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Original article

Loop-mediated isothermal amplification assay versus polymerase chain reaction for detection of *bla*NDM-1 and *bla*KPC genes among Gram negative isolates

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ABSTRACT

Background: New Delhi Metallo-b-lactamase (NDM- 1) and *Klebsiella pneumoniae* carbapenemase (KPC) are enzymes associated with resistance to many β- lactam agents. Their early detection is very important for controlling the spread of drug- resistant bacteria. This study aimed to evaluate the use of LOOP-mediated isothermal amplification technique (LAMP) assay for rapid and cost-effective detection of bla_{NDM-1} and bla_{KPC} genes among Gram-negative bacteria in comparison with conventional PCR. **Methods:** A total of 156 gram-negative bacterial isolates [*Escherichia coli* (43), *Klebsiella* spp. (66), *Pseudomonas* spp. (47)] were screened for the presence of carbapenemases (bla_{NDM-1} and bla_{KPC}) using molecular methods such as conventional PCR and LAMP assay. **Results:** bla_{NDM1} was positive in 94/156 (60.2%) isolates and bla_{KPC} was positive in 37/156 (23.7%) isolates by conventional PCR and LAMP. **Conclusion:** The LAMP technique is an excellent option for the rapid detection of bla_{NDM-1} and bla_{KPC} genes. The amplification is faster and cheaper than other molecular techniques. It is easy to implement. The thermocycler is not necessary.

Introduction

Carbapenem resistance among gram-negative bacteria is a global problem and has led to a worldwide epidemic, which continues to increase [1].

New Delhi metallo-beta-lactamase-1 gene (bla_{NDM-1}) encodes New Delhi metallo-beta-lactamase 1 (NDM-1) enzyme which causes multi-drug resistance [2].

NDM-1 causes inactivation of a broad range of b-lactam antibiotics involving carbapenems by hydrolysis of β-lactam rings. NDM-1 was detected in 11 bacterial species such as *Shigella boydii, Escherichia coli, Klebsiella sp.* and *Vibrio cholera* [3].

Klebsiella pneumoniae carbapenemase (KPC) enzymes have become a worldwide problem among *Enterobacteriaceae, Acinetobacter baumannii* and *Pseudomonas aeruginosa*[4]. They belong to class A (serine carbapenemases).

Plasmids that encode carbapenemases have been shown to have a central role in the

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dissemination of carbapenem-resistant pathogens [5], with *bla*$_{NDM}$ and *bla*$_{KPC}$ being the primary carbapenemases of concern [6].

In the treatment of infectious diseases, the administration of sufficient antibiotics is necessary for preventing the occurrence and spread of resistant pathogens. However, conventional antibiotic sensitivity testing is time consuming; as a result, a simple, rapid test such as loop-mediated isothermal amplification (LAMP) is required for the rapid selection of suitable antibiotics [7]**.**

LAMP is a new gene amplification technique that can amplify DNA under isothermal conditions[8]. LAMP is a strand displacement amplification technique [7]**,** that uses a specific DNA polymerase (Bst) and 4 to 6 primers. During the strand displacement amplification process, a dumbbell DNA structure is formed that acts as a template for cycle amplification. The speed of the reaction and the lack of a need for a thermocycler, make LAMP an encouraging technique for the development of a sensitive and simple test for the molecular detection of genes in endemic countries [8]**.**

Rapid detection of carbapenem-resistant genes as *bla*$_{NDM-1}$and *bla*$_{KPC}$ genes is of great importance for treatment then PCR assays which are usually expensive and difficult to be used in less developed areas. The LAMP method is widely used as a nucleic acid amplification test compared to other options such as the polymerase chain reaction (PCR) [9-11]. The process of molecular testing involves extracting nucleic acid from the samples, amplifying genes, and detecting them. These steps necessitate costly equipment and expertise, thereby posing challenges for conducting appropriate testing in any setting. A novel method is needed to surmount these constraints. The LAMP technique is widely regarded as a remarkable technology for amplification and detection, exhibiting numerous advantages in comparison to conventional detection methodologies. The LAMP assay exhibits heightened sensitivity compared to PCR, ranging from 10 to 100 times, thereby establishing a detection threshold of 10 copies. Additionally, DNA amplification can occur within a time frame of 30 to 60 minutes, resulting in an amplification magnitude of 10^9 to 10 [10]. Furthermore, in comparison to the PCR reaction, the LAMP assay offers a reduction of up to 1 hour [12].LAMP reaction is characterized by simplicity, rapidity and high specificity [13]**.** Therefore, evaluation the use of LAMP assay for rapid and cost-effective detection of *bla*$_{NDM-1}$ and *bla*$_{KPC}$ genes among Gram-negative bacteria in comparison with conventional PCR is needed.

Methods

This research was authorized by the Ethical Committee of the Faculty of Medicine at Assiut University in Assiut, Egypt, (Code: 17200475), in accordance with the ethical guidelines established by the World Medical Association (Declaration of Helsinki). Prior informed consent was obtained from all participants.

In this cross sectional study, all urine samples were collected in sterile containers from patients admitted to the Assiut University Urology Hospital after 48 hours of hospital admission under complete aseptic conditions from December 2019 to July 2022.

For non-catheterized patients; clean catch midstream urine sample were obtained in sterile closed labelled cups. For catheterized patients; catheter tube was disconnected from the collection bag, few milliliters were discarded and the sample was then obtained.

Eligible participants in the study fulfilled one or more of the following criteria suggesting urinary tract infection (UTI): fever>38℃, flank and/or suprapubic tenderness, dysuria and urine turbidity.The exclusion criteria included:Patients refused to participate in the study, Patients taking some drugs that affect the results, these drugs include: Metronidazole, Riboflavin, Methocarbamol and Nitrofurantoin., Patients under antibiotic therapy and Patients with inserted medicated catheters.

All clinical samples were used in this study after performing a conventional microbiological diagnosis [14]. Out of 250 samples, 156 Gram-negative bacteria were detected. The sample size was computed using this formula:

$$ss = \frac{Z^2 * (p) * (1-p)}{c^2}$$

Where:Z = Z value (e.g. 1.96 for 95% confidence level), p = percentage picking a choice, expressed as decimal (.5 used for sample size needed), c = confidence interval, expressed as decimal (e.g., .04 = ±4).

Correction for Finite Population

$$\text{new ss} = \frac{\text{ss}}{1 + \frac{\text{ss}-1}{\text{pop}}}$$

Where: pop = population

Population = 206, Confidence interval=95% → sample size needed = 106 and we raised it to 156.

Molecular detection of *bla*$_{NDM-1}$ and *bla*$_{KPC}$ genes

DNA extracts from all Gram-negative isolates (156) were used for *bla*$_{NDM-1}$ *and bla*$_{KPC}$ detection.

Three to four colonies were picked from an overnight culture plate, suspended into 100 µl of distilled water in eppendorf tubes, and placed in a dry block heater at 95°C for 15 minutes, then they were centrifuged at 14.000 rpm for 5 min to pellet the cellular debris [15]. The supernatant was transferred into eppendorf tubes. The tubes were stored at -20 °C.

Conventional PCR assay

The sequence of the primers (Thermo Fisher Scientific - US) used for amplification of *bla*$_{NDM-1}$ [16] and *bla*$_{KPC}$ [17] genes are shown in Table (1).

The PCR conditions were as follows: an initial denaturation for 3 min at a temperature of 95°C followed by 40 cycles of denaturation for 30 sec at a temperature of 95°C, primer annealing for 1 min at a temperature of 55°C, and primer extension for 1 min at a temperature of 72°C, followed by a final extension of 5 min at a temperature of 72°C.

The resulting PCR products were analyzed in a 2% agarose gel with ethidium bromide staining. The bands were subsequently visualized under UV illumination. *Bla*$_{NDM-1}$ positive isolates showed 621 bp band and *bla*$_{KPC}$ isolates showed 893 bp band.

LAMP assay

LAMP reactions were done in a 25 µl reaction mixture containing the following reagents in awater bath following the manufacturer's instructions: 3 µl Distilled water, 3.5 µl dNTP Mixture (Thermo Fisher Scientific - US), 2.5 µl 10X Bsm Buffer, 1 µl of each FIP/BIP primers, 0.5 µL of each F3/B3 primers, 1 µl of each LoopF/B primers, 2 µl Betaine, 1 µl Bsm DNA Polymerase, 4 µl $MgCl_2$ and 2 µl DNA.

The sequence of the primers (Thermo Fisher Scientific - US) used for *bla*$_{NDM-1}$ and *bla*$_{KPC}$ genes amplification[17]are shown in Table (2)

Statistical analysis:

Categorical variables were delineated by their numerical representation and percentage (N, %). The comparison between categorical variables was conducted using the Chi-square test. A two-tailed p-value of less than 0.05 was deemed to possess statistical significance. The entirety of the analyses were executed utilizing the IBM SPSS 26.0 software.

Results

By using modified Kirby Bauer disc diffusion method, 100 (64.1%) of the Gram-negative isolates were carbapenem resistant and 56 (35.9%) of the Gram-negative isolates were carbapenem sensitive. The frequency of carbapenem resistance was highest in *Pseudomonas* spp. (41 isolates, 87.2%) and lowest in *E.coli* (19 isolates, 44.2%) (table 3). There was a significant statistical difference between the frequency of carbapenem resistance and the type of some uropathogens (*Klebsiella* spp. and *Pseudomonas* spp).

Molecular detection of *bla*$_{NDM-1}$ *and bla*$_{KPC}$ genes by conventional PCR

All Gram negative bacilli isolates (156) were tested for the presence of genes encoding carbapenemases (*bla* $_{KPC}$ and *bla* $_{NDM1}$) by PCR.

In the current study, *bla*$_{NDM1}$ was positive in 94/156 (60.2%) isolates (Fig.1). It was positive in 89 (89%) of Carbapenem resistant Gram negative isolates and 5 (8.9 %) of Carbapenem sensitive isolates. *Bla*$_{KPC}$ was positive in 37/156 (23.7%) isolates (Fig.2). It was positive in 33 (33 %) of Carbapenem resistant Gram negative isolates and 4 (7.1 %) of Carbapenem sensitive isolates. (table 4). *Bla*$_{NDM-1}$ positive isolates showed 621 bp band and *bla*$_{KPC}$ isolates showed 893 bp band.

In the current study, *Pseudomonas* spp**.** was the most common pathogen containing *bla*$_{NDM-1}$ *and bla*$_{KPC}$ genes that was detected in 38 (80.9%) and 15 (31.9%) of isolates, respectively. On the other hand, *E.coli* was the least common pathogen containing *bla*$_{NDM-1}$ *and bla*$_{KPC}$ genes that were detected in 17 (39.5%) and 2 (4.7%) of isolates, respectively (table 5). There was a significant

statistical difference between the frequency of carbapenemase-encoding genes and the type of uropathogen (*E.coli*, *Klebsiella* spp. and *Pseudomonas* spp).

Molecular detection of *bla*$_{NDM-1}$ *and bla*$_{KPC}$ genes by LAMP

LAMP was done for all Gram negative bacilli isolates to detect genes encoding carbapenemases (*bla*$_{KPC}$ and *bla*$_{NDM1}$). *bla*$_{NDM1}$ was positive in 94/156 (60.2%) isolates and *bla*$_{KPC}$ was positive in 37/156 (23.7%) isolates.

The gel electrophoresis image of these products displayed ladder-like arrangements as a result of the LAMP gene amplification, which induces the formation of cauliflower-like structures characterized by numerous loops and stem-loop DNA structures featuring multiple inverted repeats.

Table 1. Sequence of primers used in conventional PCR amplification of *bla*$_{NDM-1}$ and *bla*$_{KPC}$ genes.

Primer name	Sequence
***bla*$_{NDM-1}$ forward**	GGTTTGGCGATCTGGTTTTC
***bla*$_{NDM-1}$ reverse**	CGGAATGGCTCATCACGATC
***bla*$_{KPC}$ forward**	ATGTCACTGTATCGCCGTCT
***bla*$_{KPC}$ reverse**	TTTTCAGAGCCTTACTGCCC

PCR was performed using the thermal cycler (BioRadT100, USA).
The PCR reactions were done in a 20 ml reaction mixture containing the following reagents: 3 µl Distilled water, 12.3µl dNTP Mixture, 2 µl 10X Taq PCR Buffer, 1 µl of each primer, 0.1 µl Taq DNA Polymerase and 2 µl DNA.

Table 2. The sequence of the primers used for amplification of *bla*$_{NDM-1}$ and *bla*$_{KPC}$ genes by LAMP technique.

Primer name	Sequence
***bla*$_{NDM-1}$ F3**	GCATAAGTCGCAATCCCCG
***bla*$_{NDM-1}$ B3**	CTTCCTATCTCGACATGCCG
***bla*$_{NDM-1}$ FIP**	GAGATCAACCTGCCGGTCGCTTTTTCCATACCGCCCATCTTGT
***bla*$_{NDM-1}$ BIP**	TCTGGGCGGTCTGGTCATCGTTTTTTCCAACGGTTTGATCGTCA
***bla*$_{NDM-1}$ FLB**	GGTGACTCACGCGCATCAGG
***bla*$_{NDM-1}$ BLP**	ACCACCAGCACGCGGCCGCCATC
***bla*$_{KPC}$ F3**	TCGAACAGGACTTTGGCG
***bla*$_{KPC}$ B3**	GGAACCAGCGCATTTTTGC
***bla*$_{KPC}$ FIP**	CACAGTGGGAAGCGCTCCTCTTTTGTGTACGCGATGGATACCG
***bla*$_{KPC}$ BIP**	TCAAGGGCTTTCTTGCTGCCGTTTTCGTAACGGATGGGTGTGTC
***bla*$_{KPC}$ BLP**	AGCAGCAGGCCGGCTTGCTG
***bla*$_{KPC}$ FLP**	TAACTACAGTTGCGCCTGAGC

Table 3. Frequency of carbapenem resistance among Gram-negative isolates.

Gram negative isolate	Carbapenem resistant	Carbapenem sensitive	P value
***Klebsiella* spp. (N=66)**	**40 (60.6%)**	**26 (39.4%)**	**0.017***
***Pseudomonas spp.* (N=47)**	**41 (87.2%)**	**6 (12.8%)**	**<0.001****
***E.coli* (N=43)**	**19 (44.2%)**	**24 (55.8%)**	**0.221**
Total (N=156)	**100 (64.1%)**	**56 (35.9%)**	

Results are expressed as n (%), $p \leq 0.05$ is significant.

Table 4. Number and percent of bla_{KPC} and bla_{NDM1} positive genes among Gram negative isolates as detected by conventional PCR.

	Carbapenem resistant (N=100)	Carbapenem sensitive (N=56)	P. value
***Bla*$_{KPC}$ positive**	**33 (33%)**	**4 (7.1%)**	**<0.001****
***Bla*$_{NDM1}$ positive**	**89 (89%)**	**5 (8.9%)**	**<0.001****

Results are expressed as n (%), $p \leq 0.05$ is significant.

Table 5. Frequencies of carbapenemase-encoding genes among Gram negative isolates as detected by conventional PCR.

Bacterial species	*bla* $_{NDM1}$	*bla* $_{KPC}$	P .value
***E.coli* (43)**	**17 (39.5%)**	**2 (4.7%)**	**<0.001****
***Klebsiella* (66)**	**39 (59.1%)**	**20 (30.3%)**	**0.005****
***Pseudomonas* (47)**	**38 (80.9%)**	**15 (31.9%)**	**<0.001****
Total	**94**	**37**	

Results are expressed as n (%), $p \leq 0.05$ is significant.

Table 6. Comparison of LAMP assay with PCR in the detection of bla_{NDM1} and bla_{KPC} genes Assay

Bacterial species	*bla*$_{NDM1}$		*bla*$_{KPC}$	
	PCR	**LAMP**	**PCR**	**LAMP**
***E.coli* (43)**	**17**	**17**	**2**	**2**
***Klebsiella* (66)**	**39**	**39**	**20**	**20**
***Pseudomonas* (47)**	**38**	**38**	**15**	**15**
Total	**94**	**94**	**37**	**37**

The sensitivity of LAMP test is 100%

Figure 1. Gel electrophoresis of the PCR-amplified products for bla_{NDM1} detection. In lane M, a 100bp DNA marker was present, while lane 1 served as a positive control, Lanes 2-4 were positive for bla_{NDM1} (621 bp)

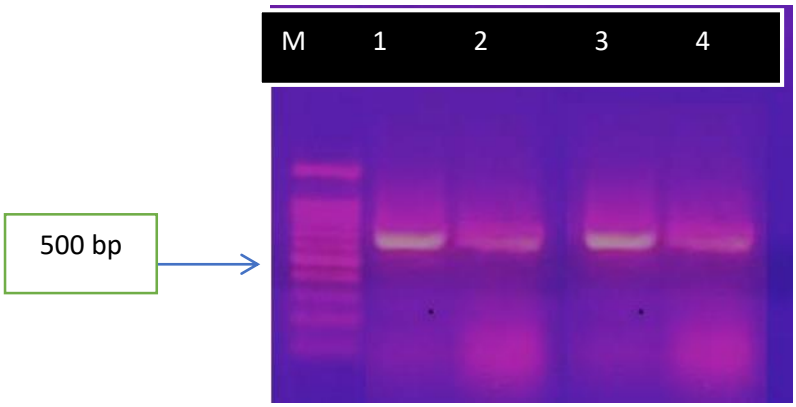


Figure 2. Gel electrophoresis of the PCR-amplified products for detection of *bla* $_{KPC}$. In lane M, a 100bp DNA marker was present, while lane 1 served as a positive control., Lanes 2-4 were positive for *bla*$_{KPC}$ (893 bp).

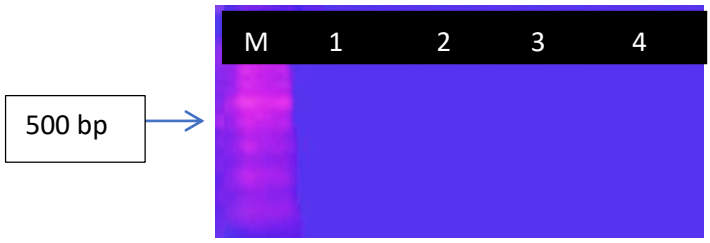
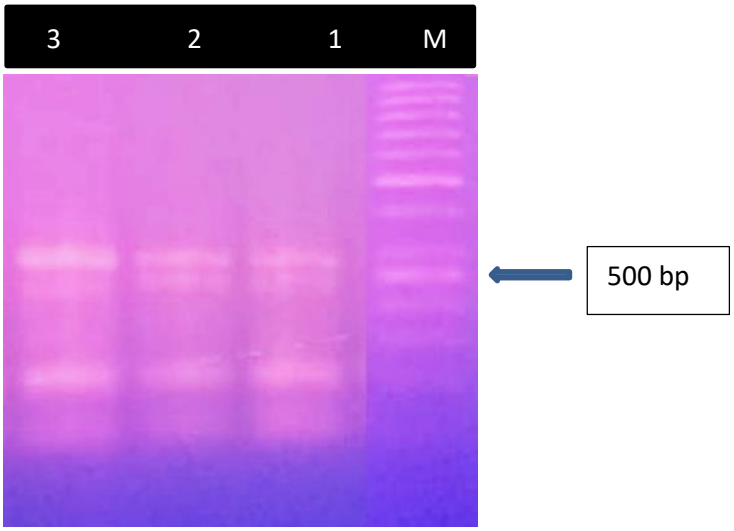


Figure 3. Gel electrophoresis of the LAMP amplified products for blaNDM1 detection. In lane M, a 100bp DNA marker was present, while lane 1 served as a positive control, Lanes 2-3 were positive for *bla*$_{NDM1}$ (ladder-like pattern).



Discussion

Antimicrobial resistance among Gram-negative bacilli increased globally in the last decade, associated with a lack of existing effective antibiotics and the absence of new antibiotics. Antimicrobial resistance causes significant economic and clinical concerns, including higher antimicrobial and hospital costs, longer hospital stay and increased mortality rates [19].

In the Global Priority Pathogens List of the World Health Organization, carbapenem-resistant *Enterobacteriaceae* and carbapenem-resistant *Pseudomonas aeruginosa* were in the pathogens group of critical priority. This shows the urgent need to develop new antibiotics to limit the resistance spread and the severity of nosocomial infections [20].

Antimicrobial resistance is a main clinical problem in the treatment of infections caused by different bacteria and has increased over time. A study reveals that urinary tract infections (UTIs) are among the prevailing infections caused by carbapenem-resistant Gram-negative bacteria [21,22]. Currently, carbapenem-resistant Gram-negative bacteria are difficult to treat and have a serious danger to the healthcare system as there are only a few treatment options. In the present study, the most common CR pathogen isolated from UTI patients was *Pseudomonas* followed by *Klebsiella* spp. This agreed with **Shields et al.,** [23] who reported that the majority of CR pathogens in UTI patients were *Pseudomonas* and *Klebsiella* spp.

In the current study, *bla*$_{KPC}$ was positive in 33/100 (33%) of Carbapenem resistant Gram negative isolates by PCR. **Satir et al., [**24] and **Han et al., [**25] detected a higher prevalence of *bla*$_{KPC}$ positive isolates by PCR 45.7% and 51.6%, respectively. However, **Solanki et al., [**26] and **Raheel et al., [**27] reported a lower prevalence of *bla*$_{KPC}$ positive isolates by PCR 15% and 7.5%, respectively.

In the present study, *bla$_{NDM1}$* was positive in 89/100 (89%) of Carbapenem resistant Gram negative isolates by PCR. This agreed with **Raheel et al., [**27] who reported the same Prevalence. However, **Solanki et al., [**26] and **Han et al., [**25] reported lower Prevalence of *bla $_{NDM1}$* positive isolates 59% and 35.7%, respectively.

The elevated rates of resistance to carbapenem and the disparity observed between our investigation and other investigations can potentially be ascribed to the inappropriate use of antibiotics in our locality, leading to an increased resistance levels.

In the current study, *bla$_{KPC}$* was positive in 7.1 % of Carbapenem sensitive isolates. *Bla $_{NDM1}$*was positive in 8.9 % of Carbapenem sensitive isolates. This agreed with **Emira et al.,**[28] who reported that *bla$_{NDM1}$* was positive in 8.7% of Carbapenem sensitive Gram negative isolates. However, **Adam and Elhag, [**28] reported that *bla$_{NDM1}$* was positive in 0% of Carbapenem sensitive Gram negative isolates.

This study, which discloses the magnitude of the dissemination of carbapenem resistance, can potentially facilitate the implementation of a proficient antibiotic stewardship program within our hospitals and the silent spread of carbapenemases in phenotypically susceptible isolates makes the implementation of strict antibiotic stewardship necessary.

In the current study, *bla$_{KPC}$* and *bla$_{NDM1}$* were positive by LAMP in the same positive isolates by PCR. This agreed with **Solanki et al., [**18] and **Moreira et al., [**30] who reported that *bla $_{NDM1}$* were positive by LAMP in the same positive isolates by PCR.

Nakano et al., [31] reported that *bla$_{KPC}$* was positive by LAMP in the same positive isolates by PCR.

However, **Chen et al., [**32] detected *bla$_{KPC}$* positive isolates by LAMP that were not detected by PCR.

This study demonstrated that the LAMP technique is a sensitive and effective method for the detection of positive samples for *bla$_{NDM1}$* and *bla$_{KPC}$*.

Conclusion

The LAMP technique is an excellent option for the rapid detection of *bla$_{NDM-1}$* and *bla$_{KPC}$* genes.PCR and DNA arrays are sensitive and specific. They need expensive equipment and experience in the analysis of the results. LAMP technique can be done without the use of thermocyclers. The time of amplification is less than other molecular techniques. It is characterized by sensitivity and specificity, easy performance and low cost It can be an excellent tool of epidemiological surveillance, especially in resource-limited settings [30].

Conflicts of interest

The authors assert that there are no conflicts of interest

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