International Journal of Botany Studies
www.botanyjournals.com
ISSN: 2455-541X
Received: 25-07-2021, Accepted: 08-08-2021, Published: 25-08-2021
Volume 6, Issue 4, 2021, Page No. 901-906

β-Lactamase producing bacterial pathogens of clinical samples detection and characterization through molecular techniques

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Abstract
To establish the predominance and genotypes of unlimited variety β-lactamases (ESBLs) amongst hospital clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*, we performed antibiotic susceptibility testing, DNA sequencing analysis. Hence, simple methods were followed herein to isolate and characterize antibiotic resistant bacteria through widespread phenotypic, morphological, biochemical and molecular characters. Among the 520 isolates collected from Prakasam (DT), Costal Andhra, Andhra Pradesh, India.
Gene sequence results in *E. coli* showed no of matches 478, in NCBI blast shows 99.31% similar to bla ndm 5 type of resistance, so below results shows genotypic conformation with ndm primer and classification of resistance type is ndm 5. bla ndm amplicon sequence of *K. pneumoniae*, based on above analysis ndm primer amplicon shows 99% similarity with bla-ndm 5 type. We confirmed that *K. pneumoniae* shows 99.09% similarity with bla ndm 5 type of resistance. *K. pneumoniae* showed no of matches 570, concluded that based on different experimental studies metallo β-lactamses resistance was identified from locally isolated *E.coli* and *K. pneumoniae* in provience of Andhra Pradesh, India.

Keywords: clinical isolates, genotypic, molecular characterzation, PCR, primer

Introduction
Antimicrobial confrontation is unsystematic and is increasing transversely dissimilar bacterial group, in opposition to every antibiotic classes, each constituency and motherland in the humanity. Predominantly in the current communicable sickness period (Shakil and Khan, 2010) [17, 18], multidrug challenging Gram -ve bacteria are most important curative confront in in cooperation hospice (Shakil *et al*., 2010) [17, 18] and population settings (Meier *et al*., 2011) [11]. Elsewhere of 6 ESKAPE pathogens, four are Gram -ve bacteria. Thus, concentration of methodical neighborhood has shifted to study antibiotic challenging Gram -ve bacteria (Bush, 2010) [3]. Enterobacteriaceae members family universally manufacture comprehensive range β-lactamases (ESBLs) that bestow confrontation to the superior invention of cephalosporins and might escort to beneficial departed split ends. ESBLs are grand apprehension since they are frequently plasmid associated (Bush, 2010) [3]. These plasmids habitually bring antimicrobial opposition genes for further antibiotics, such as aminoglycosides, tetracyclines, fluoroquinolones, chloramphenicol and sulfamethoxazole-trimethoprim. Outstandingly, in attending is a prospective menace of annoyed variety distribution of these plasmids, consequential in numerous antibiotic fighting genes organism broaden to unusual bacterial species (Ny *et al*., 2017) [14]. The β-lactamases produced through bacteria are recognized to shelter alongside the deadly produce of cephalosporins, penicillins, or monobactams on the synthesis of cell wall. β-lactamase construction is the solitary the majority widespread instrument conscientious for conflict to β-lactams in the midst of experimental isolates of Enterobacteriaceae family (Sanders *et al*., 1992) [16]. An assortment of β-lactamases confidential hooked on classes A, B, C, D based on homology of their amino acids (AAS) (Bush *et al*., 1995) [4]. Unlimited spectrums β-lactamases (ESBLs) are clavulanate disposed enzymes confer expansive confrontation to aztreonam, penicillins and cephalosporins and are detected most commonly in *Escherichia coli* and *Klebsiella pneumoniae* (Livermore, 1995) [10]. ESBLs are frequently plasmid mediated, and the majorities are mutants of the characteristic SHV and TEM enzymes, with single or supplementary AAS substitution approximately the vigorous location (Paterson *et al*., 2001) [15]. These changes consent to hydrolysis of cephalosporins extended spectrum (e.g., cefotaxime and ceftazidime) and monobactams (e.g., aztreonam), which are constant to standard SHV and TEM enzymes (DuBois *et al*., 1995) [7].
Current study to isolation, identification, distribution of antibiotic resistant genes in multi-antibiotic resistant bacteria isolated from Southern region of Prakasam (DT), Andhra Pradesh, India. Advanced methods to isolate and characterize the antibiotic resistant bacteria through molecular techniques.

Materials and methods
Sample collection
The clinical isolates of 520 samples from patients in prakasam (DT), Coastal Andhra, Andhra Pradesh, India. The samples were collected between 2015 to 2017, these bacterial isolates were identified and categorized into two species, *Escherichia coli, Klebsiella pneumonia*.

Bacterial genomic DNA isolation
Phenol/chloroform method was used for isolation, purification of bacterial genomic DNA, transfer overnight *E.*

coli (1.5 ml) culture (grown in LB medium) to a Eppendorf (1.5 ml) tube and centrifuged (10,000 rpm) for 3 min. Discarded the supernatant and lysis buffer (600 µl) was added to pellet and vortexes and incubated at 37°C for one hour. Equal volume of phenol/chloroform was added, incubated pellet and mix well through inverting the tube until the phases are completely mixed and then centrifuged (13,000 rpm) for 5. Carefully transferred the upper aqueous phase to a new tube through using pipetman (1 ml) added and then remove phenol, an equal volume of chloroform: IAA was added to the aqueous layer, mix well through inverting the tube and centrifuged (13,000) rpm for 5 min. Supernatant was collected into a new tube then added 3 volumes of cold 200 proof ice cold ethanol and kept it in -20°C for 1 hour, then centrifuged (13,000 rpm) for 15 min at 4°C. Discarded the supernatant and ethanol (70%) 1 ml was added to the DNA pellet and centrifuged at 13,000 rpm for 2 min. then DNA pellet was air dried and added T.E. buffer (50 µl) to the pellet. Isolated genomic DNA was quantified through using agarose gel (1 %) (Kamatchi *et al*., 2009; Cuzon *et al*., 2010) [9, 5].

Designed primers

Table 1

1.	TEM front P1 Primer	5'-GCGGAACCCCTATTTG-3'
	TEM-C-R-ny Primer	5'-ACC AAT GCT TAA TCA GTG AG-3'
2.	SHV OS5 Primer	5'-TTATCTCCCTGTTAGCCACC-3'
	SHV OS6 Primer	5'-GATTTGCTGATTTCGCTCGG-3'
3.	KPC-Fm imer	5'-ATGTCACTGTATCGCCGTCT-3'
	KPC-Rm Primer	5'-TTTTCAGAGCCTTACTGCCC-3'.
4.	IMP-F Primer	5'-GGAATAGAGTGGCTTAAYTCTC-3'
	IMP-R Primer	5'-GGTTTAAYAAAACAACCACC-3'
5.	NDM-F Primer	5'-GGTTTGGCGATCTGGTTTTC-3'
	NDM-R Primer	5'-CGGAATGGCTCATCACGATC-3'

Detection of antibiotic genes by PCR

BLA PCR amplification for detection the MDR antibiotic genes bla –NDM, BLA- IMP, BLA-KPC, CTX-M1, CTX-M2 and was carried out, PCR amplification of BLA-NDM/. Was carried out using the following primers (Ahmed *et al*., 2014) [1, 2], 5`-GGGCAGTCGCTTCCAACGGT-3` (forward) and 5`- GTAGTGCTCAGTGTCGGCAT-3` (reverse) with an amplicon size of 490 bp. PCR mixtures (25 µl) contained DNA (1 µl) template, master mix (12.5 µl) (Promega), each primer (1 pM), sterilized distilled water (9.5 µl). PCR amplifications were performed in effendorf Thermo Cycler using the following program for bla NDM: initial denaturation at 95 °C for 5 min, followed through 35 cycles of 45s at 94 °C, 45s of annealing at 55 °C, and 1 min of extension at 72 °C, with a final extension of 7 min at 72 °C. PCR products were run on agarose gels (1.5%), stained with ethidium bromide and visualized through UV illumination and were photographed through a UV-TEK Gel Doc device (Bora *et al*., 2014) [2].

Result and discussion

bla-ndm amplified product sequence of *E.coli*

GGCGGATGGAAGCAGGACGCATGCTGTTGGTCGATACCGCCTGGGACCGATGACCAGACCGCCCAGATCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCACGCGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGCTGGTTGCGGCGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTCAAGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGCACCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAATCTCGGTGATGCCGACACTGAAGCACTACAAC.

E. coli strain UECSK041 New Delhi metallo β-lactamase NDM5 (blaNDM5) gene, partial cds Sequence ID: MH444922.1Length: 478 Number of Matches: Range 1: 32 to 470 GenBank Graphics Next Match Previous Match Alignment statistics for match # 1 Score ExpectIdentities Gaps Strand 791 bits (428) 0.0438/442 (99%) 4/442 (0%) Plus/Plus

Table 2

<table>
<tr><td rowspan="9">Query7</td><td>GGAGGCGGCCGCGTGCTGTTGGTCGATACCGCCTGGGACCGATGACCAGACCGCCCAGA65||||||||||||| ||||||||||||||||||||||||||||||||||||||||</td></tr>
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<tr><td>CCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAATC 450</td></tr>
<tr><td>Query 426 TCGGTGAATGCCGACACATGAA 447 ||||||| |||||||| ||||| Sbjct 451</td></tr>
<tr><td>TCGGTGA-TGCCGACA-ATGAA 470</td></tr>
</table>

Ncbi blast shows 99.31% similar to bla ndm 5 type of resistance, so below results shows genotypic conformation with ndm primer and classification of resistance type is ndm five. Sequences producing significant alignments:
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Description

Max Score Total Score Query Cover E value Per. Ident Accession Select seq CP043230.1
E. coli strain Ec-050 plasmid pEc-050-NDM-5, complete sequence 789, 789, 95% 0.0-99.31% gi|1732214322|CP043230.1, Select seq LC492467.1; *E. coli* ivkd16 plasmid ivrikol16 DNA, partial sequence; 789 789 96% 0.0-99.09%. gi|1731306910|LC492467.1 Select seq LC492143.1, *E. coli* ivkdr6 plasmid pivrikol-5 DNA, partial sequence 96% 0.0 - 99.09%, gi|1731306885|LC492143.1 Select seq LC494039.1
Select seq CP042339.1, *E. coli* strain GZ04-0086 plasmid pNDM5-GZ04_1, complete sequence
96% 0.0 - 99.09% gi|1725342924|CP042339.1.
E. coli strain UECSK041 New ID Delhi metallo β-lactamase NDM5 (blaNDM5) gene, partial cds Sequence: MH444922.1 Length: 478 Number of Matches: Range 1: 32 to 470GenBankGraphicsNext Match Previous Match
Alignment statistics for match #1ScoreExpectIdentities Gaps Strand 791 bits (428) 0.0438/442 (99%) 4/442 (0%) Plus/Plus

Table 3

<table>
<tr><td rowspan="14">Query 7 GGA-</td><td>GGCGGCCGCGTGCTGTTGGTCGATACCGCCTGGGACCGATGACCAGACCGCCCAGA 65 |||
|||||||||||||||||||||||||||| ||||||||||||||||||||||| Sbjct 32</td></tr>
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Query 126</td></tr>
<tr><td>CGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTATG
185 |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| Sbjct 151</td></tr>
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210 Query 186</td></tr>
<tr><td>CCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGCTGGTTGCGGCGCAACACAGCC
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<tr><td>CCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGCTGGTTGCGGCGCAACACAGCC
270 Query 246</td></tr>
<tr><td>TGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTCA 305
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<tr><td>CCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAATC 425
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<tr><td>CCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAATC 450
Query 426</td></tr>
<tr><td></td><td>TCGGTGAATGCCGACACATGAA 447 ||||||| |||||||| ||||| Sbjct 451 TCGGTGA-TGCCGACA-ATGAA 470.</td></tr>
</table>

Bla ndm amplicon sequence of *K. pneumoniae*

GGAGTTGGAGGCGGCCGCGTGCTGTTGGTCGATAC
CGCCTGGGACCGATGACCAGACCGCCCAGATCCTC
AACTGGATCAAGCAGGAGATCAACCTGCCGGTCGC
GCTGGCGGTGGTGACTCACGCGCATCAGGACAAGA
TGGGCGGTATGGACGCGCTGCATGCGGCGGGGATT
GCGACTTATGCCAATGCGTTGTCGAACCAGCTTGC
CCCGCAAGAGGGGCTGGTTGCGGCGCAACACAGCC
TGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCA
ACCGCGCCCAACTTTGGCCCGCTCAAGGTATTTTAC
CCCGGCCCCGGCCACACCAGTGACAATATCACCGT
TGGGATCGACGGCACCGACATCGCTTTTGGTGGCT
GCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGC
AATCTCGGTGAATGCCGACACATGAAGACACCTAC
AACC.

Based on above analysis ndm primer amplicon shows 99% similarity with bla-ndm 5 type. so *K. pneumoniae* shows 99.09% similarity with bla ndm 5 type of resistance.

Sequences producing significant alignments

Select for downloading or viewing reports Description Max Score Total Score Query Cover E value Per. Ident *K. pneumoniae* NDM-5 gene for metallo-beta lactamase, complete cds 96%0.0-99.09%. *K. pneumoniae* Sab-2 blaNDM-5 gene for New Delhi metallo-beta-lactamase NDM-5, partial cds 96%0.0-99.09%.

Sequence analysis of isolate 2 (*K. pneumoniae*)

K. pneumoniae Sab-2 blaNDM-5 gene for New Delhi metallo-beta-lactamase NDM-5, partial cdsSequence ID: LC494039.1 Length: 570 Number of Matches: 1 Range 1: 68 to 505GenBankGraphicsNext MatchPrevious Match

Alignment statistics for match #1Score ExpectIdentities Gaps Strand 789 bits (427) 0.0437/441 (99%) 4/441 (0%) Plus/Plus

Table 4

Query 7 GGA-	GGCGGCCGCGTGCTGTTGGTCGATACCGCCTGGGACCGATGACCAGACCGCCCAGA 65 ||| |||||||||||||||||||||||||||| |||||||||||||||||||| Sbjct 68
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	TCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCACG 125 |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| Sbjct 127
	TCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTCGCGCTGGCGGTGGTGACTCACG 186 Query 126
	CGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTATG 185 |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| Sbjct 187
	CGCATCAGGACAAGATGGGCGGTATGGACGCGCTGCATGCGGCGGGGATTGCGACTTATG 246 Query 186
	CCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGCTGGTTGCGGCGCAACACAGCC 245 |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| Sbjct 247.
	CCAATGCGTTGTCGAACCAGCTTGCCCCGCAAGAGGGGCTGGTTGCGGCGCAACACAGCC 306 Query 246
	TGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTCA 305 |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| Sbjct 307
	TGACTTTCGCCGCCAATGGCTGGGTCGAACCAGCAACCGCGCCCAACTTTGGCCCGCTCA 366 Query 306
	AGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGCA 365 |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| Sbjct 367
	AGGTATTTTACCCCGGCCCCGGCCACACCAGTGACAATATCACCGTTGGGATCGACGGCA 426 Query 366
	CCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAATC 425 |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| Sbjct 427
	CCGACATCGCTTTTGGTGGCTGCCTGATCAAGGACAGCAAGGCCAAGTCGCTCGGCAATC 486 Query 426 TCGGTGAATGCCGACACATGA 446 ||||||| ||||||||| ||| Sbjct 487 TCGGTGA-TGCCGACAC-TGA 505.

To avoid the risk of hospital acquired and patient to patient spread of these pathogens proper hygiene should be implemented. Regular training of nursing staff should be practiced, strict infection-control measures controlling the potential spread of this resistance at a local level. Patients with a history of travel to high-risk areas and carriage of these bacteria should be sought in all hospital admissions. With limited treatment options available, slowing and preventing the spread of blaNDM is essential. Risk factors for hospital-associated infection include presence of co-morbid disease, mechanical ventilation and antibiotic exposure.

NDM producers bring several factors that are deeply disconcerting for public health worldwide.

First, the blaNDM gene has been identified in not a single species but in unrelated species (including *Acinetobacter* spp.).

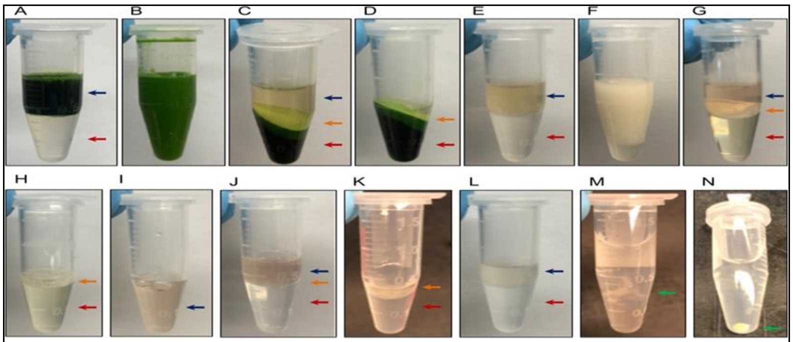

Fig. 1. Images of microcentrifuge tubes used in genomic DNA isolation from gram –ve bacteria.
Blue arrows denote aqueous (upper) phase.
While red arrows denote organic (lower) phase.
Orange arrows denote interphase material between aqueous and organic phases.
In all steps, only the aqueous phase was saved or used (i.e., the organic phase and inter phase material were discarded in each step). *Escherichia coli* cells re-suspended.

Fig 1

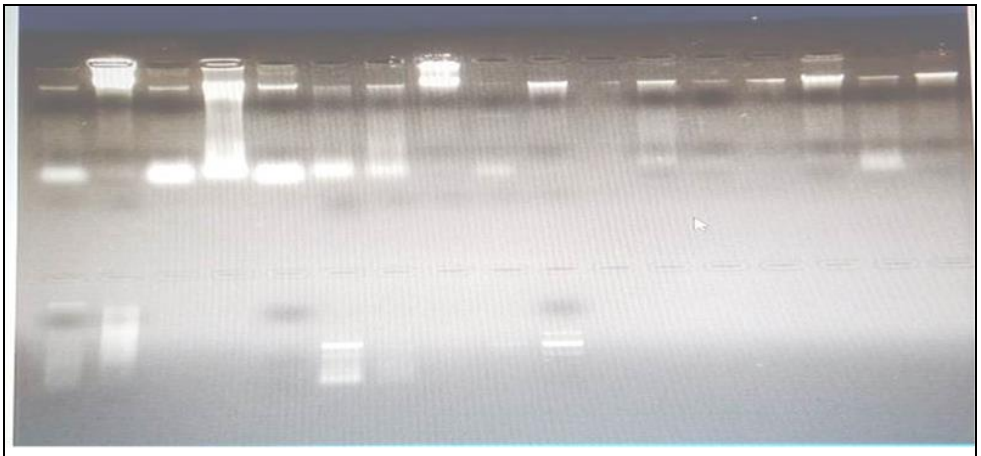

Fig. 2. DNA quantification 25 isolated DNA samples.
(Gel was done agarose gel -1%).

Fig 2

Second, it is present not only in *K. pneumoniae*, but also in *E. coli*, which is also a major community-acquired pathogen. Third, *E. coli* is also the number one cause of diarrhea in children in India. The blaNDM gene spreading in Enterobacteriaceae is an alarming risk because these novel multidrug-resistant bacteria could disseminate worldwide very quickly. The NDM-1 encoding gene is located on different large plasmids (a 180-kb plasmid for *K. pneumoniae* and a 140-kb plasmid for *E. coli*) that are easily transferable to susceptible *E. coli* J53 at a high frequency. These plasmids are also harbor genes conferring resistance to almost all antibiotics. The rapid dissemination of NDM-1 in clinically relevant bacteria has become a serious threat for therapy. In infection due to highly resistant pathogens such as NDM-positive bacteria, antimicrobial choice is limited.

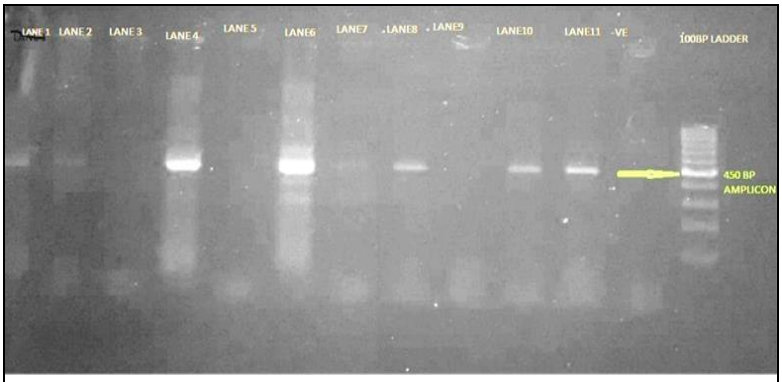

Fig. 3. Gel electrophoresis of NDM PCR products from Lane 1 to 11.
The sizes of the amplicon of NDM genes are 490 bp.
Ladder indicates 100 bp DNA.

Fig 3

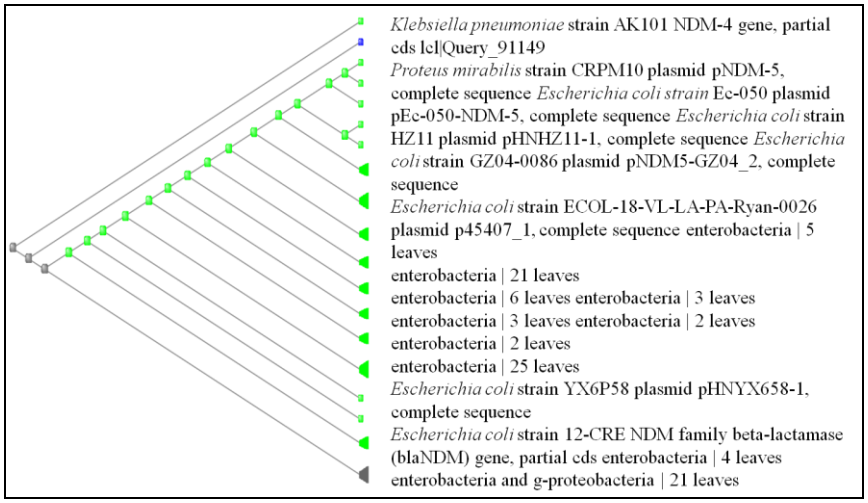

Fig 4

NDM-producing organisms frequently carry other resistance enzymes including ESBL and AmpC β-lactamases, these organisms are usually susceptible to polymyxin (including colistin and polymuxin B) and tigecycline. More importantly, carbapenem resistant bacteria such as blaNDM-1 *E. coli* and blaNDM-1 *K. pneumoniae* have become resistant to colistin (Deshpande *et al*., 2010) [6]. Tigecycline is a glycylcycline with a wide spectrum of activity against aerobic and anaerobic Gram-positive and Gram-negative bacteria. In addition, tigecycline is probably not appropriate in urinary infections owing to largely biliary excretion and low urinary levels (Nordmann *et al*., 2009) [13]. Albur's research indicated that adding tigecycline to colistin does not produce increased bacterial killing (Miriagou *et al*., 2010) [12]. Instead, it may cause antagonism at lower concentrations. The membrane-active macromolecules restore the antibacterial efficacy of tetracycline antibiotics toward blaNDM-1 *K. pneumonia* and blaNDM-1 *E. coli* clinical isolates. These findings have potentially important therapeutic implications in the management of patients with infections caused by NDM-producing pathogen (Hudson *et al*., 2014) [8].

Conclusion

Based on the study metallo β-lactamses resistance was identified from locally isolated clinical samples of *E.coli* and *kelbsiella pneumoniae*. *E. coli* showed 99.63% similar to bla ndm 5 type of gene and 0.37% mutant to ndm 5 type. In *K. pneumoniae* shows 99.09% similar to bla ndm 5 type and 0.81% mutant to ndm 5 type.

Conflict of Interest

The authors do not have any conflict of interest.

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