Fig. 4). As a result, the limitation of detection was ascertained as 50–500 fg, which corresponds to approximately 7–70 copies of template DNA. The amplification was reached at the maximum in less than 35 min reaction even with the lowest concentration (500 fg of genomic DNA template) showing positive fluorescence.

Specificity was verified using supernatants of heat-killed bacilli of various mycobacterial species and other genus bacteria (Table 2 and S1). Only *M. marinum* complex species (*M. marinum*, *M. pseudoshottsii*, and *M. ulcerans*) showed positive amplification: other species represented no amplification strictly.

4. Discussion

Our results suggest that DNA detection with both high sensitivity and specificity for the *M. marinum* complex is applicable for various surveillance tasks: fish aquaculturing, ecological, and clinical purposes. The species complex possesses characteristics as a zoonotic agent with difficulty of culturing, which indicates the importance of technical improvement of molecular surveillance for targets of various kinds. Among the complex species, *M. ulcerans* has been regarded as the most important target because of its high virulence with human. The species is readily apparent in high copy numbers of IS2404 and IS2606 in the genome, which has been used for the amplification target (Fyfe et al.,

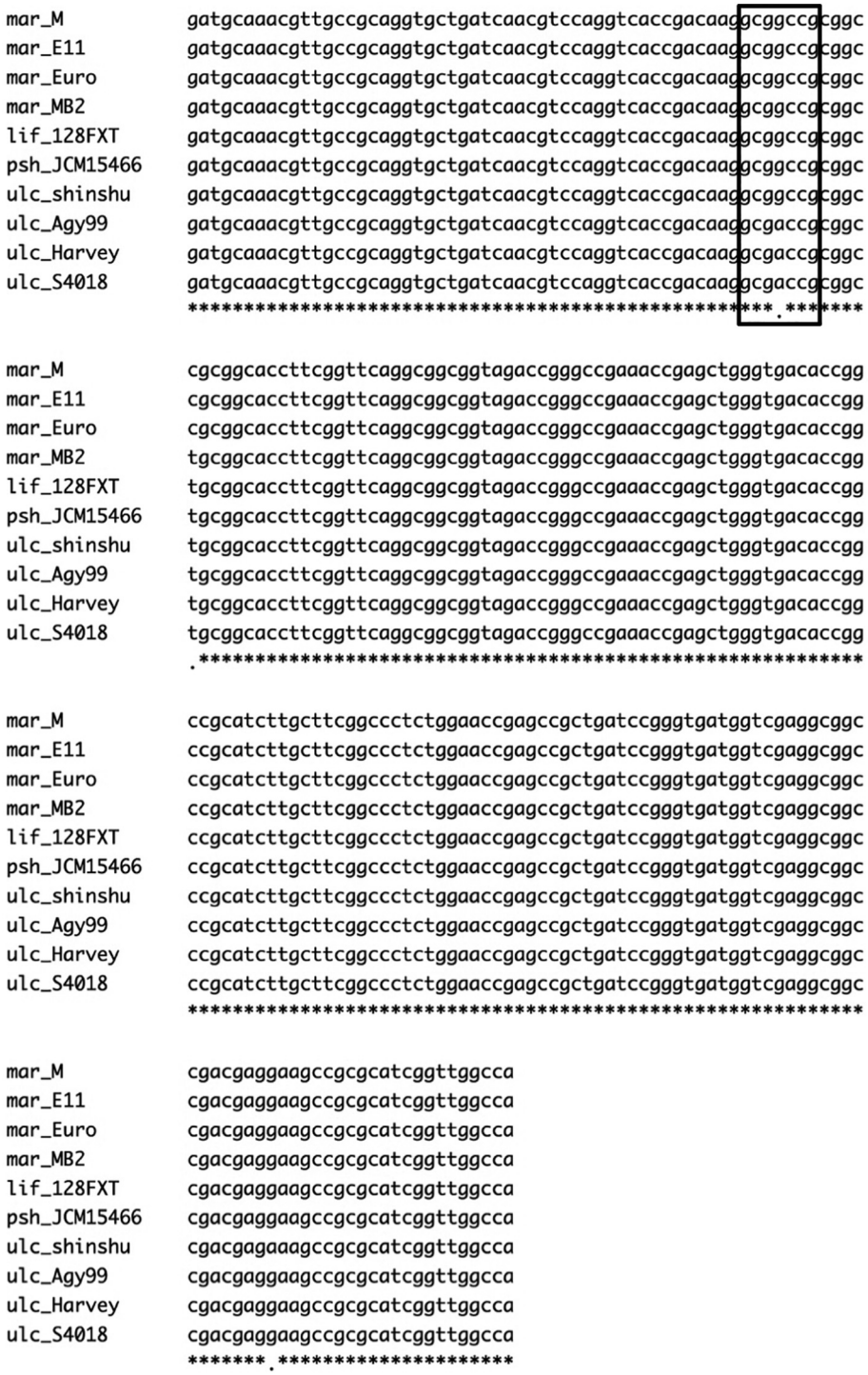

Fig. 1. Alignment of the amplified region of DNA sequences of *M. marinum* complex (*M. marinum*: M (retrieved from NC_010612.1), E11 (NZ_HG917972.2), and Europe (NZ_ANPL00000000.1), *M. liflandii* 128FXT (NC_020133.1), *M. pseudoshottsii* JCM15466 (NZ_BCND00000000.1), and *M. ulcerans*: subsp. shinshuense (NZ_AP017624.1), Agy99 (NC_008611.1), Harvey (JAOL00000000.1), and S4018 (NZ_MDUB00000000.1)) in this study. The restriction site used for distinguishing them is boxed.

2007; Stinear et al., 1999). WHO recommends a conventional PCR for IS2404 as a diagnostic method of Buruli ulcer (WHO, 2001). Earlier reports describe the use of IS2606 for a target to classify *M. marinum* (no or low copy number) and *M. ulcerans* (high copy number) (Fyfe et al., 2007). Such mobile elements can be sensitive targets because of their high copies in a genome, but it is notable that it is less specific because of their horizontal transmissibility. For instance, reportedly, IS2606 was detected from a different mycobacterial species *M. lentiflavum* (Stinear et al., 1999). It can reduce the reliability of detection of *M. marinum* complex by these mobile elements, especially in surveillance of environmental or fish/animals. To avoid possible low specificity, repetitive domains of mycolactone-producing gene *mls*AB were used as realtime PCR targets in some studies (Fyfe et al., 2007; Williamson et al., 2008). For the present study, we used a single copy gene, *mrs*A, specific to *M. marinum* complex, which provided high specificity with the isothermal amplification system (Table 2 and S1).

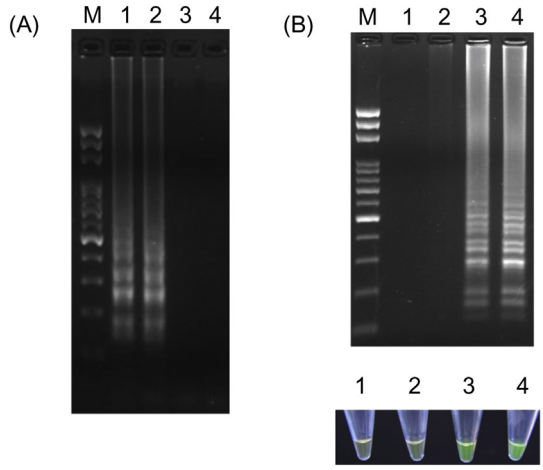

Fig. 2. Optimization of amplification conditions. (A) Determination of temperature conditions. Lane M, 100 bp ladder. Lanes 1–4 conducted for 60 min: Lane 1, 65 °C; Lane 2, 60 °C; Lane 3, 54.5 °C; Lane 4, 52 °C. (B) Determination of the time condition at the optimized temperature (60 °C). The reaction was examined using electrophoresis (upper) and visual inspection by SYBR Green I (lower). 1, 15 min; 2, 30 min; 3, 45 min; and 4, 60 min. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

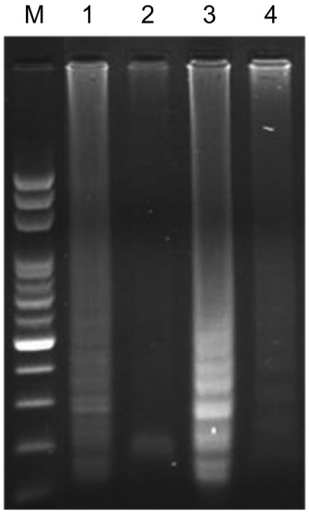

Fig. 3. Restriction analysis of amplified DNA fragments by isothermal amplification: Lane M, 100-bp ladder marker; Lanes 1 and 3, Amplified DNA from *M. marinum* strain AOD99017 and *M. ulcerans* strain AOD96005; Lanes 2 and 4, Amplified DNA from each species after digestion with *Not* I.

As a limitation of this study, we could not include field-sampling study to validate the method thoroughly. However, isothermal amplification with high specificity will be beneficial for use out of the laboratory, which might be suitable for screening tests in ecological field sciences or aquaculture needs.

Genetic descriptions of the ecology and diversity of *M. marinum* remain inadequate because of its lesser virulence, although it can indeed cause aquaculture difficulties and zoonotic infection with humans. Broad genetic diversity of *M. marinum* can be inferred from results of earlier studies (Yip et al., 2007). As described in this report, there is dispersed relatedness among nine sequence types of strains isolated from clinical cases (human), environmental water, and other animals. This result indicates the importance of isolation of *M. marinum* in various resources to elucidate its entire features of genetic diversity. Because of difficulty of culturing mycobacteria from crude samples, a genetic detection system with high specificity is expected to be

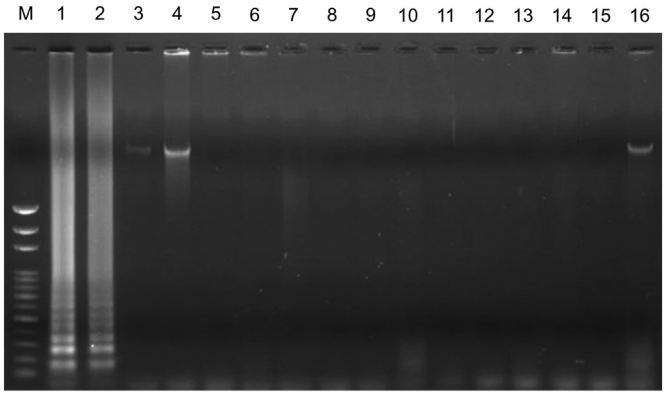

Fig. 4. Specificity of isothermal amplification for *M. marinum* complex was examined using agarose gel electrophoresis: Lane M, 100-bp ladder; Lanes 1 and 2, *M. marinum* isolated from infected fish; Lane 3, *M. gordonae* ATCC 14470; Lane 4, *Nocardia seriolae* BCRC 13745; Lane 5, *N. farcinica* BCRC 13722; Lane 6, *N. salmonicida* BCRC 12441; Lane 7, *Lactococcus garvieae* ATCC 45921; Lane 8, *Chryseobacterium meningosepticum* BCRC 10677; Lane 9, *Streptococcus dysgalactiae* BCRC 12577; Lane 10, *Enterococcus faecalis* BCRC 10789; Lane 11, *Staphylococcus aureus* BCRC 10781; Lane 12, *Streptococcus pyogenes* BCRC 10797; Lane 13, *Aeromonas hydrophila* BCRC 13018; Lane 14, *Edwardsiella tarda* BCRC 10670; Lane 15, *Flavobacterium* sp. BCRC 10098; Lane 16, *Vibrio parahaemolyticus* BCRC 12864.

necessary for ecological surveillance of this species. It is noteworthy that our isothermal amplification is the first system able to detect *M. marinum* complex irrespective of the existence of mycolactone-producing plasmids. This feature provides an efficient and useful means of detecting the bacterial species complex with broad diversity.

In addition to specificity, sensitive detection is important for ecological studies of bacteria, because bacterial concentration is not always high under environmental conditions, which might be inappropriate for their proliferation. Our isothermal system provides high sensitivity (7–70 copies/reaction, Fig. 4), which was indeed less sensitive than earlier PCR methods targeting to high-copy sites of the *M. marinum* complex (Fyfe et al., 2007). The target of our detection system was a chromosomal gene *mrs*A, only one copy of which was harbored by a genome, leading to the difference. Generally, water samples can be concentrated by filtration and purification of DNA. For instance, Williamson et al. (2008) filtered 100–200 mL of environmental water samples to purify DNA for surveillance of *M. ulcerans*. DNA samples are purified into 10–100 μL by commercial kits: the water samples are thereby concentrated 1000–10,000 times. For our isothermal amplification (such as normal DNA amplification protocols), which can detect about 7–70 copies of DNA successfully in a reaction, DNA samples of several microliters are used. Our rough estimation suggests that at least several bacilli of *M. marinum* complex should exist in 1 mL of a water sample. The minimum detection limit should be examined when the method is applied to future studies.

It is noteworthy that *M. ulcerans* subsp. *shinshuense* could not been determined based on the restriction polymorphism as other *M. ulcerans* strains were (Fig. 1). The restriction site of *mrs*A of the subspecies belonged to the intact allele, corresponding to the other *M. marinum* complex species. The subspecies has been identified uniquely from endemic cases in Japan (Nakanaga et al., 2011; Yotsu et al., 2012). Although the demographic history has remined entirely unknown, *M. ulcerans* subsp. *shinshuense* was regarded as the earliest divergent from the common ancestor of the species phylogenetically (Yip et al., 2007). Such phylogenetic background suggests that the subspecies separated from typical *M. ulcerans* strains, such as Agy99, Harvey, and S4018 (mainly isolated from Africa and Australia), before the polymorphism had occurred. Yip et al. (2007) showed that strains isolated in Mexico also diverged at an early stage, which presents the possibility that endemic lineages of *M. ulcerans* might be misassigned by the restriction

pattern of *mrs*A.

Based on LAMP assay, immediate control measures can be undertaken before the onset of infection and financial losses in aquaculture industry caused by *M. marinum* complex. Fish mycobacteriosis, also termed piscine tuberculosis, was first isolated from freshwater carp (Bataillon et al., 1897); over 150 fish species have been diagnosed as having mycobacteriosis to date. Fish inhabiting either freshwater, brackish water or seawater as well as temporal or tropical areas are potentially susceptible to mycobacterial infections (Talaat et al., 1998). Especially *M. marinum* is the most common kind of mycobacterium species of fish (Kusar et al., 2017; Sirimalaisuwan et al., 2017). Not only does it threaten fish of economic importance; it also exhibits a potent etiological agent for atypical tuberculosis in humans (Bonamonte et al., 2016). Those who are frequently exposed to contaminated water, such as fishery workers, fish fanciers, fish dealers, fish farmers, and pond cleaners, are susceptible to the disease especially when their immune function is low (Barker et al., 1997; Decostere et al., 2004). Aquarium hobbyists often obtain their fish of interest via field collection, peer exchange, or trading. Fish kept in captivity or with poor water quality are readily affected adversely by stress. Consequently, their immunity is depressed with accompanying ill management: both are causative factors of mycobacteriosis (Gauthier and Rhodes, 2009). The international trade that has prospered to date has made ornamental fish into a growing business, driving endemic fish species to become invasive species in other countries. Unpredictable risks might arise from effects on the immigrant ecosystem, and invisibly, from the unknown pathogens they harbor (Passantino et al., 2008). Emaciation, poor growth, skin ulcers, and fin erosion might not be the only clinical signs of mycobacteriosis; granulomatous symptoms might be present on the internal organs that include the peritoneum, spleen, kidney, liver, and intestines in diseased fish (Gauthier and Rhodes, 2009). A detection method with rapidity, specificity, and sensitivity for fish mycobacteriosis is anticipated as an efficient procedure to reduce not only zoonotic incidence of the infection of humans, but also industrial damage by preventing the spread of the agents.

Funding information

This work was supported by Japan Society for the Promotion Science (JSPS) KAKENHI Grant Number 15H05263.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.105671.

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