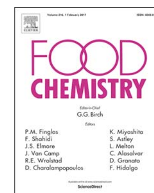
Food Chemistry 246 (2018) 26–31

Contents lists available at ScienceDirect

Food Chemistry

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



Direct duplex real-time loop mediated isothermal amplification assay for the simultaneous detection of cow and goat species origin of milk and yogurt products for field use

Mi-Ju Kim, Hae-Yeong Kim*

Institute of Life Sciences & Resources and Department of Food Science & Biotechnology, Kyung Hee University, Yongin 17104, Republic of Korea

ARTICLE INFO

Keywords:
Direct duplex real-time LAMP
On-site detection
Milk and yogurt products
Cow
Goat

ABSTRACT

A multiple loop-mediated isothermal amplification (LAMP) method was developed to detect cow and goat milk in the field using a portable fluorescence device. For rapid on-site detection, this duplex LAMP assay was used in combination with direct amplification, without DNA extraction. The cow- and goat-specific LAMP primer sets were designed based on the mitochondrial *cytochrome b* gene, and showed specificity against 13 other animal species in the reactions. The sensitivity of the duplex LAMP assay for cow and goat was 0.1 and 1 pg, respectively. The detection limit for both target species in milk mixtures was 2%. This assay successfully amplified and identified the two target species in 24 samples of commercial milk and yogurt products, with 30 min sampling-to-result analysis time. Therefore, this direct duplex real-time LAMP assay is useful for on-site simultaneous detection of cow and goat milk in commercial products, a capability needed to confirm accurate labeling.

1. Introduction

Milk and dairy products play an important role in the food industry due to their nutritional characteristics (Hrbek, Vaclavik, Elich, & Hajslova, 2014). In particular, goat milk is preferred by consumers because of its superior mineral composition, digestibility, and biological value (Liao, Liu, Ku, Liu, & Huang, 2017). In addition, people allergic to components of cow milk should consume milk-based products derived from other species, such as goats (Agrimonti, Pirondini, Marmiroli, & Marmiroli, 2015). Because of the higher price of goat milk compared to cow milk, adulteration in the dairy industry is economically attractive (Jung, Jhon, Kim, & Hong, 2011; Zachar et al., 2011). Therefore, correct labeling of the species origin of milk and dairy products is important to protect human health and ensure customer satisfaction. To prevent fraudulent species labeling in milk and milk-based products, sensitive, specific, and rapid detection method tools are needed to control, label, and authenticate the species origin of milk.

PCR-based techniques such as conventional PCR and real-time PCR assays are widely used for species discrimination in milk and dairy products. Not only are these methods capable of detecting very small amounts of cow milk in goat milk, but the DNA molecules that are the target analytes are thermally more stable than proteins (Di Domenico, Di Giuseppe, Wicochea Rodríguez, & Cammà, 2016; Liao et al., 2017). Real-time PCR in particular has been used as a quantitative tool for assaying milk-based products (Agrimonti et al., 2015; Di Domenico et al., 2016; Liao et al., 2017). However, these methods are time-consuming and require bulky, expensive equipment. In recent years, isothermal nucleic acid amplification techniques have been applied to species identification. These techniques have a shorter reaction time and higher sensitivity than PCR (Wang et al., 2015). Moreover, these techniques use a constant temperature during the amplification reaction, and can be applied as a tool for point-of-care (POC) diagnosis using minimal equipment (Craw & Balachandran, 2012; Foudeh, Didar, Veres, & Tabrizian, 2012). The loop-mediated isothermal amplification (LAMP) assay is one of the most common isothermal amplification techniques (Foudeh et al., 2012), and has been used for species identification of meat and milk (Deb et al., 2016a,b; Yang et al., 2014). Since this method employs four primers to recognize six distinct sequences of target DNA template as well as two additional primers to accelerate the LAMP reaction, it is highly specific, and sensitively detects the species origin of the sample (Wang et al., 2015). To simultaneously amplify two targets in a single reaction, multiplex LAMP assays can be applied for more efficient molecular diagnosis. Recently, a real-time duplex LAMP assay using an assimilating probe was reported for pathogen detection (Kubota & Jenkins, 2015). The assimilating probe, which is based on fluorescence resonance energy transfer (FRET), has an advantage in that it does not require additional procedures, such as restriction enzyme treatment (Kubota, Alverez, Su, & Jenkins, 2011; Kubota & Jenkins, 2015).

As the demand for rapid on-site detection has increased, LAMP

* Corresponding author.
E-mail addresses: mijukim@khu.ac.kr (M.-J. Kim), hykim@khu.ac.kr (H.-Y. Kim).

http://dx.doi.org/10.1016/j.foodchem.2017.11.014
Received 23 July 2017; Received in revised form 2 October 2017; Accepted 3 November 2017
Available online 04 November 2017
0308-8146/ © 2017 Elsevier Ltd. All rights reserved.

assays combined with direct amplification methods, which simplify the procedures needed to obtain DNA, have been reported (Abdulmawjood et al., 2014; Lee, Kim, Hong, & Kim, 2016). However, there have been no reports of a field-based approach that simultaneously detects multiple targets using multiplex real-time LAMP assay being combined with these direct amplification methods.

In this study, a direct duplex real-time LAMP assay was first developed for the simultaneous detection of cow and goat DNA using a portable fluorescence device, with no need for DNA isolation. The optimized assay was also evaluated using binary milk mixtures for the LOD analysis, and applied to detect cow and goat species in commercial milk and yogurt products. This method can deliver a sample-preparation-to-result time of approximately 30 min, demonstrating its usefulness as a rapid on-site detection method for cow and goat milk.

2. Materials and methods

2.1. Sample preparation

Cow and goat milk samples were donated by the National Institute of Animal Science (NIAS) in Korea, and used as reference materials. To test the primers' specificity, animal tissues (cow, goat, lamb, pig, horse, red deer, roe deer, chicken, duck, turkey, goose, ostrich, and pheasant) were collected from the Conservation Genome Resource Bank (CGRB) and local markets in Korea.

Two sets of binary milk mixtures containing 1, 2, 5, and 10% (v/v) of cow milk in goat milk, or vice versa, were prepared to evaluate the detection limit of the direct duplex LAMP assay. Commercial milk and yogurt products that were derived from cow and/or goat sources were purchased at local and online markets in Korea.

2.2. DNA extraction

To evaluate primer specificity, DNA was extracted from raw meat samples by the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, with minor modification. The purity and concentration of isolated DNA were measured by a UV spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). DNA with a 260/280 nm ratio between 1.8 and 2.0 was used as template DNA.

2.3. Design of LAMP primers

The sequences of mitochondrial *cytochrome b* genes for various animals, including cow and goat, were obtained from the GenBank database. To select a cow specific and a goat specific region, the sequences were aligned using the Clustal Omega program (Fig. 1). Two sets of LAMP primers were designed de novo using Primer Explorer software, version 4 (Eiken Chemical Co., Tokyo, Japan). The sequences of LAMP primers used in this study, including assimilating probes and the quench strand, are shown in Table 1. LAMP primers, assimilating probes, and the quench strand were synthesized by Bionics (Seoul, Korea) and Bioneer (Daejeon, Korea).

2.4. Simplex and duplex LAMP reactions

In order to test primer specificity and sensitivity, a simplex LAMP assay was performed in a reaction containing 15 μL of Isothermal Master Mix ISO-001nd (OptiGene, West Sussex, UK), 0.2 μM of each outer primer (F3 and B3), 1.6 μM of each inner primer (FIP and BIP), 0.4 μM of loop primer (LB), 0.04 μM of assimilating probe, 0.08 μM of the quench strand, and 10 ng of DNA template. The final reaction volume was 25 μL. The same concentrations of LAMP primers were used for the detection of cow and goat DNA. A duplex LAMP reaction was performed using the same concentrations of LAMP primers (F3, B3, FIP, BIP, and LB), including probes, with the exception that the reaction contained 1.4 μM of goat-specific inner primers (FIP and BIP) and 0.2 μM of quench strand. Both simplex and duplex LAMP reactions were performed at 65 °C for 30 min using a Genie III LAMP detector with dual channels (OptiGene, West Sussex, UK). Data was analyzed with Genie Explorer. This software was used for peak detection with the following settings: the threshold and smoothing values for cow were 0.001 and 11, respectively, and those for goat were 0.010 and 8, respectively. A non-template control was used as a negative control in all LAMP reactions.

2.5. Direct duplex LAMP reactions

All samples for the direct duplex LAMP assay, which was performed without DNA isolation, were prepared in the same way. First, 5 μL of sample was placed into a tube containing 55 μL of phosphate-buffered saline (PBS), and incubated at 98 °C for 2 min. Then, 1 μL of supernatant of sample in PBS was used as a template in the LAMP reaction and applied to the duplex LAMP reaction under the same conditions. Cow and goat DNA samples were used as positive controls in all direct duplex LAMP reactions to check detection time and fluorescence intensity.

2.6. Specificity and sensitivity

The specificity of the newly-designed LAMP primers for cow and goat DNA were evaluated against DNA extracted from 13 meat species. The sensitivity of each assay for simplex and duplex LAMP reactions was tested by serially diluting DNA from 10 to 0.00001 ng per reaction. The detection limit for direct simplex and duplex LAMP assay was determined using binary milk mixtures.

3. Results and discussion

3.1. Specificity of the LAMP primers

Two sets of species-specific LAMP primers were designed de novo to target the mitochondrial *cytochrome b* genes of cow (Accession No. KF926377.1) and goat (Accession No. DQ514544.1). These primers were designed after aligning the cow and goat sequences with sequences from other species, as shown in Fig. 1. A set of five oligonucleotide primers, including two outer primers, two inner primers, and a backward loop primer, were created to bind to seven regions of the target template. General loop primer accelerated LAMP reaction speed under isothermal conditions (Wang et al., 2015). Details of the primer sequences and binding sites used in this study are displayed in Fig. 1 and Table 1. The specificity of the new species-specific primers was evaluated using DNA isolated from 13 animal species, including cow and goat. No cross-reactivity was detected for either target species, due to high specificity of this seven-binding-site LAMP assay (Fig. S-1).

3.2. Sensitivity of simplex and duplex LAMP assays

The concentration of each primer set for the duplex LAMP assay was optimized with respect to amplification time and fluorescence intensity. To simultaneously obtain fluorescent signals, each assimilating probe for cow and goat DNA was labeled on FAM and Texas red, respectively. This decision was made based on the specifications of the real-time fluorescence detector used in this study, which had dual channels. The first channel detected excitation at 470 nm and emission at 510–560 nm, while the second detected excitation at 590 nm and emission at 620 nm. Assimilating probe included complementary nucleotide sequences of quench strands, which were bound together before LAMP reaction was initiated. When each assimilating probe labeled on FAM and Texas red was bound to target species, the two strands were separated from each other. Then, fluorescence signal amplified by each species-specific probe was measured by the real-time fluorescence detector (Kubota & Jenkins, 2015; Yi, Zhang, & Zhang, 2006). The sensitivity of the duplex LAMP assay was compared with that of the

Download English Version:

https://daneshyari.com/en/article/7586080

Download Persian Version:

https://daneshyari.com/article/7586080

Daneshyari.com