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ORIGINAL PAPER

Rapid Detection of Hepatitis A Virus in Foods Using a Bioluminescent Assay in Real-Time (BART) and Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) Technology

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Abstract

Foodborne hepatitis A infections have been considered as a major threat for public health worldwide. Increased incidences of hepatitis A virus (HAV) infection has been associated with growing global trade of food products. Rapid and sensitive detection of HAV in foods is very essential for investigating the outbreaks. Real-time RT-PCR has been most widely used for the detection of HAV by far. However, the technology relies on fluorescence determination of the amplicon and requires sophisticated, high-cost instruments and trained personnel, limiting its use in low resource settings. In this study, a robust, affordable, and simple assay, reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay in combination with a bioluminescence-based determination of amplification in real-time (BART), was developed for the detection of HAV in different food matrices, including green onion, strawberry, mussel, and milk. The efficiencies of a one-step RT-LAMP-BART and a two-step RT-LAMP-BART were investigated for the detection of HAV in different food matrices and was compared with that of real-time RT-PCR. The sensitivity of the RT-LAMP-BART assay was significantly affected by Mg^{2+} concentration ($P < 0.05$), in addition to primer quality. The optimal Mg^{2+} concentration was 2 mM for one-step RT-LAMP-BART and 4 mM for two-step RT-LAMP-BART. Compared with cartridge-purified primers, HPLC-purified primers could greatly improve the sensitivity of the RT-LAMP-BART assay ($P < 0.05$). For detecting HAV in different food matrices, the performance of two-step RT-LAMP-BART was comparable with that of real-time RT-PCR and was better than that of one-step RT-LAMP-BART. The detection limit of the two-step RT-LAMP-BART for HAV in green onion, strawberry, mussel, and milk was 8.3×10^0 PFU/15 g, 8.3×10^1 PFU/50 g, 8.3×10^0 PFU/5 g, and 8.3×10^0 PFU/40 mL, respectively. The developed RT-LAMP-BART was an effective, simple, sensitive, and robust method for foodborne HAV detection.

Keywords Hepatitis A virus · Detection · RT-LAMP-BART · Food

Introduction

Foodborne illnesses are usually caused by consumption of food or water contaminated with harmful bacteria, viruses, parasites or chemical substances (WHO, 2020). Human pathogenic viruses are the most frequent causative agents and hepatitis A virus (HAV) is one of the greatest concerns which has caused numerous foodborne disease outbreaks in the world (Di Cola et al., 2021). HAV causes hepatitis A disease that is an inflammation of liver. The symptoms can range from mild to severe, including fever, malaise, loss of appetite, diarrhea, nausea, abdominal discomfort, dark-colored urine and jaundice (EFSA, 2014; Fleet et al., 2000; Sánchez et al., 2002). It was estimated that 7134 people died from hepatitis A worldwide in 2016 alone (WHO, 2021). Foodborne outbreaks of hepatitis A have been on the rise in recent years because of increasing numbers of international travelers, mass global migration, and the fast growth of global food trade (Bhaskar, 2017; Bosch et al., 2018; Cheftel, 2011; Hu et al., 2020; Kannan et al., 2020). The worldwide increase in the occurrence of foodborne

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HAV outbreaks has necessitated the development of novel approaches for rapid identification of foods contaminated with HAV, which is essential for implementing intervention strategies to prevent and reduce illnesses caused by the virus.

Real-time RT-PCR was used in the ISO 15216–2:2019 as a standard method for the detection of HAV in different food matrices, such as food surface (bell pepper pieces), raspberries, lettuce, green onion, oysters (*Crassostrea gigas*), mussels (*Mytilus edulis*), and bottled water (ISO, 2019). Although the method is reliable and accurate, it requires trained personnel and sophisticated equipment which can regulate temperature cycling and detect fluorescent signals emitted from the excited fluorophore (Li et al., 2017). These drawbacks restrict its widespread application in resource-limited settings. It is hard to detect HAV in foods because the viral contamination level may be low and significant inhibitors in food can prevent real-time PCR assay from working properly (Sánchez et al., 2007). Therefore, there is a growing demand for devising a novel strategy for rapid, robust, sensitive, and cost-effective detection of HAV in food items using simple equipment.

The recent development of isothermal amplification techniques for nucleic acid provides a variety of alternatives of PCR-based methods (Zhou et al., 2014). Among the methods, loop-mediated isothermal amplification (LAMP) has shown to be the most promising due to its rapidity, simplicity, high efficiency and specificity (Niessen et al., 2013). LAMP assay was first described for the detection of nucleic acids of hepatitis B virus (HBV) in 2000 (Notomi et al., 2000). This method employs *Bst* DNA polymerase, an enzyme derived from *Geobacilus stearothermophilus* (formally *Bacillus stearothermophilus*), with strand displacement activity (Nagamine et al., 2002; Notomi et al., 2000). The amplification is conducted at a constant temperature between 60 and 65 °C and does not need an expensive thermal cycler. Aside from its isothermal character, LAMP has several important features: (1) LAMP shows exquisite specificity because of the involvement of four primers: two inner primers (FIP and BIP) and two outer primers (F3 and B3), which can specifically recognize six regions of the target. Additional primers, Loop primers (Nagamine et al., 2002), and/or STEM primers (Gandelman et al., 2011), can be used to accelerate the LAMP reaction; (2) the product of LAMP consists of a mixture of stem-loop DNAs with various sizes, giving rise to distinct ladder-like banding patterns on an agarose gel (Notomi et al., 2000); (3) the LAMP reaction can be easily detected by visual endpoint observation of a white precipitate of magnesium pyrophosphate (Mori & Notomi, 2009), or of the color change of calcein (Tomita et al., 2008), SYBR Green I (Njiru et al., 2012; Tao et al., 2011), hydroxynaphthol blue (Goto et al., 2009; Vu et al., 2016), or phenol red (Amaral et al., 2021) involved in the reaction; (4) LAMP product can be monitored by a real-time measurement of the turbidity of magnesium pyrophosphate, a byproduct of DNA amplification (Mori et al., 2004); (5) the LAMP reaction is more tolerant to substances that typically inhibit PCR (Francois et al., 2011; Kaneko et al., 2007; Ou et al., 2012); and (6) portable devices, such as coin-size microfluidic chips or smartphone-based mobile detection platform, can be designed to fulfill the need for on-site detection using the LAMP technique in remote areas where resources are limited (Ahmad et al., 2011; Hsieh et al., 2012; Liu et al., 2011; Lucchi et al., 2010; Song et al., 2018; Wang et al., 2020; Wu et al., 2011; Yi et al., 2014). Due to these features, LAMP assay has stimulated extensive research interest and has been applied for food analysis, such as for the detection of allergens (Mao et al., 2020; Sheu et al., 2018; Yuan et al., 2018), genetically modified crops (Li et al., 2013; Zhou et al., 2014), and for meat species identification (Aartse et al., 2017; Abdulmawjood et al., 2014; Girish et al., 2020; Kumari et al., 2020; Wang et al., 2019; Xiong et al., 2020; Zahradnik et al., 2015). In addition, LAMP assay has been widely used for the detection of viral, bacterial, fungal, and parasitic pathogens (Cao et al., 2019; Ferrara et al., 2015; Frisch & Niessen, 2019; Li et al., 2017; Mei et al., 2019; Niessen et al., 2013).

In LAMP assay, the large amounts of DNA produced during amplification lead to liberation of enormous concentration of a side product, inorganic pyrophosphate ions (PPi). Each time a nucleotide base is added during the polymerization reaction, a molecule of PPi is released. The amount of PPi produced is proportional to the amount of polynucleotide synthesized, and hence the concentration of the target template in the test material. The synthesized PPi can be converted to ATP by ATP sulfurylase using adenosine 5'-phosphosulfate (APS) as the substrate. The ATP generated is simultaneously used by firefly luciferase to oxidize its substrate luciferin to emit light. Based on this mechanism as shown in Fig. 1, the dynamic changes of PPi, and hence, the DNA amplification can be monitored by testing the light output in a real-time mode. During DNA amplification, with the increase of PPi liberated, the light signal increases rapidly firstly, and later the PPi molecules accumulated in the reaction inhibit luciferase. Together with the depletion of the substrate adenosine 5′phosphosulphate (APS) in the reaction, the light output decreases. The time at which the peak is detected (time-to-peak) is inversely proportional to the concentration of the initial template (Gandelman et al., 2010; Hardinge et al., 2020; Kiddle et al., 2012). The combination of LAMP reaction and bioluminescence assay in real-time is described as LAMP-BART technique for the detection of DNA template; when RNA is the detection target, the method is named RT-LAMP-BART (Gandelman et al., 2010). This luminescence-based assay is more cost-effective than a fluorescence-based assay such as real-time PCR, and it is more tolerant to substances that typically inhibit PCR

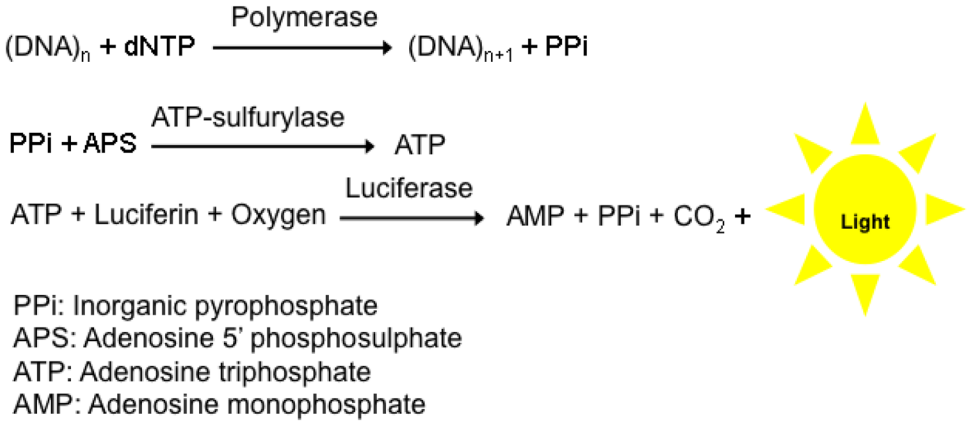

Fig. 1 Chemical mechanism of LAMP-BART assay (Gandelman et al., 2010) (Color figure online)

(Kiddle et al., 2012). The LAMP-BART assay has been used in the detection of genetically modified maize (Hardinge et al., 2018; Kiddle et al., 2012), human parvovirus B19 (Mirasoli et al., 2018), and SARS-CoV-2 (severe acute respiratory syndrome coronavirus-2) during the COVID-19 pandemic (Fei et al., 2021).

In this study, we for the first time developed a RT-LAMP-BART assay for rapid detection of HAV in different food matrices including green onion, strawberry, mussel, and milk, which have been associated with foodborne HAV outbreaks (Wu et al., 2019, 2022).

Materials and Methods

Virus Propagation and Cell Line

The cytopathogenic HM175/24A strain of HAV was propagated in fetal rhesus monkey kidney cells (FRhk-4), and the virus was enumerated by a plaque assay as described in our previous report (Wu et al., 2019). The viral titer was expressed as plaque forming unit (PFU) per mL. The viral stock was stored at − 80 °C until further analysis.

Food Sample Contamination

HAV was inoculated onto solid foods including green onion (15 g), strawberry (50 g), mussel (5 g), and into liquid food (40 mL of cow milk) according to our previous report (Wu et al., 2019, 2022). Briefly, HAV suspension (100 µL) prepared in PBS (pH 7.4) containing 8.3×10^5, 8.3×10^3, 8.3×10^1, or 8.3×10^0 PFU of viral particles were pipetted evenly onto the surface of each solid food sample in a Petri dish with 3 µL inoculated at each spot. The inoculum was dried onto the surface of each solid food by leaving at room temperature for 1 h. As to liquid food matrix (milk), 40 mL of the milk in 50 mL centrifuge tube was inoculated with 100 µL of HAV dilutions (8.3×10^5, 8.3×10^3, 8.3×10^1, or 8.3×10^0 PFU) prepared in PBS (pH 7.4). The artificially contaminated milk was mixed well and then left at room temperature for 1 h. For all foods, each HAV dilution was inoculated onto three independent samples, and one uninoculated sample was used as a negative control.

HAV Concentration and RNA Extraction

Viral particles on surface of each of the solid foods were eluted using 50 mL of glycine buffer (0.05 M glycine, 0.14 M NaCl, 0.2% (v/v) Tween 20, pH 9.0) according to our previous report (Wu et al., 2022). HAV particles in the liquid milk samples and in the glycine buffer (collected from the solid foods) were captured and concentrated by protamine-coated magnetic nanoparticles (PMNPs) as described previously (Wu et al., 2019, 2022). Viral RNA was extracted from the concentrated samples using the QIAamp MinElute Virus Spin Kit (Cat. No. 57704, Qiagen, Toronto, ON, Canada) according to the manufacturer's instructions. RNA was eluted in 40 µL of RNase-free water (Cat. No. AM9937, Applied Biosystems) and quantified using real-time RT-PCR, RT-LAMP assay, and RT-LAMP-BART assay as described in the sections below.

HAV RNA was also extracted from 200 µL of the virus stock containing 8.3×10^6 PFU/mL of virus strain HM175/24A. The RNA was serially diluted in tenfold increments and each dilution was subjected to real-time RT-PCR, RT-LAMP assay, and RT-LAMP-BART assay.

Real-Time RT-PCR

HAV RNA was reverse transcribed to cDNA using the High-capacity cDNA Reverse Transcription Kit (Cat. No. 4374966, Applied Biosystems) in accordance with the manufacturer's instructions. The cDNA was quantified by real-time PCR using a ViiA 7 Real-Time PCR system (Applied Biosystems) as described by Wu et al. (2019). The sequence of primer pairs and TaqMan probe are shown in Table 1. A

Table 1 Details of primers used for HAV assays by real-time RT-PCR and RT-LAMP-BART

Assay	Primer name	Primer sequence (5′-3′)	Primer position	Polarity	References
Real-time RT-PCR	Forward	ATAGGGTAACAGCGGCGGATAT	448–469	+	Gardner et al. (2003)
	Reverse	CTCAATGCATCCACTGGATGAG	516–537	-	
	Probe	FAM-CCATTCAACGCCGGAGG-MGB	492–508	+	This study
RT-LAMP-BART	F3	GCATGGAGCTGTAGGAGTCT	293–312	+	Yoneyama et al. (2007)
	B3	CACTCAATGCATCCACTGGA	520–539	-	
	FIP	F1C: ACCCGTAGCCTACCTCTTGTGG F2: TGTTGGGAACGTCACCTTG	385–406 329–347	- +	
	FIP031	F1C: ACCCGTAGCCTACCCCTTGTGG F2: TGTTTGGGACGTCGCCTTG	385–406 329–347	- +	
	BIP	B1C: TTGGATAGGGTAACAGCGGCG B2: CTCCGGCGTTGAATG	444–464 493–507	+ -	
	FLOOP	TGAAAGCCAAGTTAACACTG	348–367	-	
	BLOOP	GATATTGGTGAGTTGTTAAGAC	465–486	+	

FIP and FIP031 primers consisted of F1C plus F2 and BIP primer consisted of B1C plus B2. The different bases between primer FIP and FIP031 are highlighted and underlined. The positions of primers are in accordance with wild-type strain of HAV (GenBank accession number: M14707.1)

tenfold dilution series of cNDA corresponding to a virus titer ranging from 8.3×10^5 PFU/mL to 8.3×10^0 PFU/mL was used for generation of the standard curve.

RT-LAMP Assay

The primers used for the RT-LAMP assay of HAV are shown in Table 1. The RT-LAMP assay was performed using the Loopamp RNA Amplification Kit (Cat. No. LMP244, Eiken Chemical Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions. Briefly, a 25 µL-reaction was composed of 12.5 µL of 2 × reaction mixture, 1 µL of enzyme mixture containing 16 U *Bst* DNA polymerase and 2 U avian myeloblastosis virus (AMV) reverse transcriptase, 0.8 µM of each of the FIP031 and FIP primer, 1.6 µM of BIP primer, 0.8 µM of each of the FLOOP and BLOOP primer, 0.2 µM of each of the F3 and B3 primer, and 5 µL of extracted RNA. The reaction mixture was incubated using a GeneAmp PCR system 9700 (Applied Biosystems) at 62.5 °C for 60 min, 80 °C for 5 min, and then at 4 °C until further analysis (Yoneyama et al., 2007). One reaction without RNA template was used as a negative control.

The RT-LAMP products (3 µL) were resolved on 1% TAE (Cat. No. 161-0743, Bio-Rad Laboratories Ltd.) agarose gel containing 0.1 µg/mL of ethidium bromide (Cat. No. E1510, Sigma-Aldrich) by electrophoresis at 75 V for 50 min, and visualized and photographed over UV light, using an UV transilluminator (Bio-Rad Laboratories Ltd.).

One-Step RT-LAMP-BART Assay

The RT-LAMP-BART master mix was prepared by adding 187.5 µL of 2 × Lumopol buffer (Lumora Ltd., Ely, Cambridgeshire, UK) to the lyophilized RT-LAMP-BART-master tube (Lumora Ltd., Ely, Cambridgeshire, UK). Each reagent was kept on ice during the operation. Primer mix was prepared using nuclease-free water (Cat. No. AM9937, Life Technologies). The concentration of primers in the primer mix was: 2.1 µM of each of FIP031 and FIP primer, 4.3 µM of BIP primer, 2.1 µM of each of FLOOP and BLOOP primer, and 0.5 µM of each of F3 and B3 primer. Then, equal volumes of the RT-LAMP-BART master mix and primer mix were mixed together. The remaining RT-LAMP-BART master mix was aliquoted and stored at − 150 °C for later use.

RT-LAMP-BART reactions were run at 55 °C in 20 µL total volume containing 15 µL of the reagent mix and 5 µL of RNA template. Each sample was run in duplicate. The reactions were performed in a 96-well plate (Cat. No. 14-230-232, Thermo Fisher Scientific). Each reaction was covered with 20 µL of molecular grade mineral oil (Lumora Ltd., Ely, Camridgeshire, UK) to prevent evaporation. The final concentration of primers in each reaction was: 0.8 µM of each of FIP031 and FIP primer, 1.6 µM of BIP primer, 0.8 µM of each of FLOOP and BLOOP primer, and 0.2 µM of each of F3 and B3 primer. The luminescence was tested at 1 min intervals using a Bison system (Lumora Ltd., Cambridgeshire, UK). A standard curve was constructed by analyzing the time-to-peak of the tenfold serial dilutions of the viral RNA.

The effect of different concentrations (2 mM, 3 mM, and 4 mM) of $MgSO_4$ on the one-step RT-LAMP-BART reaction efficiency was tested. The effect was tested using two RNA dilutions corresponding to the viral titers of 8.3×10^5 PFU/mL and 8.3×10^3 PFU/mL. Each test was done in three replicates. The efficiency of the reaction using primers purified

with high-performance liquid chromatography (HPLC) and cartridge technique was compared. HPLC-purified primers were synthesized by Life Technologies Corporation (Burlington, ON, Canada) and cartridge-purified primers were obtained from Laboratory Services at the University of Guelph (Guelph, ON, Canada). For testing HAV in food samples, the cartridge-purified primers were used for green onions and strawberries, while HPLC-purified primers were used for mussels and milk.

Two-Step RT-LAMP-BART Assay

The LAMP-BART master mix was prepared by adding 187.5 µL of 2 × Lumopol buffer (Lumora Ltd., Ely, Cambridgeshire, United Kingdom) to the lyophilized LAMP-BART-master tube (Lumora Ltd., Ely, Cambridgeshire, UK) in a similar way as for preparing the RT-LAMP-BART master mix. Then, equal volumes of the LAMP-BART master mix and primer mix were mixed together.

LAMP-BART reactions were run at 62 °C in 20 µL total volume containing 15 µL of the reagent mix, 1 µL of nuclease-free water, and 4 µL of cDNA template. Each sample was run in duplicate. Each reaction was covered with 20 µL of molecular grade mineral oil (Lumora Ltd., Ely, Camridgeshire, UK) to prevent evaporation. The final concentration of primers in each reaction was the same as that used for one-step RT-LAMP-BART. The luminescence was tested at 1 min intervals using a Bison system (Lumora Ltd., Cambridgeshire, UK). A standard curve was constructed by analyzing the time-to-peak of the tenfold serial dilutions of HAV cDNA. The effect of different concentrations (0 mM, 2 mM, 3 mM, and 4 mM) of $MgSO_4$ on the two-step RT-LAMP-BART reaction was tested. The effect was tested using two cDNA dilutions corresponding to an initial viral titer of 8.3×10^5 PFU/mL and 8.3×10^3 PFU/mL. Each test was done in three replicates.

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with IBM SPSS Statistics (version 21; IBM Corporation, New York, USA). In all cases, differences were considered significant when the *P*-value was less than 0.05.

Results

Real-Time RT-PCR of HAV

A real-time RT-PCR was used to amplify tenfold serial dilutions of HAV cDNA. The fluorescence intensity of 6-carboxyfluorescein (FAM) was recorded over time during the amplification of cDNA templates (Fig. 2). The shape of the amplification curve monitored by fluorescence in real-time RT-PCR is different from the amplification curve measured by luminescence in RT-LAMP-BART assay (Figs. 4, 5, 6). The amplification curve for real-time RT-PCR is sigmoidal whereas the curve for BART assay is with an identifiable peak.

One-Step RT-LAMP Assay of HAV

As observed through agarose gel electrophoresis, amplification product of RT-LAMP was with a ladder-like pattern (Fig. 3). This is due to the formation of a mixture of stem-loop DNAs with various stem lengths (Notomi et al., 2000). The detection limit of RT-LAMP assay was 8.3×10^1

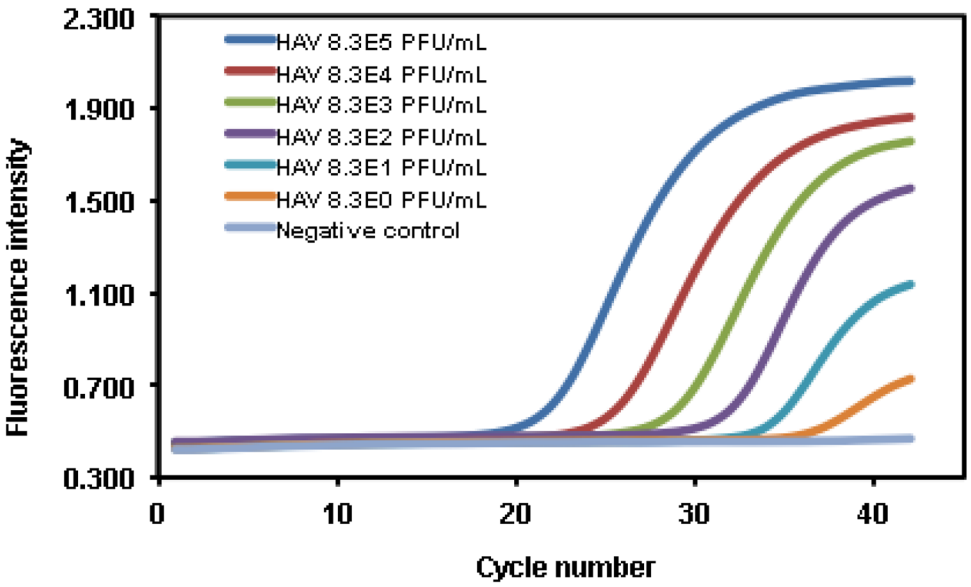

Fig. 2 Amplification of HAV cDNA in real-time RT-PCR monitored by the measurement of fluorescence (Color figure online)

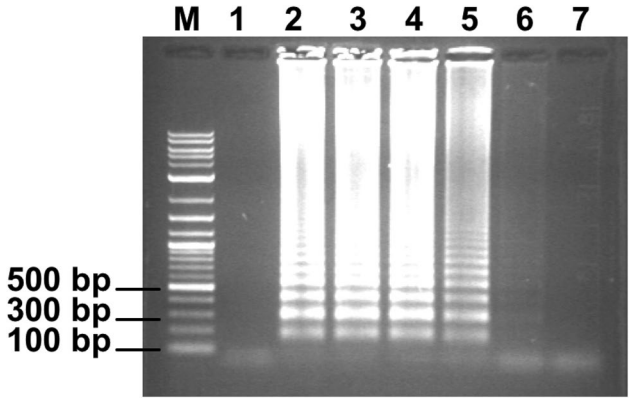

Fig. 3 Analysis of RT-LAMP amplicon of HAV RNA by 1% agarose gel electrophoresis. Lane M, 2-log DNA ladder (Cat. No. N0469L, New England Biolab); Lane 1, no template control; Lane 2–7, HAV RNA dilutions corresponding to 8.3×10^5, 8.3×10^4, 8.3×10^3, 8.3×10^2, 8.3×10^1, and 8.3×10^0 PFU/mL of the initial virus titer, respectively

PFU/mL, which was higher than that of real-time RT-PCR (8.3×10^0 PFU/mL).

Optimization of Mg^{2+} Concentration in One-Step and Two-Step RT-LAMP-BART Assay

The enzymatic reactions of RT-LAMP-BART assay were complicated, as shown in Fig. 1. Optimization of the reaction system is necessary to obtain higher sensitivity. For the tested both RNA dilutions (Fig. 4A, B), the $MgSO_4$ concentration at 2 mM demonstrated the optimal amplification effect on one-step RT-LAMP-BART. It was apparent that the time-to-peak of RT-LAMP-BART reaction performed in the presence of 2 mM $MgSO_4$ was detected earlier than those of the reaction performed with 3 mM or 4 mM $MgSO_4$, indicating that with the increase of Mg^{2+} concentration, the reaction sensitivity decreased significantly ($P<0.05$).

As shown in Fig. 5A, B, for both cDNA dilutions, the $MgSO_4$ concentration at 4 mM showed the optimal amplification effect on two-step RT-LAMP-BART. The reaction sensitivity increased significantly ($P<0.05$) with the increase of $MgSO_4$ concentration. Within the test time, the $MgSO_4$ concentration of 0 mM failed to produce an amplification signal for the cDNA dilution corresponding to a viral titer of 8.3×10^3 PFU/mL (Fig. 5).

Comparison of HPLC-Purified and Cartridge-Purified Primers in One-Step RT-LAMP-BART Assay

The effect of primers purified with HPLC and cartridge technology on the efficiency of one-step RT-LAMP-BART assay was investigated. It was found that HPLC-purified primers produced more sensitive results than cartridge-purified primers (Fig. 6). As shown in Fig. 6, for each RNA dilution, the time-to-peak of the reaction using HPLC-purified primers was earlier than that of the counterpart reaction using cartridge-purified primers and the difference is statistically significant ($P<0.05$).

Sensitivity Analysis of One-Step and Two-Step RT-LAMP-BART Assay

For one-step RT-LAMP-BART assay, a standard curve constructed by plotting the time-to-peak of the luminescence signal against the logarithm of the concentration of tenfold serial dilutions of virus titer is shown in Fig. 7. The results showed that HAV RNA was detected to at least 10^{-5} dilution corresponding to 2.1 PFU/reaction of the virus. The values indicated that the concentration of HAV with the titer of 8.3×10^1 PFU/mL could be detected by the one-step RT-LAMP-BART. This limit of detection was the same as that for one-step RT-LAMP and was one log higher than that obtained using real-time RT-PCR (Fig. 2).

A standard curve for two-step RT-LAMP-BART assay was constructed by plotting the time-to-peak of the luminescence signal against the logarithm of the concentration of the virus titer and is shown in Fig. 8. The results indicated that the virus with a titer as low as 8.3×10^0 PFU/mL, corresponding to 0.083 PFU/reaction, could be detected by the two-step RT-LAMP-BART assay. This limit of detection was the same as that obtained using real-time RT-PCR and was one log lower than that of one-step RT-LAMP-BART assay.

Comparison of Real-Time RT-PCR, One-Step RT-LAMP-BART, and Two-Step RT-LAMP-BART Assay for the Detection of HAV from Different Foods

HAV separated and concentrated from green onions, strawberries, mussels, and milk artificially contaminated with different levels of the viral particles was analyzed using real-time RT-PCR, one-step RT-LAMP-BART, and two-step RT-LAMP-BART assay. The detection limit of the three methods varies according to food type (Table 2). Any food sample uncontaminated with the virus did not give an amplification signal for any of the three detection methods. For green onions, real-time RT-PCR, two-step RT-LAMP-BART, and one-step RT-LAMP-BART assay demonstrated a detection limit of 8.3×10^0 PFU/15 g, 8.3×10^0 PFU/15 g, and 8.3×10^1 PFU/15 g, respectively. For strawberries, both real-time RT-PCR and two-step RT-LAMP-BART were able to detect HAV level as low as 8.3×10^1 PFU/50 g. The one-step RT-LAMP-BART was apparently inferior to the other two methods in terms of detection limit, with virus at the inoculum level of 8.3×10^1 PFU/50 g or 8.3×10^0 PFU/50 g

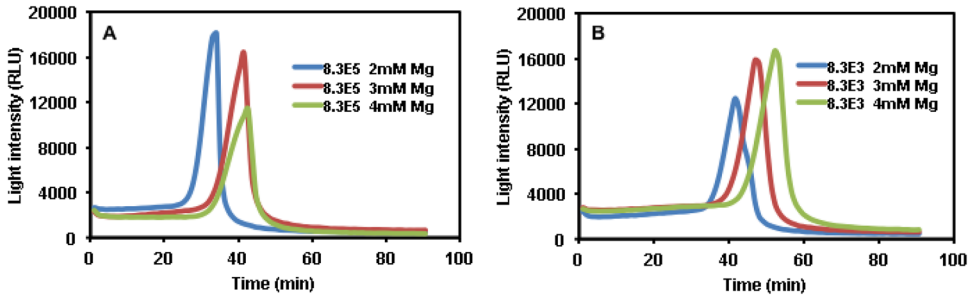

Fig. 4 Effect of $MgSO_4$ concentrations on one-step RT-LAMP-BART reaction of HAV. Each curve represents one of three replicates. **A** RNA was from 8.3×10^5 PFU/mL of HAV; **B** RNA was from 8.3×10^3 PFU/mL of HAV (Color figure online)

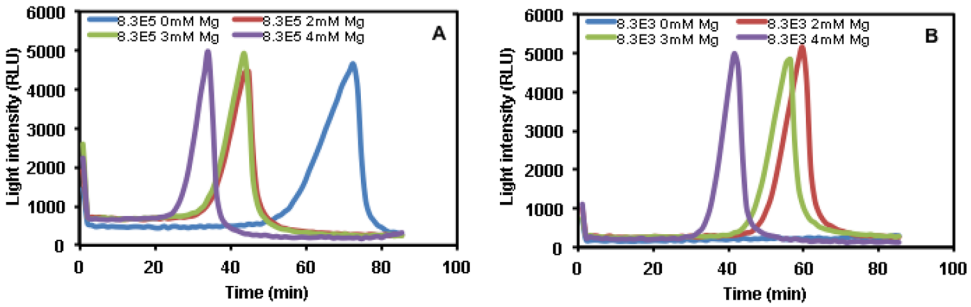

Fig. 5 Effect of $MgSO_4$ concentrations on two-step RT-LAMP-BART reaction of HAV. Each curve represents one of three replicates. **A** cDNA was from 8.3×10^5 PFU/mL of HAV; **B** cDNA was from 8.3×10^3 PFU/mL of HAV (Color figure online)

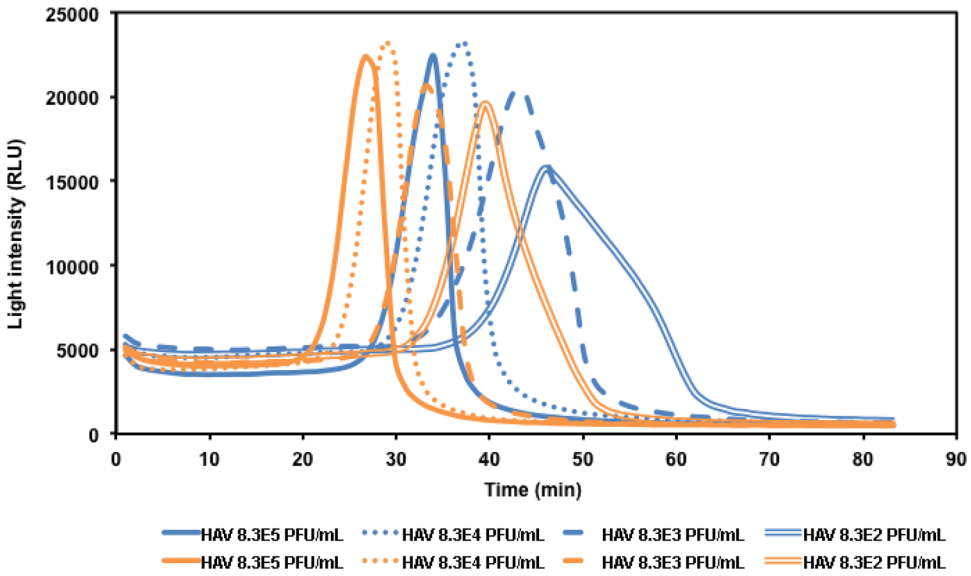

Fig. 6 Comparison of HPLC-purified and cartridge-purified primers in RT-LAMP-BART assay. Four HAV RNA dilutions corresponding to 8.3×10^5, 8.3×10^4, 8.3×10^3, and 8.3×10^2 PFU/mL of the initial virus titer were tested. The orange lines indicate the reactions using HPLC-purified primers. The blue lines indicate the reactions using cartridge-purified primers. Each curve represents one of three replicates (Color figure online)

not detectable. As for mussels and milk, all three methods showed a detection limit of 8.3×10^0 PFU/sample.

Discussion

Molecular techniques based on the amplification of genomic DNA/RNA of microorganisms have been used for the specific and sensitive detection of pathogens in foods. The most widely used detection method is real-time PCR, a technique developed in the early 1990s (VanGuilder et al., 2008). Since its invention, this technique has been improved dramatically. For example, different real-time chemistries have been used (e.g., SYBR green-based and TaqMan-based detection), different formats of the instrument have been developed (e.g., ABI 7900HT, ViiA 7, and QuantStudio real-time PCR system), and the master mix used in the technique has been optimized to minimize the amplification time and enhance tolerance to inhibitors. In spite of so much improvement, this technique requires sophisticated equipment and is expensive, making it impractical to be applied in resource-limited settings. Therefore, there is a rapidly increasing demand for

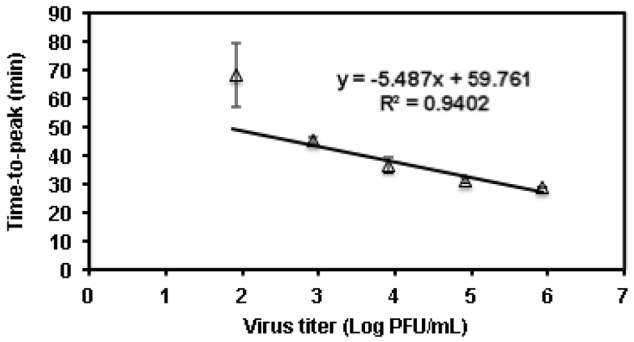

Fig. 7 Standard curve of one-step RT-LAMP-BART assay generated by testing the time-to-peak of tenfold serial dilutions of HAV RNA. Each value is the mean of three independent replicates. Bars show standard deviation. The assay was performed at 55 °C for 100 min

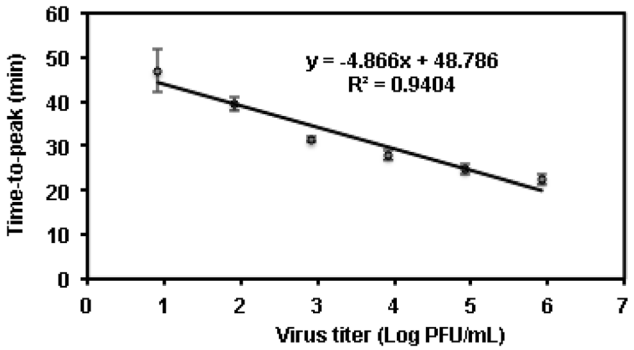

Fig. 8 Standard curve of two-step RT-LAMP-BART assay made by testing the time-to-peak of tenfold serial dilutions of HAV cDNA. Each value is the mean of three independent replicates. Bars show standard deviation. The assay was performed at 62 °C for 100 min

more affordable alternatives providing speed, simplicity, accuracy, and robustness in both molecular assay and the equipment for detecting foodborne pathogens (e.g., HAV).

As an alternative to PCR technology strictly requiring a sophisticated thermal cycler and fluorescence excitation and emission measurement equipment, LAMP reaction is conducted at a constant temperature, negating the need for a thermocycler. Because of its simplicity, this technique has provided a very convenient way for the detection of organisms including bacteria, viruses, fungi, parasites, animals, and plants (Aartse et al., 2017; Focke et al., 2013; Lalle et al., 2018; Li et al., 2017; Niessen et al., 2013; Parida et al., 2004; Sheu et al., 2018). Since its invention in 2000 (Notomi et al., 2000), this technique has also been improved dramatically. For example, conventional LAMP assay that was based on gel electrophoresis of the amplification products has been upgraded to real-time detection of the turbidity of magnesium pyrophosphate (Mori et al., 2004), of the fluorescence of chelating reagents (Tomita et al., 2008), or of the bioluminescence generated from PPi (Gandelman et al., 2010). The LAMP assay coupled with the detection of bioluminescence was named LAMP-BART in 2010 (Gandelman et al., 2010) and has shown great performance in detecting *Chlamydia trachomatis* (Gandelman et al., 2010), classical swine fever virus (Gandelman et al., 2010), genetically modified maize (Kiddle et al., 2012), human parvovirus B19 (Mirasoli et al., 2018), and SARS-CoV-2 (Fei et al., 2021).

The optimization of reaction system is essential in achieving detection sensitivity. We optimized Mg^{2+} concentration in the current study to improve the sensitivity of RT-LAMP-BART assay. The concentration of Mg^{2+} has also been optimized in previous studies. Liu et al. (2013) found

Table 2 Comparison of real-time RT-PCR, one-step RT-LAMP-BART, and two-step RT-LAMP-BART assays in detecting HAV in green onions, strawberries, mussels, and milk

Food sample	Method	No. of positive samples / No. of tested samples at inoculation level (PFU) of			
		8.3×10^5	8.3×10^3	8.3×10^1	8.3×10^0
Green onion	Real-time RT-PCR	6/6	6/6	6/6	4/6
	One-step RT-LAMP-BART	6/6	6/6	2/6	0/6
	Two-step-RT LAMP-BART	6/6	6/6	2/6	2/6
Strawberry	Real-time RT-PCR	6/6	6/6	6/6	0/6
	One-step RT-LAMP-BART	6/6	4/6	0/6	0/6
	Two-step-RT LAMP-BART	6/6	6/6	4/6	0/6
Mussel	Real-time RT-PCR	6/6	6/6	4/6	2/6
	One-step RT-LAMP-BART	6/6	6/6	4/6	4/6
	Two-step-RT LAMP-BART	6/6	6/6	2/6	2/6
Milk	Real-time RT-PCR	6/6	6/6	4/6	2/6
	One-step RT-LAMP-BART	6/6	6/6	6/6	6/6
	Two-step-RT LAMP-BART	6/6	6/6	6/6	2/6

For each food, virus was recovered on three distinct occasions and detection of viral RNA by real-time RT-PCR, one-step RT-LAMP-BART, and two-step RT-LAMP-BART was performed in duplicate resulting in six determinations for each load of virus. Virus inoculum level per food sample was 8.3×10^5 PFU, 8.3×10^3 PFU, 8.3×10^1 PFU, or 8.3×10^0 PFU. The RT-LAMP-BART assay was performed for 130 min

that 5.75 mM of Mg^{2+} was optimal in detection of *Leifsonia xyli* subsp. *xyli* in sugarcane using LAMP. Liu et al. (2011) reported that the optimal concentration of Mg^{2+} in using LAMP to detect *Bacillus cereus* in milk was 2 mM. Nie (2005) reported that 6–8 mM of Mg^{2+} were optimal in detecting *Potato virus Y* using LAMP. Aside from Mg^{2+} concentration, other factors influencing the sensitivity of LAMP reaction, such as primer concentration, dNTP (deoxyribonucleotide triphosphate) concentration, enzyme concentration, reaction temperature, and reaction time, were also optimized in these studies. However, Mg^{2+} concentration was found to be the most critical factor when optimizing LAMP reaction (Liu et al., 2011, 2013). The concentration of Mg^{2+} is important to the RT-LAMP-BART reaction because Mg^{2+} serves the cofactor of the enzymes used in the RT-LAMP-BART assay, such as reverse transcriptase, *Bst* polymerase, ATP sulfurylase, and luciferase (Cowan, 2002). ATP can only become active upon binding with Mg^{2+} (Cowan, 2002; Nakatsu et al., 2006), increasing the production of luminescence. Thus, inadequate Mg^{2+} in the reaction could lead to inactive enzymes and ATP and hence slow down chemical reaction rates. On the other hand, too much Mg^{2+} can bring many drawbacks to chemical reactions in RT-LAMP-BART, such as decreasing fidelity and specificity of DNA polymerase, interfering with complete denaturation of DNA strands during amplification, leading to primers annealing to incorrect sites of DNA template and causing nonspecific amplified products, as well as inhibiting DNA amplification through chelation by dNTPs (Goto et al., 2009; Kuffel et al., 2021). In this study, 2 mM Mg^{2+} and 4 mM Mg^{2+} was the optimal concentration for one-step RT-LAMP-BART and two-step RT-LAMP-BART, respectively.

In the current study, the primers used in RT-LAMP-BART and real-time RT-PCR for the detection of HAV were selected from the same conserved region (5'-UTR) of HAV genome. The specificity of primers used in LAMP for HAV detection has been examined by testing several genotypes of HAV and other enteric viruses, and they only amplify HAV target sequences (Yoneyama et al., 2007). For LAMP primers, the inner primers generate hairpin loops, and the outer primers displace the DNA strands. Aside from inner and outer primers, the use of loop primers (Nagamine et al., 2002) or stem primers (Gandelman et al., 2011) could accelerate the speed of amplification, and improve sensitivity of the reaction. We found that the sensitivity of the assay could also be improved by primer quality. HPLC-purified primers were used in some reports (Luo et al., 2011; Yang et al., 2011), and there were others using cartridge-purified primers (Yoda et al., 2007) in the LAMP assay. By comparing the results from HPLC-purified and cartridge-purified primers, we found that HPLC-purified primers demonstrated improved sensitivity of RT-LAMP-BART reaction. For primer synthesis, the longer the sequence, the higher the error rate and a larger number of failed truncated sequences are produced. HPLC can not only remove impurities such as salt and organic solvent, but also eliminate truncated sequences to a greater extent than cartridge purification technique, producing primers with higher yield and purity (Biolegio, https://www.biolegio.com/products-services/purification/). The inner primers (~40 bases) used in LAMP reaction are at least twice as long as the primers (~20 bases) used in PCR. HPLC technique could guarantee the lower error rate in inner primer sequences. Hardinge et al. (2018) reported that primer quality significantly affected the amplification performance of LAMP-BART assay in detection of DNA from genetically modified maize. They concluded that using HPLC-purified primers was very important because they could especially give reliable amplification of low copy number of DNA in a reaction.

The sensitivity of one-step and two-step RT-LAMP-BART assay were measured by testing tenfold serial dilutions of RNA obtained from a viral stock or cDNA. It was found that the limit of detection of two-step RT-LAMP-BART and one-step RT-LAMP-BART was 0.083 PFU/reaction and 2.1 PFU/reaction of HAV, respectively. The two-step RT-LAMP-BART showed higher sensitivity than one-step RT-LAMP-BART. The amplification temperature for LAMP assay is usually between 60 and 65 °C (Kokkinos et al., 2014). In the current study, the amplification temperature for two-step RT-LAMP-BART was 62 °C, but for one-step RT-LAMP-BART, the amplification temperature was 55 °C. This is because the reverse transcriptase in the RT-LAMP-BART-master developed by Lumora Ltd. was not stable above 60 °C. This lower amplification temperature might contribute to the lower sensitivity of the one-step RT-LAMP-BART in our experiment. The sensitivity of two-step RT-LAMP-BART and real-time RT-PCR used in our study were the same: 0.083 PFU/reaction, corresponding to the virus titer of 8.3×10^0 PFU/mL. Compared with the limited published data, the value was lower than the 0.5 PFU/reaction reported by Jothikumar et al. (2005) who used TaqMan real-time PCR, lower than 1 PFU/reaction reported by El Galil et al. (2004) for using molecular-beacon real-time RT-PCR, lower than 2 PFU/reaction reported by Jean et al. (2002) for using nucleic acid sequence-based amplification (NASBA) method, and lower than 0.1 PFU/reaction reported by Hu and Arsov (2014) for using nested real-time PCR for the detection of HAV RNA.

HAV seeded on green onions, strawberries, mussels, or in milk, was detected using real-time RT-PCR, one-step RT-LAMP-BART and two-step RT-LAMP-BART. The sensitivities of two-step RT-LAMP-BART and real-time RT-PCR were comparable in testing HAV in each food (Table 2). One-step RT-LAMP-BART assay showed better performance in testing HAV in mussels and milk than in green onions and strawberries, which might be due to the

fact that primers purified with different methods were used when detecting HAV in different foods. The cartridge-purified primers were used for testing HAV in green onions and strawberries, while HPLC-purified primers were used for testing the virus in mussels and milk. For strawberries, HAV at an inoculum level of 8.3×10^0 PFU/50 g was not detectable by any of the three methods, which might be due to the low efficiency of the virus separation and concentration step or the strong inhibition of the reactions from strawberry compounds. The detection limit of RT-LAMP-BART assay developed in the current study may be able to satisfy the need for diagnostic purpose, because HAV infectious dose is presumably between 10 and 100 viral particles and the contamination level of virus is low in food (Sánchez et al., 2007; Yezli & Otter, 2011). The detection limit of HAV in different foods obtained in our study using PMNP separation and concentration method coupled with RT-LAMP-BART is comparable with or better than that reported by other research groups using different virus detection methods (Table 3).

The amplification time of the RT-LAMP-BART (130 min) was longer than that of real-time RT-PCR (40 min) used in the current study. The reaction system for PCR, including reagents and instrument, has been improved considerably since the method was invented. For example, in our previous study, we used ABI 7900HT Fast Real-Time PCR System coupled with Absolute QPCR ROX mix to detect HAV (Wu et al., 2022). The amplification time was 100 min with the use of this system, while the amplification time was reduced to 40 min with the application of ViiA 7 system and TaqMan Fast Advanced Master Mix (Wu et al., 2022). RT-LAMP-BART assay is a relatively novel technology and the amplification time can also be reduced through optimizing the reaction conditions, such as the master mix and equipment. It was reported that the LAMP amplification time could be reduced by about 50% when the new *Bst* 2.0 or *Bst* 2.0 WarmStart DNA polymerase was used (Tanner et al., 2012) compared to the use of the wild-type *Bst* DNA polymerase. In future, the components of the master mix used for the RT-LAMP-BART reaction may be optimized to further improve the sensitivity, speed, and robustness of the method.

The RT-LAMP-BART assay has several advantages over real-time RT-PCR. The instrument used for RT-LAMP-BART and the software used for data interpretation are simple, because RT-LAMP-BART relies on the time-to-peak of light output not the absolute light intensity (Gandelman et al., 2010). An instrument containing a heating block capable of controlling temperature and a photodiode or a charge-coupled device (CCD) camera for detecting light can satisfy the requirements of the method (Gandelman et al., 2010). Real-time PCR is based on the detection of absolute fluorescence intensity during a thermal cycling reaction and requires an instrument consisting of a light-emitting diode (LED) for emitting a broad spectrum of light, filters for selecting the excitation and emission wavelength of specific fluorophores, mirrors for reflecting light, a photodiode, CCD or photomultiplier tube for detecting emitted light, and a device for heating and cooling the reaction plate. The instrument can only be operated by trained personnel and needs to be calibrated frequently to guarantee accurate experimental results. Instead, any person can operate the Bison system (Lumora Ltd.) used for RT-LAMP-BART assay after a short training session and the maintenance of the instrument is simple and easy. The instrument software used for real-time PCR protocol setup, data collection, and data analysis are much more complicated than that for RT-LAMP-BART.

Table 3 Detection limit of HAV in different foods using different detection methods

Food	Detection method	Detection limit	Reference
Green onion	Real-time RT-PCR	10^2 PFU/25 g	Zheng and Hu (2017)
Green onion	RT-PCR	1 $TCID_{50}$ (approximately 1.4 PFU)/25 g	Guevremont et al. (2006)
Green onion	Nested real-time PCR	1 PFU/25 g	Hu and Arsov (2014)
Green onion	Real-time RT-PCR	Not determined	Lowther et al. (2019)
Green onion	RT-LAMP-BART	8.3×10^0 PFU/15 g	This study
Strawberry	RT-PCR	10^4 RT-PCR unit/90 g	Rzezutka et al. (2006)
Strawberry	Real-time RT-PCR	1.2 $TCID_{50}$ (approximately 1.7 PFU)/15 g	Butot et al. (2007)
Strawberry	RT-LAMP-BART	8.3×10^1 PFU/50 g (10 RT-PCR unit/50 g)	This study
Shellfish	Real-time RT-PCR	10^0 $TCID_{50}$ (approximately 1.4 PFU)/1.5 g	Ko et al. (2018)
Shellfish	RT-PCR	10^0 $TCID_{50}$ (approximately 1.4 PFU)/1 g	Ko et al. (2015)
Shellfish	RT-PCR	1.5 PFU/3.75 g	Kingsley and Richards (2001)
Shellfish	Real-time RT-PCR	25 $TCID_{50}$ (approximately 35 PFU)/25 g	Casas et al. (2007)
Shellfish	Real-time RT-PCR	Not determined	Lowther et al. (2019)
Shellfish	RT-LAMP-BART	8.3×10^0 PFU/5 g	This study
Milk	RT-LAMP-BART	8.3×10^0 PFU/40 mL	This study

Moreover, due to the isothermal characteristic of the reaction, the RT-LAMP-BART instrument has the potential to be miniaturized to become a portable device that is suited for field applications. But PCR can only be applied in the laboratory and not on-site. Various formats of portable devices have been designed to perform the LAMP reaction in remote areas where resources are limited. A portable ESE Quant tube scanner has been used for detection of fluorescence signal of SYBR Green I in the amplification of the genome of *Vibrio parahaemolyticus* and malaria using LAMP (Lucchi et al., 2010; Surabattula et al., 2013; Yi et al., 2014). It was also reported that the LAMP reaction could be performed on a silicon chip for the detection of virulence genes of *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella* (Duarte et al., 2013). Microfludic devices have also been developed for the detection of foodborne pathogens such as *Campylobacter jejuni, Shigella, Salmonella* Typhimurium, and *Vibrio cholerae* using the LAMP technique (Hsieh et al., 2012; Tourlousse et al., 2012). All these portable devices are based on fluorescence detection. The portable device for RT-LAMP-BART will be easier to design because luminescence detection is much simpler than fluorescence. Lumora Ltd. has manufactured a portable instrument capable of testing eight samples at one time to enable the RT-LAMP-BART technique to be performed in the field (Kiddle et al., 2012). Song et al. (2018) designed a smartphone-based mobile detection platform for rapid detection of Zika virus in urine and saliva and HIV in blood using BART-LAMP technology. The platform is suitable for use at home and in the field. With the great potential of pathogen specific and ready-to-use reagent (e.g., detection kit) to be developed and the applicability of less expensive and easy-to-use portable devices, the RT-LAMP-BART technique is a promising new tool in detection of foodborne pathogens (e.g., HAV) in future.

In conclusion, RT-LAMP-BART showed good performance in the detection of HAV concentrated from different foods including green onions, strawberries, mussels, and milk in the current study. The developed methods need to be compared with the procedures in ISO 15216 for foodborne HAV detection in future. RT-LAMP-BART showed comparable sensitivity with real-time RT-PCR for detecting HAV in different foods. The reaction time of the RT-LAMP-BART may be dramatically reduced through optimizing the master mix in the future. The short testing time in combination with the miniaturized simple instruments may make this technique very powerful in detection of foodborne pathogens.

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Data Availability The data used to support the findings of this study are provided in full in the results section of this paper.

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