Evaluation of loop-mediated isothermal amplification assays for rapid detection of *bla*KPC producing *Serratia spp.* in clinical specimens: A prospective diagnostic accuracy study

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Abstract. The prevalence of carbapenem-resistant *Serratia* spp. is increasing owing to the propagation of β lactamase *Klebsiella pneumoniae* carbapenemase (*bla*KPC) and it has become one of the major global health concerns. As effective therapies for such resistant pathogens are limited, there is a great need for the rapid and sensitive characterization of the pathogen. In the present study, a loop-mediated isothermal amplification (LAMP) method for the rapid detection of *Serratia* spp. with *bla*KPC in pure cultures and clinical specimens was developed. A calcein indicator and real-time turbidity recording system were used to assess the LAMP reaction. The LAMP assay was compared with conventional PCR and real-time PCR kits for the target pathogen. The desired amplification was achieved using selected primers and detection was possible using both the calcein indicator method and the real-time turbity recording system at 65˚C for 60 min. The sensitivity of the detection system for *bla*KPC-producing *Serratia* spp. reached a detection limit of 3.92 pg/µl DNA, which was 10 times more sensitive than conventional PCR. Specificity testing indicated that the primers were highly specific. Compared with conventional culture methods and real-time PCR, the LAMP assay was more sensitive, easier for laboratory staff to master and less influenced by the clinical specimen matrix. In conclusion, a LAMP assay for *bla*KPC-producing *Serratia* spp. that permitted rapid, sensitive and economical detection for this pathogen was successfully developed. Comparisons with alternative methods indicated that the LAMP assay was more feasible in a clinical setting.

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Abbreviations: LAMP, loop-mediated isothermal amplification; BSI, bloodstream infection; CRE, carbapenem-resistant *Enterobacteriaceae*; KPC, *Klebsiella pneumoniae* carbapenemase

Key words: Serratia spp., loop-mediated isothermal amplification, specificity, carbapenem resistance

Introduction

Serratia spp., particularly the species *Serratia marcescens* (*S. marcescens*), are ubiquitous environmental gram-negative bacteria. They are members of the *Enterobacteriaceae* family that flourish in all kinds of environments and have become important pathogens and were included in those accounting for hospital-acquired infections in recent years. In general, *Serratia* spp. does not pose a threat to individuals with normal immunity. However, patients who are hospitalized in the neonatal department, (pediatric) intensive care unit (ICU) or bone marrow transplant and oncology units are immunocompromised or immunosuppressed and the severity of infection is markedly increased (1-3). *Serratia* spp. infections are also associated with other complications, such as infections in the lower respiratory tract, urinary tract, wounds and the bloodstream (4). Studies have also indicated that *Serratia* spp. may give rise to corneal ring infiltrates in patients with human immunodeficiency virus through contaminated contact lenses (5,6). Among the infections caused by *Serratia* spp., bloodstream infection (BSI) is the most prevalent and fatal (7). Hence, a rapid and sensitive characterization method is urgently needed to guide anti-infection therapies (8).

Carbapenem-resistant *Enterobacteriaceae* (CRE) have become a serious problem worldwide. Resistance to carbapenems is rising year on year due to the spread of carbapenemases, the mutation of penicillin-binding proteins, decreased membrane permeability and overexpression of efflux pumps (9,10). For CRE, the predominant mechanism contributing to such resistance involves carbapenemases, particularly β lactamase *Klebsiella pneumoniae* carbapenemase (*bla*KPC), and type B and type D carbapenemases. Genes encoding these carbapenemases have also been

detected in *S. marcescens* (11,12). In addition, other types of carbapenemases, including *S. marcescens* enzymes, *Serratia* metallo-β-lactamase, *Guiana* extended-spectrum β-lactamase and Germany imipenemase, were detected in *S. marcescens* isolated in different regions (13-18). The high hydrolytic efficiency of these carbapenemases when acting on carbapenem substrates combined with the natural resistance of *S. marcescens* to polymyxin B and polymyxin E (two restricted antibiotics used for multidrug-resistant bacteria infections), makes the treatment of *S. marcescens* infections challenging in the clinical setting (19).

Loop-mediated isothermal amplification (LAMP) is a high-specificity, high-efficiency and rapid amplification technology based on PCR. It uses a DNA polymerase with high displacement strand activity and three pairs of specially designed primers to amplify the target sequence (20). LAMP is frequently used to detect infectious diseases among humans, livestock and plants. There are various detection methods for the reaction endpoint, including the turbidity method, agarose gel electrophoresis and UV light detection method. The turbidity method is widely used due to its simple operation and real-time mode. This method detects the by-product of the LAMP reaction ($Mg_2P_2O_7$, magnesium pyrophosphate, a white precipitate) at an optical density of 650 nm every 6 sec. A variety of microorganisms have been successfully detected by using this method, such as *Mycoplasma pneumoniae, Salmonella* spp. and *Clostridium difficile* (21-23).

Due to the prevalence of carbapenem-resistant *Serratia* spp. in China (24-28), the present study aimed to develop a LAMP method for the rapid characterization of *bla*KPC producing *Serratia* spp. (from pure cultures and clinical specimens) with the calcein/Mn^{2+} complex and a constant turbidity recording system and the reaction characteristics were assessed.

Materials and methods

Bacterial strains and preparation of templates. In the present study, 31 bacterial strains isolated from patients were used, including three different strains of *Serratia* spp. (*S. marcescens* DY12303, *S. liquefaciens* DY12165 and *S. rubidaea* DY12122). The characteristics of these 31 bacterial strains are listed in Table I. *S. marcescens* DY12303 with *bla*KPC-2 was obtained from a patient who was admitted to the ICU at Henan Province Hospital of Traditional Chinese Medicine (TCM; Second Affiliated Hospital of Henan University of TCM, Zhengzhou, China) and validated by a Vitek-2 Compact automated system for ID/AST (Bio Merieux). This strain was used as the positive control for *Serratia* spp. identification and *bla*KPC detection. Sequencing of 16s ribosomal (r)RNA and *bla*KPC-2 were performed using the PCR primers PCR F27, R1492, KPC-forward (F) and KPC-reverse (R) (see Table III). The PCR mixture contained 10 μl PCR 2X master mix reagent (Tiangen Biotech Co., Ltd.), 1 μl 2 μM forward and reverse primers, 1 μl (2 ng) of template and 7 μl double-distilled (dd)H_2O. The procedure was as follows: Initial denaturation at 94˚C for 5 min, followed by 35 cycles of 94˚C for 30 sec as the denaturation step, 58˚C for 30 sec as the annealing step and 72˚C for 30 sec as the extension step. For the final extension step, the reaction was held at 72˚C for 10 min. The product was sequenced by Sangon Biotechnology Co., Ltd. and the sequencing results of 16s rRNA and *bla*KPC-2 were 100% identical to the *S. marcescens* and *bla*KPC-2 sequences in GenBank. Genomic DNA from bacteria was extracted using a QIAamp DNA Mini kit (cat. no. 51304; Qiagen GmbH) following the manufacturer's protocol. Purified DNA was diluted 100-fold and saved as a template for the experiments.

Primer design. As the LuxS gene of *Serratia* spp. diverged from other luxS genes of bacterial species, such as *Escherichia coli*, *Salmonella enterica*, *Pantoea ananatis*, *Listeria monocytogenes*, *Yersinia pestis*, and *Erwenia amylovora* the luxS gene, which is associated with the quorum sensing system of *Serratia* spp., was selected as the target sequence. The complete luxS gene sequences of *S. marcescens* ATCC274 (GenBank accession no. AJ628150), *S. odorifera* (GenBank accession no. GG753567), *S. lique faciens* ATCC27592 (GenBank accession no. CP006252) and *S. rubidaea* (GenBank accession no. CP014474) were obtained from the National Center for Biotechnology Information (NCBI) GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) and compared using CLUSTAL W2.1. The sequence alignment results (Fig. S1) were further analyzed using Primer Explorer software V5 (http://primerexplorer.jp/lampv5e/index.html), and forward outer (F3), backward outer (B3), forward internal (FIP), backward internal (BIP), forward loop (LF) and backward loop (LB) primers were designed to optimize the amplification reaction.

To design universal LAMP primers for *bla*KPC, KPC-1 to KPC-32 (the GenBank accession no. of each *bla*KPC subtype is listed in Table II) were compared using CLUSTAL W2.1 (29). The sequence alignment results were tackled with the same procedure for *Serratia* spp. To obtain the most appropriate primers, three sets of primers were designed for *Serratia* spp. and *bla*KPC. The sensitivity and specificity of the LAMP assay were then compared with those of conventional PCR. To complete this comparison experiment, luxS-F and luxS-R primers for *S. marcescens* identification and KPC-F and KPC-R for *bla*KPC identification were designed. The primers were synthesized by Sangon Biotechnology Co., Ltd. and the primer sequences are listed in Table III.

Specificity and sensitivity evaluation of the LAMP assay. To evaluate the sensitivity of the LAMP assay, pure genomic DNA of *S. marcescens* DY12303 was extracted by using a QIAamp DNA Mini Kit (Qiagen GmbH). DNA quantity and quality were determined with a NanoDrop-1000 Spectrometer (Thermo Fisher Scientific Inc.). The pure genomic DNA was further subjected to serial 10-fold dilutions over a log7 scale with ddH_2O. Template concentrations ranging from 392.0 ng/μl to 0.392 pg/μl were measured to compare the detection limit difference between the LAMP assay and conventional PCR.

To determine the specificity, 18 common non-*Serratia* clinical isolates were evaluated using the *Serratia* spp. identification LAMP assay. In addition, 12 clinical isolates carrying prevalent resistance genes were used for the specificity evaluation of *bla*KPC LAMP assays (details of the bacterial strains are presented in Table I).

LAMP assay protocol and product detection. A 25-μl volume of the reaction system of the DNA amplification kit (Eiken

Table I. Bacterial strains used in the present study.

Species	Resistance mechanism	Source
S. marcescens DY12303	KPC-2	Clinical isolate
S. liquefaciens DY12165	-	Clinical isolate
S. rubidaea DY12122	-	Clinical isolate
E. coli DY12123	-	Clinical laboratory[a]
K. pneumoniae DY12326	-	Clinical isolate
A. baumannii DY12522	-	Clinical isolate
P. vulgaris DY12184	-	Clinical laboratory
P. aeruginosa DY12122	VIM-2	Clinical laboratory
S. sonnel DY12531	-	Clinical laboratory
Y. pestis DY12189	-	Clinical laboratory
C. freundii DY12218	-	Clinical laboratory
S. typhi DY12161	-	Clinical laboratory
M. morganii DY12439	-	Clinical laboratory
P. rettgeri DY12210	-	Clinical laboratory
A. baumannii FY12252	OXA-23	Clinical isolate
P. aeruginosa DY12476	-	Clinical laboratory
K. oxytoca DY12151	-	Clinical isolate
E. cloacae DY12105	-	Clinical isolate
E. coli DY12228	NDM-1	Clinical laboratory
K. pneumoniae DY12274	-	Clinical isolate
P. mirabilis DY12407	-	Clinical laboratory
S. maltophilia DY12143	-	Clinical laboratory
E. coli DY12295	MCR-1	Clinical laboratory
B. cepacia DY12521	VIM-1	Clinical isolate
K. pneumoniae DY12384	MCR-1	Clinical isolate
A. baumannii DY12223	NDM-1	Clinical isolate
S. maltophilia DY12143	*bla*L1	Clinical isolate
S. typhi DY12161	TEM-1	Clinical laboratory
*E. aerogen*es DY12121	CTX-M-3	Clinical isolate
E. cloacae DY12315	NDM-1	Clinical isolate
P. aeruginosa DY12476	IMP-9	Clinical isolate

[a]Department of Clinical Laboratory of Henan Provincial Hospital of Traditional Chinese Medicine. *bla*, β lactamase; KPC, *Klebsiella pneumoniae* carbapenemase; VIM, Verona imipenemase; OXA, oxacillinase; NDM, New Delhi metallo-β-lactamase; MCR, plasmid-mediated colistin resistance gene; IMP, imipenemase.

Chemical Co., Ltd.) was applied to the LAMP assay. The main components of the reaction system were recorded in the instruction book for the d kit. The final concentrations of primers required for one reaction were as follows: For FIP and BIP 40 pmol, for LF and LB 20 pmol, and for F3 and B3 5 pmol. Finally, 1 μl genomic DNA template was added to the reaction tube (Eiken Chemical Co., Ltd.) containing the materials required for the LAMP reaction. The reaction proceeded at 65˚C for a duration of 60 min. For inactivated products, a reaction temperature of 80˚C for 5 min in a constant-temperature dry bath was used. To detect LAMP products, two different methods were adopted. The first was direct visual inspection; 1 μl of calcein/Mn^{2+} complex (Eiken Chemical Co., Ltd.) as the indicator was added to 24 μl of LAMP reactants prior to the LAMP reaction. In positive reactions, the color changed from orange to green, whereas in negative reactions, there was no color change. The color change was easily observed under natural light or with the aid of ultraviolet light (365 nm wavelength). An analytical method was also used for reaction monitoring. This method used an LA-230 Loopamp real-time turbidimeter (Eiken Chemical Co., Ltd.) to monitor the turbidity (the white precipitation caused by the presence of magnesium pyrophosphate, $Mg_2P_2O_7$). The LAMP assay was assessed in real-time by recording the optical density at 650 nm every 6 sec and the turbidity was recorded with the turbidimeter software.

Conventional PCR protocol and product detection. For conventional PCR, 50-μl reaction mixtures were employed. The main components were as follows: 25 μl PCR 2X master mix reagent (Tiangen Biotech Co., Ltd.), 1 μM forward and reverse primers (luxS and KPC; see Table II) and 1 μl of the

Table II. Subtypes of *bla*KPC used in *bla*KPC primer design.

Subtype of *bla*KPC	GenBank accession no.
KPC-1	AF297554
KPC-2	AY034847
KPC-3	AB557734
KPC-4	FJ473382
KPC-5	EU400222
KPC-6	EU555334
KPC-7	EU729727
KPC-8	FJ234412
KPC-9	FJ624872
KPC-10	GQ140348
KPC-11	HM066995
KPC-12	HQ641422
KPC-13	HQ342889
KPC-14	JX524191
KPC-15	KC433553
KPC-16	KC465199
KPC-17	KC465200
KPC-18	NG049251
KPC-19	KJ775801
KPC-21	NG049254
KPC-22	NG049255
KPC-24	NG049256
KPC-25	KU216748
KPC-26	KX619622
KPC-27	KX828722
KPC-28	NG052581
KPC-30	KY646302
KPC-31	NG055494
KPC-32	NG055495

*bla*KPC, β lactamase *Klebsiella pneumoniae* carbapenemase.

DNA template. The procedure for conventional PCR was an initial incubation at 94˚C for 2 min, 35 cycles at 94˚C for 30 sec as a denaturation step, 58˚C for 30 sec as the annealing step, and 72˚C for 30 sec as the extension step. For the final extension step, the reaction was held at 72˚C for 10 min. The PCR products were separated by electrophoresis using 1% agarose gels (Amresco) and GelRed (Biotium) as a nucleic acid dye at the recommended concentrations. A Gel Doc XR+ imaging system (Bio-Rad Laboratories, Inc.) was used to image the PCR products.

Evaluation of inhibition in clinical specimens. To evaluate inhibition in clinical specimens, pure DNA extracted by QIAamp DNA Mini Kit (Qiagen GmbH) from the sputum and blood of healthy donors were mixed 1:1 with serially diluted genomic DNA extracted from the clinical isolate *S. marcescens* DY12303 with *bla*KPC as templates for the LAMP assay. Thus, the final concentration of templates ranged from 19.6 to 0.0196 pg/μl. Subsequently, 1 μl of DNA template was added to 24 μl of the reaction mixture; the concentrations in the mixture for LAMP were described above. Finally, 25 μl of the LAMP assay mixture was used to evaluate the inhibitory characteristics of the pure DNA extracted from sputum and blood.

LAMP assay evaluation of clinical specimens. Clinical specimens required for the present study were collected at the Henan Province Hospital of TCM (Zhengzhou, China) from January to December 2018. The age range of patients was 4-82 years old, with a mean age of ± SD is 43.67±23.44. The sex ratio was 208 (male): 151 (female). Sputum specimens obtained from the ICU, Neurosurgery department and Oncology department were collected to investigate the efficacy of the LAMP assay for detecting *Serratia* spp. with *bla*KPC. First, a conventional culture method was adopted-all clinical specimens were inoculated, cultured, separated and identified in accordance with the national clinical inspection operating procedures of China and the results were used as a gold standard. In short, the clinical samples were inoculated into 5% blood agar medium and cultured at 37˚C for 18-24 h. For Gram-negative bacilli, the isolated strains were biochemically identified according to Bergey's Manual Systematic Bacteriology (30) and compared with positive control strains. In short, the difference between *Serratia marcescens* and other bacteria in the same genus was arabinose-negative, raffinose-negative and xylose-negative; it may ferment sugars including maltose, glucose, mannose and sucrose. Each specimen was then stored at -20˚C for DNA extraction. To reduce DNA loss during the extraction process, sputazyme (Kyokuto) was used to reduce the viscosity. In brief, the proper volume of sputazyme was added to 500 μl sputum, then boiled for 5 min and subsequently centrifuged at 6,600 x g for 10 min at room temperature. The DNA in the supernatants was used as templates. A QIAamp DNA Microbiome Kit (Qiagen GmbH) was used to extract bacterial DNA from the sputum specimens according to the manufacturer's instructions. Both unpurified and purified DNA were used as templates for LAMP assays and real-time PCR assay (*Serratia marcescens* genesig Standard kit, Primerdesign Co., Ltd.) to compare the two methods.

Real-time PCR protocol and product detection. A *Serratia marcescens* genesig Standard kit (Primerdesign Co., Ltd.) was used for real-time PCR in accordance with the manufacturer's protocol. The final volume of the *S. marcescens* Probe-based Fluorescent quantitative PCR was 20 μl, which contained 10 μl Precision PLUS 2X qPCR Master Mix, 1 μl *S. marcescens* primer/probe mix, 5 μl (2 ng) of templates and 4 μl RNase-free water. PCR cycling parameters were as follows: 95˚C for 2 min, followed by 50 cycles at 95˚C for 10 sec and 60˚C for 1 min.

Data analysis. Sensitivity, specificity, positive predictive value (PPV), negative predicted value (NPV) and positive likelihood ratio (PLR) were calculated by using Clinical Calculator 1 (http://vassarstats.net/clin1.html#return). The calculations were as follows: Sensitivity=A/(A+C) x100%, Specificity=D/(D+B) x100%, PPV=A/(A+B) x100%, NPV=D/(D+C) x100%, PLR= Sensitivity/(1-Specificity) x100%, where A indicates positivity according to LAMP and conventional culture, B positivity according to LAMP and negativity on conventional culture,

Table III. Sequences of primers used for amplification of LuxS and *bla*KPC.

Primer	Type/definition	Sequence (5'-3')
U5-FIP	Forward inner	GCTTTCCAGGCATCGGCAACGCACCGGTTTCTACATGAG
U5-BIP	Reverse inner	ATGGCCGACGTGCTGAAAGTACTGGTACTCGTTCAGCTCA
U5-F3	Forward outer	GGCGTGGAGATTATCGACAT
U5-B3	Reverse outer	CGAGTGCATGTGGTAGGTAC
U5-LF	Loop forward	TTCCGGCACGCCGATCA
U5-LB	Loop reverse	GACCGACCAGCGCAAGA
K17-FIP	Forward inner	CAGCACAGCGGCAGCAAGACTGAGGAGCGCTTCCCA
K17-BIP	Reverse inner	TCCGTTACGGCAAAAATGCGCTCGTCATGCCTGTTGTCAGAT
K17-F3	Forward outer	GGCGCAACTGTAAGTTACCG
K17-B3	Reverse outer	TCACTGTATTGCACGGCG
K17-LF	Loop forward	GCCCTTGAATGAGCTGCAC
K17-LB	Loop reverse	GGTTCCGTGGTCACCCA
LuxS-F	PCR forward	TTGATCTGCGCTTTTGCCGC
LuxS-R	PCR reverse	TTCACCACCACGCCGTTATC
KPC-F	PCR forward	CGGTCAGTCCGTTTGTTC
KPC-R	PCR reverse	CTTGGTCGGTCTGTAGGG
F27	16S rRNA PCR	AGAGTTTGATCCTGGCTCAG
R1492	16S rRNA PCR	ACGGCTACCTTGTTACGACTT

rRNA, ribosomal RNA; KPC, *Klebsiella pneumoniae* carbapenemase.

C negativity on LAMP and positivity on conventional culture and D negativity on LAMP and conventional culture.

Results

Optimal primers for the LAMP assay of Serratia spp. *with blaKPC*. A total of three sets of candidate primers were designed for the detection of *Serratia* spp. and *bla*KPC. Under the same reaction conditions, it was observed that the primer U5 set amplified the target sequence at 15, 18 and 21 min for *S. marcescens* DY12303, *S. liquefaciens* DY12165 and *S. rubidaea* DY12122, respectively. For the remaining two sets, either low efficiency or amplification failure was observed. Therefore, the U5 sets were chosen, which were able to amplify the target gene in the shortest time, as the optimal reaction primers for *Serratia* spp. identification (Fig. 1A). For *bla*KPC detection, primer K17 was confirmed as the fastest and most appropriate primer (Fig. 1B). Accordingly, the U5 and K17 primer sets (listed in Table III) were chosen as the optimal primers for the detection of *Serratia* spp. with *bla*KPC in the LAMP assay.

Optimal temperatures for the LAMP assay of Serratia spp. *with blaKPC*. To determine the most suitable reaction conditions for the selected primers in the LAMP reaction for the detection of *Serratia* spp. with *bla*KPC, several temperatures from 61 to 68˚C at 1˚ intervals were evaluated. As indicated in Fig. 2A and B, 64-67˚C was the most suitable temperature range. Based on these results, 65˚C was chosen as the reaction temperature.

Specificity of the LAMP assay for Serratia spp. *with blaKPC*. To estimate the specificity of the LAMP assay, *S. marcescens* DY12303 was used as the positive control and ddH_2O as the negative control. Furthermore, 18 non-*Serratia* spp. bacterial strains and 12 clinical isolates without *bla*KPC were tested in the specificity experiments. Fig. 3A and B indicate that an increase in the turbidity curve only occurred when DNA from *S. marcescens* DY12303 was used as the template. For the negative control (ddH_2O) and the other reference strains, no significant amplification was detected. These results suggested that the primers selected had excellent specificity.

Sensitivity of the LAMP assay for Serratia spp. *with blaKPC*. As indicated in Fig. 4A-1 and B-1, the detection limit of the LAMP assay for *Serratia* spp. and *bla*KPC was 3.92 pg/μl when monitored by a real-time turbidimeter recording system. In addition, a direct visual method was adopted to monitor the results of the LAMP assay for *Serratia* spp. and *bla*KPC. Prior to the LAMP reaction, 1 μl of calcein/Mn^{2+} complex was added to 24 μl of the prepared LAMP reaction mixture. All positive reactions turned green, whereas the negative reactions still remained orange (Fig. 4A-2 and B-2). It was concluded that these two detection methods had similar sensitivities. To compare sensitivities, conventional PCR was also performed by using *luxS* and *bla*KPC primers. The amount of template added in the conventional PCR was the same as that for the LAMP assay. It was observed that the detection limit of conventional PCR for luxS and KPC was 39.2 pg/μl (Fig. 4A-3 and B-3). Taken together, these results suggested that the LAMP assay was 10 times more sensitive than conventional PCR.

Inhibition analysis of clinical specimens. Pure genomic DNA extracted from the clinical isolate *S. marcescens* DY12303,

Table IV. Sensitivity and specificity of *Serratia* spp. with β lactamase *Klebsiella pneumoniae* carbapenemase in the loop-mediated isothermal amplification assay.

	Culture (n)						
Sample/result	Positive	Negative	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	PLR (%)
Sputum directly							
Positive	66	15	94.3 (85.3-98.2)	94.8 (91.4-97.0)	81.5 (71.0-88.9)	98.6 (96.1-99.5)	18.26 (11.1-29.8)
Negative	4	274					
Extracted DNA							
Positive	66	16	94.3 (85.3-98.2)	94.5 (91.0-96.7)	80.5 (70.0-88.1)	98.6 (96.1-99.5)	17.0 (10.5-27.5)
Negative	4	273					

Percentages are provided with the 95% confidence interval in brackets. PPV, positive predictive value; NPV, negative predictive value; PLR, positive likelihood ratio.

Figure 1. Primer screening of *Serratia* spp. harboring *bla*KPC. in the loop-mediated isothermal amplification assay. (A) Three sets of primers were used for luxS gene amplification under the same conditions: U3, U5 and U142. Samples: S.M, *S. marcescens* DY12303; S.L, *S. liquefaciens* DY12165; S.R, *S. rubidaea* DY12122. (B) Three sets of primers for *bla*KPC gene amplification in *S. marcescens* DY12303 under the same conditions: K9, K17 and K21. Turbidity was monitored by a real-time turbidimeter recording system at 650 nm, the recording frequency set at 6 sec. *bla*KPC, β lactamase *Klebsiella pneumoniae* carbapenemase.

which has *bla*KPC-2, was used as a positive control and seeded into DNA extracted from the blood and sputum of healthy donors and reaction curves were compared with those of samples from the pure culture. As indicated in Fig. 4A-1 and B-1, compared with the pure culture samples, the sensitivity of the luxS and KPC LAMP assay for the spiked samples was unchanged, at 1.96 pg/μl (Fig. 5A and B). Thus, it was concluded that the DNA extracted from the sputum and blood did not interfere with the LAMP assay.

Performance comparison of the LAMP assay, conventional culture and real-time PCR in detecting clinical specimens of Serratia spp. *with blaKPC*. In the present study, sputum specimens collected at the Henan Province Hospital of TCM (Zhengzhou, China) were used to examine the feasibility, sensitivity and specificity of the LAMP assay. The LAMP assay was compared with conventional culture and the results of real-time PCR kits. From January to December 2018, a total of 359 sputum specimens were collected. For conventional culturing combined with Taqman probe real-time PCR

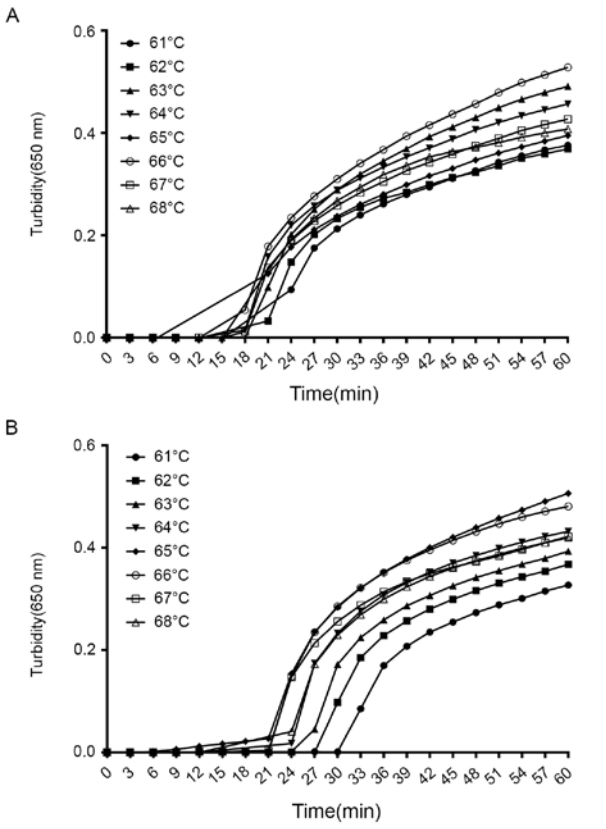

Figure 2. Optimal reaction temperature screening of the LAMP assay for *bla*KPC harbored in *Serratia* spp. (A) The LAMP reaction for *S. marcescens* identification at different temperatures. (B) Reaction curve of the LAMP reaction for *bla*KPC identification at different temperatures. Turbidity was monitored by a real-time turbidimeter recording system at 650 nm every 6 sec. LAMP, loop-mediated isothermal amplification; *bla*KPC, β lactamase *Klebsiella pneumoniae* carbapenemase.

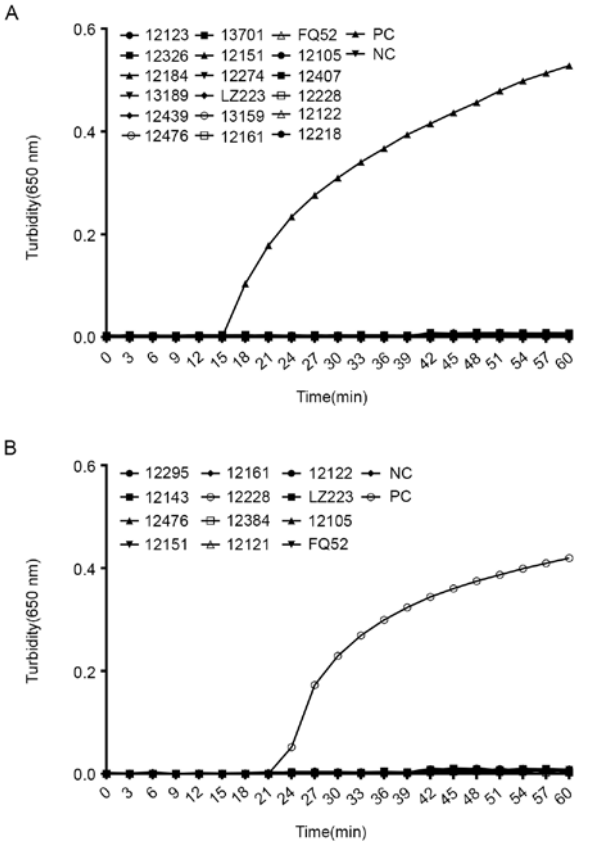

Figure 3. Specificity of the LAMP assay for *Serratia* spp. harboring *bla*KPC. (A) Specificity of the LAMP reaction for *S. marcescens* identification. Samples: PC, positive control (*S. marcescens* DY 12303); NC, negative control (double-distilled water); 12123, *E. coli* DY12123; 12326, *K. pneumoniae* DY12326; 12223, *A. baumannii* DY12223; 12184, *P. vulgaris* DY12184; 12122, *P. aeruginosa* DY12122; 12531, *S. sonnel* DY12531; 12189, *Y. pestis* DY12189; 12218, *C. freundii* DY12218; 12161, *S. typhi* DY12161; 12439, *M. morganii* DY12439; 12210, *P. rettgeri* DY12210; 12252, *A. baumannii* FY12252; 12476, *P. aeruginosa* DY12476; 12151, *K. oxytoca* DY12151; 12105, *E. cloacae* DY12105; 12228, *E. coli* DY12228; 12274, *K. pneumoniae* DY12274; 12407, *P. mirabilis* DY12407. (B) Specificity of the LAMP reaction for *bla*KPC detection. Samples: PC, positive control (*S. marcescens* DY 12303); NC, negative control (double-distilled water); 12295, *E. coli* DY12295; 12521, *B. cepacia* DY12521; 12384, *K. pneumoniae* DY12384; 12223, *A. baumannii* DY12223; 12143, *S. maltophilia* DY12143; 12161, *S. typhi* DY12161; 12121, *E. aerogene*s DY12121; 12315, *E. cloacae* DY12315; 12476, *P. aeruginosa* DY12476; 12228, *E. coli* DY12228; 12122, *P. aeruginosa* DY12122; 12252, *A. baumannii* FY12252. Turbidity was monitored by a Loopamp real-time turbidimeter at 650 nm every 6 sec. Amplification was performed at 65˚C for 60 min. LAMP, loop-mediated isothermal amplification; *bla*KPC, β lactamase *Klebsiella pneumoniae* carbapenemase.

for *bla*KPC, 70 positive specimens of *bla*KPC producing *Serratia* spp. were detected. For the LAMP assay, with non-purified DNA (boiling method) from sputum specimens as templates, 62 positive specimens of *bla*KPC producing *Serratia* spp. were detected. The sensitivity and specificity of the LAMP assay were 94.3 and 94.8%, respectively. However, when the same templates were used for real-time PCR directly, no amplification products were detected due to the existence of interfering substances in the sputum. When purified DNA extracted from the sputum specimens was used as a template for the LAMP assay, the sensitivity and specificity was 94.3 and 94.5%, respectively. When the same templates were used in a real-time PCR assay, the sensitivity was 94.3% and the specificity was 96.0%, which was similar to those of the LAMP assay. The results of the LAMP assay are presented in Table IV. The present results revealed that the LAMP reaction had excellent sensitivity and specificity, and was less susceptible to interfering substances in clinical specimens than the real-time PCR method.

Discussion

Serratia spp. are the smallest gram-negative bacilli in the environment and belong to the *Enterobacteriaceae* family. In the clinical setting, the most commonly encountered species are *S. marcescens* and *S. liquefaciens*. *S. marcescens* is generally considered as an innocuous, non-pathogenic, environmental organism. As its red colonies are easily recognized by the naked eye, it is frequently used as a popular biological marker (31). However, this organism has recently been thought to be an important opportunistic pathogen that may lead to various types of infectious disease in vulnerable populations, such as patients at ICUs and hematopathy departments. Nosocomial infections caused by *S. marcescens* have reached 1-2%. Infections may take place in any part of the body, with the respiratory tract and the blood stream being the most common (32). Furthermore, in pediatric patients, *S. marcescens may give rise to* meningitis according to case

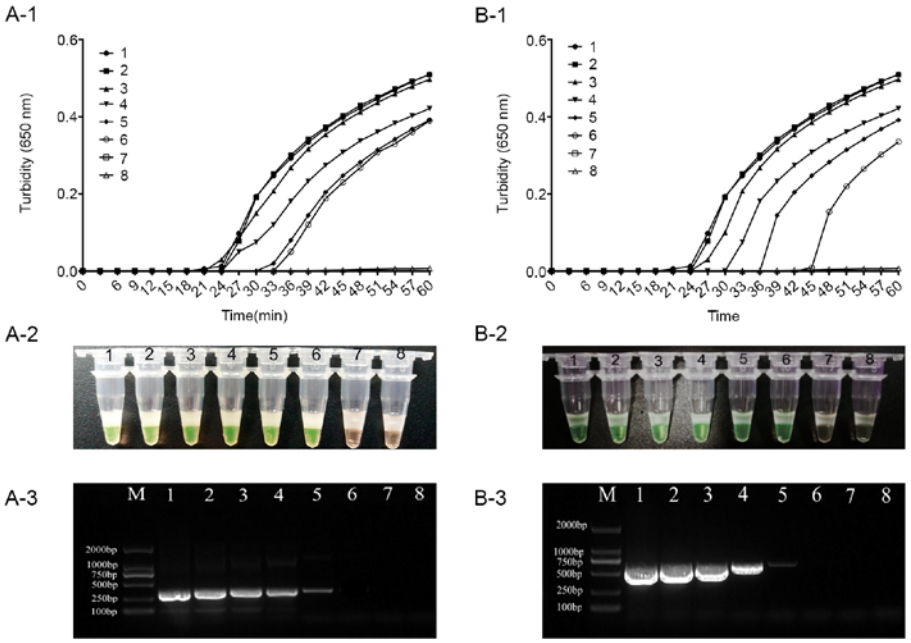

Figure 4. Sensitivity comparison between the LAMP assay and conventional PCR for *Serratia* spp. harboring *bla*KPC. The pure genomic DNA extracted from *S. marcescens* DY12303 was diluted in a serial 10-fold dilution. Both LAMP reactions and PCRs were carried out in duplicate for each dilution point. Tubes and lanes: 1, 392.0 ng/μl; 2, 39.2 ng/μl; 3, 3.92 ng/μl; 4, 392.0 pg/μl; 5, 39.2 pg/μl; 6, 3.92 pg/μl; 7, 0.392 pg/μl; 8, negative control (double-distilled H_2O). (A-1 and B-1) The turbidity of the LAMP assays for *S. marcescens* (A-1) and *bla*KPC (B-1) were monitored by a Loopamp real-time turbidimeter at 650 nm every 6 sec. (A-2 and B-2) 1 μl of fluorescent detection reagent was added to 25 μl of LAMP reaction mixture prior to the LAMP reaction. In positive reactions, the color changed from orange to green. Otherwise, there was no color change. (A-3 and B-3) The PCR products were analyzed by 1% agarose gel electrophoresis and stained with GelRed. LAMP, loop-mediated isothermal amplification; *bla*KPC, β lactamase *Klebsiella pneumoniae* carbapenemase; M, marker.

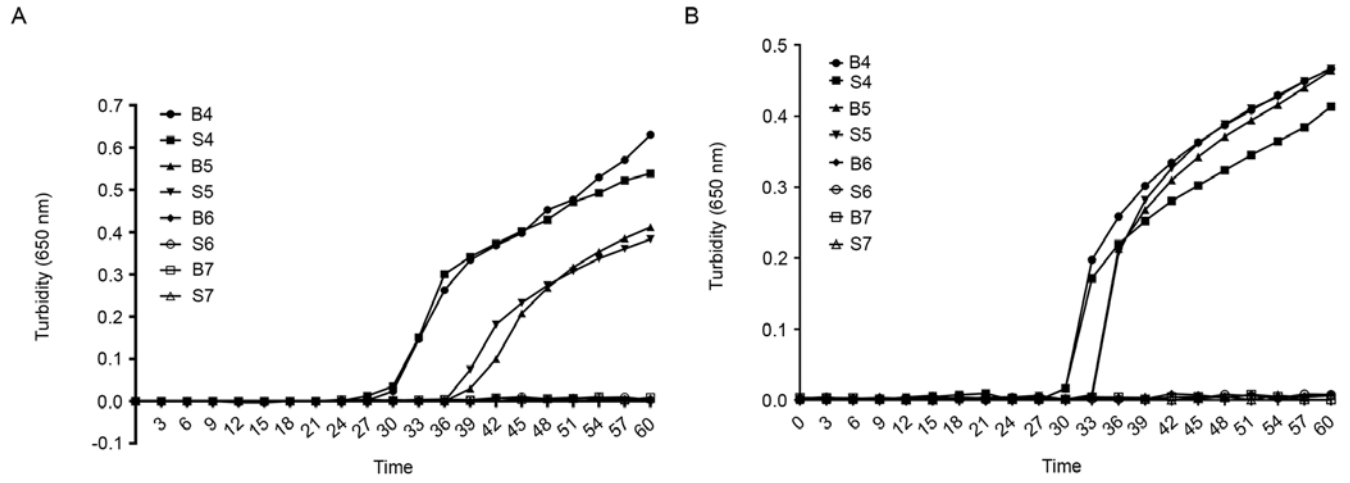

Figure 5. Detection of *Serratia* spp. harboring *bla*KPC in spiked blood specimens and sputum specimens. (A) Detection of the luxS gene in spiked human blood specimens and sputum specimens by a Loopamp real-time turbidimeter at 650 nm every 6 sec. (B) Detection of the *bla*KPC gene in spiked human blood specimens and sputum specimens by a Loopamp real-time turbidimeter at 650 nm every 6 sec. Samples: B4-B7, concentration of genomic DNA in human blood extract of 19.6, 1.96, 0.196 and 0.0196 pg/μl, respectively; S4-S7, concentration of genomic DNA in sputum extract of 19.6, 1.96, 0.196 and 0.0196 pg/μl, respectively. *bla*KPC, β lactamase *Klebsiella pneumoniae* carbapenemase.

reports (33). *S. marcescens* infection may also cause endocarditis and osteomyelitis in patients with heroin addiction (34).

Serratia spp.-associated BSI usually occurs due to contaminated intravenous solutions and is a life-threatening condition that must be diagnosed and treated as soon as possible (35). In recent decades, routine BSI diagnosis has included continuous monitoring of the blood culture by automated systems, subcultivating on an appropriate solid medium and bacterial identification using commercial kits, requiring ~72 h. Although the detection time has been shortened by the introduction of mass spectrometry as an alternative method for bacterial identification directly from positive blood cultures, the specialized equipment and high cost of this method restrict its use in general hospitals and health care units (36). Furthermore, this method cannot be used to detect resistance markers, such as *bla*KPC, in *Serratia* spp. Therefore, the development of a convenient, rapid, sensitive detection method for *Serratia* spp. with *bla*KPC is required

for the diagnosis and treatment of *Serratia* spp.-associated BSI.

In our experience, lassical culturing used to detect *Serratia spp* requires >48 h to identify the target pathogen and antibiotic spectrum. Furthermore, the sensitivity of the culture method is insufficient for the detection of *Serratia* spp. with *bla*KPC. To the best of our knowledge, a method based on LAMP assays for detecting *bla*KPC harboring *Serratia* spp. has not been previously reported. Compared with conventional bacterial culture and identification methods, LAMP assays may proceed at a constant temperature and the time required is only 1 h, saving time and effort. Furthermore, heparin, a commonly used anticoagulant in serum and plasma, which is well-known as an inhibitory substance for conventional PCR, does not affect LAMP assays (37). It's also possible for LAMP assays to detect a pathogen directly from biological fluids, such as blood, pleuroperitoneal fluids and cerebrospinal fluid (38).

After consideration of the epidemiology of *Serratia* spp. and the advantages of the LAMP assay, the present study aimed to design primers specifically for *Serratia* spp. with the most prevalent carbapenemase resistance gene, *bla*KPC, for use in a LAMP assay. The LAMP assay allows clinical specimens to be screened more easily and rapidly than existing techniques.

The sensitivity analysis suggested that the detection limit of the LAMP method reached 3.92 pg/μl for *Serratia* spp. with *bla*KPC. A sensitivity comparison with conventional PCR revealed that the LAMP was 10 times more sensitive than conventional PCR. In the specificity analysis, the *luxS* gene from *Serratia* spp. (~516 bp) was selected as the target sequence for *Serratia* spp. identification. It was indicated that *Serratia* spp. was able to be detected within 30 min and 19 non-*Serratia* spp. family bacterial strains tested negative, demonstrating good specificity. Furthermore, 12 bacterial strains without *bla*KPC also produced negative results. In summary, the *bla*KPC primers designed in the present study were able to detect all subtypes of *bla*KPC.

It was previously demonstrated that for the LAMP assay, it is unnecessary to purify the DNA from clinical specimens (39). However, exogenous DNA and inhibitors may markedly reduce the sensitivity of PCR. To assess the anti-interference performance of the LAMP method, DNA was extracted from the sputum and blood of healthy donors and spiked specimens were prepared using genomic DNA extracted from the DY12303 bacterial strain and extracts from sputum and blood. The results indicated that the limit of bacterial detection in the LAMP assay with spiked specimens was similar to that for the pure culture.

To investigate the adaptability of the LAMP assay for clinical specimens, 359 sputum specimens were collected and the DNA was extracted with a commercial DNA extraction kit and the boiling method. The detection results for the LAMP assay and real-time PCR were compared with conventional culture methods. The results indicated that the detection efficiency of the LAMP assay was almost the same as that of the conventional culture method and was not affected by the DNA extraction method. For the real-time PCR, the detection efficiency was the same as for the conventional culture method when purified DNA from sputum was used as templates, but no amplification products were detected when non-purified DNA extracted by the boiling method was used as the template. From these results, it was concluded that the LAMP assay had good sensitivity, specificity and efficiency compared with conventional culture and real-time PCR methods. Furthermore, in the present study, the fluorescent reagent (calcein/Mn^{2+} complex) that was added to the reaction mixture made the detection results more visual and easier to determine. The advantages of the LAMP assay suggested that this method was more suitable than conventional molecular methods, such as regular PCR and real-time PCR, for the rapid detection of *bla*KPC-producing *Serratia* spp. in clinical specimens.

In the LAMP reaction, it is not necessary for all of the primers to link with the target sequence. FIP and BIP primers are of great importance and if these primers bind to the target sequence, the signal is positive. However, further studies are required to confirm this assumption (40). Although the amplification principle of the LAMP method is complex, the performance of this method is more feasible and efficient than that of other methods. The operation of the assay may be accomplished under isothermal conditions, which may be achieved in a primary laboratory. Due to its favorable characteristics, this LAMP assay may be adopted by inspection and quarantine departments to perform site inspections for *bla*KPC-producing *Serratia* spp. In ICUs of hospitals, the LAMP assay may be used as a means of point-of-care testing to detect resistant bacteria and guide antibiotic usage.

In summary, a detection method based on a LAMP assay for *bla*KPC-producing *Serratia* spp. was successfully established. The sensitivity, specificity and feasibility were indicated to be superior to conventional culture methods and molecular biotechnology methods. The most significant feature of the LAMP assay presented in the present study is that it is easy for the staff of a primary laboratory to master. Thus, this assay may have a positive impact in primary hospitals, disease control and prevention departments, as well as inspection and quarantine departments. Furthermore, the present study offers a novel perspective on the infectious characteristics of *Serratia* spp. and their resistance mechanisms, leading to a greater understanding of antibiotic screening for the treatment of critical care patients.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YL and WL conceived the study. XL, DZ, and CW performed the experiments. XZ and DP collected the clinical strains and performed the data analysis. DP and WL wrote the manuscript.

YL and XL confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The procedures in the present study were approved and documented by the Ethics Committee of Henan Province Hospital of TCM (Zhengzhou, China). All research complied with the declaration of Helsinki and the relevant rules of the Ethics Committee. Informed consent for participation in the study was obtained from all participants (or their parent or legal guardian).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Ogtrop ML, van Zoeren-Grobben D, Verbakel-Salomons EM and van Boven CP: *Serratia marcescens* infections in neonatal departments: Description of an outbreak and review of the literature. J Hosp Infect 36: 95-103, 1997.
2. Manning ML, Archibald LK, Bell LM, Banerjee SN and Jarvis WR: *Serratia marcescens* transmission in a pediatric intensive care unit: A multifactorial occurrence. Am J Infect Control 29: 115-119, 2001.
3. Dessì A, Puddu M, Testa M, Marcialis MA, Pintus MC and Fanos V: *Serratia marcescens* infections and outbreaks in neonatal intensive care units. J Chemother 21: 493-499, 2009.
4. Hsieh S and Babl FE: *Serratia marcescens* cellulitis following an iguana bite. Clin Infet Dis 28: 1181-1182, 1999.
5. Chaidaroon W and Supalaset S: Corneal ring infiltrates caused by *Serratia marcescens* in a patient with human immunodeficiency virus. Case Rep Ophthalmol 7: 359-363, 2016.
6. Gastmeier P: *Serratia marcescens*: An outbreak experience. Front Microbiol 5: 81, 2014.
7. Knowles S, Herra C, Devitt E, O'Brien A, Mulvihill E, McCann SR, Browne P, Kennedy MJ and Keane CT: An outbreak of multiply resistant *Serratia marcescens*: The importance of persistent carriage. Bone Marrow Transplant 25: 873-877, 2000.
8. Gupta N, Hocevar SN, Moulton-Meissner HA, Stevens KM, McIntyre MG, Jensen B, Kuhar DT, Noble-Wang JA, Schnatz RG, Becker SC, *et al*: Outbreak of *Serratia marcescens* bloodstream infections in patients receiving parenteral nutrition prepared by a compounding pharmacy. Clin Infect Dis 59: 1-8, 2014.
9. Sullivan KV, Deburger B, Roundtree SS, Ventrola CA, Blecker-Shelly DL and Mortensen JE: Pediatric multicenter evaluation of the Verigene gram-negative blood culture test for rapid detection of inpatient bacteremia involving gram-negative organisms, extended-spectrum beta-lactamases, and carbapenemases. J Clin Microbiol 52: 2416-2421, 2014.
10. Tojo M, Fujita T, Ainoda Y, Nagamatsu M, Hayakawa K, Mezaki K, Sakurai A, Masui Y, Yazaki H, Takahashi H, *et al:* Evaluation of an automated rapid diagnostic assay for detection of Gram-negative bacteria and their drug-resistance genes in positive blood cultures. PLoS One 9: e94064, 2014.
11. Nasri E, Subirats J, Sànchez-Melsió A, Mansour HB, Borrego CM and Balcázar JL: Abundance of carbapenemase genes (bla_{KPC}, bla_{NDM} and bla_{OXA-48}) in wastewater effluents from Tunisian hospitals. Environ Pollut 229: 371-374, 2017.
12. Srisrattakarn A, Lulitanond A, Wilailuckana C, Charoensri N, Wonglakorn L, Saeniamla P, Daduang J and Chanawong A: Rapid and simple identification of carbapenemase genes, bla_{NDM}, bla_{OXA-48}, bla VIM, bla_{IMP-14} and bla_{KPC} groups, in Gram-negative bacilli by in-house loop-mediated isothermal amplification with hydroxynaphthol blue dye. World J Microbiol Biotechnol 33: 130, 2017.
13. Hopkins KL, Findlay J, Meunier D, Cummins M, Curtis S, Kustos I, Mustafa N, Perry C, Pike R and Woodford N: *Serratia marcescens* producing SME carbapenemases: An emerging resistance problem in the UK? J Antimicrob Chemother 72: 1535-1537, 2017.
14. Pollett S, Miller S, Hindler J, Uslan D, Carvalho M and Humphries RM: Phenotypic and molecular characteristics of carbapenem-resistant *Enterobacteriaceae* in a health care system in Los Angeles, California, from 2011 to 2013. J Clin Microbiol 52: 4003-4009, 2014.
15. Wachino J, Yoshida H, Yamane K, Suzuki S, Matsui M, Yamaqishi T, Tsutsui A, Konda T, Shibayama K and Arakawa Y: SMB-1, a novel subclass B3 metallo-beta-lactamase, associated with ISCR1 and a class 1 integron, from a carbapenem-resistant *Serratia marcescens* clinical isolate. Antimicrob Agents Chemother 55: 5143-5149, 2011.
16. de Vries JJ, Baas WH, van der Ploeg K, Heesink A, Degener JE and Arends JP: Outbreak of *Serratia marcescens* colonization and infection traced to a healthcare worker with long-term carriage on the hands. Infect Control Hosp Epidemiol 27: 1153-1158, 2006.
17. Nodari CS, Siebert M, Matte UDS and Barth AL: Draft genome sequence of a GES-5-producing *Serratia marcescens* isolated in southern Brazil. Braz J Microbiol 48: 191-192, 2017.
18. Wendel AF, Kaase M, Autenrieth IB, Peter S, Oberhettinger P, Rieber H, Pfeffer K, Mackenzie CR and Willmann M: Protracted regional dissemination of GIM-1-producing *Serratia marcescens* in western germany. Antimicrob Agents Chemother 61: e01880-16, 2017.
19. Mahlen SD: Serratia infections: From military experiments to current practice. Clin Microbiol Rev 24: 755-791, 2011.
20. Wong YP, Othman S, Lau YL, Son R and Chee HY: Loop mediated isothermal amplification (LAMP): A versatile technique for detection of microorganisms. J Appl Microbiol 124: 626-643, 2017.
21. Sakai J, Maeda T, Tarumoto N, Misawa K, Tamura S, Imai K, Yamaguchi T, Iwata S, Murakami T and Maesaki S: A novel detection procedure for mutations in the 23S rRNA gene of *Mycoplasma pneumoniae* with peptide nucleic acid-mediated loop-mediated isothermal amplification assay. J Microbiol Methods 141: 90-96, 2017.
22. Mashooq M, Kumar D, Niranjan AK, Agarwal RK and Rathore R: Development and evaluation of probe based real time loop mediated isothermal amplification for Salmonella: A new tool for DNA quantification. J Microbiol Methods 126: 24-29, 2016.
23. Lin M, Liu W, Wang P, Tan J, Zhou Y, Wu P, Zhang T, Yuan J and Chen Y: Rapid detection of ermB gene in *Clostridium difficile* by loop-mediated isothermal amplification. J Med Microbiol 64: 854-861, 2015.
24. Cai JC, Zhou HW, Zhang R and Chen GX: Emergence of *Serratia marcescens*, *Klebsiella pneumoniae*, and *Escherichia coli* Isolates possessing the plasmid-mediated carbapenem-hydrolyzing beta-lactamase KPC-2 in intensive care units of a Chinese hospital. Antimicrob Agents Chemother 52: 2014-2018, 2008.
25. Lin X, Hu Q, Zhang R, Hu Y, Xu X and Lv H: Emergence of *Serratia marcescens* isolates possessing Carbapenem-hydrolysing β-lactamase KPC-2 from China. J Hosp Infect 94: 65-67, 2016.
26. Zhou T, Zhang X, Guo M, Ye J, Lu Y, Bao Q and Chi W: Phenotypic and molecular characteristics of carbapenem-non-susceptible Enterobacteriaceae from a teaching hospital in Wenzhou, southern China. Jpn J Infect Dis 66: 96-102, 2013.
27. Zhang R, Liu L, Zhou H, Chan EW, Li J, Fang Y, Li Y, Liao K and Chen S: Nationwide surveillance of clinical carbapenem-resistant Enterobacteriaceae (CRE) strains in China. EBioMedicine 19: 98-106, 2017.
28. Li B, Feng J, Zhan Z, Yin Z, Jiang Q, Wei P, Chen X, Gao B, Hou J, Mao P, *et al*: Dissemination of KPC-2-encoding IncX6 plasmids among multiple *Enterobacteriaceae* Species in a Single Chinese Hospital. Front. Microbiol 9: 478, 2018.
29. Thompson JD, Higgins DG and Gibson TJ: CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673-4680, 1994.
30. Garrity G, Staley JT, Boone DR, De Vos P, Goodfellow M and Rainey FA: Bergey's manual® of systematic bacteriology. In: Vol 2 The Proteobacteria, Part B: The Gammaproteobacteria. Springer, New York, NY, 2006.

31. Joyner J, Wanless D, Sinigalliano CD and Lipp EK: Use of quantitative real-time PCR for direct detection of *Serratia marcescens* in marine and other aquatic environments. Appl Environ Microbiol 80: 1679-1683, 2014.
32. Ferrer R, Martin-Loeches I, Phillips G, Osborn TM, Townsend S, Dellinger RP, Artiqas A, Schorr C and Levy MM: Empiric antibiotic treatment reduces mortality in severe sepsis and septic shock from the first hour: Results from a guideline-based performance improvement program. Crit Care Med 42: 1749-1755, 2014.
33. Khanna A, Khanna M and Aggarwal A: *Serratia marcescens*-a rare opportunistic nosocomial pathogen and measures to limit its spread in hospitalized patients. J Clin Diagn Res 7: 243-246, 2013.
34. Farhoudi HO, Banks T and Ali N: A case report of serratia endocarditis with review of the literature. Med Ann Dist Columbia 43: 401-406, 1974.
35. Su JR, Blossom DB, Chung W, Gullion JS, Pascoe N, Heseltine G and Srinivasan A: Epidemiologic investigation of a 2007 outbreak of *Serratia marcescens* bloodstream infection in Texas caused by contamination of syringes prefilled with heparin and saline. Infect Control Hosp Epidemiol 30: 593-595, 2009.
36. Chemaly RF, Rathod DB, Sikka MK, Hayden MK, Hutchins M, Horn T, Tarrand J, Adachi J, Nguyen K, Trenholme G and Raad I: *Serratia marcescens* bacteremia because of contaminated prefilled heparin and saline syringes: a multi-state report. Am J Infect Control 39: 521-524, 2011.
37. Enosawa M, Kageyama S, Sawai K, Watanabe K, Notomi T, Onoe S, Mori Y and Yokomizo Y: Use of loop-mediated isothermal amplification of the IS900 sequence for rapid detection of cultured Mycobacterium avium subsp. paratuberculosis. J Clin Microbiol 41: 4359-4365, 2003.
38. Dhama K, Karthik K, Chakraborty S, Tiwari R, Kapoor S, Kuamr A and Thomas P: Loop-mediated isothermal amplification of DNA (LAMP): A new diagnostic tool lights the world of diagnosis of animal and human pathogens: A review. Pak J Biol Sci 17: 151-166, 2014.
39. Notomi T, Mori Y, Tomtia N and Kanda H: Loop-mediated isothermal amplification (LAMP): Principle, features, and future prospects. J Microbiol 53: 1-5, 2015.
40. Lin WH, Zou BJ, Song QX and Zhou GH: Progress in multiplex loop-mediated isothermal amplification technology. Yi Chuan 37: 899-910, 2015.