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Sensitive and Rapid Detection of the New Delhi Metallo-Beta-Lactamase Gene by Loop-Mediated Isothermal Amplification

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New Delhi metallo-β-lactamase 1 (NDM-1), which is associated with resistance to carbapenem, was first reported in 2008. A sensitive and rapid molecular assay to detect the plasmid *bla*$_{NDM-1}$ in clinical isolates is needed to control its spread. We describe a loop-mediated isothermal amplification (LAMP) assay for the rapid detection of *bla*$_{NDM-1}$ from pure culture and sputum, urine, and fecal samples. Eight sets of primers were designed to recognize six or eight distinct sequences on target *bla*$_{NDM-1}$, and one set was selected as the most appropriate set of primers for its rapid detection. The specificity and sensitivity of the primers in the LAMP reactions for *bla*$_{NDM-1}$ detection were determined. The sensitivity of the LAMP assay for *bla*$_{NDM-1}$ detection in sputum, urine, and fecal samples was also tested. Two methods, namely, monitoring of turbidity and addition of calcein to the reaction tube, were used to determine negative and positive results. The results showed that target DNA was amplified and visualized by the two detection methods within 70 min at an isothermal temperature of 65°C. The sensitivity of LAMP, with a detection limit of 10.70 pg/μl DNA, was 100-fold greater than that of PCR. Thirteen infection bacterial strains without *bla*$_{NDM-1}$ were selected for testing of specificity, and the results of the amplification were negative, which showed that the primers had good levels of specificity. The LAMP method reported here is demonstrated to be a potentially valuable means for the detection of *bla*$_{NDM-1}$ and rapid clinical diagnosis, being fast, simple, and low in cost.

Pathogens carrying the plasmid *bla*$_{NDM-1}$ are known for their high rates of resistance to carbapenems and were first reported in August 2010. Kumarasamy et al. identified 44 isolates carrying New Delhi metallo-β-lactamase 1 (NDM-1) in Chennai, India; 26 in Haryana, India; 37 in the United Kingdom; and 73 in other sites in India and Pakistan (14). NDM-1 was mostly found among *Escherichia coli* and *Klebsiella pneumoniae* strains (6, 18) which were highly resistant to all antibiotics except tigecycline and colistin. NDM-1 was reported worldwide within 6 months (1, 25, 30; European Antimicrobial Resistance Surveillance Network database [http://ecdc.europa.eu/en/activities/surveillance/EARS-Net/Pages/Database.aspx]). Bacteria with resistance to carbapenems conferred by *bla*$_{NDM-1}$ are potentially a major global health problem. The WHO has urged countries to implement infection control measures in hospitals to limit the spread of multidrug-resistant strains and to reinforce national policies on the prudent use of antibiotics (32). Thus, a sensitive and rapid method for detection of *bla*$_{NDM-1}$ is needed to prevent its spread. We isolated *Acinetobacter baumannii* XM with *bla*$_{NDM-1}$ for the first time in 2010 from an advanced lung cancer patient and used this strain in the present study.

Recently, real-time PCR assays for the rapid detection of *bla*$_{NDM-1}$ have been reported (13). While these assays for rapid, sensitive, and specific detection appear to be promising, PCR requires specialized high-cost instruments and consumables. In addition, *Taq* DNA polymerase in PCR assays can be inactivated by inhibitors present in crude biological samples (5). Thus, another rapid, simple, and cost-effective assay is needed to complement current PCR methods. The recently developed loop-mediated isothermal amplification (LAMP) method requires only a temperature-controlled water bath. It is based on autocycling strand displacement DNA synthesis in the presence of *Bst* DNA polymerase under isothermal conditions within 1 h (19, 29). The LAMP method was evaluated and optimized for *bla*$_{NDM-1}$ detection. Because four or six specific primers that recognize six or eight different sequences on the DNA target are used, LAMP amplifies DNA with a high specificity; and LAMP technology has been widely used in clinical diagnosis (6, 9, 21); qualitative and quantitative detection of epidemic bacteria (7, 20, 28, 34), viruses (10, 27, 31), and parasites (3, 12); as well as in fetal sex identification (8), among other applications. In the study described in this communication, we first designed eight sets of primers and optimized the LAMP assay for detection of *bla*$_{NDM-1}$. Second, the specificity and sensitivity of the primers in the LAMP reactions for *bla*$_{NDM-1}$ detection were determined. Finally, the sensitive LAMP assay for *bla*$_{NDM-1}$ detection in sputum, urine, and fecal samples was tested.

MATERIALS AND METHODS

Bacterial strains and preparation of templates. A total of 14 bacterial strains were used in this study, and their sources are listed in Table 1. Isolate *A. baumannii* XM was from an advanced lung cancer patient in Fujian Province and was responsible for China's first NDM-1 infection reported by the Academy of Military Medical Sciences. *bla*$_{NDM-1}$ was validated by PCR-based sequencing, and the sequence of the *bla*$_{NDM-1}$ gene showed 100% identity with the sequences of previously reported genes. *bla*$_{NDM-1}$ was located on the chromosome by analysis of the pulsed-field gel electrophoresis (PFGE) profile and Southern blot hybridization (data

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TABLE 1 Bacterial strains used in this study

Species	Source
Acinetobacter baumannii XM (with bla_{NDM-1})	Clinical isolate
A. baumannii H949	Our microorganism center
A. baumannii F398	Our microorganism center
A. baumannii B260	Our microorganism center
A. baumannii H18	Our microorganism center
Shigella sonnei 2531	Our microorganism center
S. flexneri 4536	Our microorganism center
Salmonella enteritidis 50326-1	Our microorganism center
Vibrio carchariae 5732	Our microorganism center
V. parahaemolyticus 5474	Our microorganism center
S. enterica serotype Paratyphi 86423	Our microorganism center
Enteroinvasive *E. coli* 44825	Our microorganism center
Enterotoxigenic *E. coli* 44824	Our microorganism center
Enteropathogenic *E. coli* 2348	Our microorganism center

not shown). These bacteria were cultured at 37°C in brain heart infusion (BHI) broth according to a standard protocol. The Chelex method was used to extract the bacterial whole genomic DNA (including plasmid; the same method was used for the assay described below) as follows: the bacterial pellet, in 200 μl phosphate-buffered saline (PBS), was mixed with an equal volume of Chelex DNA extraction buffer (25 mM NaOH, 10 mM Tris-HCl, 1% Triton X-100, 1% NP-40, 0.1 mM EDTA, 2% Chelex-100). The mixture was incubated at 100°C for 10 min and then immediately placed on ice. Finally, the mixture was centrifuged at 14,000 × *g* for 2 min. The supernatant was used as the template in both the LAMP assay and PCRs (17). In order to estimate the sensitivity and specificity of the LAMP assay under real conditions, pure genomic DNA was extracted from *A. baumannii* XM using a Wizard genomic DNA purification kit from Promega. The genomic DNA was then prepared by serial 10-fold dilutions to give concentrations ranging from 1,070 ng/μl to 0.107 pg/μl.

Preparation of pure culture. A 200-μl sample from an overnight culture of bacteria was subjected to centrifugation at 8,000 rpm for 10 min, and the supernatant was discarded. The bacterial cells were resuspended in 200 μl PBS, and then the Chelex method (discussed above) was used to extract the bacterial genomic DNA.

Preparation of sputum samples, urine samples, and stool samples. Sputum samples, urine samples, and stool samples were collected from healthy donors. For sputum and urine samples, a 200-μl sample was extracted directly with the Chelex method. For stool samples, 100 mg of the fecal sample was suspended in 0.9 ml distilled water by vigorous shaking for 5 min. After 7 min, precipitation took place and 200 μl of supernatant was mixed with an equal volume of Chelex DNA extraction buffer to prepare the templates. Then, the pure genomic DNA extracted from *A. baumannii* XM was placed into the different sample templates and concentrations of pure genomic DNA were made up as follows: 107.0 ng/μl, 10.70 ng/μl, 1.070 ng/μl, 107.0 pg/μl, 10.70 pg/μl, 1.070 pg/μl, 0.107 pg/μl, and 0.010 pg/μl.

Primer design. To design bla_{NDM-1}-specific LAMP primers, the sequence of bla_{NDM-1} with accession number FN396876 was downloaded from the NCBI GenBank database (35). The sequence was further analyzed by Primer Explorer (version 4) software (http:/primerexplorer.jp/lamp), and the outer forward primer (F3), outer backward primer (B3), forward inner primer (FIP), and backward inner primer (BIP) (19) were designed. An additional two loop primers (loop F and loop B) were designed to accelerate the amplification reaction. To obtain the most appropriate primers, we designed eight sets of primers. The FIP and BIP primers recognize both sense and antisense strands and were linked by a four-thymidine spacer (TTTT). To compare the sensitivity and specificity of PCR, normal PCR was performed with the primers named NDM1-F and NDM1-R. The primers were synthesized commercially (Beijing AUGCT DNA-SYN Biotechnology Co., Ltd., Beijing, China).

LAMP reaction. The LAMP reactions were carried out in 25-μl reaction mixtures (DNA amplification kit; Eiken Chemical Co., Ltd., Tochigi, Japan) containing the following reagents (final concentrations): 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.8 M betaine, 8 mM $MgSO_4$, 1.4 mM each deoxynucleoside triphosphate, and 8 U *Bst* DNA polymerase. The amount of primer needed for one reaction was 40 pmol for FIP and BIP, 20 pmol for LF and LB, and 5 pmol for F3 and B3. Finally, an appropriate amount of template genomic DNA was added to the reaction tube. The reaction was carried out in the reaction tube (Reaction Tube; Eiken Chemical Co., Ltd., Tochigi, Japan) at 65°C for 50 min and inactivated at 80°C for 5 min in dry bath incubators.

Detection of LAMP products. Two different methods were used to detect LAMP products. For direct visual inspection, 1 μl of calcein (fluorescent detection reagent; Eiken Chemical Co., Ltd., Tochigi, Japan) was added to 25 μl of LAMP products before the LAMP reaction. For a positive reaction, the color changed from orange to green, while a negative reaction failed to turn green and remained orange. The color change could be observed by the naked eye under natural light or with the aid of UV light at 365 nm. For monitoring of turbidity (15), real-time amplification by the LAMP assay was monitored through spectrophotometric analysis by recording the optical density at 400 nm every 6 s with the help of a Loopamp real-time turbidimeter (LA-230; Eiken Chemical Co., Ltd., Tochigi, Japan).

PCR detection. PCRs were carried out with 25-μl reaction mixtures containing 12.5 μl PCR master mix reagents (Tiangen Biotech Co., Ltd., Beijing, China), 1 μmol/liter NDM1-F and NDM1-R primers, and the same amount of DNA template used in the LAMP reaction. The reaction was initially carried out at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The final extension step was carried out at 72°C for 10 min. The PCR-amplified products were analyzed by 2% agarose gel (Amresco) electrophoresis and stained with ethidium bromide. Images were documented by a Bio-Rad Gel Doc EQ imaging system.

RESULTS

The most appropriate primers for rapid detection of bla_{NDM-1}. We designed eight sets of primers for detection of bla_{NDM-1}. Under the same reaction conditions, we observed that four turbidity curves occurred after reaction for 15 min, that four of eight sets amplified the target sequence, and that the primers in the CJXJ1 set, which could amplify the target gene in the shortest time, were the fastest and the most optimal reaction primers (Fig. 1). Our data show that the use of loop primers shortened the amplification time by approximately one-third, which agreed with reported data (16). The primers in the CJXJ1 primer set (Table 2) were chosen as the final primers for bla_{NDM-1} detection by LAMP.

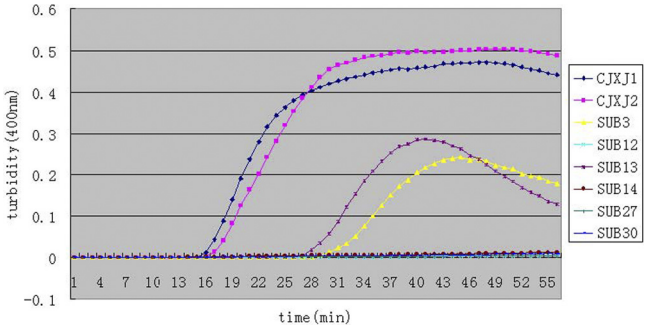

FIG 1 Eight sets of primers amplified the target gene under the same conditions. Turbidity was monitored by a Loopamp real-time turbidimeter at 400 nm every 6 s. Assays with the CJXJ1 and CJXJ2 primer sets were performed with Loop primers; assays with the other sets were performed without Loop primers.

TABLE 2 Sequence of primers used for specific amplification of bla_{NDM-1}

Primer	Type	Sequence (5′–3′)
CJXJ1F3	Forward outer	GCATAAGTCGCAATCCCCG
CJXJ1B3	Backward outer	GGTTTGATCGTCAGGGATGG
CJXJ1FIP	Forward inner	CTGGCGGTGGTGACTCACGTTTTG CATGCAGCGCGTCCA
CJXJ1BIP	Backward inner	CGCGACCGGCAGGTTGATCTTTTG GTCGATACCGCCTGGAC
CJXJ1LF1	Loop forward	GCATCAGGACAAGATGGGC
CJXJ1LB1	Loop backward	TCCAGTTGAGGATCTGGGC
NDM1-F	PCR forward	CAGCACACTTCCTATCTC
NDM1-R	PCR backward	CCGCAACCATCCCCTCTT

Temperature of bla_{NDM-1} LAMP reaction. To optimize the reaction conditions of the primers for bla_{NDM-1} in the LAMP reaction, we observed different temperatures from 55°C to 69°C at 2°C intervals. As shown in Fig. 2, 63°C to 67°C is the most suitable reaction temperature range. Finally, we chose 65°C as the reaction temperature.

Specificity of NDM-1 LAMP. To test the specificity of LAMP for bla_{NDM-1}, we used *A. baumannii* XM with bla_{NDM-1} as the positive strain and distilled water as the negative control. Thirteen infectious bacterial strains without bla_{NDM-1} were selected. As depicted in Fig. 3, we observed that the increased turbidity curve appeared only when *A. baumannii* XM with bla_{NDM-1} was used as the template and not with the negative control (double-distilled water) and other bacterial species, including *A. baumannii* H949, *A. baumannii* F398, *A. baumannii* B260, *A. baumannii* H18, *S. sonnei* 2531, *S. flexneri* 4536, *S. enterica* serotype Enteritidis 50326-1, *S. enterica* serotype Paratyphi 86423, enteroinvasive *E. coli* 44825, enterotoxigenic *E. coli* 44824, enteropathogenic *E. coli* 2348, *V. carchariae* 5732, and *V. parahaemolyticus* 5474. These results suggest that these primers could be used to detect bla_{NDM-1}.

Sensitivity of NDM-1 LAMP. To determine the sensitivity of primers in LAMP detection of bla_{NDM-1}, pure genomic DNA was extracted from *A. baumannii* XM using a Wizard genomic DNA purification kit, and the genomic DNA was subject to serial 10-fold dilutions to give concentrations ranging from 1,070 ng/μl to 0.107 pg/μl. As shown in Fig. 4A, the detection limit of the LAMP assay for bla_{NDM-1} was 10.70 pg/μl. Moreover, we monitored the results of the bla_{NDM-1} analysis by LAMP by a direct visual method. Before the LAMP reaction, we added 1 μl of fluorescent

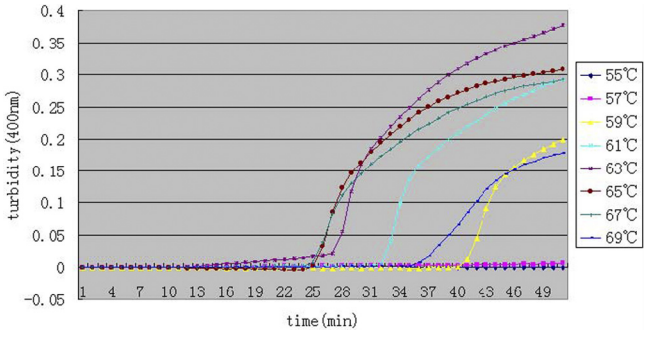

FIG 2 Different temperatures of the LAMP reaction for detection of NDM-1. Turbidity was monitored by a Loopamp real-time turbidimeter at 400 nm every 6 s.

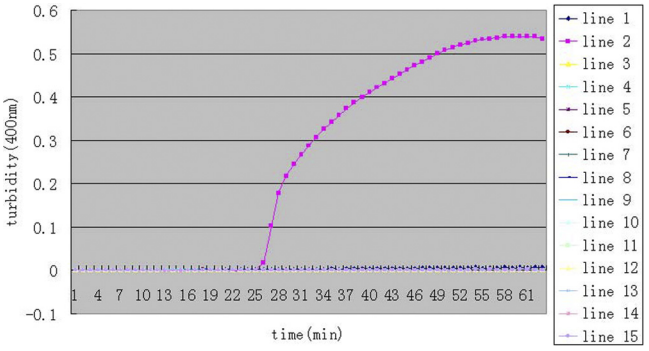

FIG 3 Specificity of the LAMP reaction for detection of bla_{NDM-1}. Turbidity was monitored by a Loopamp real-time turbidimeter at 400 nm every 6 s. Amplification was performed at 65°C for 65 min. Lines: 1, negative control (double-distilled water); 2, *A. baumannii* XM; 3, *A. baumannii* H949; 4, *A. baumannii* F398; 5, *A. baumannii* B260; 6, *A. baumannii* H18; 7, *S. sonnei* 2531; 8, *S. flexneri* 4536; 9, *S. enterica* serotype Enteritidis 50326-1; 10, *V. carchariae* 5732; 11, *S. enterica* serotype Paratyphi 86423; 12, enteroinvasive *E. coli* 44825; 13, enterotoxigenic *E. coli* 44824; 14, enteropathogenic *E. coli* 2348; and 15, *V. parahaemolyticus* 5474.

detection reagent to 25 μl of the LAMP reaction mixture. When the reaction was finished, all positive reactions turned green, while the negative ones remained orange (Fig. 4B). Therefore, we concluded that these two detection methods had the same sensitivity. For comparison purposes, PCR using the NDM1-F and NDM1-R primers with the same amount of *A. baumannii* with bla_{NDM-1} was also carried out. We observed that the detection limit for PCR was 1.07 ng/μl (Fig. 4C).

Evaluation of bla_{NDM-1} LAMP with sputum, urine, and fecal samples. The bla_{NDM-1} analysis with the LAMP assay was then further evaluated with sputum, urine, and fecal samples. The preparation of three samples was described previously. We can see from Fig. 5A to C that, compared with the pure samples (Fig. 4A), the sensitivity for the simulated samples was unchanged (10.70 pg/μl). We conclude that LAMP is less affected by extraneous components in samples than is PCR.

Clinical sample detection. A total of 336 clinical samples and clinical swabs of the environment in intensive care units were collected for LAMP-based surveillance of bla_{NDM-1} from 7 hospitals. We detected the other three strains with bla_{NDM-1}, which were *A. lwoffii* SJH, *Stenotrophomonas maltophilia* JKYJ-01, and *Enterococcus faecalis* PIJ. These results were validated by PCR-based sequencing, and the sequences of the bla_{NDM-1} genes showed 100% identity with those of previously reported genes. These three NDM-1-producing strains were resistant to carbapenems, cephalosporins, and the β-lactam inhibitor combinations tested and susceptible to colistin. Transfer of bla_{NDM-1} from the three isolates was done with *E. coli* J53, the clinical strains of *S. flexneri* 4536 and *S. enterica* serotype Enteritidis as recipients. However, no transconjugant was observed. The genetic locations of the bla_{NDM-1} in the three strains were determined by analysis of PFGE profiles and Southern blot hybridization. The data clearly showed that bla_{NDM-1} is on the chromosome in *A. lwoffii* SJH, *S. maltophilia* JKYJ-01, and *E. faecalis* PIJ (data not shown). Further experiments are in progress to investigation the molecular nature of these strains. These findings prove the validity of the method that we have established. We are now working on larger-scale screening using the LAMP method in the hope of better understanding NDM-1 prevalence.

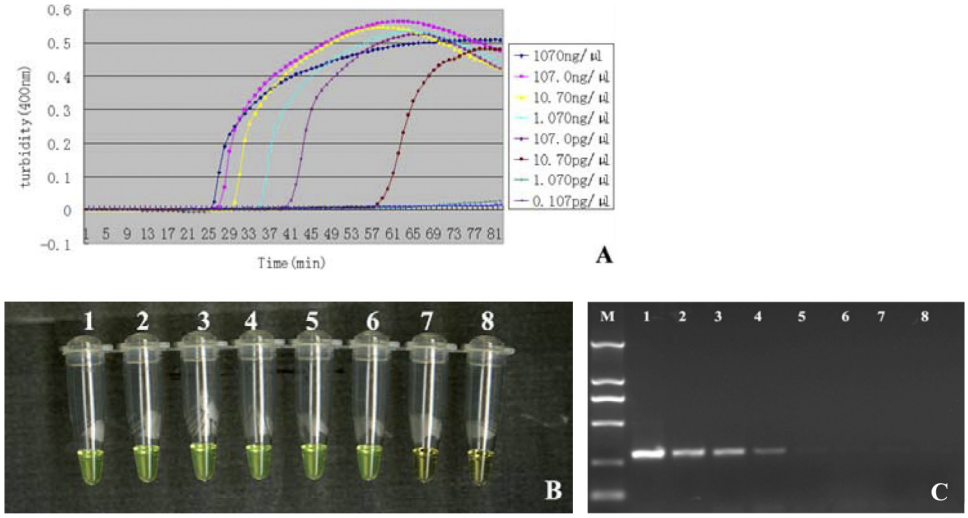

FIG 4 Comparison of sensitivity between the LAMP reaction and PCR for detection of the bla_{NDM-1} gene. The pure genomic DNA extracted from *A. baumannii* XM was diluted in a serial 10-fold dilution. Both LAMP reactions (A and B) and PCRs (C) were carried out in duplicate for each dilution point. Tubes and lanes: 1, 1,070 ng/μl; 2, 107.0 ng/μl; 3, 10.70 ng/μl; 4, 1.070 ng/μl; 5, 107.0 pg/μl; 6, 10.70 pg/μl; 7, 1.070 pg/μl; 8, 0.107 pg/μl. (A) Turbidity was monitored by a Loopamp real-time turbidimeter at 400 nm every 6 s; (B) 1 μl of fluorescent detection reagent was added to 25 μl of LAMP reaction mixture before the LAMP reaction; (C) the PCR products were analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide.

DISCUSSION

In the past several years, numerous multiple-drug-resistant bacteria have been identified: methicillin-resistant *Staphylococcus aureus*, the so-called ESKAPE organisms (an acronym for *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), and others. They are notorious because of their resistance to multiple antimicrobial agents rather than enhanced virulence or pathogenicity. More recently, bla_{NDM-1}, which is a new mobile class B enzyme or metallo-β-lactamase, has emerged and has added to the antibiotic resistance problem. Bacteria with resistance to carbapenem conferred by New Delhi metallo-β-lactamase 1 (NDM-1) are now becoming a major global health problem. The majority of cases have been reported in India (14) and Pakistan (23). Cases have also been reported in Australia (25), Greece (30), Canada (22), Singapore (2), and the United States (1)

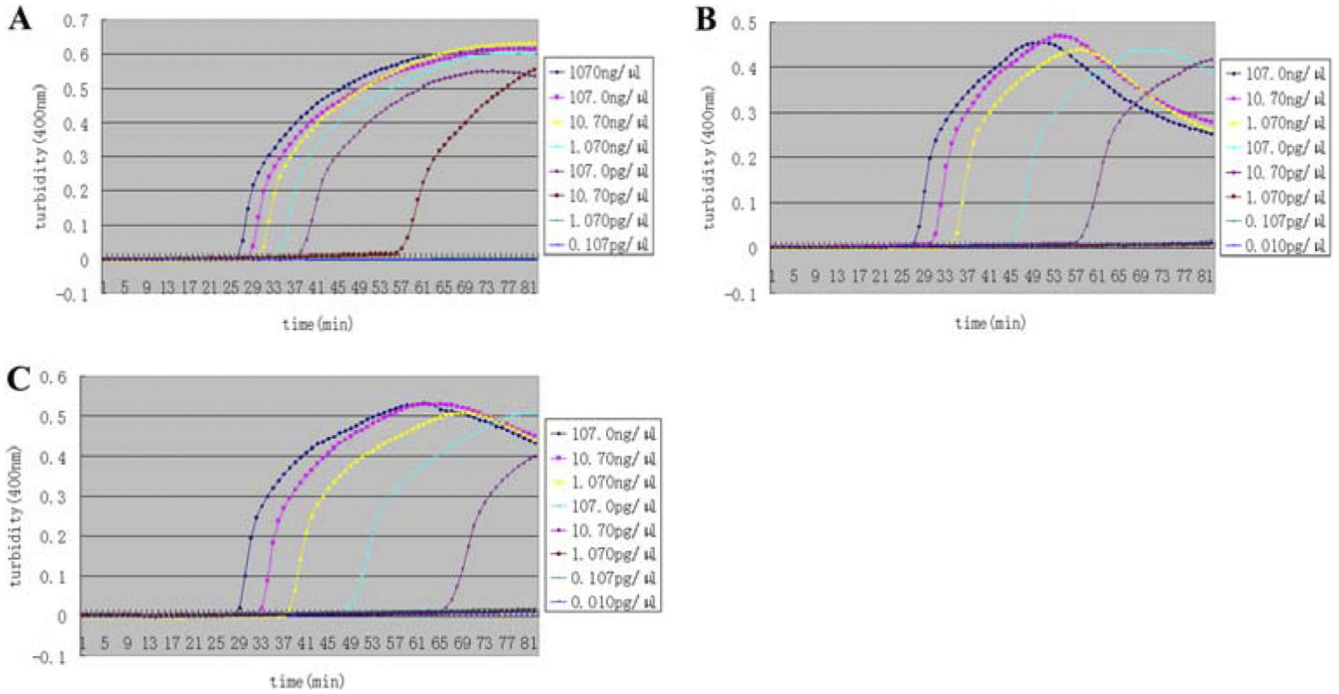

FIG 5 Detection of the bla_{NDM-1} gene in simulated sputum samples (A), simulated urine samples (B), and simulated fecal samples (C) by a Loopamp real-time turbidimeter at 400 nm every 6 s. The concentration of pure genomic DNA extracted from *A. baumannii* XM in each simulated sputum sample is shown.

and, according to recent reports, in China (36), Japan (4), Kenya (26), Oman (24), and China's Taiwan region (33). Development of a sensitive and reliable test for NDM-1 is therefore a priority for early diagnosis and control.

To meet this challenge, we evaluated and optimized a novel LAMP assay for $bla_{\text{NDM-1}}$ detection that was able to specifically detect $bla_{\text{NDM-1}}$ in pathogens that carry $bla_{\text{NDM-1}}$ within 90 min, including DNA extraction. In evaluating the sensitivity of the LAMP assay for $bla_{\text{NDM-1}}$ detection, we observed that the LAMP assay was 100-fold more sensitive than the PCR assay. In addition, the PCR is carried out under temperature-cycling conditions, which is time-consuming, and the reaction also depends on the high precision of the PCR instruments. Compared to PCR, the LAMP reaction was carried out in a constant-temperature environment, and it does not require temperature cycling, so a temperature-controlled water bath or other device that can heat stably is sufficient. Moreover, LAMP reaction primers specifically recognize target sequences of four or six of the six or eight independent target sequence regions, whereas PCR primers recognize target sequences of two independent regions. The specificity and sensitivity are thereby greatly enhanced, and the probability of false-positive results is decreased. Kaneko et al. found that the LAMP reaction is not susceptible to the influence of different components in clinical samples, and so purification of DNA from the sample is not necessary (11). On the other hand, the sensitivity of the PCR can be greatly reduced in the presence of exogenous DNA and inhibitors. Therefore, the LAMP method is more suitable than PCR for rapid detection of $bla_{\text{NDM-1}}$ in clinical samples. Although the amplification principle of the LAMP method is complex, it is a simple procedure whose rapidity, high sensitivity, and specificity make it suitable for $bla_{\text{NDM-1}}$ detection, especially for routine diagnostic and infection control purposes.

Although there are many advantages of LAMP assay (19) (LAMP amplifies DNA with high efficiency under isothermal conditions, LAMP is highly specific for the target sequence, and LAMP is simple and easy to perform), it shows a high rate of false-positive results in its current performance. This is because the amplification efficiency of the LAMP assay is extremely high and the LAMP assay is capable of synthesizing 20 μg of specific DNA in a 25-μl reaction mixture within 60 min (15). Strict spatial separation of reagent preparation and performance of the test is very necessary to avoid contamination. At present, we added low-melting-point paraffin wax to the reaction tubes, after adding reaction solution, to prevent the spread of amplification products. Now it seems that this approach works well for avoidance of contamination.

In conclusion, a specific, sensitive, rapid, and cost-effective LAMP assay for $bla_{\text{NDM-1}}$ detection in pathogens was established. The LAMP assay will be very useful for rapid detection of $bla_{\text{NDM-1}}$ in primary health care units.

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We have no conflicts of interest to declare.

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