International Journal of Food Microbiology 178 (2014) 107–112

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Simple and rapid method for the detection of *Filobasidiella neoformans* in a probiotic dairy product by using loop-mediated isothermal amplification

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ARTICLE INFO

Article history:
Received 12 November 2013
Received in revised form 21 February 2014
Accepted 6 March 2014
Available online 16 March 2014

Keywords:
Rapid detection
Filobasidiella neoformans
Loop-mediated isothermal amplification (LAMP)
rDNA internal transcribed spacer 2 (ITS2)
Dairy product
Rapid DNA extraction

ABSTRACT

Yeast contamination is a serious problem in the food industry and a major cause of food spoilage. Several yeasts, such as *Filobasidiella neoformans*, which cause cryptococcosis in humans, are also opportunistic pathogens, so a simple and rapid method for monitoring yeast contamination in food is essential. Here, we developed a simple and rapid method that utilizes loop-mediated isothermal amplification (LAMP) for the detection of *F. neoformans*. A set of five specific LAMP primers was designed that targeted the 5.8S–26S rDNA internal transcribed spacer 2 region of *F. neoformans*, and the primer set's specificity was confirmed. In a pure culture of *F. neoformans*, the LAMP assay had a lower sensitivity threshold of 10^2 cells/mL at a runtime of 60 min. In a probiotic dairy product artificially contaminated with *F. neoformans*, the LAMP assay also had a lower sensitivity threshold of 10^2 cells/mL, which was comparable to the sensitivity of a quantitative PCR (qPCR) assay. We also developed a simple two-step method for the extraction of DNA from a probiotic dairy product that can be performed within 15 min. This method involves initial protease treatment of the test sample at 45 °C for 3 min followed by boiling at 100 °C for 5 min under alkaline conditions. In a probiotic dairy product artificially contaminated with *F. neoformans*, analysis by means of our novel DNA extraction method followed by LAMP with our specific primer set had a lower sensitivity threshold of 10^3 cells/mL at a runtime of 60 min. In contrast, use of our novel method of DNA extraction followed by qPCR assay had a lower sensitivity threshold of only 10^5 cells/mL at a runtime of 3 to 4 h. Therefore, unlike the PCR assay, our LAMP assay can be used to quickly evaluate yeast contamination and is sensitive even for crude samples containing bacteria or background impurities. Our study provides a powerful tool for the primary screening of large numbers of food samples for yeast contamination.

1. Introduction

Yeasts are important contaminants in the food industry that cause food spoilage and produce unpleasant smells and carbon dioxide gas. Unlike bacteria, yeasts can grow at low pH, low temperatures, and low water activities. Some species of yeast are also opportunistic pathogens in humans (Barnett, 2008; Fleet and Balia, 2006; Walsh et al., 2004). *Filobasidiella neoformans* (anamorph: *Cryptococcus neoformans*) is a yeast that is capable of infecting healthy persons and causes disseminated pulmonary cryptococcosis or cryptococcal meningitis (Casadevall and Perfect, 1998; Fleet, 2007; Perfect and Casadevall, 2002). Therefore, accurate and rapid methods for the detection of yeasts, especially those that are opportunistic pathogens in humans, are needed to ensure product safety during the early stages of food production.

Traditional culture-based methods for identifying yeast contamination remain in widespread use in the food industry despite the development of molecular technologies. Although effective, culture-based methods are extremely laborious and time-consuming, and they may not detect viable but unculturable cells present in food samples (Millet and Lonvaud-Funel, 2000). Culture-based methods are also unsuitable for identifying yeasts that are considered emerging opportunistic pathogens, because those methods do not always correspond to the current taxonomy. García et al. (2004) reported an enzyme-linked immunosorbent assay that is able to detect yeasts in contaminated yogurt at a concentration of 10^5 cfu/mL. Furthermore, advances in molecular techniques now enable the detection of yeasts based on matrix-assisted laser desorption ionization-time of flight mass spectrometry (Marklein et al., 2009) and Fourier-transform infrared microspectroscopy (Wenning et al., 2002), and nucleic acid amplification techniques are in widespread use for the detection and identification of a variety of yeast species (Casey and Dobson, 2003; Gray et al., 2011; Salinas et al., 2009). These methods are relatively quick to perform compared with traditional culture-based methods.

Because fermented milk spoils relatively quickly, methods for the rapid detection and enumeration of yeast contaminants are essential. Mayoral et al. (2006) reported a reverse transcriptase-polymerase chain reaction (RT-PCR) assay that is able to detect viable *Kluyveromyces*

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http://dx.doi.org/10.1016/j.ijfoodmicro.2014.03.008
0168-1605/

marxianus in contaminated yogurt at concentrations of 10^2 cfu/mL. More recently, Makino et al. (2010) reported a real-time quantitative PCR (qPCR) assay that was able to detect nine opportunistic yeast species in dairy products at concentrations between 10^1 and 10^2 cells/mL at runtimes of 4 to 5 h. However, it is difficult to analyze PCR products by means of electrophoresis or to use qPCR systems during food manufacture because monitoring for yeast contamination is conducted on a daily basis. Therefore, simple, rapid methods are preferable for the preparation of samples for nucleic acid amplification and routine assay.

In 2000, Notomi et al. (2000). developed a nucleic acid amplification method called loop-mediated isothermal amplification (LAMP). The LAMP reaction is performed under isothermal conditions with a set of four to six specially designed primers, and specific amplification and detection of target DNA can be completed in a single step without the need for advanced instruments (Mori and Notomi, 2009). Moreover, the sensitivity of the LAMP assay is less affected by the presence of inhibitory substances than are PCR-based assays (Kaneko et al., 2007). LAMP assays have been used to detect pathogenic bacteria and viruses (Itano et al., 2006; Kurosaki et al., 2009; Yamazaki et al., 2008) as well as bacteria and yeasts that cause food contamination (Hayashi et al., 2007; Wang et al., 2012); however, to our knowledge, there are currently no reports of the use of LAMP for the detection of yeasts in dairy products. It is laborious and time-consuming to extract DNA from yeast cells by means of freezing, sonication, or bead beating or by applying biochemical, enzymatic, or organic solvent treatments (Cheng and Jiang, 2006; Karakousis et al., 2006; Watanabe et al., 2010). Therefore, the rapid and simple method for extracting DNA from yeast cells further highlights the advantages of LAMP-based methods.

In the present study, we (i) assessed the suitability of LAMP for the rapid detection of *F. neoformans* in a probiotic dairy product and (ii) developed a simple and rapid method for DNA extraction to improve the LAMP assay system for the dairy industry.

2. Materials and methods

2.1. Microbial strains and culture conditions

The yeast strains used in this study were obtained from the Culture Collection of the Yakult Central Institute for Microbiological Research (Tokyo, Japan) (Table 1). The yeasts were grown in YM broth (BD Difco; Becton, Dickinson and Co., Sparks, MD, USA) for 18 to 48 h at 26 °C.

2.2. Development of an F. neoformans-specific LAMP primer set

Five LAMP primers (Fneo-F3, Fneo-B3, Fneo-FIP, Fneo-BIP, and Fneo-LB) targeting the internal transcribed spacer 2 (ITS2) region of *F. neoformans* ATCC 32045 (GenBank accession no.: EU240005) were designed by using PrimerExplorer V4 (Eiken Chemical Co., Ltd., Tokyo, Japan; https://primerexplorer.jp/lamp4.0.0) (Table 2, Fig. 1).

2.3. DNA extraction

DNA was extracted from yeast samples as described (Makino et al., 2010). Briefly, 2 mL of overnight yeast cultures was pelleted by centrifugation at 20,000 ×*g* for 5 min, and the cell pellet was resuspended in a 250 μL extraction buffer (100 mM Tris–HCl, 40 mM EDTA; pH 9.0). To remove milk fat from yeast cells suspended in the probiotic dairy product, 0.2% (final concentration) sodium hydroxide was added to 1 mL of the product before pelleting and resuspension in extraction buffer as described above. Glass beads (700 mg; diameter, 0.1 mm: Tomy Seiko, Tokyo, Japan) and 500 μL benzyl chloride were added to the suspension, and the mixture was vortexed vigorously for 30 s by using a FastPrep FP120 homogenizer (Bio101, Vista, CA, USA) at a setting of 6.5 m/s. Next, 50 μL of 10% sodium dodecyl sulfate was added to the suspension, and the mixture was vortexed vigorously at 50 °C for 20 min in a microincubator (M-36; Taitec, Tokyo, Japan). Then, 150 μL of 3 M sodium acetate was added, and the mixture was cooled on ice for 3 min. After centrifugation at 20,000 ×*g* for 8 min, the supernatant was collected and DNA was precipitated with isopropyl alcohol. Finally, the DNA

Table 1
Yeast strains tested and LAMP results.

Species	Strain	Original designation	LAMP Tt (min)
Candida albicans	YIT 8272^T	IFO 1385^T	ND
Candida diversa	YIT 8277^T	JCM 1848^T	ND
Candida krusei	YIT 10248^T	JCM 1609^T	ND
Candida dubliniensis	YIT 12348^T	CBS 7987^T	ND
Candida etchellsii	YIT 8278^T	JCM 8066^T	ND
Candida glabrata	YIT 8280^T	JCM 3761^T	ND
Candida intermedia	YIT 12375^T	JCM 1607^T	ND
Candida magnoliae	YIT 12368^T	NBRC 0705^T	ND
Candida metapsilosis	YIT 12411^T	CBS 10907^T	ND
Candida orthopsilosis	YIT 12412^T	CBS 10906^T	ND
Candida parapsilosis	YIT 10333^T	DSM 5784^T	ND
Candida rugosa	YIT 8221^T	IFO 0750^T	ND
Candida stellata	YIT 8285	IFO 0857	ND
Candida tropicalis	YIT 12376^T	JCM 1541^T	ND
Candida versatilis	YIT 8287^T	JCM 8065^T	ND
Candida viswanathii	YIT 12347^T	NBRC 10321^T	ND
Candida zeylanoides	YIT 12369^T	NBRC 10324^T	ND
Clavispora lusitaniae	YIT 12370^T	NBRC 10059^T	ND
Cryptococcus curvatus	YIT 12377^T	JCM 1532^T	ND
Cryptococcus heveanensis	YIT 12151^T	NBRC 10764^T	ND
Debaryomyces etchellsii	YIT 8300^T	JCM 3656^T	ND
Filobasidiella neoformans	YIT 12009^T	CBS 132^T	17.8 ± 0.2
Kluyveromyces lactis	YIT 8103^T	AJ 5298^T	ND
Kluyveromyces marxianus	YIT 11365^T	NBRC 10005^T	ND
Lodderomyces elongisporus	YIT 12344^T	NBRC 1676^T	ND
Pichia guilliermondii	YIT 12371^T	NBRC 10106^T	ND
Saccharomyces cerevisiae	YIT 8264^T	IFO 10217^T	ND
Trichosporon asahii	YIT 11996^T	JCM 2466^T	ND
Trichosporon asahii	YIT 12461	JCM 1809	ND
Trichosporon asahii	YIT 12462	JCM 1810	ND
Trichosporon asteroides	YIT 12378^T	JCM 2937^T	ND
Trichosporon brassicae	YIT 12034^T	NBRC 1584^T	ND
Trichosporon coremiiforme	YIT 12463^T	JCM 2938^T	ND
Trichosporon cutaneum	YIT 12372^T	NBRC 1198^T	ND
Trichosporon dermatis	YIT 12468^T	NBRC 102675^T	ND
Trichosporon faecale	YIT 12467^T	NBRC 103892^T	ND
Trichosporon gracile	YIT 12373^T	NBRC 103905^T	ND
Trichosporon inkin	YIT 12379^T	JCM 9195^T	ND
Trichosporon japonicum	YIT 12464^T	JCM 8357^T	ND
Trichosporon loubieri	YIT 12124^T	JCM 3939^T	ND
Trichosporon mucoides	YIT 12380^T	JCM 9939^T	ND
Trichosporon ovoides	YIT 12381^T	JCM 9940^T	ND
Trichosporon porosum	YIT 12382^T	JCM 1458^T	ND

Value is mean ± SD; ND: not detected within 60 min; LAMP: loop-mediated isothermal amplification; Tt: Threshold time; ^{T}: Type strain; AJ: Central Research Laboratories, Ajinomoto Co. Ltd, Japan; CBS: Centraalbureau voor Schimmelcultures, Netherlands; DSM: German Collection of Microorganisms and Cell Cultures, Germany; IFO, Institute for Fermentation, Japan; JCM, Japan Collection of Microorganisms, Japan; NBRC: National Institute of Technology and Evaluation Biological Resource Center, Department of Biotechnology, Japan.

Table 2
Primers used in this study.

Method	Primer name	Sequence (5′–3′)	Reference
LAMP	Fneo-F3	GCCCTTTGGTATTCCGAAGG	This study
	Fneo-B3	GGTCAAACAAAAAGAGATGGTTGT	
	Fneo-FIP	TTTGCAGGTCGCGGCAAACATCAATC CCTCGGGTTTTATTACC	
	Fneo-BIP	TGGGAAGGTGATTACCTGTCAGCAA GCCGAAGACTACCCCAT	
	Fneo-LB	GCGTAATAAGTTTCGCTGGGCC	
qPCR	sp-Fneo-F	TTTGAAATCTGGCGTCCTCCGGGC	Makino et al. (2010)
	sp-Fneo-R	GCTATAACACACCCCGAAGAGT	

LAMP: loop-mediated isothermal amplification; qPCR: quantitative polymerase chain reaction.