Development of uracil-DNA-glycosylase-supplemented loop-mediated isothermal amplification coupled with nanogold probe (UDG-LAMP-AuNP) for specific detection of *Pseudomonas aeruginosa*

ORAPAN MANAJIT[1,2], SIWAPORN LONGYANT[1,2], PAISARN SITHIGORNGUL[1,2] and PARIN CHAIVISUTHANGKURA[1,2]

[1]Department of Biology, Faculty of Science; [2]Center of Excellence for Animal, Plant and Parasite Biotechnology, Srinakharinwirot University, Bangkok 10110, Thailand

Abstract. *Pseudomonas aeruginosa* (*P. aeruginosa*) is an important opportunistic pathogen that causes serious infections in humans, including keratitis in contact lens wearers. Therefore, establishing a rapid, specific and sensitive method for the identification of *P. aeruginosa* is imperative. In the present study, the uracil-DNA-glycosylase-supplemented loop-mediated isothermal amplification combined with nanogold labeled hybridization probe (UDG-LAMP-AuNP) was developed for the detection of *P. aeruginosa*. UDG-LAMP was performed to prevent carry over contamination and the LAMP reactions can be readily observed using the nanogold probe. A set of 4 primers and a hybridization probe were designed based on the *ecfX* gene. The UDG-LAMP reactions were performed at 65˚C for 60 min using the ratio of 40% deoxyuridine triphosphate to 60% deoxythymidine triphosphate. The detection of UDG-LAMP products using the nanogold labeled hybridization probe, which appeared as a red-purple color, was examined at 65˚C for 5 min with 40 mM $MgSO_4$. The UDG-LAMP-AuNP demonstrated specificity to all tested isolates of *P. aeruginosa* without cross reaction to other bacteria. The sensitivity for the detection of pure culture was 1.6×10^3 colony-forming units (CFU) ml^{-1} or equivalent to 3 CFU per reaction while that of polymerase chain reaction was 30 CFU per reaction. The detection limit of spiked contact lenses was 1.1×10^3 CFU ml^{-1} or equivalent to 2 CFU per reaction. In conclusion, the UDG-LAMP-AuNP assay was rapid, simple, specific and was effective for the identification of *P. aeruginosa* in contaminated samples.

Introduction

Pseudomonas aeruginosa is a gram-negative, rod-shaped, belonging to the family of *Pseudomonadaceae*. It can be found in many natural environments including warm and moist atmospheres containing very low levels of organic material (1). Therefore, *P. aeruginosa* can contaminate contact lens, cosmetic and several hospital niches, including taps, drains, water pipes, medical equipment and several other devices leading to nosocomial infections (2). This organism is an opportunistic pathogen that can cause serious infections including septicemia, pneumonia, endocarditis, otitis and keratitis (3). *P. aeruginosa* is an increasingly frequent organism found in humans with bacterial keratitis particularly among contact lens wearers (4). This organism can adhere on the surface of contact lens and form the biofilm, as a result, ability to grow is increasing and it is difficult to dispose. *P. aeruginosa* is an important cause of keratitis and may lead to permanent loss of vision if not treated promptly and appropriately (5). Therefore, the rapid and accurate method for determination of *P. aeruginosa* is required.

Identification of *P. aeruginosa* in the clinical laboratory is generally performed by growing the bacteria on nalidixic acid-cetrimide (NAC) agar or cetrimide agar, Pseudomonas CN selective agar (PCN) and Pseudomonas isolation agar (PIA). Although this method is credible but the time required for performing is 24-72 h, which is a long time and other *Pseudomonas* species may display similar growth that limit the identification of species (6). Using polymerase chain reaction (PCR) methods, which allow for more rapid identification of *P. aeruginosa*, have been shown to be highly specific and less time-consuming than classical method (3,7,8) and PCR have also been reported (9,10). However, the PCR based methods require expensive device and skilled personnel to avoid cross contamination between samples.

Correspondence to: Dr Parin Chaivisuthangkura, Department of Biology, Faculty of Science, Srinakharinwirot University, 23 Sukhumvit, Bangkok 10110, Thailand
E-mail: parin@g.swu.ac.th

A novel nucleic acid amplification method termed loop-mediated isothermal amplification (LAMP) using a set of four oligonucleotide primers and the strand displacement enzyme for amplification of target gene at constant temperature ranging from 60 to 65°C was invented (11). LAMP method has been successfully used to detect various bacterial pathogens such as *Vibrio vulnificus* (12), *Vibrio parahaemolyticus* (13), and *Salmonella* spp. (14). In the case of *P. aeruginosa* detection, traditional LAMP methods based on various genes such as outer membrane lipoprotein I (*opr I*) gene (15), outer membrane lipoprotein L (*opr L*) gene (16), *ecfX* gene in bottled water samples (17), *exoS* and *exoU* of Type III Secretion System (18) were reported. In order to increase specificity, the visualization of LAMP product using a labelled-hybridization probe is an alternative method. The LAMP assay combined with DNA-labeled gold nanoparticles (AuNP) probe were established to detect various viral pathogens such as shrimp yellow head virus (YHV) (19), white spot syndrome virus (WSSV) (20), or identification of human DNA in forensic evidence (21). In this system, the DNA-AuNP probe aggregates with a color shift from red to blue or colorless at optimal salt concentration if there is no hybridization reaction (18). However, hybridization of the AuNP probe to a complementary target DNA prevents aggregation in salt environment and solution remained red.

Since LAMP method is highly sensitive for nucleic acid amplification; therefore, the assay is likely susceptible to carryover contamination. To overcome this problem, a one-pot, closed-vessel enzymatic assay that prevent carryover contamination during LAMP reaction was reported (22). This system used uracil-DNA-glycosylase (UDG) to digest uracil-containing LAMP amplicons from previous reaction prior to performing LAMP amplification in the same tube. This system had been successfully used with traditional LAMP assay for detection of *Salmonella* Typhimurium (22). In the present study, a modified UDG-LAMP combined with colorimetric AuNP probe assay (UDG-LAMP-AuNP) for specific identification of *P. aeruginosa* based on *ecfX* gene was developed. The specificity and sensitivity for detection of *P. aeruginosa* was also investigated.

Materials and methods

Bacterial isolates and DNA extraction. A total of 39 bacterial isolates including 16 isolates of *P. aeruginosa*, 8 isolates of *Pseudomonas* spp. and 15 isolates of other bacteria were used in the present study (Table I). The 16S rRNA gene amplifications were performed to verify the bacterial identification as described in a previous report (23). The origins and sources of all 40 bacterial isolates tested were indicated in Table I. All bacterial isolates were grown for overnight at 37°C in trypticase soy agar (TSA). A single colony from TSA was picked and inoculated into trypticase soy broth (TSB) at 37°C for overnight.

DNA was extracted from bacteria cultured in TSB by using QIAamp DNA mini kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacture's manual. The extracted DNA was stored at -20°C until use and an isolate of *P. aeruginosa* (PA07) ATCC was used for the assay of optimization and sensitivity testing with pure culture.

General PCR. The DNA extracted from bacterial samples was used as a template for PCR amplification. The PCR amplification of *ecfX* gene was performed as described in previous reports (3). Briefly, the PCR was carried out with primers ECF 1 (ATGGATGAGCGCTTCCGT) and ECF 2 (TCATCCTTCGCCTCCCTG) for 35 cycles, each of which consisted of denaturation at 94°C for 45 sec, annealing at 58°C for 45 sec and extension at 72°C for 30 sec. The PCR amplicon size was 528 bp. PCR products were separated by electrophoresis on 1% (w/v) agarose gel and visualized using a gel documentation system (Dynamica GelView Master).

Design of LAMP primers and probe for the LAMP assay. A set of four primers consisting of 2 outer primers (F3 and B3) and 2 inner primers (FIP and BIP) recognized six distinct regions on *ecfX* gene (GenBank, accession no. DQ 996559.1) of *P. aeruginosa* was designed using PrimerExplorer ver. 4 (http://primerexplorer.jp/elamp4.0.0/index.html). The FITC-labeled oligonucleotide probe was synthesized and labeled with a thiol group at 5'-end (Bio Basic Inc., Markham, Ontario, Canada). The sequences of primers and probe are shown in Table II.

Optimization of UDG-LAMP conditions. In order to prevent the carryover contamination of LAMP product, the UDG-LAMP reactions were performed as described by a previous study with some modification. The dTTP in a standard dNTP mix was partially replaced with dUTP and UDG was used to degrade uracil-labeled LAMP amplicons (22).

The UDG-LAMP assay was carried out in a total of 25 μl reaction mixture consisted of 40 pmol each of the inner primers (FIP and BIP), 5 pmol each of the outer primers (F3 and B3), 1.4 mM each of dATP, dGTP, dCTP and dUTP: dTTP (different ratios at 100% dUTP; 80% dUTP + 20% dTTP; 60% dUTP + 40% dTTP; 40% dUTP + 60% dTTP; 20% dUTP + 80% dTTP; and 100% dTTP) (New England Biolabs, Inc., Ipswich, MA, USA), 5.0 mM of $MgSO_4$, 0.8 M Betaine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 8 U of *Bst* 2.0 DNA polymerase (New England Biolabs), 5 U of UDG (New England Biolabs), 1X of supplied buffer and DNA template. The reaction mixture was incubated at 37°C for 5 min. and then further incubated at 60, 63 and 65°C for 60 min to determine the optimal temperature. To establish the optimal time, the UDG-LAMP reaction was carried out at pre-determined temperature of 65°C for 30, 45, 60, 75 and 90 min and heated at 80°C for 10 min to terminate the reaction. The products were analyzed by 2% agarose gel electrophoresis and visualized using a gel documentation system (Dynamica GelView Master).

Preparation of ssDNA-labeled gold nanoparticles probe (AuNPs-probe). The AuNPs probe was prepared according to a previous report (21). Briefly, 4 ml of colloidal AuNPs with diameter of 10 nm (Sigma-Aldrich; Merck KGaA) was added to 2.5 nmol of the thiol-probe and incubated with shaking at 150 rpm, 45°C for 16 h. The following solutions consisted of 4 μl of 10% SDS, 400 μl of 100 mM phosphate buffer, pH 7.5 (0.02 M $NaH_2PO_4 \cdot H_2O$, 0.08 M $Na_2HPO_4 \cdot 7 H_2O$) and 200 μl of 2 M NaCl were added to the mixture and incubated at 45°C, 150 rpm shaking for 48 h. The AuNPs-probe was precipitated

Table I. Bacterial isolates used in the present study.

Bacterial isolates	Origin	DNA amplification $ecfX$		Source
		LAMP	PCR	
A, *Pseudomonas aeruginosa* (n=16)				
P. aeruginosa (PA02)	Unknown	+	+	DMST
P. aeruginosa (PA04)	Urine	+	+	DMST
P. aeruginosa (PA05)	Sputum	+	+	DMST
P. aeruginosa (PA06)	Compost	+	+	DMST
P. aeruginosa (PA07)	Outer-ear infection	+	+	ATCC
P. aeruginosa (PA08)	Animal room water bottle	+	+	ATCC
P. aeruginosa (PA10)	Bacterial resistance testing of latex paint	+	+	ATCC
P. aeruginosa (PA11)	Sputum	+	+	DMST
P. aeruginosa (PA12)	Blood culture	+	+	DMST
P. aeruginosa (PA14)	Pus	+	+	TISTR
P. aeruginosa (PA16)	Intercostal Drainage	+	+	DMST
P. aeruginosa (PA17)	Cerebrospinal fluid	+	+	DMST
P. aeruginosa (PA19)	Heart blood	+	+	DMST
P. aeruginosa (PA20)	Endo tracheal	+	+	DMST
P. aeruginosa (PA21)	Unknown	+	+	DMST
P. aeruginosa (PA22)	Unknown	+	+	TISTR
B, *Pseudomonas* spp. (n=8)				
P. japonica 1526	Flower of *Haliconia sp.*	-	-	TISTR
P. putida 23201	Unknown	-	-	DMST
P. fluorescens 358	Unknown	-	-	TISTR
P. olevorans 1097	Epoxidizes terminal olefins	-	-	TISTR
P. syringae 19310	*Syringa vulgaris*, Great Britain	-	-	ATCC
P. stutzeri 22487	Blood culture	-	-	DMST
P. boreopolis 33662	Unknown	-	-	ATCC
P. acidovorans	Unknown	-	-	Unknown
C, Other bacteria (n=15)				
Escherichia coli 25922	Unknown	-	-	ATCC
Plesiomonas shigelloides 22107	Rectal swab	-	-	DMST
Photobacterium damselae sub *piscicida*	Unknown	-	-	Unknown
Vibrio ordalii VIB02	Unknown	-	-	DABU
Vibrio anguillarum AVL01	Unknown	-	-	GB
Vibrio campbellii 21361	Unknown	-	-	GB
Vibrio alginolyticus 24047	Stool	-	-	DMST
Vibrio cholerae 22117	Stool	-	-	DMST
Vibrio shilonii 4907012	*Penaeus vannamei*	-	-	SWU
Vibrio harveyi 639	*Penaeus monodon*	-	-	CENTEX
Salmonella Typhimurium 14029	Unknown	-	-	ATCC
Salmonella Enteritidis 7108	Unknown	-	-	DMST
Staphylococcus aureus 25923	Unknown	-	-	ATCC
Aeromonas veronii 21255	Unknown	-	-	DMST
Yersinia rukeri	Unknown	-	-	DABU

ATCC, American Type Culture Collection, Rockville, Maryland, USA; CENTEX, Centex Shrimp, Faculty of Science, Mahidol University, Thailand; DABU, Department of Aquatic Science, Burapa University, Thailand; DMST, Department of Medical Science, Ministry of Public Health, Thailand; GB, Ghent University, Belgium; SWU, Department of Biology, Srinakharinwirot University, Thailand; TISTR, Thailand Institute of Scientific and Technological Research; PA/*P. aeruginosa, Pseudomonas aeruginosa*; PCR, polymerase chain reaction; LAMP, loop-mediated isothermal amplification.

Table II. Primers and the probe for loop-mediated isothermal amplification designed from the *ecfX* gene of *Pseudomonas aeruginosa*.

Primer	Sequence (5'-3')
Forward outer primer (F3)	TCCGTGGTTCCGTCTCG
Backward outer primer (B3)	AAGTTGCGGGCGATCTG
Forward inner primer (FIP)	TGCCCAGGTGCTTGCGCATTTTCATGCCTATCAGGCGTTCC
Backward inner primer (BIP)	GCCGACCTCGCCCAGGATATTTTGCTCGACCGATTGCCG
Thiol probe	(SH-) A_{10}-GGATACTTTCGACCAGTGGC

with centrifugation at 20,000 x g, 4°C for 30 min. The pellet was washed twice with 700 μl of solution containing 10 mM phosphate buffer, 100 mM NaCl, and 0.01% (w/v) SDS. Finally, the pellet was resuspended in 700 μl of 10 mM phosphate buffer, monitored for absorbance at 525 nm in the range of 0.3-0.4, stored at 4°C and protected from light.

Optimization of the AuNPs-probe for detection of UDG-LAMP products. To establish the optimal salt concentration for induction of free probe aggregation, 5 μl of MgSO$_4$ was added to the mixture (the total volume of 15 μl) to achieve the final concentration of 5, 10, 20, 40, 100 and 200 mM. The positive reaction (red-purple color) and negative reaction (blue-gray or colorless) were observed and recorded by naked eyes and by UV-visible analysis (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

To optimize the hybridization temperature for detection of UDG-LAMP products, 2.5 nmol of AuNPs-probe solution was added to the UDG-LAMP products in a 1:1 ratio at 57, 62 and 65°C for 5 min. The optimal hybridization time was investigated at 5, 10 and 20 min under pre-determined temperature (65°C).

Specificity testing of PCR and UDG-LAMP-AuNPs assays. The specificity of UDG-LAMP-AuNPs and PCR based on *ecfX* gene was performed to examine the 39 bacterial isolates including 16 isolates of *P. aeruginosa*, 8 isolates of *Pseudomonas* spp. and 15 isolates of other bacteria as shown in Table I. DNA templates isolated from bacterial cultures by QIAamp DNA mini kit (Qiagen) were used to evaluate the specificity test.

Sensitivity of UDG-LAMP-AuNPs and PCR assays in pure culture. The sensitivity of the UDG-LAMP-AuNPs assay for the detection of *P. aeruginosa* in pure culture was performed according to a previous study (24) with some modifications using known amounts of *P. aeruginosa* (PA 07). Briefly, bacterial cells from a single colony on TSA were inoculated into 4 ml of trypticase soy broth (TSB; Difco) and incubated at 37°C for overnight. Approximately 40 μl of TSB culture was added to a new 4 ml of TSB and incubated at 37°C with shaking at 225 rpm to mid-log phase (OD 600 nm=0.5-0.6). Then, 10-fold serial dilutions of the cultures were prepared in phosphate-buffered saline solution (PBS).

To extract DNA from pure culture, 100 μl of each dilution was transferred into a new microcentrifuge tube and centrifuged at 20,000 x g for 5 min. Then, the supernatant

was removed and the pellet was resuspended in 50 μl of 25 mM NaOH and subsequently heated at 95°C for 5 min. After neutralization with 4 μl of 1 M Tris-HCl buffer (pH 7.5), the suspension was centrifuged at 4°C, 20,000 x g for 5 min and used as a template (1 μl) with optimization condition for UDG-LAMP-AuNPs and PCR assays. The detection limit of UDG-LAMP-AuNPs detection methods was compared with that of PCR assay.

In parallel, to count the bacteria colony number, 100 μl of each dilution was spread on TSA in duplicate and incubated at 37°C for overnight. The bacterial colonies were counted at the dilution yielding 30-300 colony-forming units (CFUs) and then the CFU ml^{-1} of bacterial suspension was calculated.

Sensitivities of UDG-LAMP-AuNPs and PCR with spiked contact lens. Contact lens samples (Pretty lens; VASSEN Co., Ltd., Pyeongtaek-si gyeonggido, Republic of Korea) were purchased at a store in Bangkok, Thailand. The contact lens samples were washed with 4 ml of sterile PBS. The DNA template was prepared as described below and tested with PCR specific to *ecfX* gene as indicated in the above section to confirm that they were negative for *P. aeruginosa*. The bacterial suspension and adherence of *P. aeruginosa* to contact lens was employed according to a previous report (25) with some modifications using known amounts of *P. aeruginosa* (PA 07). Briefly, a single bacterial colony on TSA was inoculated into a 5 ml of trypticase soy broth and incubated for overnight at 37°C. Then, bacterial cells were collected by centrifugation at 2,000 x g for 10 min and the pellet was washed with sterile PBS twice before resuspended in PBS. The absorbance at 660 nm was adjusted to the range of 0.1 which is ~10^8 CFU ml^{-1}. Sterile contact lenses were transferred into 2 ml of 10^8 CFU ml^{-1} of PA07 and incubated at 37°C with shaking at 125 rpm for 24 h.

After 24 h, the contact lenses were removed aseptically and washed gently with PBS to remove loosely attached microorganisms before transferred to 4 ml sterile PBS and vortexed for 1 min to remove the adhered microorganisms. Then, 10-fold serial dilutions of the cultures were prepared in PBS. For DNA extraction, the 100 μl of each dilution was centrifuged at 20,000 x g for 5 min, the supernatant was removed and the pellet was resuspended in 50 μl of 25 mM NaOH and subsequently heated at 95°C for 5 min. After neutralization with 4 μl of 1 M Tris-HCl buffer (pH 7.5), the suspension was centrifuged at 4°C, 20,000 x g for 5 min and the supernatant was used as a template (1 μl) for UDG-LAMP-AuNPs and PCR assay. The sensitivity of UDG-LAMP-AuNPs was compared
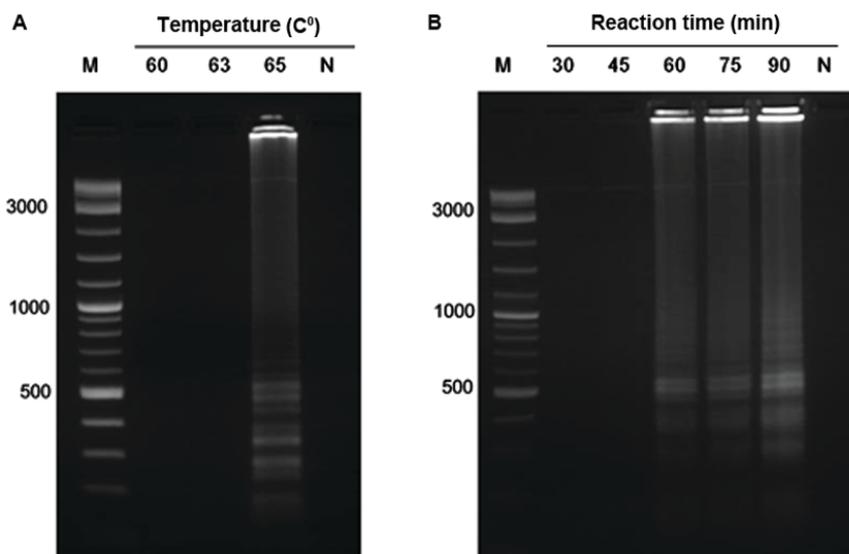
Figure 1. Determination of loop-mediated isothermal amplification conditions at different temperatures and time. (A) Various temperatures, as indicated on top of the gel, were tested for 60 min, and (B) various reaction times, as indicated, were performed at 65°C, using DNA extracted from the *Pseudomonas aeruginosa* 07 bacterial isolate. Lane N, negative control (no DNA template); lane M, molecular weight marker.

with that of PCR assay. In parallel, the bacterial colonies were counted as described above.

Statistical analysis. The results were analysed using the descriptive statistics tests of SPSS version 23.0 software (IBM Corp., Armonk, NY, USA). Data are presented as the mean ± standard deviation of three independent experiments.

Results

Optimization of the temperature, reaction time and ratio of dUTP to dTTP for Pseudomonas aeruginosa detection by UDG-LAMP. To determine the optimal conditions for UDG-LAMP assay, various temperatures for the UDG-LAMP assay were conducted at 60, 63 and 65°C for 60 min. The ladder-like pattern characteristic of LAMP reaction was only observed at 65°C tested temperature; therefore, the temperature at 65°C was chosen for the subsequent UDG-LAMP assays (Fig. 1A).

To determine the optimal time for UDG-LAMP assay, the reaction was conducted at 65°C for 30, 45, 60, 75 and 90 min. The LAMP amplicons could be clearly observed at 60, 75 and 90 min but no amplification was detected at 30 and 45 min. Therefore, the reaction time at 60 min was chosen as optimal time for the UDG-LAMP assay to minimize the reaction time (Fig. 1B).

The LAMP reaction using *Bst* 2.0 DNA polymerase was well progressed using dUTP in place of dTTP; however, the concentration of dUTP affected the visible of ladder-like pattern characteristic on agarose gel. When dTTP was totally replaced with dUTP, the UDG-LAMP reaction produced no ladder-like pattern (Fig. 2, lane 1). Therefore, the ratio between dUTP to dTTP was varied from 80% dUTP + 20% dTTP; 60% dUTP + 40% dTTP; 40% dUTP + 60% dTTP; 20% dUTP + 80% dTTP; and 100% dTTP. The ladder-like pattern was observed at the ratios of 60% dUTP + 40% dTTP; 40% dUTP + 60% dTTP; 20% dUTP + 80% dTTP; and 100%
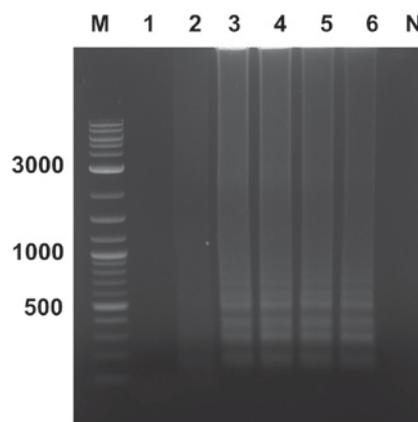
Figure 2. Optimization of dUTP to dTTP for loop-mediated isothermal amplification. The ratios of dUTP to dTTP were as follows: Lane 1, 100% dUTP; lane 2, 80% dUTP + 20% dTTP; lane 3, 60% dUTP + 40% dTTP; lane 4, 40% dUTP + 60% dTTP; lane 5, 20% dUTP + 80% dTTP; and lane 6, 100% dTTP. Lane M, molecular weight marker; lane N, negative control (no DNA template); dUTP, deoxyuridine triphosphate; dTTP, deoxythymidine triphosphate.

dTTP (Fig. 2). Therefore, the ratio of 40% dUTP to 60% dTTP was chosen for the subsequent UDG-LAMP assays to study the specificity and sensitivity.

Optimization of the AuNPs-probe for detection of UDG-LAMP products. To study the effect of salt concentration (MgSO$_4$), the UDG-LAMP product were hybridized to AuNPs probe under various concentrations of MgSO$_4$ including 5, 10, 20, 40, 100 and 200 mM. The results showed that 40 mM MgSO$_4$ yielded the clear difference between positive sample (red-purple) and negative control (blue-gray or colorless) (Fig. 3A and B). These results were corresponded to LAMP reactions followed by UV-visible analysis (Fig. 3C and D) and the absorbance at 525 nm clearly confirmed that results (Fig. 3E). Therefore, 40 mM MgSO$_4$ was used for all subsequent assays.
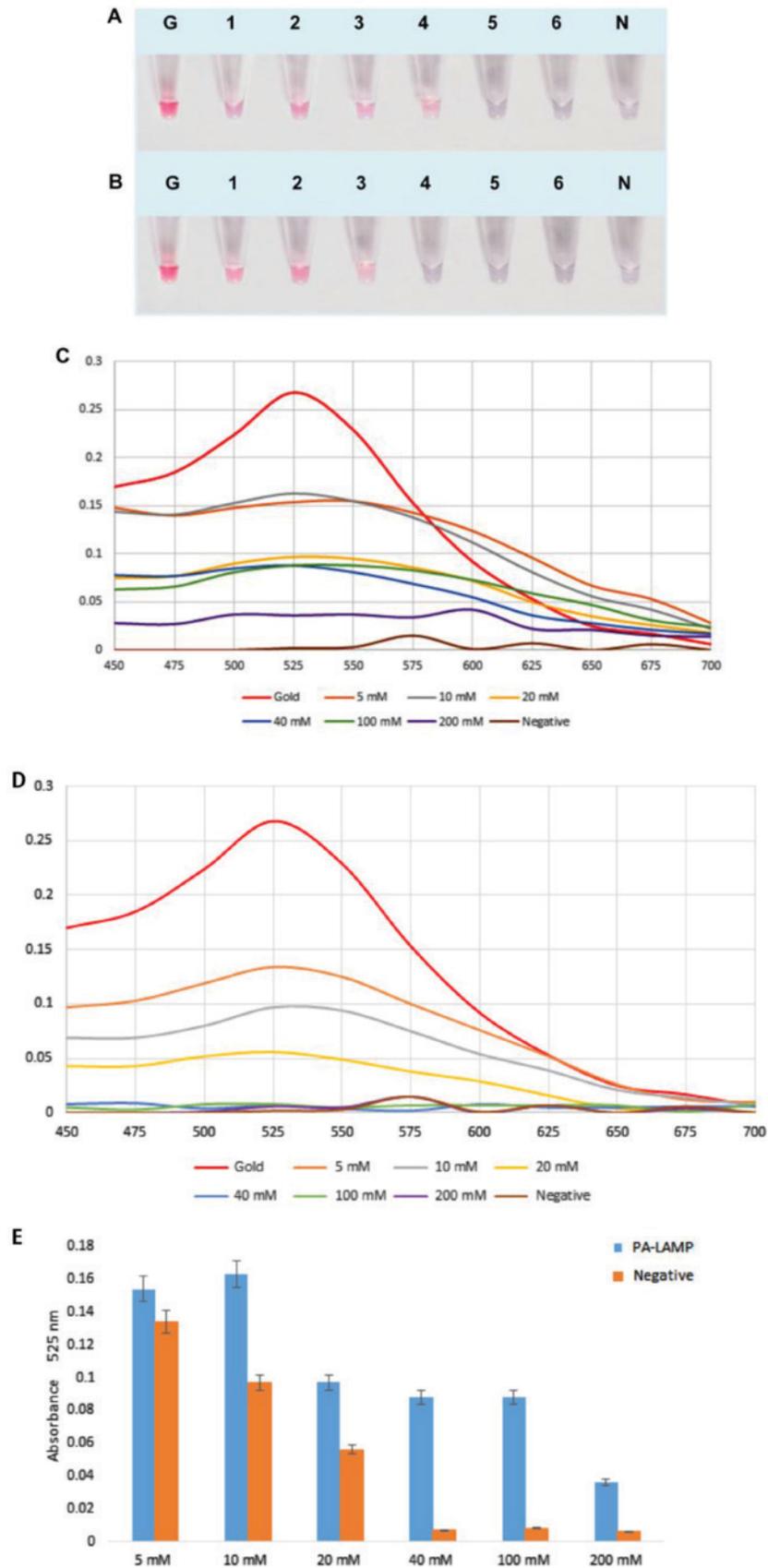
Figure 3. Optimization of the $MgSO_4$ concentration for UDG-LAMP-AuNPs. (A) Various concentrations of $MgSO_4$ were added with the AuNPs probe to *P. aeruginosa* UDG-LAMP products and (B) non-*P. aeruginosa* UDG-LAMP products (negative control). Hybridization was performed at 65˚C for 5 min. Lane G, AuNPs probe only (without salt solution); lane 1-6, $MgSO_4$ concentration at 5, 10, 20, 40, 100 and 200 mM, respectively; lane N, negative control (no DNA template). Absorption spectra of the colloidal AuNP probe in the presence of (C) *P. aeruginosa* UDG-LAMP products and (D) non-*P. aeruginosa* UDG-LAMP products under various concentrations of $MgSO_4$. (E) Absorption of UV-visible spectrophotometer at the wavelength of 525 nm. Blue bar indicates positive samples and the orange bar indicates negative samples. LAMP, loop-mediated isothermal amplification; UDG-LAMP-AuNPs, uracil-DNA-glycosylase-supplemented LAMP coupled with nanogold probe; AuNPs, gold nanoparticles; PA/*P. aeruginosa, Pseudomonas aeruginosa*.
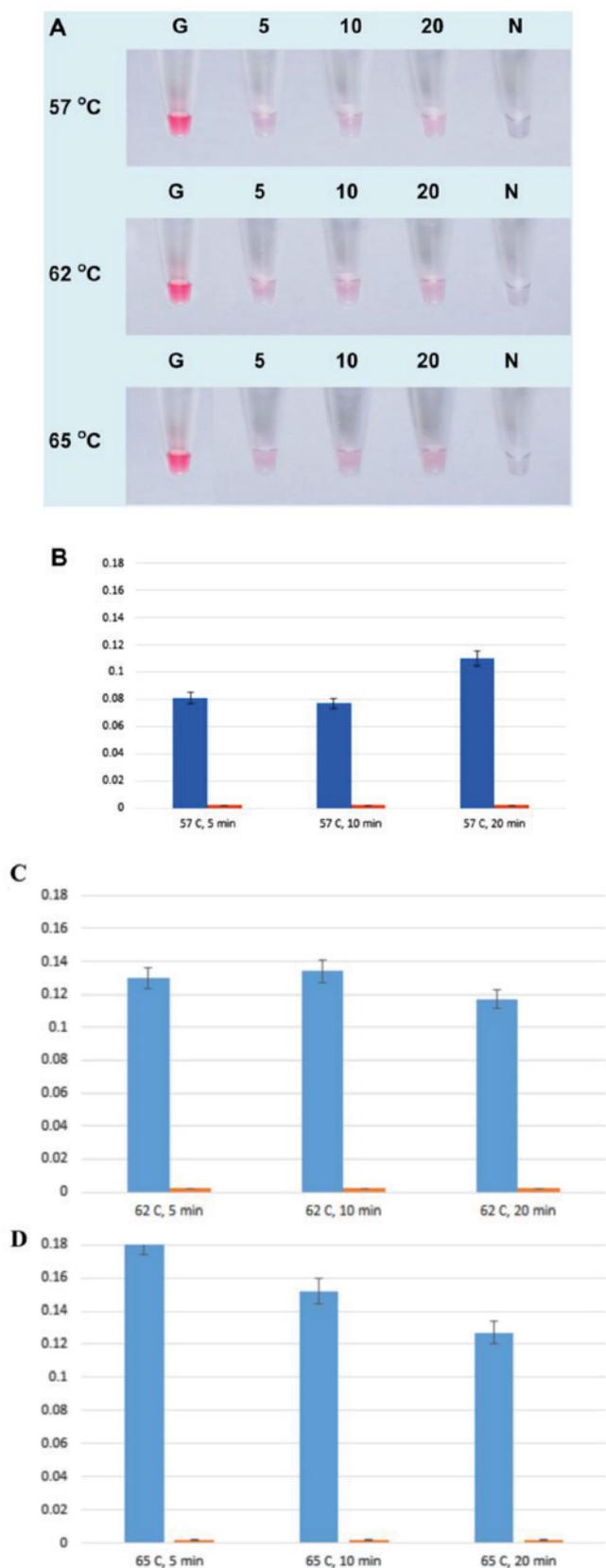
Figure 4. (A) Optimization of hybridization temperatures and durations for the detection of UDG-LAMP products at 57, 62 and 65°C for 5, 10 and 20 min, respectively. Lane G, AuNPs probe only (without salt solution); lane N, negative control (no DNA template). Absorption of ultraviolet-visible spectrophotometer at a wavelength of 525 nm for the detection of UDG-LAMP products using hybridization time at 5, 10 and 20 min for (B) 57°C, (C) 62°C and (D) 65°C. Blue bars indicate positive sample and orange bars indicate negative samples. UDG-LAMP, uracil-DNA-glycosylase-supplemented loop-mediated isothermal amplification; AuNPs, gold nanoparticles.
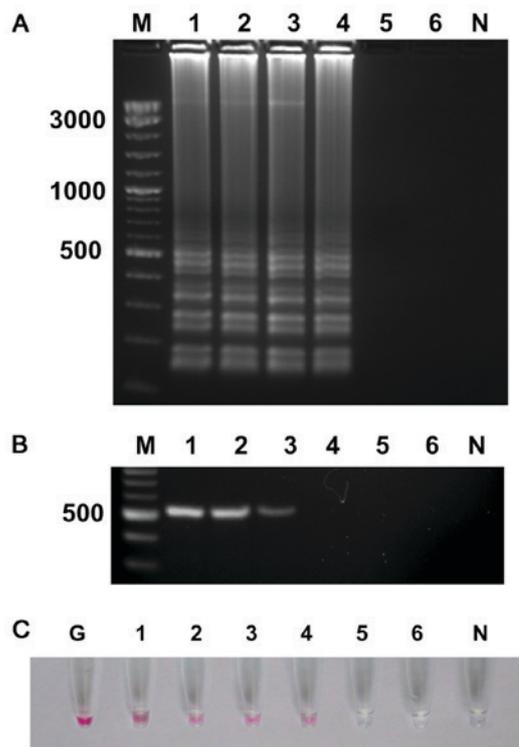
Figure 5. Sensitivity testing for *P. aeruginosa* pure culture detection by (A) UDG-LAMP, (B) polymerase chain reaction and (C) UDG-LAMP-AuNP. Lanes 1-6 represent the 10-fold serial dilution of *P. aeruginosa* from 1.6×10^6 CFU ml^{-1} to 1.6×10^1 CFU ml^{-1}. Lane M, molecular weight DNA marker; lane N, negative control; lane G, gold nanoparticle probe only; *P. aeruginosa*, *Pseudomonas aeruginosa*; CFU, colony-forming units; UDG, uracil-DNA-glycosylase; LAMP, loop-mediated isothermal amplification; AuNPs, gold nanoparticles.

To determine the optimal temperature and time for hybridization between AuNPs probe and UDG-LAMP product, the hybridization was conducted at 57, 62 and 65°C for 5, 10, and 20 min at each temperature. The color reactions demonstrated similar results in all ranges of the tested temperature and time (Fig. 4A). However, the UV-visible analysis revealed that the hybridization at 65°C for 5 min yielded the highest absorbance at 525 nm (Fig. 4D). Therefore, the hybridization performed at the temperature of 65°C for 5 min was chosen for the subsequent reactions.

Specificity of the UDG-LAMP-AuNPs and PCR assays. The specific testing demonstrated that UDG-LAMP-AuNPs and PCR specific to *ecfX* gene revealed positive results to all tested isolates of *P. aeruginosa*. While 8 other *Pseudomonas* species and all non-*Pseudomonas* bacteria yielded negative results (Table I).

Detection limit of UDG-LAMP-AuNPs and PCR assays with pure culture. To determine the sensitivity of UDG-LAMP-AuNPs and PCR assays for detection of *P. aeruginosa* (PA07), the stock solution of bacterial culture (1.6×10^8 CFU ml^{-1}) was diluted in 10-fold serial dilution and DNA extracted from each dilution was used in the UDG-LAMP-AuNPs and PCR assays. The UDG-LAMP and UDG-LAMP-AuNPs assay exhibited the positive result at 1.6×10^3 CFU ml^{-1} or equivalent to ~3 CFU per reaction (Fig. 5A and C), whereas, the PCR assay
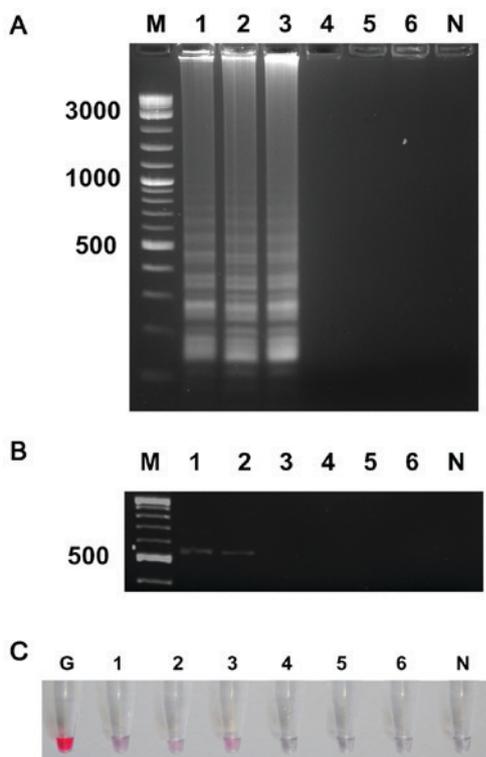
Figure 6. Sensitivity testing for *P. aeruginosa* detection in contact lens samples by (A) UDG-LAMP, (B) polymerase chain reaction and (C) UDG-LAMP-AuNP. Lanes 1-6 represent the 10-fold serial dilution of *P. aeruginosa* from $1.1x10^5$ CFU ml^{-1} to 1.1 CFU ml^{-1}. Lane M, molecular weight DNA marker; lane N, negative control; lane G, gold nanoparticle probe only; *P. aeruginosa, Pseudomonas aeruginosa*; CFU, colony-forming units; UDG, uracil-DNA-glycosylase; LAMP, loop-mediated isothermal amplification; AuNPs, gold nanoparticles.

showed the detection limit at $1.6x10^4$ CFU ml-1 or equivalent to 30 CFU per reaction (Fig. 5B).

Sensitivity of UDG-LAMP-AuNPs and PCR assay with spiked contact lens samples. The detection limit of UDG-LAMP-AuNPs and PCR assays for PA07 spiked into contact lenses was examined. After removal of loosely attached bacteria, the determined number of bacteria in washed solution was $1.1x10^6$ CFU ml^{-1}. After 10-fold serial dilutions of washed solution was performed, the total DNA extracted from each dilution was used to investigate the detection limit. The results showed the sensitivity of UDG-LAMP and UDG-LAMP-AuNPs at $1.1x10^3$ CFU ml^{-1} or equivalent to 2 CFU per reaction (Fig. 6A and C). Whereas, the PCR assay showed detection limit at $1.1x10^4$ CFU ml^{-1} or equivalent to 20 CFU per reaction (Fig. 6B).

Discussion

P. aeruginosa is one of the most important pathogens causing human infections such as pneumonia, bacteremia, urinary tract infections and keratitis (26,27). Microbial keratitis is a serious complication of contact lens wear and *P. aeruginosa* is a common causative agents associated with infectious keratitis reported in many countries (28). In order to manage patient infection, a rapid and specific detection method is required to differentiate keratitis caused by *P. aeruginosa* from other microorganism infections.

In the present study, a UDG-LAMP-AuNP method targeting to the *ecfX* gene of *P. aeruginosa* was successfully developed. The optimum temperature and time for UDG-LAMP reaction were 65°C and 60 min, respectively. Upon hybridization with nanogold labeled probe and salt addition, the positive reaction (red-purple color) of UDG-LAMP products could be observed within 5-10 min. This is more rapid than the PCR method and more suitable for small laboratory since the sophisticated equipment is not required. The LAMP assay is highly sensitive for DNA amplification; therefore, it is prone to carryover contamination. In our study, the UDG was effectively used to digest the uracil incorporated amplicons in a one-tube, closed vessel reaction. This UDG-LAMP strategy was successfully employed with *Salmonella* Typhimurium (22) and multiplex LAMP for simultaneous detection of white spot syndrome virus (WSSV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) in shrimp (29). In the present study, the optimum ratio of 40% dUTP to 60% dTTP was used. This ratio was in agreement with that of previous reports for detection of *Salmonella* Typhimurium (the ratio of 1:1) (22) and WSSV-IHHNV (the ratio of 5/8) (29).

The gold nanoparticle can interact with disulfide modified DNA probe and the ability to change color (from red to blue) on self-assembly is due to aggregation at an optimal salt concentration. In our study, under the low-salt concentration (5 to 10 mM MgSO$_4$ solution), the AuNP probes were still stabilized in both control samples and the samples containing *P. aeruginosa*. However, at 40 mM MgSO$_4$ the aggregation of AuNP probes in the control sample was clearly observed (blue-gray or colorless) and could be distinguishable from positive sample containing *P. aeruginosa* (red color). This result was in good agreement with previous report on specific detection of WSSV (20).

Previous studies on detection of *P. aeruginosa* showed that the false-negative results could be obtained from PCR assays targeting to the *toxA* and *algD* genes due to the highly polymorphic characteristic and sequence variation of *P. aeruginosa* (3,30). However, the PCR assay targeting to *ecfX* for specific detection of *P. aeruginosa* has been reported (3). In our UDG-LAMP-AuNPs assay, four specific LAMP primers and DNA probe targeting to *ecfX* gene of *P. aeruginosa* were designed and employed to ensure high specificity of nucleic acid amplification (3). Positive results were obtained for all tested *P. aeruginosa* isolates without cross-reaction to other bacterial species, demonstrating that these LAMP primers and DNA probe were specific for identification of *P. aeruginosa*.

In the present study, the sensitivity of UDG-LAMP-AuNPs assay in pure culture was $1.6x10^3$ CFU ml^{-1} or equivalent to 3 CFU per reaction, 10 times more sensitive than that of PCR. The detection limit of UDG-LAMP-AuNPs at 3 CFU per reaction was more sensitive than traditional LAMP method for detection of *P. aeruginosa* based on *oprI* gene (10 CFU per reaction) (15).

In the case of artificially inoculated contact lenses, the detection limit of UDG-LAMP-AuNPs was $1.1x10^3$ CFU ml^{-1} or equivalent to 2 CFU per reaction, 10 times more sensitive than that of PCR assay ($1.1x10^4$ CFU ml^{-1} or equivalent to 20 CFU per reaction). The sensitivity of UDG-LAMP-AuNPs

in both pure culture and spiked contact lenses were more sensitive than that of the conventional PCR assay. These results agreed with previous studies that demonstrate a better sensitivity of LAMP compared to that of PCR (13,15,16,31). The detection limit of UDG-LAMP-AuNPs for spiked contact lenses at 2 CFU per reaction was comparable to that of colorimetric LAMP detection of *P. aeruginosa* inoculated in mouse feces at 3.25 CFU per reaction (16) and LAMP assay for direct detection of *P. aeruginosa* from equine genital swabs at 11 DNA copies per reaction (32).

Recently, a microchip electrical sensing for detection of *P. aeruginosa* was developed. The detection limit of spiked eye wash samples at 10 CFU ml^{-1} was reported (33). Another study based on magnetic relaxation switch (MRSw) aptasensor for detection of *P. aeruginosa* with the sensitivity of 50 CFU ml^{-1} was also reported (34). In our study, an inferior detection limit of 1.1×10^3 CFU per ml was obtained. However, the UDG-LAMP-AuNPs reaction could be performed with simple and inexpensive heating block while the MRSw method required a large size and high cost of magnetic relaxation scanners which is improper for field study.

In conclusion, the first UDG-LAMP-AuNPs method for detection of *P. aeruginosa* based on *ecfX* gene was successfully established. The assay demonstrated high specificity and high sensitivity of ~3 CFU per reaction with pure culture and 2 CFU per reaction with spiked contact lens samples. The developed UDG-LAMP-AuNPs assay is a sensitive, simple, rapid and valuable tool for specific diagnosis of *P. aeruginosa* in contaminated biological samples.

Acknowledgements

References

1. van der Kooij D, Oranje JP and Hijnen WA: Growth of *Pseudomonas aeruginosa* in tap water in relation to utilization of substrates at concentrations of a few micrograms per liter. Appl Environ Microbiol 44: 1086-1095, 1982.
2. Romling U, Kader A, Sriramulu DD, Simm R and Kronvall G: Worldwide distribution of *Pseudomonas aeruginosa* clone C strains in the aquatic environment and cystic fibrosis patients. Environ Microbiol 7: 1029-1038, 2005.
3. Lavenir R, Jocktane D, Laurent F, Nazaret S and Cournoyer B: Improved reliability of *Pseudomonas aeruginosa* PCR detection by the use of the species-specific *ecfX* gene target. J Microbiol Methods 70: 20-29, 2007.
4. Onlen Y, Tamer C, Oksuz H, Duran N, Altug ME and Yakan S: Comparative trial of different anti-bacterial combinations with propolis and ciprofloxacin on *Pseudomonas* keratitis in rabbits. Microbiol Res 162: 62-68, 2007.
5. McLaughlin-Borlace L, Stapleton F, Matheson M and Dart JK: Bacterial biofilm on contact lenses and lens storage cases in wearers with microbial keratitis. J Appl Microbiol 84: 827-838, 1998.
6. Weiser R, Donoghue D, Weightman A and Mahenthiralingam E: Evaluation of five selective media for the detection of *Pseudomonas aeruginosa* using a strain panel from clinical, environmental and industrial sources. J Microbiol Methods 99: 8-14, 2014.
7. Jimenez L, Smalls S and Ignar R: Use of PCR analysis for detecting low levels of bacteria and mold contamination in pharmaceutical sample. J Microbiol Methods 41: 259-265, 2000.
8. Deschaght P, Van daele S, De Baets F and Vaneechoutte M: PCR and the detection of *Pseudomonas aeruginosa* in respiratory samples of CF patient. A literature review. J Cyst Fibros 10: 293-297, 2011.
9. Anuj SN, Whiley DM, Kidd TJ, Bell SC, Wainwright CE, Nissen MD and Sloots TP: Identification of *Pseudomonas aeruginosa* by a duplex real-time polymerase chain reaction assay targeting the *ecfX* and *gyrB* genes. Diagn Microbiol infect Dis 63: 127-131, 2009.
10. McCulloch E, Lucas C, Ramage G and Williams C: Improved early diagnosis of *Pseudomonas aeruginosa* by real-time PCR to prevent chronic colonization in a pediatric cystic fibrosis population. J Cyst Fibros 10: 21-24, 2011.
11. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N and Hase T: Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28: e63, 2000.
12. Surasilp T, Longyant S, Rukpratanporn S, Sridulyakul P, Sithigorngul P and Chaivisuthangkura P: Rapid and sensitive detection of *Vibrio vulnificus* by loop-mediated isothermal amplification combined with lateral flow dipstick targeted to *rpoS* gene. Mol Cell Probes 25: 158-163, 2011.
13. Prompamorn P, Sithigorngul P, Rukpratanporn S, Longyant S, Sridulyakul P and Chaivisuthangkura P: The development of loop-mediated isothermal amplification combined with a lateral flow dipstick for detection of *Vibrio parahaemolyticus*. Lett Appl Microbiol 52: 344-351, 2011.
14. Li J, Zhai L, Bie X, Lu Z, Kong X, Yu Q, Lv F, Zhang C and Zhao H: A novel visual loop-mediated isothermal amplification assay targeting gene 62181533 for the detection of Salmonella spp. in foods. Food control 60: 230-236, 2016.
15. Zhao X, Wang L, Li Y, Xu Z, Li L, He X, Liu Y, Wang J and Yang L: Development and application of a loop-mediated isothermal amplification method on rapid detection of *Pseudomonas aeruginosa* strains. World J Microbiol Biotechnol 27: 181-184, 2011.
16. Goto M, Shimada K, Sato A, Takahashi E, Fukasawa T, Takahashi T, Ohka S, Taniguchi T, Honda E, Nomoto A, *et al*: Rapid detection of *Pseudomonas aeruginosa* in mouse feces by colorimetric loop-mediated isothermal amplification. J Microbiol Methods 81: 247-252, 2010.
17. Zhang S, Xu X, Wu Q and Zhang J: Rapid and sensitive detection of *Pseudomonas aeruginosa* in bottled water by loop-mediated isothermal amplification. Eur Food Res Technol 236: 209-215, 2013.
18. Shi H, Chen Z and Kan J: Development of loop-mediated isothermal amplification assays for genotyping of Type III Secretion System in *Pseudomonas aeruginosa*. Lett Appl Microbiol 61: 361-366, 2015.
19. Jaroenram W, Arunrut N and Kiatpathomchai W: Rapid and sensitive detection of shrimp yellow head virus using loop-mediated isothermal amplification and a colorogenic nanogold hybridization probe. J Virol Methods 186: 36-42, 2012.
20. Seetang-Nun Y, Jaroenram W, Sriurairatana S, Suebsing R and Kiatpathomchai W: Visual detection of white spot syndrome virus using DNA-functionalized gold nanoparticles as probes combined with loop-mediated isothermal amplification. Mol Cell Probes 27: 71-79, 2013.
21. Watthanapanpituck K, Kiatpathomchai W, Chu E and Panvisavas N: Identification of human DNA in forensic evidence by loop-mediated isothermal amplification combined with a colorimetric gold amplification combined with a colorimetric gold nanoparticle hybridization probe. Int J Legal Med 128: 923-931, 2014.
22. Hsieh K, Mage PL, Csordas AT, Eisenstein M and Soh HT: Simultaneous elimination of carryover contamination and detection of DNA with uracil-DNA-glycosylase-supplemented loop mediated isothermal amplification (UDG-LAMP). Chem Commun 50: 3747-3749, 2014.
23. Weisburg WG, Barns SM, Pelletier DA and Lane DJ: 16S ribosomal DNA amplification for phylogenic study. J Bacteriol 173: 697-703, 1991.
24. Yamazaki W, Seto K, Taguchi M, Ishibashi M and Inoue K: Sensitive and rapid detection of cholera toxin producing *Vibrio cholerae* using a loop-mediated isothermal amplification. BMC Microbiol 8: 94, 2008.
25. Chan KY, Cho P and Boost M: Microbial adherence to cosmetic contact lenses. Cont Lens Anterior Eye 37: 267-272, 2014.

26. Jarvis WR and Martone WJ: Predominant pathogens in hospital infections. J Antimicrob Chemother 29 (Suppl A): S19-S24, 1992.
27. Schwartz T, Volkmann H, Kirchen S, Kohnen W, Schön-Hölz K, Jansen B and Obst U: Real-time PCR detection of *Pseudomonas aeruginosa* in clinical and municipal wastewater and genotyping of the ciprofloxacin-resistant isolates. FEMS Microbiol Ecol 57: 158-167, 2006.
28. Willcox MD: Management and treatment of contact lens-related *Pseudomonas* keratitis. Clin Ophthalmol 6: 919-924, 2012.
29. He L and Xu HS: Development of a multiplex loop-mediated isothermal amplification (mLAMP) method for the simultaneous detection of white spot syndrome virus and infectious hypodermal and hematopoietic necrosis virus in penaeid shrimp. Aquaculture 311: 94-99, 2011.
30. Qin X, Emerson J, Stapp J, Stapp L, Abe P and Burns JL: Use of real-time PCR with multiple targets to identify *Pseudomonas aeruginosa* and other nonfermenting gram-negative bacilli from patients with cystic fibrosis. J Clin Microbiol 41: 4312-4317, 2003.
31. Srisuk C, Chaivisuthangkura P, Rukpratanporn S, Longyant S, Sridulyakul P and Sithigorngul P: Rapid and sensitive detection of *Vibrio cholerae* by loop-mediated isothermal amplification targeted to the gene of outer membrane protein ompW. Lett Appl Microbiol 50: 36-42, 2010.
32. Diribe O, Fitzpatrick N, Sawyer J, La Ragione R and North S: A Rapid and simple loop-mediated isothermal amplification assay for the detection of *Pseudomonas aeruginosa* from equine genital swabs. J Equine Vet Sci 35: 929-934, 2015.
33. Pandya HJ, Kanakasabapathy MK, Verma S, Chug MK, Memic A, Gadjeva M and Shafiee H: Label-free electrical sensing of bacteria in eye wash samples: A step towards point-of-care detection of pathogens in patients with infectious keratitis. Biosens Bioelectron 91: 32-39, 2017.
34. Jia F, Xu L, Yan W, Wu W, Yu Q, Tian X, Dai R and Li X: A magnetic relaxation switch aptasensor for the rapid detection of *Pseudomonas aeruginosa* using superparamagnetic nanoparticles. Microchim Acta 184: 1539-1545, 2017.