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Original Article

Rapid detection of carbapenem resistance genes using multiplex LAMP and melt curve analysis in clinical specimens

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Abstract

Introduction: Carbapenem-resistant Enterobacteriaceae have become a major clinical and public health challenge due to the need for rapid administration of effective antimicrobials and implementation of supplemental infection control practices. Identifying genes associated with carbapenem resistance is crucial for managing these cases. The timely initiation of effective antimicrobial therapy and infection control interventions to prevent spreading are critical. Therefore, rapid diagnostic tests for detecting carbapenemase-producing carbapenem-resistant Enterobacteriaceae (CP-CRE) are necessary.

Methodology: This study aimed to evaluate the use of LAMP and melting curve analysis in real-time polymerase chain reaction (RT-PCR) to identify carbapenem resistance genes New Delhi metallo-β-lactamase (*NDM*) and oxacillinase *(OXA*) in clinical specimens.

Results: This study emphasized the benefits of the LAMP strategy in comparison to traditional methods, demonstrating its effectiveness and practicality. LAMP was proven to be more sensitive than RT-PCR and conventional multiplex PCR, with a detection rate of 96.7% and 91.6% respectively. While LAMP had a slightly lower specificity rate of 88.7%, it remains a promising method for rapid and accurate identification. Furthermore, the study found that LAMP could detect bacterial DNA even in low quantities, with a limit of detection of 10^2 CFU/mL for both *K. pneumoniae* and *E. coli*. This capability is crucial for early diagnosis and treatment in settings where multidrug-resistant (MDR) bacteria are prevalent.

Conclusions: RT-PCR systems that combine melting curve analysis with LAMP offer promise for quick and precise identification of carbapenem resistance genes in clinical specimens. This approach can enhance the diagnosis and management of multidrug-resistant bacterial infections.

Key words: LAMP; carbapenem resistance; *NDM*; *OXA*; real time PCR; melt curve analysis.

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Introduction

Antimicrobial resistance (AMR) has become a persistent global public health issue, with an estimated 10 million deaths annually worldwide by 2050 [1]. According to the World Health Organization (WHO), bacterial antimicrobial resistance was solely responsible for 1.27 million fatalities in 2019 alone, and it was also partially responsible for 4.95 million deaths [2]. The widespread use of antimicrobial drugs has paved the way for selecting bacterial strains against these therapies, causing a tremendous rise in cases of AMR.

WHO recognized the severity of this worldwide health concern, and published a prioritized list of antibiotic-resistant bacteria in 2017 [3]. Enterobacteriaceae, which include key pathogens such as *Escherichia coli* and *Klebsiella pneumonia*, are classified as critically significant within this list [4]. These two infections put a heavy burden on healthcare resources and have a major effect on death rates.

This circumstance reflects a worrying trend where certain bacteria have evolved resistance to vitally needed drugs, such as carbapenems and third generation cephalosporins [5]. The broad-spectrum action of carbapenems, a class of beta-lactam antibiotics, makes them essential for treating infections brought on by bacterial strains that are resistant to drugs. They are frequently regarded as antibiotics of last resort, saved for infections that are really severe or in situations when no other treatments have worked. Beta-lactam antibiotics, such as carbapenems, share structural similarities with penicillins and cephalosporins. Their mode of action is to prevent the production of bacterial cell walls [6]. Since carbapenem drugs are frequently seen as the last line of defense against serious bacterial infections, bacteria generate enzymes called carbapenemases that impart resistance to these medicines. These enzymes cause carbapenems to

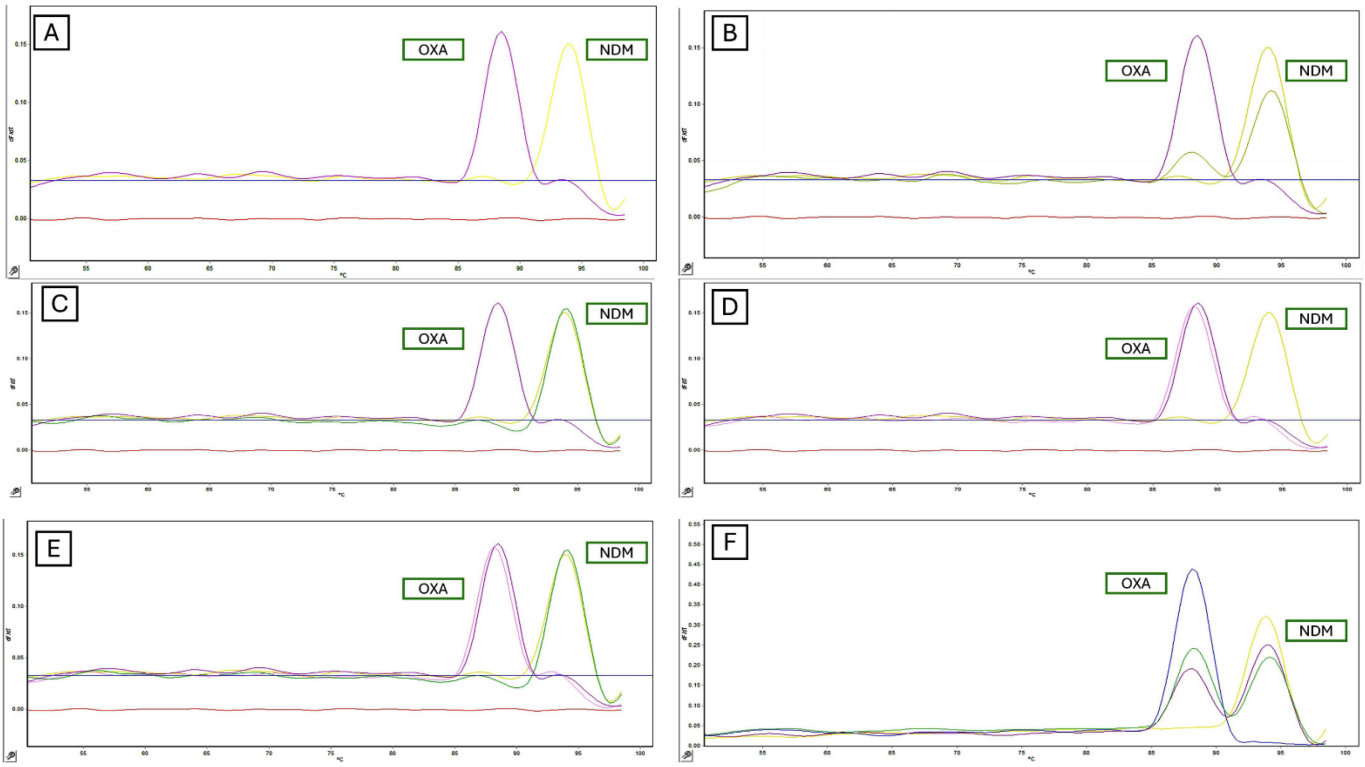
hydrolyze, which makes them useless and adds to the worrying increase of bacterial strains that are resistant to the antibiotic. Carbapenemases may induce resistance to a wide range of β-lactam antibiotics; therefore, they pose a substantial danger to the efficacy of antimicrobial treatment and should be taken seriously as a public health concern [7].

The Ambler classification is frequently utilized to comprehend and arrange carbapenemases in a methodical manner. β-lactamases, which include carbapenemases, are classified into four different classes: A, B, C, and D. Classes A and B are especially important in the setting of carbapenemases. Serine carbapenemases, or class A carbapenemases, are characterized by the use of a serine residue in their active site for hydrolytic action. Notable examples of this kind of enzyme include *Klebsiella pneumoniae* carbapenemase (KPC). Conversely, class B carbapenemases are metallo-β-lactamases (MBLs) that need metal ions—typically zinc—in order to catalyze reactions. Imipenemase (IMP), verona integron-encoded metallo-β-lactamase (VIM), and New Delhi metallo-β-lactamase (NDM) are notable examples of class B members [8,9]. NDM and oxacillinases (OXA) are identified as leading indicators of carbapenem resistance in *Escherichia coli* and *Klebsiella pneumoniae* [10].

The synthesis as well as the impact of NDM and OXA on multidrug resistance (MDR) highlight the significance of creating unique diagnostic approaches in addition to novel treatments derived from the knowledge of clinical isolates of *K. pneumoniae* and *E. coli*. It has been customary to regularly screen these isolates for the *NDM* [8] and *OXA* [9] genes utilizing molecular methods such as polymerase chain reaction (PCR). Nevertheless, research into other techniques has been spurred by the need for a speedier and more affordable diagnostic solution.

Loop-mediated isothermal amplification (LAMP) is an emerging diagnostic method for the detection of NDM and OXA enzymes in these pathogenic strains. It has several advantages over conventional PCR methods [11]. The isothermal nature of the assay eliminates complex thermal cycling mechanisms thus being cost-

Figure 1. Representative melt curve profiles from RT-LAMP amplification targeting NDM and OXA resistance genes in clinical samples and controls.



A. Positive control showing distinct melt peaks for both NDM and OXA. **B.** Positive control for NDM and OXA along with primer controls (PC) for both targets. **C.** Clinical sample showing amplification of NDM only. **D.** Clinical sample showing amplification of OXA only. **E.** Distinct clinical samples each showing amplification of either NDM or OXA. **F.** Clinical sample positive for both NDM and OXA, showing two specific melt peaks corresponding to each target. Each melt curve demonstrates target-specific amplification, with distinct melting temperatures confirming the specificity and presence or absence of the respective resistance genes.

effective and easy to use, especially in resource limited settings. As a result, it can be said that the efficiency of LAMP for fast amplification targeting DNA sequences allows detecting *NDM* and *OXA* genes within a short time, which is valuable for patient management [12].

This study combines real-time polymerase chain reaction (RT-PCR) and melting curve analysis to enhance the diagnostics of LAMP. RT-PCR is used to determine quantitative measures of target gene presence and can be used for understanding the prevalence and distribution of *NDM* and *OXA* genes in bacterial strains. This method allows for the distinction of particular DNA sequences according to their varying melting temperatures by examining the melting behavior of amplified DNA. By using this capability, the diagnostic procedure may be further refined by identifying and differentiating between various *NDM-1* and *OXA* variations. The study investigates the possibilities of combining melting curve analysis with LAMP in RT-PCR devices, comparing the results with those obtained from conventional PCR and RT-PCR. This study adds to the constantly developing toolkit against MDR bacteria, and, in the process, aims to tilt the odds back in favor of humankind in the continuous fight against the threat of antimicrobial resistance.

Methodology

Sample collections

Samples were collected at the Ramaiah Memorial Hospital in Bengaluru from September 2022 to February 2023. These samples underwent diagnostic microbiological analysis, i.e., pathogen identification and susceptibility testing to a panel of antimicrobials using the Vitek II system (bioMérieux, Marcy-l'Étoile, France). The results were interpreted based on the Clinical and Laboratory Standards Institute (CLSI) guidelines from 2020 [13].

DNA extraction

The QIAamp DNA extraction mini kit (Qiagen, Hilden, Germany) was utilized for DNA extraction, following the manufacturer's protocol.

RT PCR

RT-PCR was performed using a Rotor-Gene Q real-time PCR instrument (Qiagen, Hilden, Germany). Each 25 µL reaction contained 12.5 µL of 2× Qiagen Master Mix (Cat. No. 330502; Qiagen, Hilden, Germany), 7.5 µL of molecular-grade water, 1 µL each of forward and reverse primers (10 µM working stock; final concentration 0.4 µM), and 3 µL of DNA template. The primers used for real-time PCR are listed in Table 1. Two positive controls with DNA templates containing *NDM* and *OXA* genes were used. The RT-PCR protocol consisted of an initial denaturation step at 95 °C for 3 minutes, followed by 30 cycles of amplification. Each cycle included denaturation at 95 °C for 1 minute, annealing at 61 °C for 30 seconds with fluorescence acquisition in the green channel, and extension at 72 °C for 40 seconds. Finally, a melting curve analysis was performed from 50 °C to 99 °C with continuous fluorescence measurement in the green channel.

Conventional multiplex PCR

Conventional multiplex PCR was conducted using a gradient PCR instrument (Bio-Rad Laboratories,

Table 1. Oligonucleotide primer sequences of target genes for RT-PCR and LAMP.

Genes	Primer sequence (5 ´to 3´)
RT-PCR and conventional PCR	
NDM-F	GACCGCCCAGATCCTCAA
NDM-R	CGCGACCGGCAGGTT
OXA-F	AAGCCATGCTGACCGAAG
OXA-R	CAAGTTCAACCCAACCAACC
LAMP	
NDM B3	AGCCACCAAAAGCGATGTC
OXA_B3	CACGATGCGCTGACTGACTACG
NDM_BF	TATTTTACCCCGGCCCCG
OXA BF	ATTTCGGCTACCCAGCAAATC
NDM F3	GCTTGCCCCGCAAGAG
OXA_F3	CCGCGATGAAGTACTCAGTT
OXA BIP	TGGCTCGATGGATGGTATTCGCTGTTGTGATACAGCTTGCGT
NDM_BIP	CCAACTTTGGCCCGCTCAAGGGGTGCCGTCGATCCCAA
OXA LF	ACTCATACGTGCCTCACCA
NDM _LF	GCGGCGAAGTCAGGCT
NDM _FIP	CGGTTGCTGGTTCGACCCAGGTTGCGGCGCAACAC
OXA_FIP	CCATAATCGAAGGCGTGCAGCACAAGAATTTGCCCGCCAAAT

Primer sequences designed for the detection of *New Delhi metallo-β-lactamase (NDM)* and *oxacillinase (OXA)* genes, using conventional polymerase chain reaction (PCR), real-time PCR (RT-PCR), and loop-mediated isothermal amplification (LAMP) assays. Each primer set was designed to specifically amplify conserved regions within the target resistance genes. The forward (F) and reverse (R) primers were used for PCR; while LAMP employed a set of 6 primers recognizing 8 distinct regions of the target gene, including outer primers (F3 and B3), inner primers (FIP and BIP), and loop primers (LF and BF). The primer sequences are presented in the 5′ to 3′ orientation.

Hercules, CA, USA). The reaction mix had a total volume of 25 µL, comprising 12.5 µL of 2× Qiagen Master Mix (Cat. No. 201443; Qiagen, Hilden, Germany), 7.5 µL of molecular-grade water, 0.2 µL each of forward and reverse primers (100 µM stock; final concentration 0.8 µM), and 3 µL of DNA. The primers used for multiplex PCR are listed in Table 1. Two positive controls containing DNA templates with *NDM* and *OXA* genes were included. The PCR protocol included an initial denaturation step at 95 °C for 3 minutes, followed by 30 amplification cycles. Each cycle consisted of denaturation at 95 °C for 1 minute, annealing at 57 °C for 30 seconds, extension at 72 °C for 40 seconds, and a final extension at 72 °C for 3 minutes.

The resulting amplicons were separated using a 1.5% agarose gel containing 0.5 µg/µL ethidium bromide. Electrophoresis was conducted in 1× TAE buffer at 100 V for 55 minutes using an electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA). A 50 bp DNA ladder was used as a molecular size marker, and DNA bands were visualized using a gel documentation system (Syngene, Cambridge, UK).

Real-time loop-mediated isothermal amplification (RT LAMP)

Real-time PCR was performed using a Rotor-Gene Q instrument (Qiagen, Hilden, Germany). Each 25 µL reaction contained 12.5 µL of 2× Qiagen Master Mix (Cat. No. 330502; Qiagen, Hilden, Germany), 8.3 µL of molecular-grade water, 0.1 µL each of forward and reverse primers (10 µM working stock; final concentration 0.04 µM), and 3 µL of DNA template. Two positive controls containing DNA templates of *NDM* and *OXA* genes were included.

The primers for real-time LAMP (RT-LAMP) were designed using the NEB LAMP Primer Design Tool (New England Biolabs, Ipswich, MA, USA) [14]. Each primer set consisted of 6 oligonucleotides: 1 pair of inner primers (FIP and BIP), 1 pair of outer primers (F3 and B3), and 1 pair of loop primers (LF and LB), as listed in Table 1.

The RT-LAMP protocol included a holding step at 60 °C for 2 minutes, followed by 55 cycles of isothermal amplification at 61 °C for 1 minute with real-time monitoring of fluorescence in the green channel. A melting curve analysis was then conducted from 55 °C to 99 °C, with continuous fluorescence detection in the green channel.

Limit of detection for LAMP assay

In order to determine the limits of detection, each microorganism was inoculated into saline to achieve final concentrations ranging from 10^7 to 10^2 colony-forming units per milliliter (CFU/mL). Alternatively, positive samples were used to create serial 10-fold dilutions in saline buffer. The final concentration for each dilution was based on the approximate initial concentration determined by culture [15].

Statistical analysis

Data were entered in Microsoft Excel (version 2013; Microsoft Corp., Redmond, WA, USA). Diagnostic sensitivities, specificities, and positive and negative predictive values of real-time PCR, LAMP, and conventional PCR were calculated using 2×2 contingency tables, with real-time PCR considered the reference gold standard method. Statistical analysis was performed using MedCalc Statistical Software (version 20.218; MedCalc Software Ltd., Ostend, Belgium). Further, data were analyzed using Bayesian latent class model considering all the three diagnostic modalities as imperfect and also: (i) each subject is independent and identically distributed; (ii) tests are independent of other tests; and (iii) all tests are detecting the parameter of interest, that is true disease. Bayesian latent class modelling was performed using the Modelling for Infectious Disease Centre, Mahidol-Oxford Research Unit (MICE) tool, freely available online upon registration [16].

Results

In this study, 91 clinical specimens from patients were analyzed, with 51 from female patients and 40 from male patients, and the average age was 33 ± 18 years. The majority were urine samples (67, 73.7%), followed by pus samples (24, 26.3%). Carbapenem resistance was observed in 56 samples (61%), with 35 samples identified as *K. pneumoniae*, 23 as *E. coli*, and 33 as negative (indicating carbapenem sensitivity). The carbapenem-sensitive samples were included for standardization purposes (Figure 1). These findings highlight the prevalence of carbapenem resistance in clinical specimens, particularly among *K. pneumoniae* and *E. coli* isolates.

The limit of detection (LOD) of LAMP was determined to be 10^2 CFU/mL for both pathogens tested in this study. This LOD indicates the lowest concentration of bacterial DNA at which the LAMP assay can reliably detect the presence of the target genes.

Significant differences were observed in the detection rates of *NDM* and *OXA* genes using individual methods across various clinical specimens. The results

Table 2. Clinical specimens and detection rates of *NDM* and *OXA* genes using individual methods.

Test	Total positives	Clinical sample		Carbapenem resistance detected		
		Urine (67)	Pus (24)	*E. coli* (19)	*K. pneumoniae* (27)	Negative (21)
RT-PCR	41	35	6	15	25	1
LAMP	45	37	8	14	26	5
Multiplex PCR conventional	39	32	7	12	24	3

Detection of *New Delhi Metallo-β-lactamase (NDM)* and *oxacillinase (OXA)* genes among clinical isolates tested by different molecular methods, including real-time polymerase chain reaction (RT-PCR), loop-mediated isothermal amplification (LAMP), and multiplex conventional PCR. A total of 91 clinical specimens comprising urine (n = 67) and pus (n = 24) samples were analyzed. The isolates included *Escherichia coli* (n = 19) and *Klebsiella pneumoniae* (n = 27). The number of carbapenem-resistant isolates detected by each method is presented, along with the number of negative samples (n = 21). LAMP demonstrated the highest detection rate, followed by RT-PCR and multiplex PCR.).

are summarized in Table 2. RT-PCR identified 41 positive samples, with 35 from urine, 6 from pus, and none from *E. coli* or *K. pneumonia*. Two positives from pus were missed. LAMP detected 45 positive samples, including 37 from urine, 8 from pus, and 5 from *K. pneumonia*; but missed 1 sample that was positive for *E. coli*. Conventional multiplex PCR found 39 positives, including 32 from urine, 7 from pus, and 3 from *K. pneumonia*. Four samples that were positive for *E. coli* were missed. Overall, LAMP had the highest total positives and showed potential as a reliable method for carbapenem resistance detection, especially in urine samples.

The gold standard model was used to compare the diagnostic methods, assuming real-time PCR as the perfect gold standard. The diagnostic sensitivity and specificity of the LAMP assay were 90.2% and 84.0%, respectively; while those of conventional multiplex PCR were 85.4% and 92.0%, respectively. The Bayesian latent class model (BLCM) showed that LAMP outperformed both RT-PCR and conventional multiplex PCR in sensitivity, with LAMP demonstrating a sensitivity of 96.7% compared to 91.6% for RT-PCR and 92.5% for conventional multiplex PCR. While LAMP's specificity was slightly lower at 88.7% compared to RT-PCR (92.6%) and conventional multiplex PCR (97.6%), it still maintained an acceptable level of specificity. (Table 3). A detailed description of the results obtained from BLCM is available in the MICE Result page (tropmedres.ac). This suggests that LAMP is more likely to detect true positives than the other methods, which is crucial for accurate diagnosis.

Discussion

The emergence and spread of MDR bacteria, particularly those exhibiting carbapenem resistance, pose a significant threat to public health worldwide [17]. In this study, the potential of combining melting curve analysis with LAMP in RT-PCR devices to detect carbapenem resistance genes (*NDM* and *OXA*) in clinical specimens were explored. The findings demonstrate the feasibility and effectiveness of this approach, highlighting its advantages over conventional methods.

One key finding of this study was the high

Table 3. Diagnostic efficacies of Real-Time PCR (RT-PCR), Loop-Mediated Isothermal Amplification (LAMP), and conventional multiplex PCR in the present study using gold standard model and Bayesian latent class model.

Parameters total (N = 91)	RT PCR was assumed as a perfect gold standard (%).	Bayesian latent class model (%) considering all tests are imperfect.
Prevalence	45.1 (34.7–55.8)	44.9 (34.1–55.7)
RT PCR		
Sensitivity	100	91.6 (79.3–98.4)
Specificity	100	92.6 (82.3–98.7)
PPV	100	91.0 (78.2–98.4)
NPV	100	93.2 (82.7–98.7)
LAMP		
Sensitivity	90.2 (75.9–96.8)	96.7 (86.8–99.9)
Specificity	84.0 (70.3–92.4)	88.7 (77.2–96.8)
PPV	82.2 (67.4–91.5)	87.5 (73.5–96.5)
NPV	91.3 (78.3–97.2)	97.1 (87.9–99.9)
Conventional multiplex PCR		
Sensitivity	85.4 (70.1–93.9)	92.5 (79.8–99.6)
Specificity	92.0 (79.9–97.4)	97.6 (88.9–100)
PPV	89.7 (74.8–96.7)	96.8 (85.8–100)
NPV	88.5 (75.9–95.2)	94.2 (83.2–99.7)

Comparative diagnostic performance of Real-Time PCR (RT-PCR), Loop-Mediated Isothermal Amplification (LAMP), and conventional multiplex PCR for the detection of *New Delhi Metallo-β-lactamase (NDM)* and *Oxacillinase (OXA)* genes among 91 clinical isolates. Two analytical frameworks were employed: (i) RT-PCR assumed as a perfect gold standard and (ii) a Bayesian latent class model considering all tests as imperfect. The table shows the estimated prevalence along with the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for each method, expressed as mean values with 95% confidence intervals (CI) or 95% credible intervals (CrI). The Bayesian approach provided adjusted estimates accounting for potential misclassification among assays. Estimated means with 95% confidence interval; Estimated median with 95% credible interval.

sensitivity of LAMP in detecting carbapenem resistance genes, with a sensitivity of 96.7% compared to 91.6% for RT-PCR and 92.5% for conventional multiplex PCR. This suggests that LAMP is more likely to detect true positives, which is crucial for accurate diagnosis and appropriate patient management. The slightly lower specificity of LAMP (88.7%) compared to RT-PCR (92.6%) and conventional multiplex PCR (97.6%) indicates that LAMP may produce more false positives. However, the overall performance of LAMP in terms of sensitivity and specificity makes it a promising tool for the rapid and accurate detection of carbapenem resistance genes in clinical specimens.

Another significant finding in this study was the LOD of LAMP, which was determined to be 10^2 CFU/mL for both *E. coli* and *K. pneumoniae*. This is notably lower than the limit found in a study focused on detecting bacteria from urine samples, which reported a limit of detection of 10^4 CFU mL [18]. This study's lower LOD indicates the ability of the LAMP assay to reliably detect bacterial DNA at exceptionally low concentrations. This capability is critical for early detection and timely treatment, particularly in environments where MDR bacteria are prevalent.

This study also highlights the importance of using a combination of diagnostic methods, such as RT-PCR with LAMP to improve the accuracy of carbapenem resistance gene detection. The BLCM analysis showed that LAMP outperformed both RT-PCR and conventional multiplex PCR in sensitivity, while maintaining an acceptable level of specificity. This suggests that a combination of these methods could provide a more comprehensive and accurate diagnosis of carbapenem resistance in clinical specimens.

The study demonstrates the potential of combining melting curve analysis with LAMP in RT-PCR devices for the rapid and accurate detection of carbapenem resistance genes in clinical specimens. This approach offers several advantages, including high sensitivity, low LOD, and the ability to detect multiple pathogens simultaneously. Further research is needed to validate these findings in larger cohorts and to explore the clinical utility of this approach in the diagnosis and management of MDR bacterial infections.

Limitations

Even though LAMP showed good sensitivity and specificity in this investigation, bigger cohort validation studies are required to corroborate these results. Additionally, the cost-effectiveness and scalability of the LAMP test in real-world clinical settings should be assessed before general adoption. Another limitation of the study was the detection of only two target genes for carbapenem resistance. This was done based on the prevalent carbapenem resistant genes in the region. The assay has scope for further addition of targets in the future studies.

Conclusions

Melting curve analysis and LAMP RT-PCR instruments, have the potential to be used for the quick and precise identification of carbapenem resistance genes (*NDM* and *OXA*) in clinical specimens. LAMP is a potential method for the early diagnosis and prompt treatment of MDR bacterial infections, especially those caused by *E. coli* and *K. pneumoniae*, due to its high sensitivity and low detection limit. Although LAMP had somewhat lower specificity than RT-PCR and conventional multiplex PCR, its overall sensitivity and specificity made it an important addition to the diagnostic toolset for carbapenem resistance.

Ethics approval

The study was approved by the University Ethics Committee for Human Trials Approval of Ramaiah University of Applied Sciences (EC-23/136-SD-FLAHS).

Consent to participate

Informed consent was obtained from all individual participants included in the study.

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Authors' contributions

VVN, experiments and manuscript writing; SK and TT, experiments; VC, sample processing and analysis; TS, planning and supervision.

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Conflict of interest

No conflict of interest is declared.

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