Journal of Microbiological Methods 132 (2017) 41–45

Contents lists available at ScienceDirect

Journal of Microbiological Methods

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

Enhancing melting curve analysis for the discrimination of loop-mediated isothermal amplification products from four pathogenic molds: Use of inorganic pyrophosphatase and its effect in reducing the variance in melting temperature values

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ARTICLE INFO

Article history:
Received 7 October 2016
Received in revised form 26 October 2016
Accepted 26 October 2016
Available online 27 October 2016

Keywords:
Loop-mediated isothermal amplification
Melting curve analysis
Thermostable inorganic pyrophosphatase
Aspergillus fumigatus
Penicillium expansum
Penicillium marneffei
Histoplasma capsulatum

ABSTRACT

Loop-mediated isothermal amplification (LAMP) is widely used for differentiating causative agents in infectious diseases. Melting curve analysis (MCA) in conjunction with the LAMP method reduces both the labor required to conduct an assay and contamination of the products. However, two factors influence the melting temperature (Tm) of LAMP products: an inconsistent concentration of Mg^{2+} ion due to the precipitation of $Mg_2P_2O_7$, and the guanine-cytosine (GC) content of the starting dumbbell-like structure. In this study, we investigated the influence of inorganic pyrophosphatase (PPase), an enzyme that inhibits the production of $Mg_2P_2O_7$, on the Tm of LAMP products, and examined the correlation between the above factors and the Tm value using MCA. A set of LAMP primers that amplify the ribosomal DNA of the large subunit of *Aspergillus fumigatus*, *Penicillium expansum*, *Penicillium marneffei*, and *Histoplasma capsulatum* was designed, and the LAMP reaction was performed using serial concentrations of these fungal genomic DNAs as templates in the presence and absence of PPase. We compared the Tm values obtained from the PPase-free group and the PPase-containing group, and the relationship between the GC content of the theoretical starting dumbbell-like structure and the Tm values of the LAMP product from each fungus was analyzed. The range of Tm values obtained for several fungi overlapped in the PPase-free group. In contrast, in the PPase-containing group, the variance in Tm values was smaller and there was no overlap in the Tm values obtained for all fungi tested: the LAMP product of each fungus had a specific Tm value, and the average Tm value increased as the GC% of the starting dumbbell-like structure increased. The use of PPase therefore reduced the variance in the Tm value and allowed the differentiation of these pathogenic fungi using the MCA method.

1. Introduction

Melting curve analysis (MCA) is an approach for assessing the dissociation properties of nucleic acid amplicons based on their variable thermal stability. Thermal stability is generally determined by the size of the amplicon, its guanine-cytosine (GC) content, and the solvent conditions, including pH and the concentration of Na^+ and Mg^{2+} (Ririe et al., 1997; Breslauer et al., 1986; Steger, 1994; Hartwig, 2001; Owczarzy et al., 2008). This method does not require electrophoresis: all reactions are conducted in a closed test tube, making this a clear, rapid and useful method for differentiating specific amplicons. MCA has been used in many laboratories to differentiation pathogenic agents and nonspecific products (such as primer dimers), mainly for use in PCR products (Shrestha et al., 2003; Bu et al., 2005).

Loop-mediated isothermal amplification (LAMP) is a rapid and highly sensitive DNA amplification method that does not require a thermal cycling process (Notomi et al., 2000; Mori and Notomi, 2009). The application of MCA to the LAMP method is desirable, just as it is for PCR methods (Njiru et al., 2008; Yamamura et al., 2009; Lenarcic et al., 2014). We previously reported that MCA may be useful for differentiating pathogenic fungal-specific products and primer dimers in the LAMP method (Uemura et al., 2008), but the melting temperature (Tm) of LAMP products generated using commonly used LAMP reaction mixtures is insufficiently reproducible to differentiate specific products produced in a given reaction. There is therefore a need to identify new

Abbreviations: LAMP, loop-mediated isothermal amplification; PCR, polymerase chain reaction; MCA, melting curve analysis; PPase, thermostable inorganic pyrophosphatase; Tm, melting temperature; Afum, *Aspergillus fumigatus*; Pexp, *Penicillium expansum*; Pmar, *Penicillium marneffei*; Hcap, *Histoplasma capsulatum*.

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http://dx.doi.org/10.1016/j.mimet.2016.10.020
0167-7012/

reaction conditions that provide stable and specific Tm values for each LAMP product generated from different fungal pathogens. The Mg^{2+} concentration greatly influences Tm and varies between LAMP reaction mixtures because magnesium pyrophosphate ($Mg_2P_2O_7$) is produced as a byproduct during DNA amplification (Mori et al., 2001). In the present research, we demonstrate the effect of thermostable inorganic pyrophosphatase (PPase), which inhibits the production of $Mg_2P_2O_7$, in obtaining stable Tm values and allowing the differentiation of LAMP products. Moreover, it is very difficult to predict the Tm values of LAMP products synthesized by strand displacement DNA polymerase reactions that continuously synthesize variable-sized products (Notomi et al., 2000), in contrast to PCR, which basically generates same-sized products with predictable GC content.

Previous reports provide evidence that all LAMP products have inverted-repeated sequences, and that these sequences may form dumbbell-like structures (Notomi et al., 2000; Tomita et al., 2008). We speculated that there is a correlation between the Tm value and GC content of the starting dumbbell-like structure, in which case we would be able to predict the Tm values of variable sized and complex LAMP products from their GC content. Accordingly, herein we examined the correlation between the Tm values of LAMP products and the GC content of these theoretical dumbbell-like structures.

2. Materials and methods

2.1. Tested fungal strains and DNA extraction

The following pathogenic molds were used as model fungi: *Aspergillus fumigatus*, a common and important pathogen in deep-seated mycosis, and *Penicillium expansum, Penicillium marneffei,* and *Histoplasma capsulatum*, which often cause cross reactions against *Aspergillus* spp. in PCR or immunological assays (Hope et al., 2005; Huang et al., 2007; Wheat et al., 2007; Lass-Florl et al., 2011). In this study, *A. fumigatus* JCM 10253, *P. expansum* TIMM 1293, *P. marneffei* TIMM 6206, and *H. capsulatum* TIMM 1846 were tested. All fungal strains were obtained from the culture collections of the Japan Culture of Microorganisms (Ibaraki, Japan), Teikyo Institute of Medical Mycology (Tokyo, Japan). The fungal DNAs were extracted using the phenol-chloroform method (Makimura et al., 1998). The templates were diluted with distilled water to provide identical salt concentrations in the samples.

2.2. LAMP primers

A LAMP primer set that can amplify the test fungal DNA extracts was designed based on the ribosomal DNA large subunit sequences obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/nucleotide/). The LAMP primer sequences used were: EO-F3 (forward outer primer): AAGAGCTCAAATTTGAAAGCTG, EO-B3 (backward outer primer): CGGCCAGTATTTAGCTTTAGATG, EO-FIP (forward inner primer, F1c + F2): GTCCCAGACGGGATTCTCACTTGTAATTTGCAGAGGATGCTT, EO-BIP (backward inner primer, B1c + B2): TGCGTCCGTGTGAAGCTCACCCATTTAGAGCTGCATT, EO-LF (forward loop primer): CGTTCCAGGGCACTTAGAC, and EO-LB (backward loop primer): CGACGAGTCGAGTTGTTTGG. This primer set was used for all tested fungi in this study.

2.3. Sequences of the tested fungi and analysis of the LAMP dumbbell-like structures

Sequencing was performed with a 3130xl Genetic Analyzer (Applied Biosystems) using the universal primers 28SF1 and 635 for fungal ribosomal DNA large subunits (Sugita et al., 2003). Multiple sequence alignment analysis was performed with GENETYX Ver.12 software (GENETYX Co., Ltd., Tokyo, Japan). The LAMP primer set was designed by designating six distinct regions on the target DNA as F3, F2, F1, B1c, B2c, and B3c, starting from the 5′ end, where 'c' represents a complementary sequence. A schematic diagram of the starting LAMP dumbbell–like structure is shown in Fig. 1 and is based on previous reports (Notomi et al., 2000; Tomita et al., 2008) and the manufacturer's website (http://loopamp.eiken.co.jp/e/lamp/principle.html). The dumbbell-like structure is constructed from a BIP primer sequence, a unique sequence, and the complementary sequence of the FIP primer (FIPc) (Fig. 1). The outer regions beyond F2c and B2 are removed during the folding process and thus the dumbbell-like structure does not contain the F3c and B3 regions (Notomi et al., 2000; Tomita et al., 2008). The theoretical LAMP dumbbell-like structure and the sequencing results depicted in Figs. 1 and 2 were used to calculate the sizes and GC content of the structures.

2.4. LAMP assay melting curve analysis (MCA)

LAMP assays were conducted in the presence and absence of PPase in a total reaction volume of 25 μL. The PPase-free reaction mixture comprised 12.5 μL of 2× reaction mixture (40 mM Tris-HCl, pH 8.8, 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2% Tween 20, 1.6 M, betaine, 2.8 mM each dNTP, 1 μL of *Bst* DNA polymerase (Loopamp DNA amplification kit, Eiken Chemical Co. Ltd., Tochigi, Japan), 40 pmol EO-FIP, 40 pmol EO-BIP, 20 pmol EO-LF, 20 pmol EO-LB, 5 pmol EO-F3, 5 pmol EO-B3 (Eurofins Genomics, Tokyo, Japan), 0.5 μg YO-PRO®-1 mL^{-1} (propidium dye; Life Technologies, Gaithersburg, MD.), 6.8 μL of distilled water, and 2 μL of template DNA. The PPase-containing reaction mixture had the same composition except that 0.8 U/mL of PPase (New England Biolabs Japan Inc., Tokyo, Japan) diluted to 6.8 μL with distilled water was added. Template DNA (2 μL) was added to the reaction mixtures, and the reactions were conducted using a Thermal Cycler Dice® (Takara, Kyoto, Japan). Fluorescence was observed using the 6-carboxyfluorescein (FAM) fluorescence channel. The thermal program consisted of a DNA amplification step (65 °C for 90 min) and a melting curve step (15 s at 95 °C, 30 s at 60 °C, and 15 s at 95 °C). The LAMP reaction was performed in quintuplicate using serial dilutions of genomic DNA (1 ng, 100 pg, 10 pg, and 1 pg/μL) as templates. MCA was performed for each LAMP product.

2.5. Statistical analysis

All statistical analyses were performed with GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA). Data are shown as the mean (±2SD) taken from all Tm values for each fungus. Welch's *t*-test was used to compare two data sets of the mean Tm values for each fungus. *F*-test was used to compare the variances of Tm values between the PPase-free group and the PPase-containing group for each fungus. A p value <0.01 was considered significant.

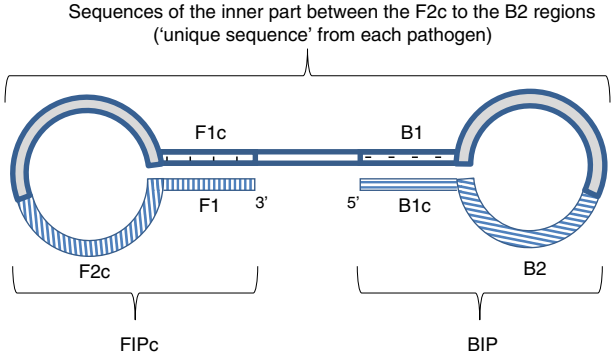

Fig. 1. Theoretical LAMP starting dumbbell-like structure. The sequences of the inner part between the F2c to the B2 regions, indicated by the bolded section, are designated as 'unique sequences' from each pathogen. The dumbbell-like structure is constructed from a BIP primer sequence, a unique sequence, and the complementary sequence of the FIP primer (FIPc).