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Comparison between Polymerase Chain Reaction and Loop Mediated Isothermal Amplification for the detection of *Staphylococcus aureus* in food

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Abstract

This research aimed to compare Polymerase Chain Reaction (PCR) and Loop Mediated Isothermal Amplification (LAMP) for the detection of *Staphylococcus aureus* in food. *Staphylococcus aureus* is a human pathogen due to widespread infection and being methicillin-resistant causing bacteraemia and tissue infections. According to six distinct sequences of the *mecA* and *femA* in *S. aureus*, four specific primers that were designed to detect *S. aureus* using LAMP. In addition, the sensitivity of LAMP was performed and compared with PCR. The results showed that the LAMP completed the reaction within 30 mins at 65°C. LAMP is considered a promising alternative method compared to the PCR technique where LAMP can be used in laboratories and fields with limited resources.

1. Introduction

An important bacteria associated with infection is *Staphylococcus aureus*, one of the important bacterial pathogens associated with *Staphylococcus aureus* infection, including the methicillin-resistant Staphylococcus *aureus* (MRSA), which produces a wide range of well-known virulence factors, one of the most important is the *Staphylococcus* enterotoxin products (SEs) and *Staphylococcal* food poisoning responsible for toxic shock syndrome (TSST) toxin (Adwan *et al*., 2006; Udo *et al*., 2009). It is still considered one of the important diseases causing bacteremia and causing high mortality and hospitalization, *Staphylococcus aureus*, the bacterium blood (Cosgrove, 2005).

Therefore, the rapid detection and differentiation between normal *S. aureus* and methicillin-resistant *staphylococci* have a great prognostic, therapeutic and economic value that determines the high mortality rate regardless of appropriate antimicrobial treatment (Lodicin and McKinnon, 2005). As public health awareness increases, it is important to develop detection mechanisms in terms of speed, ease, cost and accuracy in showing bacteriological results, as the PCR assays and real-time PCR, which were developed during the last period, are now used (Leblond-Bourget *et al*., 1996). However, obstacles to real-time PCR and PCR applications (such as consumption of time, expensive equipment and reagents, risk of contamination, reduction in low detection levels, requirements for trained personnel, and operating space) hinder its widespread use.

LAMP is based on the use of thermostable DNA polymerase with strand displacement effective (Bstpolymerase from *Bacillus stearothermophilus*) and 4 or 6 primers designed specifically to identify the six distinct specific regions layout in a target gene, the isothermal DNA amplification method consists of (1) pair of outside prefixes (F3, B3) (2) an internal prefixes (FIP, BIP) (3) a loop primer. Either for the Loop Primer F or for the Loop Primer B, which contains a region with a complementary sequence for a single-loop located within both regions (B1, B2 and F1, F2) (Notomoi *et al*., 2000). LAMP is considered a promising alternative method compared to the PCR technique. Therefore, this study aimed to compare PCR and LAMP in detecting *S. aureus* in food.

2. Materials and methods

2.1 Bacterial isolation

Food samples were collected from different local markets in Baghdad between December 2018 and March 2019. The samples were transferred to the laboratory for further processing. The food samples were added with buffered peptone water and then incubated in a shaker incubator for 24 hrs at 37°C. The growth suspension was then inoculated onto mannitol salt agar plates. Presumptive colonies of *S. aureus* were picked and

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further tested for coagulase.

2.2 Antimicrobial susceptibility test

The sensitivity of *S. aureus* isolates to diverse antimicrobials were determined according to the modified Kirby-Bauer method (Benson, 2001; Morello, 2006) by using Muller-Hinton agar. In addition, to identify methicillin-resistant *S. aureus*, isolates were tested with Oxacillin (5 µg) as per the method by Kampf *et al*. (1998)

2.3 Chromosomal DNA isolation

A 24-hour culture of the isolates were prepared. The chromosomal DNA of the isolates were extracted using the DNA genomic kit (Promega, USA). A total of 1.5 mL of the culture was precipitated at 14,000 rpm/25°C and was re-suspended with 500 µL EDTA (50 mM). A total of 100 µL of 30 mg/mL lysozyme solution was added and then the suspension was incubated for 30 – 60 mins at 37°C. Next, the suspension was centrifuged at 14,000 rpm for 1 min and re-suspended by gently pipetting 850 µL of the solution. The suspension was then held in liquid nitrogen for 30 – 60 s and subsequently incubated in a 80°C water bath for 2 mins and back into the liquid nitrogen. The liquid was removed and RNase was added to the mixture followed by incubation for 30 mins at 37°C. During incubation, the tubes were inverted to enhance mixing. About 200 – 300 µL solution was added to the mixture to precipitate roughly 4 mg/mL proteins. The mixture as rotated for 10 s on medium speed. The mixture was then centrifuged at 25°C, 14,000 rpm for 10 mins. The supernatant was transferred into a new 1.5 mL tube. In order to remove all the protein residual out of the extract, the lysate was centrifuged at 25°C, 14,000 rpm for 5 mins. Room-temperature-isopropanol (600 µL) was added to deposit the DNA. The tube was then centrifuged at 25°C, 14,000 rpm for 10 mins to pellet and removing any remaining contaminants. The pellet was washed with 70% ethanol and resuspended in 50 – 100 µL with 10 mM Tris HCl (pH 8.5).

2.4 Polymerase chain reaction

Table 1 displays the primer sequences used in the polymerase chain reaction (PCR). The PCR master mix of 25 µL was prepared as shown in Table 2. The PCR was performed following the protocols described by Johnson *et al.* (2000) as shown in Table 3. The PCR products were subjected to agarose gel electrophoresis (Sambrook and Russell, 2001; Wang *et al.*, 2001; Gerrit *et al*., 2010). A 1.5% agarose gel incorporated with 5 µL ethidium bromide was prepared. The PCR product (5 µL) was loaded into the wells and the electrophoresis was carried out at 80 V for 90 mins. After the electrophoresis, the gel was viewed under transient UV light using a gel documentation system (BIO-RAD) imaging device. The expected base pairs of the PCR products were compared with a 100 bp DNA Ladder.

2.5 Loop Mediated Isothermal Amplification

The LAMP assay was performed using the primer sequences and concentrations in Table 4 and Table 5, respectively. Warm Start LAMP kit was used to prepare the reaction mixture as shown in Table 6. The tubes were

Table 1. Primers for PCR for the detection of *femA* and *mecA* in *S. aureus.*

Gene	Primer code	Oligo Sequences direction (5'→3')	Amplified products size	Reference
femA	G*FEMA*R-1	AAAAGCACATAACAAGCG	132 bp	Johnson *et al*. (2000)
	G*FEMA*R-2	GATAAAGAAGAAACCAGCAG		
mecA	G*MECA*R-1	ACTGCTATCCACCCTCAAAC	163 bp	
	G*MECA*R-2	CTGGTGAAGTTGTAATCTGG		

Table 2. PCR master mix for the detection of *femA* and *mecA* in *S. aureus.*

PCR reaction mixture	Final Concentration	Volume for one reaction (µL)
Free nucleic water		4.5
Master Mix (2X)	1X	12.5
$MgCl_2$	1 mM	1
DNA	0.2 µg	5
Forward Primer	0.2 / µm	1
Reverse Primer	0.2 / µm	1
Final Volume reaction		25

Table 3. PCR conditions for the detection of *femA* and *mecA* in *S. aureus.*

Step			Optimal Temperature and Time		No. of Cycles
1	Initial denaturation		94°C	5 mins	1
2	Amplification	Denaturation	94°C	2 mins	35
		Annealing	57°C	2 mins	
		Elongation	72°C	1 mins	
3	Extension		72°C	7 mins	1

Table 4. Primers for LAMP for the detection of *femA* and *mecA* in *S. aureus.*

Gene	Primer	Oligonucleotide sequence (5'→3')	Primer source
femA	F3	ATGCTGGTGGTACATCAA	Xu *et al*. (2012)
	B3	TGGTTTAATAAAGTCACCAACAT	
	BIP	GGTCAATGCCATGATTTAATGCATAGCATTCCGTCATTTTGCC	
	FIP	CAGAAGATGCTGAAGATGCTGGTCAATAATTTCAGCATTGTAACC	
	LF	AATCATTTCCCATTGCACT	
	LB	TGTAGTTAAATTCAA	
mecA	F3	AAGATGGCAAAGATATTCAACT	
	B3	AGGTTCTTTTTTATCTTCGGTTA	
	BIP	GTGGATAGCAGTACCTGAGCCTTGATGCTAAAGTTCAAAAGAGT	
	FIP	CCTCAAACAGGTGAATTATTAGCACCTTCGTTACTCATGCCATAC	
	LF	TAATCATTTTTCATGTTG	
	LB	TGTAAGCACACCTTCATATGACGT	

incubated at 65°C for 30 mins. In the reaction tube, 1 µL from 0.1% SYBR® Green (Promega) was added. A positive result for LAMP was observed with the naked eye or ultraviolet (UV) imaging.

Table 5. LAMP primers stocks and working dilutions for the detection of *femA* and *mecA* in *S. aureus.*

Primers of LAMP	Stock (10× concentration)	Final (1× concentration)
FIP	16 µm	1.6 µm
BIP	16 µm	1.6 µm
F3	2 µm	0.2 µm
B3	2 µm	0.2 µm
LF	4 µm	0.4 µm
LB	4 µm	0.4 µm

Table 6. LAMP reaction mixture for the detection of *femA* and *mecA* in *S. aureus.*

LAMP reaction mixture	DNA target detection (µL)	No template control (NTC) (µL)
2X LAMP Warm-Start Master Mix	12.5	12.5
50% Fluorescent dye	0.5	0.5
10x LAMP Primer Mix	2.5	2.5
Target DNA	1	-
Free nucleic water	8.5	9.5
Total Volume	25	25

2.6 Sensitivity

Template DNA for the *Staphylococcus aureus subsp. aureus* Rosenbach ATCC 700699 reference strain was diluted with D.W from 1 up to 10 µL which was used for determining the limit of detection (sensitivity) for both PCR and LAMP assays. A total of 5 µL of samples were used as a template DNA for PCR assay, another 5 µL of samples were used in LAMP assay as a DNA dilution template (King *et al.,* 2013).

3. Results and discussion

The results of the polymerase chain reaction (PCR) were confirmed by the electrophoresis showed similarity with the specific detection of *S. aureus*. No magnifying products listed for any other bacterial strain have been noticed in the results. Considered as an indicator for diagnosis of *Staphylococcus* isolates, the *femA* is a specific marker for detection of S. *aureus* (Rajesh *et al*., 2015). The result in Figure 1 showed that the *S. aureus* isolates gave positive results for the *femA* specified for *S. aureus* at 132 bp. Several studies reported that quantitative PCR assays targeted the small subunit rRNA (*16S rRNA*) and *femA* were used to detect *S. aureus* bacteria from isolated sample (Al-Khafaji and Flayyih, 2014; Al–Alak and kadhim, 2016).

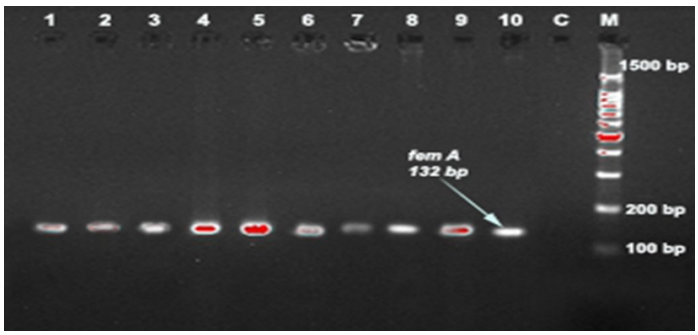

Figure 1. Gel electrophoresis of the PCR product of *femA* (132 bp) of *S. aureus*. Line M: 100 bp DNA marker, Line C: control negative, Line 1 – 10: *S. aureus* isolates.

To ensure the LAMP test was more economical compared to the conventional PCR, the annealing temperature of the primers in LAMP was maintained at 65°C with the additional of 2 extra loop primers which improves the amplification specificity (Nagamine *et al*., 2002). In addition, the reaction time was greatly reduced to 30 mins compared to PCR which requires 3 hours to complete the reaction. The results were also visible by the naked eye (Figure 2).

Due to the use of 6 prefixes to identify 6 regions in the *femA* sequence and are specially designed, the specificity of LAMP is considered high, which is specific to *S. aureus* bacteria. The results in LAMP were

more detectable than conventional PCR and at a limit of 22.8 pg/µL for the genomic DNA, which is 100 times better. LAMP-based methodologies are favourable due to the rapid and relatively simple template DNA purification process (Kaneko *et al.,* 2007; Mori and Notomi, 2009). In previous studies, no amplification or low sensitivity (as low as 1.7×10^5 CFU/mL) for PCR assays had been reported when template DNA was not purified, which had been raised to 27–180 CFU/mL following a 2-hour incubation (Alarcon *et al*., 2006; Pitcher *et al*., 1989). However, the simple template preparation process in this study takes only 20 mins and had been previously proved applicable in various food samples in preliminary studies (Zhao, Li, Wang *et al.,* 2010; Zhao, Wang, Chu *et al.*, 2010; Zhao *et al*., 2011). Therefore, the current LAMP assays should be applicable for pathogen detection in real food samples. As aforementioned, laborious demand, expensive reagents and equipment restrict the broad application of RT-PCR in clinical routine laboratories. However, with the reaction performed under isothermal conditions without a thermal cycler, only simple equipment like a heat block and water baths were needed for the operation of LAMP assays at low expense. In comparison, the real-time turbid meter is commonly required in previous publications on LAMP detection (Misawa *et al*, 2007). Sun *et al*. (2010) reported that the LAMP assay had a higher detection capacity of 10 CFU/mL than conventional PCR and this result agrees with our study results as shown in Figure 3 and Table 7.

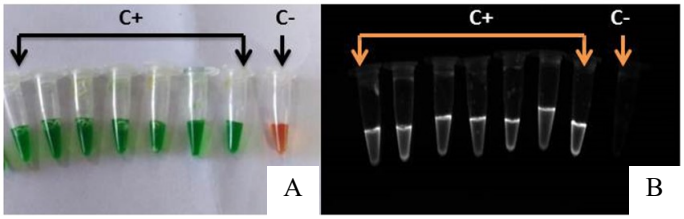

Figure 2. LAMP reaction tubes observed (A) normal light and (B) UV light. C-: control negative, C+:control positive.

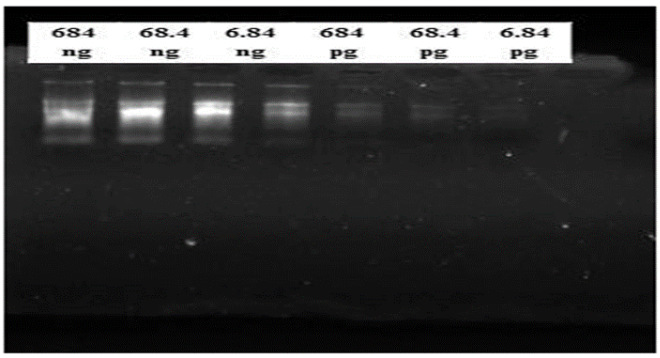

Figure 3. Sensitivity test of the LAMP reaction of *S. aureus*.

4. Conclusion

The developed direct LAMP assay is a rapid detection method for identifying *S. aureus* with high specificity. Using optimal conditions, LAMP is equally specific in identifying when compared to PCR. Based on this study, for the detection of *S. aureus*, LAMP is more efficient than conventional PCR due to its simplicity, specificity, sensitivity, and economically. This technique can be used during the outbreak of foodborne diseases for rapid detection of emergent microorganisms.

Conflict of interest

The authors declare no conflict of interest.

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Table 7. Comparison between the sensitivity of LAMP and PCR

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6
DNA concentration	684/ng	68.4/ng	6.84/ng	684/pg	68.4/pg	6.84/pg
LAMP	+	+	+	+	+	-
PCR	+	+	+	-	-	-

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