LAMP, PCR, and real-time PCR detection of *Acetobacter aceti* in yogurt

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Abstract Acetic acid bacteria (AAB) can spoil food. *Acetobacter aceti* as a core subgroup of AAB is usually isolated from yogurt. *A. aceti* should be timely and effectively detected to prevent yogurt contamination. The present study focused on *A. aceti* to establish an assay that can be performed to detect AAB in yogurt. LAMP, PCR, and real-time PCR were applied and compared for detecting *A. aceti* from pure culture and artificially contaminated yogurt samples. In pure culture, LAMP showed the highest detection sensitivity with 10^{-1} CFU/mL. For yogurt samples, the sensitivity limit of LAMP was 10^2 CFU/mL, which was lower than that of real-time PCR (10^1 CFU/mL). The results indicated that these methods could be quickly and efficiently applied to detect *A. aceti*. As LAMP technology has low cost and high detection efficiency, it can potentially be applied for detecting *A. aceti* in production and quality control programs of yogurt.

Keywords: *Acetobacter aceti*, yogurt, LAMP, PCR, real-time PCR

Introduction

Acetic acid bacteria (AAB) are gram-negative bacilli closely related to human life. These bacteria can regulate carbohydrate metabolism and generate acetic acid from ethanol or monosaccharides. AAB are widely used in the food industry for applications such as producing vinegar and fermenting cocoa beans (1–4). *A. aceti* can completely oxidize acetic acid to carbon dioxide and water (5). However, numerous reports revealed that food contamination is commonly caused by AAB. AAB are typically present on the surface of damaged fruits, thus resulting in the rancidity of fruits, pollution of wine, and change of wine quality, as well as triggering other adverse reactions contributing to losses in the food industry (6–9).

A. aceti as a core subgroup of AAB are most widely used in the food industry (1–3). However, they can also influence food quality as rancidity deterioration ingredients, such as in beer and other alcoholic drinks (6,8). Yogurt is a popular food, and its consumption has increased in recent years mainly because of its acclaimed health benefits. However, yogurt contaminated with AAB can cause great losses to the yogurt industry. Considering the metabolic characteristics, *A. aceti* can produce acetic acid mainly by consuming the nutrients in yogurt and can completely oxidize acetic acid to carbon dioxide and water. Consequently, it can change the structure of probiotics, correspondingly resulting in yogurt rancidity, discoloration, swollen

container, and an off-flavor (10–12). Therefore, *A. aceti* should be timely and effectively detected to prevent yogurt contamination during production. The present study focused on *A. aceti* to establish an assay that can be performed to detect AAB in yogurt.

Current detection methods of AAB mainly focus on traditional physiological and biochemical techniques (13). These methods are time consuming, tedious, and unsuitable for practical applications with low sensitivity. Gene detection methods based on molecular biology techniques are the common methods that are currently used for microbiological tests such as loop-mediated isothermal amplification (LAMP) (14) and polymerase chain reaction (PCR) techniques (15–17). Molecular biology techniques for detecting AAB also include denaturing gradient gel electrophoresis (18), amplified ribosomal restriction fragment analysis (19), amplified fragment length polymorphism (20), and other DNA molecular marker techniques. However, these methods are expensive and more complicated, thus limiting their practical applications.

LAMP as a constant temperature amplification technology for nucleic acids is continuously being developed since it was established (14). Target genes can be amplified up to 10^9 copies by a LAMP assay in 1 h using four specifically designed primers based on the six regions of the target fragment DNA in the reaction of auto cycling strand displacement DNA synthesis (Bst DNA polymerase) (21). LAMP has advantages such as high specificity and sensitivity, simple

operation, and low cost, which are suitable for rapid detection. Recently, the LAMP technology is successfully applied in the detection of viruses (22) and other pathogens (21,23,24) as well as in clinical diagnoses (25).

The PCR technology is an *in vitro* enzymatic amplification technology of specific genes or cloned sequences caused by the modulation of a specific oligonucleotide. It is widely used to detect specific genes of a variety of pathogens (26–28). Similarly, the PCR technology is established for the rapid test of bacteria in the current standards such as International Organization for Standards and other Chinese standards. The real-time PCR technology can execute real-time monitoring of the PCR process through the accumulated fluorescent signals in the PCR system; therefore, it is more convenient and has better specificity and sensitivity than other methods. During DNA replication, the SYBR Green I as a fluorescent dye can be incorporated into a specific DNA double strand to emit fluorescent signal. Moreover, the fluorescent signal is synchronized with increasing PCR products.

In the present study, the LAMP, PCR, and SYBR Green I real-time PCR methods were applied, and the specificity, sensitivity, and feasibility of these methods were compared. The aim of this study was to establish a fast, accurate, and highly sensitive detection method of *A. aceti* contamination at the early stage of yogurt production to decrease economic losses. The present study provides a useful reference for the future investigation of detecting *A. aceti* and other AAB in the food industry.

Materials and Methods

Strains and culture conditions Twenty strains were purchased from the China Center of Industrial Culture Collection (CICC). Four strains of *A. aceti* were included. The culture conditions and strain species are not shown.

DNA extraction The strains were cultured in the corresponding media for 24–48 h. Their genomic DNA was extracted using the Ezup Column Genomic DNA Extraction Kit (Shanghai Sangon Biological Engineering Co., Ltd., Shanghai, China), according to the manufacturer's instructions. In this method, 1 mL of the sample was centrifuged. The extracted genomic DNA was stored at -20°C until further use. A ten-fold serial dilution of *A. aceti* was prepared to obtain a culture broth with a strain concentration of 10^0 - to 10^{-8} -fold compared with the initial broth. The extracted DNA was used to measure sensitivity.

LAMP detection The 16-23S rRNA internal transcribed spacer gene sequence (NBRC14818, GI: 39573569) with high specificity was used to design primers from the conserved parts using the BioEdit software and <https://primerexplorer.jp/lamp4.0.0/index.html>. The amplified fragment was approximately 200 bp. The outer (F3 and B3) and inner primers [Forward Inner Primer (FIP) and Back Inner Primer (BIP)] are

Table 1. LAMP primer sequences

Primer names	Primer sequences (5'→3')
F3	CGGTTGGTGCGTATGTGG
B3	CCATGCACAGAAACCATCCA
FIP	ACGCTTCCGCCAAAATCCCTTGGGTTGGTCTGACCCATGA
BIP	AATGTGACGCGCTTGAATGAGCCCAAGGCAGAACCCCGATA

listed in Table 1. The LAMP reaction system (25 μL) contained 6 mM MgCl_2 , 1.4 mM dNTPs (Takara, Kusatsu, Japan), 0.8 μM inner primers (FIP and BIP), 0.2 μM outer primers (F3 and B3), 1 $\times$ Bst DNA polymerase reaction buffer (New England Biolab, Ipswich, MA, USA), 8 U Bst enzyme (New England Biolab), 0.8 mM betaine (Sigma-Aldrich, St. Louis, MI, USA), 2 μL of DNA template, and appropriate sterile distilled water to a total volume of 25 μL . The LAMP reaction system was placed in a water bath at 62°C for 30 min, and the reaction was terminated at 80°C for 3 min. After amplification, the LAMP products were digested by the XbaI enzyme (37°C for 1 h). The 20 μL system included 1 μL XbaI, 2 μL 10 $\times$ M buffer, 2 μL 0.1% BSA, 2 μL LAMP product, and appropriate sterile distilled water to obtain a total volume of 20 μL . The reaction was terminated after adding 2 μL 10 $\times$ loading buffer.

PCR and real-time PCR detection The outer primers (F3 and B3) of the LAMP reaction were used for PCR amplification. The PCR system (25 μL) included 1 $\times$ PCR buffer, 1.25 U Taq DNA polymerase, 0.2 mM dNTPs, 1.5 mM MgCl_2 (Takara), 0.4 μM outer primers (F3 and B3), 2 μL of DNA template, and appropriate sterile distilled water to obtain a total volume of 25 μL . The PCR program was performed as follows: 95°C for 5 min followed by 35 cycles of 95°C for 45 s, 57°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 10 min.

For the real-time PCR, the amplification was performed in 20 μL of the reaction mixture, including 10 μL SYBR Premix Ex Taq (ABI), 0.5 μL of 10 μM outer primer for each (F3 and B3), 2 μL of DNA template, and appropriate sterile distilled water to obtain a total volume of 20 μL . All real-time PCR amplifications were performed on ABI7300 as follows: 95°C for 3 min, 40 cycles of 95°C for 3 s, and 60°C for 31 s. The conditions for the melting curve analysis are 95°C for 15 s and 60°C for 60 s.

Specificity test The DNA templates extracted from the 20 strains were amplified to evaluate the specificity of primers. The amplified products of LAMP and PCR were analyzed by 2% agarose gel electrophoresis using a Bio-Rad Imaging system (1 $\times$ TAE buffer, 100V, 45 min). The specificity of the real-time PCR was evaluated by the melting curve.

Sensitivity test The *A. aceti* strain CICC21682 was the model strain chosen for the sensitivity test. First, 0.1 mL culture was shaken, and the *A. aceti* suspension was then gradually diluted using sterile distilled water from 3.63×10^6 to 3.63×10^{-2} CFU/mL with a dilution

ratio of 10. Genomic DNA was extracted for the sensitivity test. The feasibility of the three methods was compared by subjecting each diluted DNA sample to three parallel detections. For detecting LAMP sensitivity, in addition to agarose gel electrophoresis, the sensitivity test also included the visual inspection of a white magnesium pyrophosphate precipitate and the SYBR Green I chromogenic method. In this experiment, the SYBR Green I fluorescent dye was added to the centrifuge tube of the LAMP system after the reaction; color change was observed in the reaction solution after mixing.

A. aceti detection in artificially contaminated yogurt Fresh and qualified yogurt without *A. aceti* contamination was purchased from a local supermarket. Ten milliliter yogurt was inoculated with *A. aceti* CICC21682 (3.63×10^6 CFU/mL), and then diluted with 90 mL sterile distilled water. After that, 10 mL diluent solution was absorbed, and centrifuged to remove supernatant, and then washed twice with 5 mL PBS buffer (pH 7.4). The precipitate was collected for DNA extraction using the Ezup Column Genomic DNA Extraction Kit (Shanghai Sangon Biological Engineering Co., Ltd.) according to the manufacturer's instruction.

All experiments were performed in triplicate and analyzed statistically.

Results and Discussion

Specificity and sensitivity test by LAMP DNA from the 20 strains was subjected to LAMP amplification using the primers for *A. aceti*. Agarose gel electrophoresis results showed that only four *A. aceti* strains had specific DNA ladders, particularly with a 200-bp amplified product band. By contrast, *Glucose bacilli* and other strains did not show DNA ladders, as shown in Fig. 1. These results suggest that this method had excellent specificity.

For the sensitivity test, the gradient concentrations of *A. aceti* were 3.63×10^6 , 3.63×10^5 , 3.63×10^4 , 3.63×10^3 , 3.63×10^2 , 3.63×10^1 , 3.63×10^0 , 3.63×10^{-1} , and 3.63×10^{-2} CFU/mL when they were subjected to the dilution ratios of 10^0 – 10^8 of the original broth. After the DNA templates of *A. aceti* at various concentrations were subjected to LAMP amplification, the agarose gel electrophoresis results clearly revealed amplified bands until the concentration of *A. aceti* was diluted by 10^7 fold, suggesting that the LAMP detection limit of *A. aceti* was 3.63×10^1 CFU/mL (Fig. 2).

A white magnesium pyrophosphate precipitate was visible at the bottom of No. 1–4 tubes to the naked eye (from 3.63×10^6 CFU/mL to 3.63×10^3 CFU/mL). However, the precipitate in No. 5–8 tubes, which were diluted from 10^4 fold to 10^7 fold, could not be seen with the naked eye (data not shown).

Although the gel electrophoresis method is a representative detection method, it can easily result in cross-contamination. Previous studies have shown that adding 0.5 μ L of 1,000 $\times$ SYBR Green I at the end point of the reaction helps identify positive results quickly based

Fig. 1. Specificity test of LAMP primers for *A. aceti* by agarose gel electrophoresis. M, 100 bp DNA ladder marker; 1, *A. aceti* (CICC21682); 2, *A. aceti* (CICC22519); 3, *A. aceti* (CICC21683); 4, *A. aceti* (CICC21684); 5, *Glucose bacilli*; 6, *Salmonella enteritidis*; 7, *Salmonella typhimurium*; 8, *Cronobacter sakazakii*; 9, *Shigella flexneri*; 10, *Lactobacillus rhamnosus*; 11, *Plant lactose bacillus*; 12, *Streptococcus thermophilus*; 13, *Lactobacillus delbrueckii subsp lactis*; 14, *Bifidobacterium longum*; 15, *Listeria monocytogenes*; 16, *Campylobacter jejuni*; 17, *Escherichia coli*; 18, *Vibrio parahaemolyticus*; 19, β -hemolytic streptococcus; and 20, *Bacillus cereus*.

Fig. 2. Sensitivity test for the detection of *A. aceti* by LAMP. M, 100 bp DNA ladder marker; 1, Negative control; 2, 3.63×10^6 CFU/mL; 3, 3.63×10^5 CFU/mL; 4, 3.63×10^4 CFU/mL; 5, 3.63×10^3 CFU/mL; 6, 3.63×10^2 CFU/mL; 7, 3.63×10^1 CFU/mL; 8, 3.63×10^0 CFU/mL; 9, 3.63×10^{-1} CFU/mL; 10, 3.63×10^{-2} CFU/mL.

on the color change (29). In this study, 0.1 μ L of 10,000 $\times$ SYBR Green I dye was chosen as the best adding concentration. A gradient concentration of the SYBR Green I dye was added to the LAMP system with 3.63×10^6 CFU/mL *A. aceti* DNA template. After adding 0.1 μ L of 10,000 $\times$ SYBR Green I dye, positive results revealed the occurrence of green fluorescence from orange color, which was visible to the naked eye (data not shown). This method is sensitive and accurate.

White magnesium pyrophosphate precipitate, gel electrophoresis, and SYBR Green I dye fluorescence methods were used for detection and the results were compared. The SYBR Green I dye fluorescence method was fast, convenient, and had the highest sensitivity. But the progress of opening the PCR tubes could easily cause aerosol pollution. At present, the most advanced detection methods are real-time fluorescence or turbidity monitoring. When the monitor data reached the level which could be detected, it revealed that the LAMP results were positive. Besides, adding proper amount of UNG enzyme in the LAMP system could also reduce contamination from

Fig. 3. Specificity test of PCR primers for *A. aceti* by agarose gel electrophoresis. M, 100 bp DNA ladder marker; 1, *A. aceti* (CICC21682); 2, *A. aceti* (CICC22519); 3, *A. aceti* (CICC21683); 4, *A. aceti* (CICC21684); 5, *Glucose bacilli*; 6, *Salmonella enteritidis*; 7, *Salmonella typhimurium*; 8, *Cronobacter sakazakii*; 9, *Shigella flexneri*; 10, *Lactobacillus rhamnosus*; 11, Plant lactose bacillus; 12, *Streptococcus thermophilus*; 13, *Lactobacillus delbrueckii subsp lactis*; 14, *Bifidobacterium longum*; 15, *Listeria monocytogenes*; 16, *Campylobacter jejuni*; 17, *Escherichia coli*; 18, *Vibrio parahaemolyticus*; 19, β -hemolytic streptococcus; and 20, *Bacillus cereus*.

amplification products to a certain extent (30-32).

In other words, the LAMP technology is convenient and fast, and the detection sensitivity is as high as 10^{-1} CFU/mL in pure culture.

Specificity and sensitivity tests by PCR The extracted DNA templates were amplified by PCR using the outer primers (F3 and B3) of LAMP. The PCR-amplified products were evaluated by 2% agarose gel electrophoresis. Only *A. aceti* revealed the amplified target fragment with 200 bp, as shown in Fig. 3. The size of the target fragment was approximately 200 bp. These results suggest that this method had excellent specificity for *A. aceti*.

For the sensitivity test of PCR, the results showed that the template DNA revealed the target band until at a concentration of 3.63×10^1 CFU/mL (No. 1–6 tubes). However, the band could not be observed at a concentration lower than 3.63×10^1 CFU/mL, as shown in Fig. 4.

Specificity and sensitivity test by SYBR Green I real-time fluorescent PCR Based on the analysis of the melting curve of the amplified products, the amplified product from *A. aceti* (CICC21682) obtained by the real-time fluorescent PCR at 80.1°C only presented a typical single peak in Fig. 5. Other strains showed no amplification and

Fig. 4. Sensitivity test for *A. aceti* by PCR. M, 100 bp DNA ladder marker; 1, 3.63×10^6 CFU/mL; 2, 3.63×10^5 CFU/mL; 3, 3.63×10^4 CFU/mL; 4, 3.63×10^3 CFU/mL; 5, 3.63×10^2 CFU/mL; 6, 3.63×10^1 CFU/mL; 7, 3.63×10^0 CFU/mL; and 8, 3.63×10^{-1} CFU/mL.

melting curve peaks. This means that the primers were specific for *A. aceti*.

For the sensitivity test of real-time PCR, the C_t values at the concentration 3.63×10^6 , 3.63×10^5 , 3.63×10^4 , 3.63×10^3 , 3.63×10^2 and 3.63×10^1 CFU/mL were 19.9, 22.9, 26.2, 30.2, 33.6, and 36.5, respectively, with a standard curve correlation coefficient of 0.9984 (data not shown). The logarithm of the copy number for the DNA template from *A. aceti* at gradient concentrations revealed an excellent linear relationship with the C_t value. When the DNA concentration was less than 3.63×10^1 CFU/mL, the quantitative detection of *A. aceti* could not be observed (data not shown).

***A. aceti* detection in artificially contaminated yogurt** The concentration of *A. aceti* obtained by the conventional separation and culture method from the contaminated yogurt was 1.3×10^6 CFU/mL. Three detection methods, LAMP, PCR, and real-time PCR, revealed strong specificity, which was not influenced by lactic acid bacteria. The detection limits of these methods were 1.3×10^2 , 1.3×10^4 , and 1.3×10^1 CFU/mL (data not shown), respectively. This result suggested that the fluorescent real-time PCR had the highest detection sensitivity for the artificially contaminated samples. However, this method is expensive and more complicated, limiting their practical applications. The sensitivity PCR detection for bacteria in pure culture can be as high as 3.63×10^1 CFU/mL. However, the detection limit of bacteria in the yogurt samples is only 10^4 CFU/mL. This result confirmed that

Fig. 5. Melting curve resulting from SYBR Green I real-time PCR analysis with *A. aceti* (CICC21682)

real-time PCR has better specificity and sensitivity.

The specificity of the SYBR Green I method for target sequences is lower than that of the real-time fluorescent TaqMan probe, but this method has the advantages of low cost and simple operation. Its specificity can be determined by the product melting curve. The SYBR Green I real-time PCR method revealed high sensitivity and its detection limit for both pure culture and artificially contaminated yogurt samples can reach up to 10^1 CFU/mL. Their quantitative detection limits can also reach up to 10^1 CFU/mL with a correlation coefficient of 0.9984.

In published references, the conserved genes of *A. aceti* included Hsp60 (groEL), and 16S rDNA (33-35). After pre-experiments (data not shown), 16-23S ITS rRNA sequence (NBRC14818, GI: 39573569) with high specificity was identified to design primers from the conserved parts using the BioEdit software. We compared the sensitivity of the 3 methods, including PCR, real-time PCR and LAMP, with the same gene and same primers.

In the present study, the LAMP, conventional PCR, and SYBR Green I real-time PCR were applied, and the findings were compared for detecting *A. aceti*. These three methods are not necessary for the pre-enrichment of bacteria and have excellent specificity. In 2007, Gammon *et al.* (15) successfully applied the real-time PCR technology to detect trace amounts of *Gluconobacter* and *Gluconacetobacter* species in electrolyte replacement drinks. In this study, we revealed that the real-time PCR technology possesses high sensitivity. It could be used to detect *A. aceti* contaminated yogurt samples. However, the application of this method is limited because of the requirements for expensive equipment and reagents, as well as high operation skills of laboratory assistants. In general, the LAMP technology has higher sensitivity, lower technological cost, and high detection efficiency for artificially contaminated samples containing *A. aceti* and naturally deteriorated samples containing *A. aceti* and pure culture of *A. aceti*. Thus, this method shows a potential application in the production and quality control of yogurt.

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Disclosure The authors declare no conflict of interest.

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