

A real-time loop-mediated isothermal amplification assay for rapid detection of *Shigella* species

Liew, P.S.^{1,2}, Teh, C.S.J.^{2,3}, Lau, Y.L.⁴ and Thong, K.L.^{1,2*}

¹Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

²Laboratory of Biomedical Science and Molecular Microbiology, Institute of Graduate Studies, University of Malaya

³Department of Medical Microbiology, Faculty of Medicine, University of Malaya

⁴Department of Parasitology, Faculty of Medicine, University of Malaya

*Corresponding author email: thongkl@um.edu.my

Received 21 January 2014; received in revised form 16 April 2014; accepted 25 April 2014

Abstract. Shigellosis is a foodborne illness caused by the genus *Shigella* and is an important global health issue. The development of effective techniques for rapid detection of this pathogen is essential for breaking the chain of transmission. Therefore, we have developed a novel loop-mediated isothermal amplification (LAMP) assay targeting the invasion plasmid antigen H (*ipaH*) gene to rapidly detect *Shigella* species. This assay could be performed in 90 min at an optimal temperature of 64°C, with endpoint results visualized directly. Notably, the method was found to be more sensitive than conventional PCR. Indeed, the detection limit for the LAMP assay on pure bacterial cultures was 5.9×10^5 CFU/ml, while PCR displayed a limit of 5.9×10^7 CFU/ml. In spiked lettuce samples, the sensitivity of the LAMP assay was 3.6×10^4 CFU/g, whereas PCR was 3.6×10^5 CFU/g. Overall, the assay accurately identified 32 *Shigella* spp. with one enteroinvasive *Escherichia coli* displaying positive reaction while the remaining 32 non-*Shigella* strains tested were negative.

INTRODUCTION

The genus *Shigella* belongs to the family *Enterobacteriaceae*, which is characterized by Gram-negative, rod-shaped, non-motile, non-spore forming facultative anaerobes. There are four species of *Shigella*: *Shigella flexneri*, *Shigella sonnei*, *Shigella boydii*, and *Shigella dysenteriae*. *Shigella* is widely acknowledged as one of the most important foodborne pathogens involved in shigellosis outbreaks. Indeed, *S. dysenteriae* is the primary pathogen causing bacillary dysentery (Trofa *et al.*, 1999). Symptoms of shigellosis include a short period of watery diarrhea, abdominal pains, and malaise, which can be followed by permanent emission of stools with blood and mucus (World Health Organization, 2009).

Shigella is more common in developing countries, particularly in Asia. In fact, it is

estimated that 125 million cases of shigellosis occur annually in Asia, resulting in 14,000 deaths (Bardhan *et al.*, 2010). Also, shigellosis is more common in children less than five years old (von Seidlein *et al.*, 2006). *Shigella* is transmitted through consumption of contaminated foods (e.g., potato salad, raw vegetables, meat, and milk) and improper food handling.

Several assays for detecting *Shigella* have been reported. The direct culture method is time consuming and involves multiple steps, such as pre-enrichment and enrichment, followed by plating on selective and diagnostic agars (FDA, 2001). In order to overcome issues related to low specificity and sensitivity, PCR-based methods were developed (Li *et al.*, 2004; Thong *et al.*, 2005). Although PCR assays can allow rapid detection of food-borne pathogens (within 24 h), they display limitations, including a

requirement for trained personnel, high risk of contamination, low detection limit, and need for expensive equipment/reagents (Wang *et al.*, 2012).

Notomi *et al.* (2000) developed a novel technique termed loop-mediated isothermal amplification (LAMP), which represents a simple, rapid, specific, and cost-effective nucleic acid amplification method. The assay is performed under isothermal conditions (constant temperature) and utilizes a strand displacement reaction, omitting the need for fluctuating temperatures to denature and re-anneal DNA strands. Moreover, the LAMP assay displays very high specificity due to the use of four primers, which recognize six distinct regions of the target gene (Notomi *et al.*, 2000; Zhang *et al.*, 2011). Furthermore, the whole process from sample collection to detection requires only 90 minutes, and endpoint results can be achieved based on simple fluorescence and turbidity visualization.

Here, we report the development of a rapid, simple, and novel LAMP assay for the detection of four *Shigella* species. Moreover, we compared the specificity and sensitivity of our LAMP assay to conventional PCR-based detection of *Shigella* using pure cultures and spiked lettuce samples.

MATERIALS AND METHODS

Bacterial strains

A total of 65 bacterial strains were used, including 13 *Shigella sonnei*, 9 *Shigella flexneri*, 1 *Shigella boydii*, 9 *Shigella dysenteriae*, and 33 non-*Shigella* spp. as negative controls. All the bacteria were previously obtained from clinical samples, food or zoonotic sources. The bacteria from glycerol stocks were cultured on Luria-Bertani (LB) agar plate and allowed to grow overnight to obtain single colonies. Then, a single colony was cultured on selective Xylose lysine deoxycholate (XLD) agar to check for purity of *Shigella* spp.

DNA extraction by boiling method

A loopful of bacterial colonies was suspended in 100 μ l of deionized water. The bacterial suspensions were heated at 99°C for 5 min and immediately chilled on ice for 10 min. Finally, the crude lysates were centrifuged at 13,400 rpm for 3 min and the supernatant was used as DNA templates for PCR and LAMP assays.

Polymerase Chain Reaction (PCR)

The primers used for PCR were Shig1 and Shig2 (targeted *ipaH* gene) previously reported by Thong *et al.* (2005) (Table 1). The PCR reaction was performed in 25 μ l volumes containing 1X PCR buffer (Promega, USA), 1 mM MgCl₂, each of the deoxynucleoside triphosphates (dNTPs) at 0.02 mM, 0.4 μ M of each Shig1 and Shig2 primers, 1U of *Taq* DNA polymerase (Promega, USA) and 5 μ l DNA template (~50 ng/ μ L). A negative control (using water as template) was included.

DNA amplification was performed in an Eppendorf Mastercycler EP Gradient Thermal Cycler. The thermal profile for PCR was 95°C at 5 min for initial denaturation, followed by 30 cycles of 95°C for 50 s, 55°C for 1.5 min and 72°C for 2 min and a final extension cycle at 72°C for 7 min. The PCR products (5 μ l each) were analyzed on a 1.5% agarose gel by electrophoresis. A 100 bp ladder was used as a molecular size marker. The gel was then stained and visualized by using Gel Doc XR documentation system (Bio-Rad Laboratories, Inc., CA, USA).

Primer design for LAMP assay

The FIP, BIP, F3, and B3 primers were designed using the LAMP Primer Explorer V4 software (<http://primerexplorer.jp/elamp4.0.0/index.html>) to target 6 distinct regions of *ipaH* gene based the published DNA sequence of the *Shigella flexneri* 2a str. 2457T (GenBank: NC004741) (Table 1). The specificity of the primers for the selected regions of *ipaH* gene was confirmed

Table 1. List of primer sequences of the PCR and LAMP

Methods	Primers	Length	Sequences (5'→3')	Source
PCR	Shig 1	20	TGGAAAACTCAGTGCCTCT	Thong <i>et al.</i> , 2005
	Shig 2	20	CCAGTCCGTAAATTCATTCT	
LAMP	F3	19	GGAGAATTCCCAGGCATTC	This study
	B3	20	TCCGGAGATTGTTCCATGTG	
	FIP (F1c – F2)	40	CGACCTGTTACGGAATCCGG- CGTTCCTTGACCGCCTTTC	
	BIP (B1c – B2)	38	CTGCGGAGCTTCGACAGCAG- CGGTCCTCACAGCTCTCA	

using BLAST of the National Center for Biotechnology Information (NCBI) server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The primers were synthesized commercially by Bioneer (Bioneer, Inc., CA, USA).

LAMP assay

The LAMP reaction was conducted as described by Notomi *et al.* (2000) using Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan). Briefly, the LAMP reaction was carried out in a final volume of 25 µl containing 12.5 µl of 2X reaction mix provided in the kit, 40 pmol of each primer FIP and BIP, 5 pmol of each primer F3 and B3, 8U of *Bst* DNA polymerase and 2.5 µl of DNA template. The same reaction mixture without template DNA (replaced by deionized water) was used as a negative control.

The reactions were carried out in the LA-500 Loopamp real-time turbidimeter (Eiken Chemical Co., Ltd., Tokyo, Japan). This device is specially designed for real-time reporting of loop-mediated isothermal amplification reaction whereby it detects and records the turbidity values every 6 s. The reactions were conducted at 64°C for 90 min, followed by enzyme inactivation at 80°C for 2 min. The experiment was performed twice to ensure reproducibility.

Visualisation of LAMP product

By using the real-time turbidimeter, positive amplification was indicated when the

turbidity exceeds the threshold (T_t) value which is 0.1 within the time of amplification (Mori *et al.*, 2004). The end-point detection of positive results could also be determined by direct visualization of the turbidity of the mixtures. After a brief centrifugation, presence of accumulated white pellet indicates positive results (Mori *et al.*, 2001). Another method of visualization of end product is the addition of SYBR green dye. An aliquot of 1 µl of 10-fold diluted SYBR Green I (Invitrogen, CA, USA) was added to 25 µl of amplicons of LAMP assay. A positive or negative result was determined through both visual observation of the color change of the solution under visible light and a fluorescence assay under ultraviolet (UV). For further confirmation, 5 µl of LAMP products were subjected to horizontal electrophoresis in 1.5% agarose gel. A 100 bp ladder was used as a molecular size marker. The gel was then stained with Gel Red for 30 min and photographed using Gel Doc XR documentation system (Bio-Rad Laboratories, Inc., CA, USA).

Specificity test of LAMP and PCR amplification

To determine the specificity of the primers, LAMP assay was applied on a total of 32 *Shigella* spp. and 33 non-*Shigella* strains. The non-*Shigella* strains included *Escherichia coli*, *Salmonella* spp., *Vibrio* spp., *Yersinia* spp., *Klebsiella pneumoniae* and *Acinetobacter baumannii*.

Sensitivity test of LAMP and PCR amplification on pure bacterial cultures

A *S. sonnei* strain (TH 2/10) was used to determine the sensitivity of LAMP and PCR detections. A single colony of bacteria was inoculated in 1 ml of Luria-Bertani (LB) broth. The culture was allowed to grow for 3-5 hours at 37°C until the OD₆₀₀ was approximately 1. The broth culture was serially diluted 10-fold and each dilution was used as DNA template for the PCR and LAMP amplification as described in Section 2.3 and Section 2.5. The same reaction mixture without template DNA (replaced by sterile water) was used as a negative control. The amplicons were analyzed by gel electrophoresis in 1.5% agarose gel. Each assay was repeated at least twice to ensure reproducibility.

Sensitivity test of LAMP and PCR amplification with spiked food

Lettuce was chosen as the food matrix to evaluate the sensitivity of LAMP assay in this study. This is because lettuce is commonly eaten raw or as part of the ready-to-eat food ingredient. Preparation of bacterial culture was performed as described in Section 2.8. Briefly, a 10 g sample of lettuce was spiked with 1 ml of bacterial culture and left at room temperature for around 30 min. The food was homogenized with 90 ml of Gram-negative (GN) broth using a stomacher to produce 1:10 lettuce-GN homogenate. The homogenate was then analyzed immediately. Briefly, the homogenate was subjected to 10-fold serial dilutions. An aliquot of 100 µl of each dilution was spread onto *Salmonella-Shigella* agar in duplicates for colony count while another 100 µl of the dilutions were centrifuged at 13,400 rpm for 3 min for crude DNA extraction for later used in LAMP and PCR assay. Besides that, enrichment was performed by incubating the *Shigella*-spiked produce homogenate overnight at 37°C. After that, the homogenate was processed similarly as described above for colony count and also the crude DNA preparation for PCR and LAMP assay.

Data analysis

Standard curves for sensitivity of LAMP assay with pure bacterial culture and spiked lettuce

were generated by plotting time threshold, *Tt* values against log CFU/ml for the real-time turbidimeter platform and the linear regression was calculated using the Microsoft Excel Software. The detection limits (number of CFU/ml in pure culture or in spiked lettuce) were presented as the lowest numbers of the cells that could be detected by the assays. Quantitative capabilities of the assays were derived based on the correlation coefficient (R^2) values from the standard curves.

RESULTS

Optimization of the LAMP condition for detection of *Shigella* spp.

The parameters tested were the temperature and time taken to amplify DNA. The temperatures tested were 60°C, 64°C and 65°C. No amplification of DNA was observed at 60°C while amplification was observed at 64°C and 65°C. Both temperatures showed almost the same amplification time, but a higher turbidity value was obtained at 64°C (data not shown). Hence, the temperature at 64°C was selected for subsequent analysis. Amplifications occurred between 49.5 to 85 min for the 65 strains tested (data not shown). Hence, the amplification time was set within 90 min.

Specificity of LAMP and PCR amplification

Positive reactions were detected in all 32 *Shigella* strains. In the real-time turbidimeter platform, the time threshold, *Tt* values fell between 49.5 to 85 min with the average of 67.2 min for DNA prepared from cell densities of 5.9×10^6 to 5.9×10^9 CFU/ml. Among the 10 *E. coli* strains tested, only enteroinvasive *E. coli* (EIEC) gave positive reaction. No *Tt* value was observed for the other 32 non-*Shigella* strains. These results obtained by using LAMP assay were comparable with PCR (Table 2). All the tubes that showed positive reactions for *Shigella* spp. were turbid and white pellets were formed after a brief centrifugation (data not shown). The LAMP products were also subjected to agarose gel electrophoresis (Fig. 2) and ladder-like DNA bands were observed. After



Figure 1. Locations and nucleotide sequence of the *ipaH* gene of *Shigella flexneri* 2a str. 2457T used for designing the PCR and LAMP primers

Table 2. Bacterial strains used and results for PCR and LAMP assay for detection of *Shigella*

Bacterial species	No. tested	No. of positive for	
		PCR	LAMP
<i>Shigella</i>			
<i>Shigella sonnei</i>	13	13	13
<i>Shigella flexneri</i>	9	9	9
<i>Shigella boydii</i>	1	1	1
<i>Shigella dysenteriae</i>	9	9	9
Non-<i>Shigella</i>			
<i>Escherichia coli</i>	10	1	1
<i>Salmonella</i> Enteritidis	2	0	0
<i>Salmonella</i> Albany	1	0	0
<i>Salmonella</i> Typhimurium	1	0	0
<i>Salmonella</i> Typhi	2	0	0
<i>Vibrio</i> spp.	2	0	0
<i>Vibrio cholerae</i>	2	0	0
<i>Vibrio parahaemolyticus</i>	2	0	0
<i>Acinetobacter baumannii</i>	2	0	0
<i>Klebsiella pneumoniae</i>	7	0	0
<i>Yersinia enterocolitica</i>	2	0	0

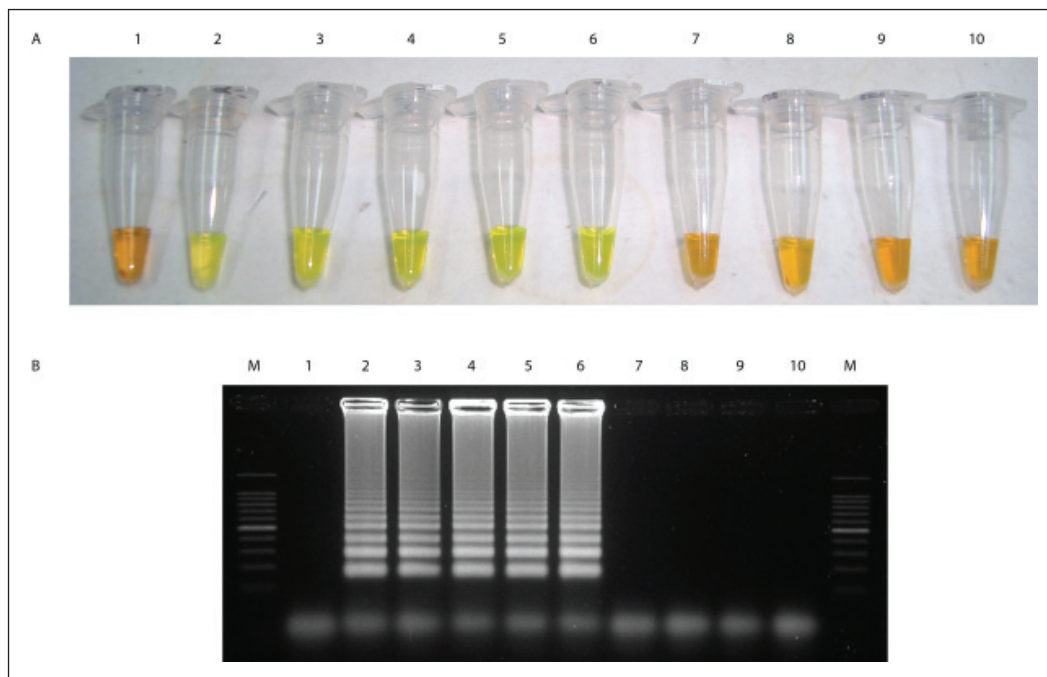


Figure 2. Specificity test of the LAMP reaction for detection of *ipaH* gene of *Shigella* (A) using SYBR Green I (B) and through gel electrophoresis.

Lane M: 100-bp DNA ladder as size markers; Lane 1: negative control (no DNA template); Lane 2-6: *Shigella sonnei* (TH 7/08), *Shigella sonnei* (TC 25/98), *Shigella dysenteriae* (TH 23/97), *Shigella flexneri* (TH 1/01), and *Shigella boydii* (TH 26/98) respectively; Lane 7: *Salmonella* Typhimurium (STM 342/04); Lane 8: *Acinetobacter baumannii* (AC 090203); Lane 9: *Vibrio cholerae* (VC 1338); Lane 10: *Escherichia coli* (EC 15).

the addition of SYBR Green I, all the tubes with positive reactions demonstrated a color change from orange to green-yellowish whereas for tubes with non-*Shigella* spp. the original orange colour of SYBR Green I was retained (Fig. 2).

Sensitivity and quantitative capabilities of LAMP and PCR amplification with pure bacterial cultures

The sensitivity of the *ipaH*-based LAMP assay and PCR assay were tested using 10-fold serial dilutions of viable culture of *Shigella sonnei* in 0.85% saline solution. A representative turbidity graph from the real-time turbidimeter platform is shown in Fig. 3A. For pure culture templates ranging from 5.9×10^5 to 5.9×10^8 CFU/ml, there were amplifications with the *Tt* values fell between 60 to 110 min. No amplification was obtained for DNA from cell densities of 5.9×10^2 to 5.9×10^4 CFU/ml. Therefore, the detection limit of the LAMP assay on pure culture analysed

in a real-time turbidimeter was 5.9×10^5 CFU/ml. In contrast, the PCR assay on pure culture had a detection limit of 5.9×10^7 CFU/ml (Fig. 4). Therefore, LAMP assay was 100-fold more sensitive than PCR assay. A positive amplification by LAMP assay was shown by the increase of turbidity while the PCR assay was indicated by the 422 bp band (Fig. 4).

A standard curve of the tests on 10-fold serially diluted pure culture of *S. sonnei* strain TH 2/10 was generated by the real-time turbidimeter with the cell numbers ranging from 5.9×10^5 to 5.9×10^8 CFU/ml. According to the standard curve, the correlation coefficient (R^2) value for LAMP assay was 0.908. The high R^2 value indicates good linear relationships between *Shigella* cell numbers (log CFU/ml) and the amplification signals (*Tt* values), whereby the *Tt* value decreases as the cell numbers of *Shigella* increases (Fig. 3). PCR, on the other hand, is not quantitative.

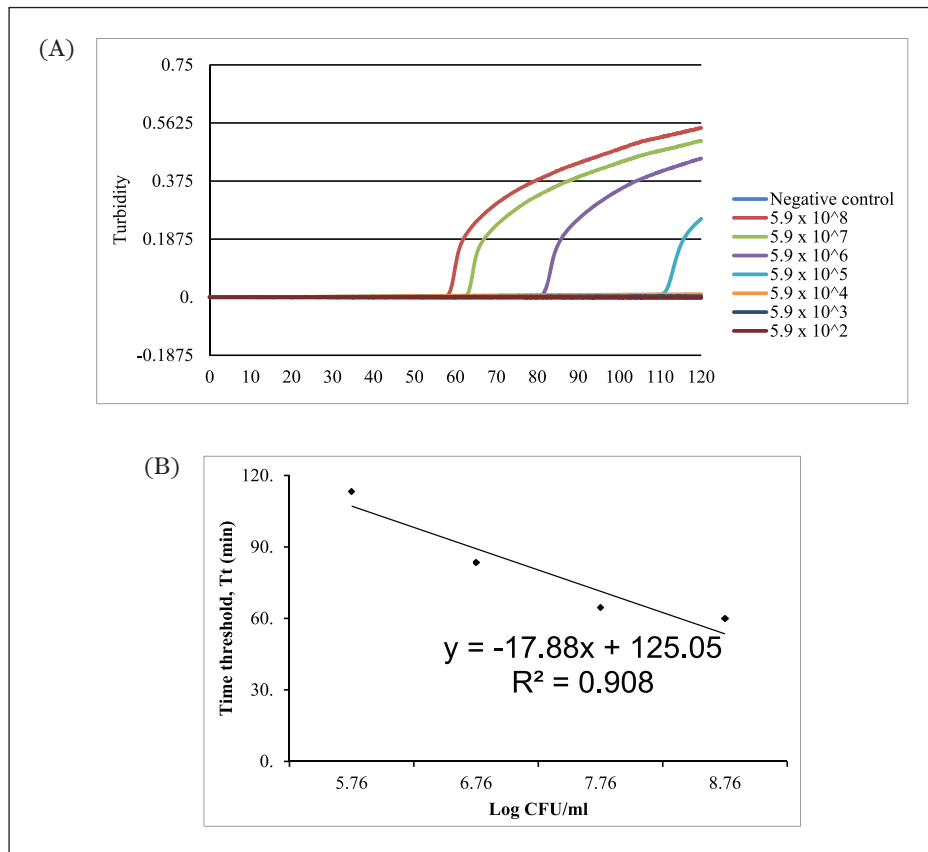


Figure 3. Sensitivity test for detection of *ipaH* gene of *Shigella* from pure cultures using real-time turbidimeter.

(A) The curves from left to right indicate the decreasing concentration of CFU from bacterial colonies ranging from 5.9×10^8 CFU/ml to 5.9×10^2 CFU/ml. The detection limit for LAMP assay was 5.9×10^5 CFU/ml. (B) LAMP standard curve was generated for cell concentrations ranging from 5.9×10^5 to 5.9×10^8 CFU/ml.

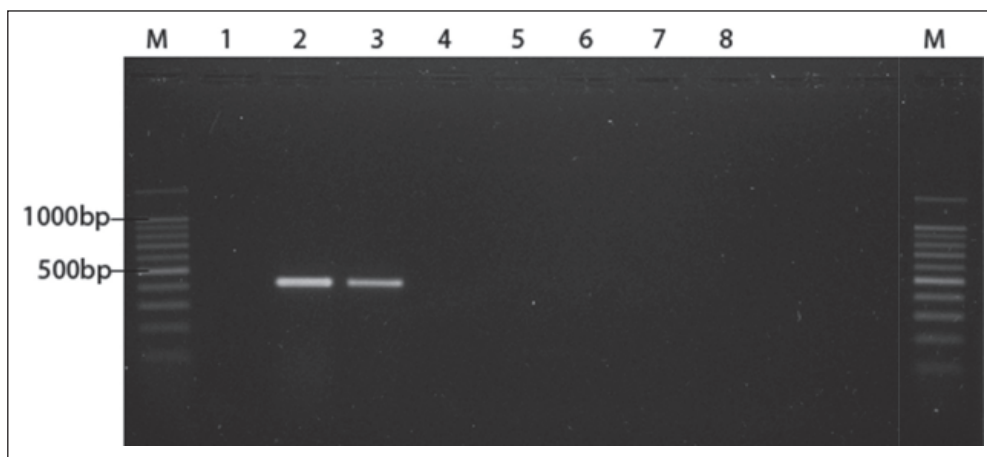


Figure 4. Sensitivity test for detection of pure culture *Shigella* by PCR assay.

Lane M: 100bp DNA ladder; Lane 1: negative control; Lanes 2-8: ten-fold serial dilutions of TH 2/10 strain from 5.9×10^8 CFU/ml to 5.9×10^2 CFU/ml. The detection limit for PCR assay was 5.9×10^7 CFU/ml.

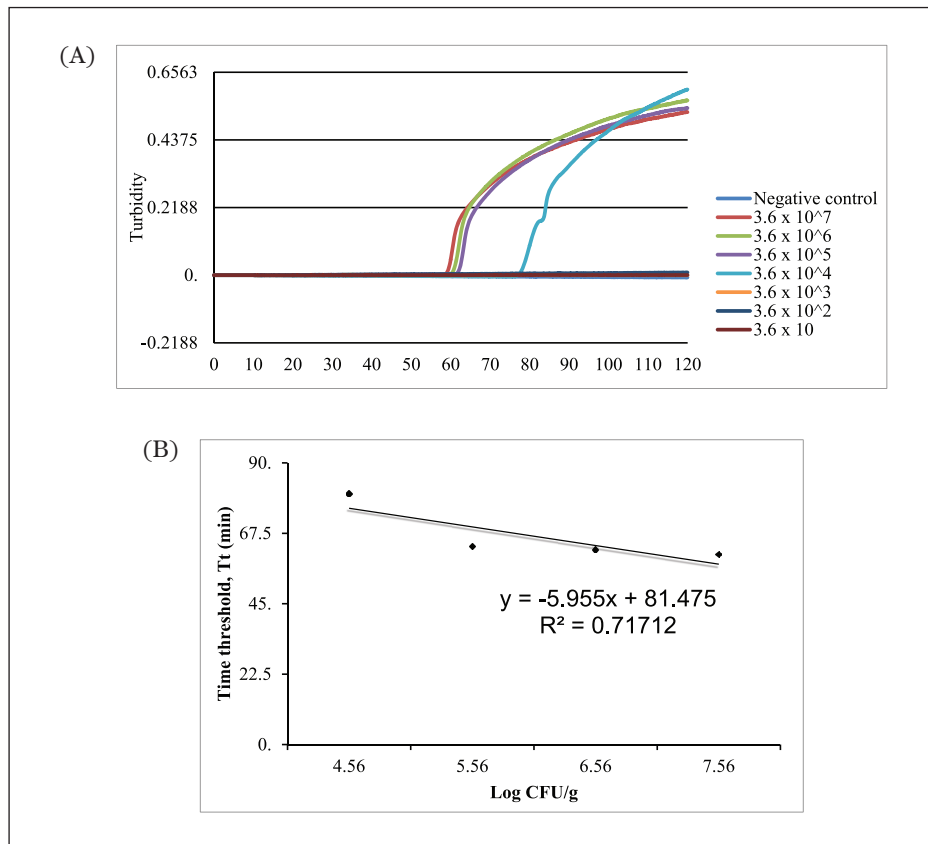


Figure 5. Sensitivity test for detection of *ipaH* gene of *Shigella* from spiked lettuce using real-time turbidimeter.

(A) The curves from left to right indicate the decreasing concentration of CFU for bacterial colonies ranging from 3.6×10^7 CFU/g to 3.6×10^1 CFU/g. The detection limit for LAMP assay was 3.6×10^4 CFU/g. (B) LAMP standard curve was generated for cell concentrations ranging from 3.6×10^4 to 3.6×10^7 CFU/g.

Sensitivity test and quantitative capabilities of LAMP and PCR amplification in spiked lettuce

When LAMP and PCR tests were done using food homogenates without an overnight incubation, no positive result was obtained for spiked samples with cell density ranging from 3.6×10^1 to 3.6×10^7 CFU/g (data not shown). However, after an overnight incubation, there were amplifications with the T_t values between 60 to 80 min for DNA prepared from cell concentration starting from 3.6×10^4 to 3.6×10^7 CFU/g (Fig. 5A). The LAMP assay gave negative results for samples with cell density ranging from 3.6×10^1 to 3.6×10^3 CFU/g. Therefore, the detection limits of the LAMP assay run in a real-time turbidimeter on spiked lettuce

samples was 3.6×10^4 CFU/g. In contrast, the PCR assay on spiked lettuce samples had a detection limit of 3.6×10^5 CFU/g (data not shown).

Figure 5B shows the standard curves of the tests on 10-fold serially diluted *S. sonnei* strain TH 2/10 in spiked lettuce homogenates generated by the real-time turbidimeter. According to the standard curve, the correlation coefficient (R^2) value for LAMP assay was calculated to be 0.717. The R^2 value was not as high as the R^2 value (0.908) for pure culture but it still indicates a linear relationship between *Shigella* cell numbers (log CFU/g) and the amplification signals (T_t values), whereby the T_t value decreases as the cell numbers increases.

DISCUSSION

The LAMP primers employed in our assay were designed to detect the *ipaH* gene of *Shigella* spp. This gene is located on a 4.6 kb *Hind*III fragment derived from the invasion plasmid, which encodes various invasion-related outer membrane polypeptides (i.e., invasion plasmid antigens). The *ipaH* gene not only exists as multiple copies on the invasion plasmid of virulent *Shigella*, but can also be found in the chromosome (Buysse *et al.*, 1995). This gene has been widely used as a target in molecular methods for detecting *Shigella* spp. (Lüscher and Altwegg, 1994; Theron *et al.*, 2001; Thong *et al.*, 2005), including LAMP method. Notably, *ipaH* can also be found in a pathotype of *E. coli*, enteroinvasive *E. coli* (EIEC) (Chapman *et al.*, 2006). While assessing the specificity of our assay primers, we observed non-*Shigella* samples that tested positive for *ipaH*, which likely reflects the presence of EIEC. Such observation was also reported by Song *et al.* (2005). The EIEC strain used in this study was a kind gift of Gomez-Daurte (Gomez-Duarte *et al.*, 2009).

The first study to use the *ipaH* gene as target gene to design the LAMP primers for detection of *Shigella* was reported by Song and his colleagues (Song *et al.*, 2005). Their LAMP method efficiently detected the gene within 2 h with high specificity and sensitivity (detection limit of 4×10^3 CFU/ml). We did not use their LAMP primers in our study because our initial aim was to design loop primers by using our own designed primers to shorten the amplification time by half or one third (Zhao *et al.*, 2010). However the loop primers could not be developed as there were no suitable sequences. Although the sensitivity in our study (5.9×10^5 CFU/ml) was not as high as that reported in Song *et al.* (2005), we used the real-time turbidimeter which detected and recorded the turbidity values every 6 s without the end-point detection such as gel electrophoresis and product visualization methods. Later on, Shao *et al.* (2011) utilized the Song's LAMP primers to test for *Shigella*. The specificity reported was in agreement with Song *et al.* (2005).

Both the studies showed high specificity and sensitivity but post amplification analysis was needed to confirm the positive results. Soli and his colleagues (Soli *et al.*, 2013) evaluated the LAMP end-point detection methods, namely turbidity visualization, use of dye such as hydroxynaphthol blue (HNB) and SYBR Green I, and readings from the Loopamp Endpoint Turbidimeter. For end-point detection, their study showed that LAMP colorimetric detection methods have equivalent or better sensitivity than visualization of precipitate or automated turbidity readers. This suggested that colorimetric methods could be used as one of the inexpensive methods to interpret the results. Although LAMP assays have been developed by others for *Shigella* spp., our study was the first to use the real-time turbidimeter for detection of this foodborne pathogen and tested on artificially spiked food and the results were comparable to those studies.

We found that temperature was a crucial parameter during development of our LAMP assay. In order to increase the efficiency of the assay, the LAMP primers (outer primers FIP and BIP [F3 and B3]) were chosen using LAMP primer design software so that the melting temperatures (T_m) ranged from 60°C to 65°C. Indeed, the *Bst* DNA polymerase employed in this study functions optimally between these temperatures, facilitating primer annealing. Also, the large fragment of *Bst* DNA polymerase displays faster strand displacement activity and is able to separate the non-template strand without use of a thermal cycler (Notomi *et al.*, 2000). Nevertheless, Lim *et al.* (2013) reported that the LAMP assay could be performed at temperatures as low as 58°C. Notably, in the present study, a Loopamp Real-time Turbidimeter was used for real-time visualization of results based on turbidity graphs.

We observed that both the PCR-based technique and the *ipaH* LAMP assay yielded 97% specificity and 100% sensitivity; however, the detection limit for LAMP was 100-fold higher than PCR when tested on pure bacterial cultures and 10-fold higher than

PCR when used on spiked lettuce samples. This may not be surprising when considering that the sensitivity of PCR assays can be affected by the presence of inhibitors, such as food components, media, or DNA extraction solutions (Rossen *et al.*, 1992). Indeed, culture medium, biological substances, and microflora have been shown to have less effect on LAMP assays (Kaneko *et al.*, 2007; Mori *et al.*, 2001; Ye *et al.*, 2011). This is in agreement with findings by Wang *et al.* (2012) and Surasilp *et al.* (2011), who illustrated the sensitivity of LAMP assays.

In the present study, we could not detect or recover bacteria when we directly tested the spiked lettuce samples without pre-enrichment (results not shown). Similar observations have been reported by Jiménez *et al.* (2010). This could be due to environmental stresses (e.g., starvation, unsuitable incubation temperatures, or oxygen concentration), which can lead to non-culturable states (Oliver, 2005). Therefore, we applied enrichment steps using Gram-negative (GN) broth, followed by plating on *Salmonella-Shigella* agar. GN broth is a selective medium that preferentially allows the growth of enteric pathogens (e.g., *Salmonella* and *Shigella*) over normal flora (Taylor and Schelhart, 1969). Indeed, performing enrichment steps can allow physiologically stressed or injured bacterial cells to multiply, thus increasing the likelihood of detection.

Overall, the time required to perform the *ipaH* LAMP assay and to determine the end point was 2 h. Thus, it is more rapid than conventional PCR, which involves multiple steps (i.e., master mix preparation, amplification, agarose gel electrophoresis, and gel visualization) and requires approximately 3 h to obtain positive results. Although the reaction time for our LAMP assay was shorter than PCR, it was longer than previously reported for other LAMP assays (> 60 min), especially when testing samples with low bacterial count. Nevertheless, loop primers could be incorporated to shorten reaction times (Zhao *et al.*, 2010). In addition, instead of using the real-time turbidimeter, LAMP assays can be

performed with simple equipment (e.g., water baths or controlled heating blocks), and the results can be determined by direct visualization of white precipitate following centrifugation or changes in color upon addition of SYBR Green I. However, among these detection methods, real-time turbidimeter monitoring is the only one that is potentially quantitative. Although previous studies have examined the quantitative capability of real-time LAMP for *Vibrio vulnificus* and *Salmonella* spp. in pure culture and spiked food samples (Chen *et al.*, 2011; Han *et al.*, 2011), our study is the first to demonstrate a good linear correlation coefficient ($R^2 = 0.7171-0.908$) for LAMP-based detection of *Shigella* in both pure culture and spiked lettuce samples, suggesting its quantitative capabilities.

In conclusion, we demonstrated that the specificity of LAMP- and PCR-based detection methods for *Shigella* was comparable. However, the sensitivity of the *ipaH* LAMP assay was much higher than conventional PCR. Therefore, we have developed a rapid and effective LAMP assay that can be easily adapted for field detection of *Shigella* spp.

Acknowledgement. This study was funded by UMRG (RP001-13BIO) and University of Malaya Post Graduate Research Fund (PPP) (P0024/2012A). We would like to thank Koh Xiu Pei for technical assistance.

REFERENCES

- Bardhan, P., Faruque, A.S., Naheed, A. & Sack, D.A. (2010). Decrease in shigellosis-related deaths without *Shigella* spp.-specific interventions, Asia. *Emerging Infectious Diseases* **16**: 1718-1723.
- Buysse, J.M., Hartman, A.B., Strockbine, N. & Venkatesan, M. (1995). Genetic polymorphism of the *ipaH* multicopy antigen gene in *Shigella* spp. and enteroinvasive *Escherichia coli*. *Microbial Pathogenesis* **19**: 335-349.

- Chapman, T.A., Wu, X.Y., Barchia, I., Bettelheim, K.A., Driesen, S., Trott, D., Wilson, M. & Chin, J.J. (2006). Comparison of virulence gene profiles of *Escherichia coli* strains isolated from healthy and diarrheic swine. *Applied and Environmental Microbiology* **72**: 4782-4795.
- Chen, S., Wang, F., Beaulieu, J.C., Stein, R.E. & Ge, B. (2011). Rapid detection of viable salmonellae in produce by coupling propidium monoazide with loop-mediated isothermal amplification. *Applied and Environmental Microbiology* **77**: 4008-4016.
- Gomez-Duarte, O.G., Bai, J. & Newel, E. (2009). Detection of *E. coli*, *Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica*, *Vibrio cholerae*, and *Campylobacter* spp. enteropathogens by three-reaction multiplex PCR. *Diagnostic Microbiology and Infectious Disease* **63**: 1-9.
- Han, F., Wang, F. & Ge, B. (2011). Detecting potentially virulent *Vibrio vulnificus* strains in raw oysters by quantitative loop-mediated isothermal amplification. *Applied and Environmental Microbiology* **77**: 2589-2595.
- Jiménez, K.B., McCoy, C.B. & Achi, R. (2010). Detection of *Shigella* in lettuce by the use of a rapid molecular assay with increased sensitivity. *Brazilian Journal of Microbiology* **41**: 993-1000.
- Kaneko, H., Kawana, T., Fukushima, E. & Suzutani, T. (2007). Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. *Journal of Biochemical and Biophysical Methods* **70**: 499-501.
- Kothary, M.H. & Babu, U.S. (2001). Infective dose of foodborne pathogens in volunteers: a review. *Journal of Food Safety* **21**: 49-73.
- Li, J.W., Shi, X.Q., Chao, F.H., Wang, X.W., Zheng, J.L. & Song, N. (2004). A study on detecting and identifying enteric pathogens with PCR. *Biomedical and Environmental Sciences* **17**: 109-120.
- Lim, K.T., Teh, C.S. & Thong, K.L. (2013). Loop-Mediated Isothermal Amplification Assay for the Rapid Detection of *Staphylococcus aureus*. *BioMed Research International* <http://dx.doi.org/10.1155/2013/895816>.
- Mori, Y., Kitao, M., Tomita, N. & Notomi, T. (2004). Real-time turbidimetry of LAMP reaction for quantifying template DNA. *Journal of Biochemical and Biophysical Methods* **59**: 145-157.
- Mori, Y., Nagamine, K., Tomita, N. & Notomi, T. (2001). Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochemical and Biophysical Research Communications* **289**: 150-154.
- Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research* **28**: E63.
- Oliver, J.D. (2005). The viable but nonculturable state in bacteria. *The Journal of Microbiology* **43**: 93-100.
- Rossen, L., Norskov, P., Holmstrom, K. & Rasmussen, O.F. (1992). Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. *International Journal of Food Microbiology* **17**: 37-45.
- Shao, Y., Zhu, S., Jin, C. & Chen, F. (2011). Development of multiplex loop-mediated isothermal amplification-RFLP (mLAMP-RFLP) to detect *Salmonella* spp. and *Shigella* spp. in milk. *International Journal of Food Microbiology* **148**: 75-79.
- Soli, K.W., Kas, M., Maure, T., Umezaki, M., Morita, A., Siba, P.M., Greenhill, A.R. & Horwood, P.F. (2013). Evaluation of colorimetric detection methods for *Shigella*, *Salmonella*, and *Vibrio cholerae* by loop-mediated isothermal amplification. *Diagnostic Microbiology and Infectious Disease* **77**: 321-323.

- Song, T., Toma, C., Nakasone, N. & Iwanaga, M. (2005). Sensitive and rapid detection of *Shigella* and enteroinvasive *Escherichia coli* by a loop-mediated isothermal amplification method. *FEMS Microbiology Letters* **243**: 259-263.
- Surasilp, T., Longyant, S., Rukpratanporn, S., Sridulyakul, P., Sithigorngul, P. & Chaivisuthangkura, P. (2011). Rapid and sensitive detection of *Vibrio vulnificus* by loop-mediated isothermal amplification combined with lateral flow dipstick targeted to *rpoS* gene. *Molecular and Cellular Probes* **25**: 158-163.
- Taylor, W.I. & Schelhart, D. (1969). Isolation of Shigellae. VII. Comparison of gram-negative broth with Rappaport's enrichment broth. *Applied Microbiology* **18**: 393-395.
- Theron, J., Morar, D., Du Preez, M., Brozel, V.S. & Venter, S.N. (2001). A sensitive seminested PCR method for the detection of *Shigella* in spiked environmental water samples. *Water Research* **35**: 869-874.
- Thong, K.L., Hoe, S.L., Puthuchery, S.D. & Yasin, R. (2005). Detection of virulence genes in Malaysian *Shigella* species by multiplex PCR assay. *BMC Infectious Diseases* **5**: 8.
- Trofa, A.F., Ueno-Olsen, H., Oiwa, R. & Yoshikawa, M. (1999). Dr. Kiyoshi Shiga: discoverer of the dysentery bacillus. *Clinical Infectious Diseases* **29**: 1303-1306.
- U.S. Food and Drug Administration (FDA). Food Bacteriological Analytical Manual: Chapter 6 *Shigella*. 2001 update. Accessed 21 Nov 2012. Available online: <http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm070789.htm>.
- von Seidlein, L., Kim, D.R., Ali, M., Lee, H., Wang, X., Thiem, V.D., Canh do, G., Chaicumpa, W., Agtini, M.D., Hossain, A., Bhutta, Z.A., Mason, C., Sethabutr, O., Talukder, K., Nair, G.B., Deen, J.L., Kotloff, K. & Clemens, J. (2006). A multicentre study of *Shigella* diarrhoea in six Asian countries: disease burden, clinical manifestations, and microbiology. *PLOS Medicine* **3**: e353.
- Wang, L., Li, Y., Chu, J., Xu, Z. & Zhong, Q. (2012). Development and application of a simple loop-mediated isothermal amplification method on rapid detection of *Listeria monocytogenes* strains. *Molecular Biology Reports* **39**: 445-449.
- World Health Organization (WHO). Initiative for vaccine research (IVR): diarrheal diseases (updated February 2009). Shigellosis. Accessed 21 Nov 2012. Available online: http://www.who.int/vaccine_research/diseases/diarrhoeal/en/index6.html.
- Ye, Y., Wang, B., Huang, F., Song, Y., Yan, H., Alam, M.J., Yamasaki, S. & Shi, L. (2011). Application of *in-situ* loop-mediated isothermal amplification method for detection of *Salmonella* in foods. *Food Control* **22**: 438-444.
- Zhang, Y., Shan, X., Shi, L., Lu, X., Tang, S., Wang, Y., Li, Y., Alam, M.J. & Yan, H. (2011). Development of a *fimY*-based loop-mediated isothermal amplification assay for detection of *Salmonella* in food. *Food Research International*, doi:10.1016/j.foodres.2011.02.015.
- Zhao, X., Wang, L., Chu, J., Li, Y., Li, Y., Xu, Z., Li, L., Shirliff, M.E., He, X. & Liu, Y. (2010). Development and application of a rapid and simple loop-mediated isothermal amplification method for food-borne *Salmonella* detection. *Food Science and Biotechnology* **19**: 1655-1659.