JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Detection of *bla*$_{NDM-1}$ gene in ESBL producing *Escherichia coli* and *Klebsiella pneumoniae* isolated from urine samples

Sohni Naeem[1], Hazrat Bilal[2], Hafsah Muhammad[1], Muhammad Asif Khan[1], Fareeha Hameed[1], Sher Bahadur[3], Tayyab Ur Rehman[1]

[1] *Institute of Basic Medical Sciences, Khyber Medical University, Peshawar, Pakistan*
[2] *Faculty of Health Sciences, Institute of Physical Sciences and Information Technology, Anhui University, Hefei PR, China*
[3] *Department of Animal Health, University of Agriculture, Peshawar, Pakistan*

Abstract

Introduction: Enterobacteriaceae such as *Escherichia coli* and *Klebsiella pneumoniae* are the most prominent bacterial species resistant to almost all commonly used antibiotics. Carbapenem is one of the last resort drugs for treating such emerging multidrug-resistant bacteria. This study aimed to detect carbapenem-resistant *bla*$_{NDM-1}$ gene in ESBL producing *E. coli* and *K. pneumoniae* isolates.

Methodology: A total of 190 *E. coli* and 350 *K. pneumoniae* isolates were screened for extended spectrumβ-lactamase (ESBL), carbapenemase and metallo β-lactamase (MBL) production via double-disk synergy test (DDST), modified Hodge test and combined-disk diffusion method. The *bla*$_{NDM-1}$ gene was detected by PCR and confirmed via Sanger sequencing method.

Results: Of the 540 isolates tested, 71.8% were found to be multidrug-resistant. Overall rate of ESBL-positive isolates were 57.89% *E. coli* and 31.42% *K. pneumoniae*. Among ESBL positive isolates, 49.09% *E. coli* and 40% *K. pneumoniae* were positive for carbapenemase production whereas MBL production was detected in 29% *E. coli* and 22% *K. pneumoniae* isolates. In MBL positive isolates, (37%) *E. coli* and (40%) *K. pneumoniae* isolates harboured *bla*$_{NDM-1}$ gene. The pair-wise DNA was aligned with the NDM-1 sequence from GenBank. The alignment score was 243 and the blast nucleotide sequencing results showed 97% sequence similarity with the sequences in GenBank for the *bla*$_{NDM-1}$ gene.

Conclusions: The *bla*$_{NDM-1}$ gene was found to be the most prevalent in urine samples. There is a dire need to conduct screening tests in hospitals and communities to find out the exact prevalence of the *bla*$_{NDM-1}$ spread in our population.

Key words: *bla*$_{NDM-1}$; carbapenemase; extended-spectrum-β-lactamase; metallo-β-lactamase; *E. coli*; *K. pneumoniae*.

J Infect Dev Ctries 2021; 15(4):516-522. doi:10.3855/jidc.12850

(Received 19 April 2020 – Accepted 08 September 2020)

Introduction

New Delhi Metallo-β-lactamase (NDM-1) type metallo-beta lactamase (MBL) is one of the most potent and prevalent enzymes of MBL in *Escherichia coli*, *Klebsiella pneumoniae* and other Gram-negative rods [1]. The *bla*$_{NDM-1}$ gene was first detected in 2009 in a Swedish patient, who acquired this gene in India by urinary tract infection, the causative agent of which was carbapenem resistant *K. pneumoniae* [2]. At first, the gene was identified in a 180kb plasmid [3,4], but later on, it was discovered that other plasmids ranged from 50-500 kb of various Gram-negative species, also harbored this gene [5]. This gene is usually present on plasmid and has the capability of transferring from one bacterium to another bacterium, from man to man and even from country to country. Poor sanitation and misuse of antibiotics provokes the spread of NDM-1 [6].

The blaNDM-1 gene is present on various plasmids, also containing other resistant genes such as ESBL genes (CTX-M-15, SHV-12), carbapenem-resistant genes or carbapenemase-encoding genes (OXA-48, OXA-181, VIM), plasmid-associated cephalosporinase genes (CMY-16, CMY-58), aminoglycoside resistance genes (16s rRNA methylase), macrolide resistance genes (esterase), rifampin resistance gene, *qnr* genes (*qnrAB*, *qnrB1*, *qnrB2*) and sulfamethoxazole resistance genes, reported from other parts of the world including Pakistan [6–9].

Carbapenem antibiotics, having a large spectrum of activity, were once considered a good option against ESBL producers but due to the emergence of resistance towards carbapenems in the form of carbapenem

hydrolyzing β-lactamases, has reduce the efficiency of carbapenems [10]. This resistance can be acquired by alteration in PBPs which is a target site for carbapenem attachment, alteration in the structure of porin proteins, carbapenem hydrolyzing β-lactamases or by increased action of efflux pumps. Several genes are also responsible for carbapenemase production, the New Delhi metallo β-lactamase-1 (bla_{NDM-1}) being one of them [11].

Carbapenem-resistant genes are rapidly increasing, and over the last few years, carbapenem resistance has become a major health issue. The high prevalence of these genes in Pakistan is due to the inappropriate use of drugs in hospitals and health care settings both public and private. Previous studies show that carbapenemase producing Gram-negative bacteria are prevalent but the magnitude of such infections has not been recorded in Peshawar, Pakistan. Whereas, a study conducted in Islamabad showed that the prevalence of this resistance is 36.7% for NDM-1 among Gram-negative bacteria [8]. The aim of the present study was to determine the prevalence and distribution of the bla_{NDM-1} gene in *E. coli* and *K. pneumoniae* isolates collected from tertiary care hospitals of Peshawar and Islamabad, Pakistan.

Methodology

Study design and sampling

This cross-sectional study involved four major tertiary care hospitals of Peshawar and Islamabad including Khyber Teaching Hospital (KTH), Combined Military Hospital (CMH), Hayatabad Medical Complex (HMC) and Pakistan Institute of Medical Sciences Islamabad (PIMS). The study was conducted during January 2018 to June 2018. A total of 540 non-duplicated clinical isolates of *E. coli* (190) and *K. pneumoniae* (350) were isolated from urine samples using standard microbiological collection techniques.

Bacterial isolates identification

The collected clinical isolates were identified as *E. coli* and *K. pneumoniae* based on morphological characteristics of bacterial colonies on Cysteine Lactose Electrolyte Deficient media (CLED) and Eosin Methylene Blue (EMB) agar media (Oxoid, UK). Further confirmation of the isolates was done by Gram staining and routine biochemical analysis such as indole test, oxidase test, urease test, citrate test and triple sugar iron test [12]. The identified isolates were stored at -80 ºC for molecular detection of resistant genes. Working cultures were maintained on nutrient agar at 8 ºC for up to four weeks.

Antimicrobial susceptibility testing

The susceptibility testing for a range of antibiotics was done by Kirby-Baur disk diffusion method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) [13]. The antibiotics used were ampicillin (AMC = 30 µg), ceftazidime (CAZ = 30 µg), cefixime (CFM = 5 µg), imipenem (IPM = 10 µg), ciprofloxacin (CIP = 5 µg), amikacin (AMK = 30 µg), ceftriaxone (CRO = 30 µg), nalidixic acid (NAL = 30 µg), amoxicillin (AMX = 10 µg), cefotaxime (CTX = 30 µg), gentamicin (CN = 30 µg), doripenem (DOR = 30 µg), levofloxacin (LEV = 5 µg), tigecycline (TGC = 15 µg), and nitrofurantoin (NIT = 10 µg). The isolates that showed resistance to more than three antibiotics together with beta-lactams, were processed further.

Detection of extended-spectrum-β-lactamases (ESBL)

ESBL detection was done by double disk synergy test using Muller Hinton Agar (MHA) media (Oxoid, UK), according to CLSI guidelines [13]. The antibiotic disks used were ceftriaxone (CRO 30 µg), cefotaxime (CTX 30 µg), ceftazidime (CAZ 30 µg), cefepime (FEP 30 µg) and amoxicillin+ clavulanic acid (AUG 20 + 10 µg). The agar plates were incubated at 37 ºC for 18 hours. An increase of ≥ 5 mm in the zone diameter of any of the antibiotic disks used towards the center was considered as ESBL positive [14]. For control strains, DDST positive *Pseudomonas aeruginosa* (ATCC 27853) strain was used as positive control whereas DDST negative *Pseudomonas aeruginosa* strain as a negative control.

Detection of carbapenemases

Modified Hodge test (MHT) was performed using MHA agar media along with 10 µg Ertapenem disk for the detection of carbapenemases production [15]. After 18 hours of incubation at 37 ºC, plates were observed for the appearance of a clover leaf-like indentation at the intersection point between the test organism and the indicator organism. The *K. pneumoniae* (ATCC BAA-1705**)** MHT positive was used as a positive control whereas *K. pneumoniae* (DSMZ 9377) MHT negative as a negative control.

Detection of metallo-β-Lactamases

MBLs were identified using combined disk diffusion method on MHA media along with 10 µg of meropenem disk and 0.5 M EDTA as an indicator. This procedure was conducted following a previously published method [16,17]. After 18-24 hours incubation time, the plates were observed for a ≥ 7 mm increase in the zone of inhibition of the combination disks

(meropenem + EDTA) as compared to meropenem disk alone in order to identify MBL positive isolates. The positive control used was MBL positive *Pseudomonas aeruginosa*, and the negative control was MBL negative *Pseudomonas aeruginosa* ATCC 27853 strain.

Molecular identification of bla$_{NDM-1}$ gene by PCR

The Plasmid DNA was extracted by alkaline lysis method [18]. The quantity and quality of the extracted DNA was determined by using Nanodrop (courtesy of Forman Christian College Biotechnology laboratory) followed by gel electrophoresis to visualize the quality and the expected size of plasmid DNA. Amplification of the DNA was done using specific primers for *bla*$_{NDM-1}$ gene, as shown in Table 1. The PCR cycling conditions were: 94ºC for 4 minutes, 35 cycles of 94 ºC for 4 seconds, 55 ºC for 40 seconds and 72 ºC for 60 seconds followed by 72 ºC for 10 minutes.

Plasmid stability assay

The plasmids of *bla*$_{NDM-1}$ gene carrying isolates were verified for its permanency. Sub-culturing of the isolates was carried out for six consecutive days on MHA media without meropenem and with meropenem disk at 0.5 µg/mL. After every sub-culturing, phenotypic detection of carbapenem resistance and presence of plasmid DNA on 0.7% agarose gel was observed.

Nucleotide sequence interpretation

The PCR products of two NDM-1 positive isolates (one *E. coli* and one *K. pneumoniae*) were sent to Macrogen (Korea), an external expertise provider. The nucleotide sequences and the presumed protein sequences were examined with Basic Local Alignments Search Tool (BLAST) [19].

Results

Clinical bacterial isolates

Out of 540 clinical isolates collected from different hospitals in Peshawar and Islamabad, 190 (35.2%) were identified as *E. coli* and 350 (64.8%) as *K. pneumoniae* being the most predominant species. The number and frequency of the isolates collected from different hospitals are shown in Table 2.

Antimicrobial susceptibility

In *E. coli* isolates, highest resistance was observed towards ceftriaxone (74.7%), followed by ampicillin (74.2%) and cefotaxime (69.4%), whereas imipenem (94.7%), doripenem (88.4%), and nitrofurantoin (86.8%) are found to be the most effective drugs in the present study. In *K. pneumoniae* isolates, lowest susceptibility rate was observed towards amoxicillin (81.7%), ampicillin (77.7%) and cefixime (71.7%). However, *K. pneumoniae* isolates showed highest susceptibility towards imipenem (98.2%) and doripenem (90%) respectively (Table 3). The overall percentage of MDR among the 540 bacterial isolates was 71.8 %. The MDR percentage was dominated in *K. pneumoniae* (73.1%) as from *E. coli* isolates (69.4%).

Prevalence of ESBL, Carbapenemase and MBL producing E. coli and K. pneumoniae

Of the total 540 isolates, 57.89% *E. coli* and 31.42% *K. pneumoniae* were ESBL positive with DDST. Among 220 ESBL positive isolates of *E. coli* and *K. pneumoniae*, 49.09% *E. coli* and 40% *K. pneumoniae* were positive for carbapenemase production. Among 98 MHT positive isolates, 29% *E. coli* and 22% *K. pneumoniae* were phenotypically positive for MBL production (Table 4).

PCR results

Out of 26 MBL positive *E. coli* and *K. pneumoniae*, 10 were found to harbor the *bla*$_{NDM-1}$ gene by PCR amplification. The details and characteristic profiles of NDM-1 positive isolates are given in Table 4 and Table 5.

Plasmid stability assay

The plasmid stability was confirmed by repeated sub-culturing of the resistant isolates of *E. coli* and *K. pneumoniae*. The plasmid isolated after every subculture was of the same size on 0.7% agarose gel.

Sequence interpretation

The pairwise DNA was aligned with the NDM-1 sequence taken from NCBI using the Sequence Manipulation Suite, a Pair-wise aligned DNA online software. The alignment score was 243, which showed similarity with NDM-1 gene.

Table 1. Primers for amplification of blaNDM-1 gene.

Sr. No	Target Gene	Primers	Primer Sequences (5'- 3')	Amplicon Size (bp)	References
1	*bla*$_{NDM-1}$	NDM F* NDM R†	GGGCAGTCGCTTCCAACGGT GTAGTGCTCAGTGTCGGCAT	475bp	[15]

* Forward; † Reverse.

Table 2. Number of *E. coli* and *K. pneumoniae* strains isolated from hospitals.

Hospitals	*E. coli*	*K. pneumoniae*
KTH	70	136
CMH	40	46
HMC	30	64
PIMS	50	104
Total	190	350

KTH = Khyber Teaching Hospital; CMH = Combined Military Hospital; HMC = Hayatabad Medical Complex; PIMS = Pakistan Institute of Medical Sciences Islamabad.

Table 3. Antibiotic susceptibility pattern of *E. coli* and *K. pneumoniae* isolates.

Antibiotics	Bacterial isolates			
	E. coli (n = 190)		*K. pneumoniae* (n = 350)	
	Susceptible (%)	Resistant (%)	Susceptible (%)	Resistant (%)
Ampicillin	25.7	74.2	22.2	77.7
Ceftazidime	53.6	46.3	65.1	34.8
Cefixime	41.05	58.9	28.2	71.7
Ciprofloxacin	52.1	47.8	67.4	32.54
Amikacin	48.4	51.5	48	52
Ceftriaxone	25.2	74.7	35.4	64.5
Nalidixic acid	38.9	61	30.8	69.1
Amoxicillin	65.7	34.2	18.2	81.7
Cefotaxime	30.5	69.4	46.8	53.1
Cefalexin	42.1	57.8	78.8	21.1
Doripenem	88.4	11.5	90	10
Levofloxacin	56.8	43.1	44.2	55.7
Tigecycline	40	60	29.4	70.5
Nitrofurantoin	86.8	13.1	62.8	37.1
Imipenem	94.7	5.2	98.2	1.71

Table 4. Prevalence of ESBL, carbapenemase, MBL-producing and blaNDM-1 positive *E. coli* and *K. pneumoniae* isolates.

Bacterial spp.	ESBL positive/ total isolates	MHT positive/ ESBL positive isolates	MBLpositive / MHT positive isolates	NDM-1 positive/ MBLpositive isolates
E. coli	110/190 (57.89%)	54/110 (49.09%)	16/54 (29%)	6/16 (37%)
K. pneumoniae	110/350 (31.42%)	44/110 (40%)	10/44 (22%)	4/10 (40%)

Table 5. Characteristic profile of NDM-1 positive *E. coli* and *K. pneumoniae* isolates.

Bacteria	Isolate no	Hospital	ESBL	MHT	MBL	NDM-1 gene
E. coli	EK10	KTH	Positive	Positive	Positive	Present
	EK14	KTH	Positive	Positive	Positive	Present
	EK33	KTH	Positive	Positive	ND	Present
	EH09	HMC	Positive	Positive	Positive	Present
	EH27	HMC	Positive	Positive	ND	Present
	EP23	PIMS	Positive	Positive	Positive	Present
K. pneumoniae	KPK11	KTH	Positive	Positive	ND	Present
	KPH06	HMC	Positive	Positive	Positive	Present
	KPH29	HMC	Positive	Positive	Positive	Present
	KPH55	HMC	Positive	Positive	Positive	Present

KTH = Khyber Teaching Hospital; HMC = Hayatabad Medical Complex; PIMS = Pakistan Institute of Medical Sciences; ESBL = Extended Spectrum beta-lactamase; MHT = Modified Hodge test; MBL = Metallo beta-lactamase; NDM = New Delhi Metallo beta-lactamase; ND = non-determinable.

Furthermore, this sequence was entered in BLAST on NCBI. The blast nucleotide sequencing showed 99% similarity with *E. coli* (accession number MT367572.1) and 98% similarity with *K. pneumoniae* (accession number MT320914.1), containing the NDM-1 gene.

Nucleotide accession numbers

The nucleotide sequences of *E. coli* and *K. pneumoniae* isolates carrying bla_{NDM-1} gene have been submitted in GenBank under accession numbers MK576132 and MK569761 respectively.

Discussion

Regulatory authority's negligence, poor enforcement efforts and the distribution of antibiotics by poorly trained people are the factors involved in the emergence of antibiotic resistance. The trend of using antibiotics is on rise in many countries [20]. Hence, inappropriate use leads to the selection of resistant bacterial strains that produce infections that are very difficult to treat, resulting in financial burden on health sectors and on society [21].

The emergence of co-resistance of ESBLs along with other β-lactamases is a serious issue worldwide. The bla_{NDM-1} gene has already been reported along with other ESBL producing isolates from Pakistan [8]. In the present study we looked for the occurrence of bla_{NDM-1} gene in ESBL producing isolates from Peshawar and Islamabad Pakistan.

Here we reported 110 (31.42%) isolates of *K. pneumoniae* as ESBL producers, the same percentage (31.7%) has been reported from India in 2014 [22]. The ESBL ratio in our region is one of the highest ESBL infections reported as compared to Canada (5%), United States (8%), Europe (23%) and Pacific Western region (25%). However, Farhat Ullah *et al*. in 2009 reported this as 54 (58.5%) [23], which is higher as compared to our study. Other studies reported 60.4% ESBL producers in Iran [24] and 50% in Israel [25]. The contradictions among the reported findings could be due to inadequate antibiotic therapy which might have resulted in an increase in the ESBL prevalence [26,27].

The number of ESBL samples of E. coli in our study is 110 (57.89%), which is in accordance with the study reported in India as 52.49% [28]. However, the results from Pakistan and India are higher than those observed in Tanzania 15.1% [29], Nepal 9.0% [30], and the Libyan community 13.4% [31]. This wide variation in prevalence is probably due to the type of specimens collected from different.

We report highest percentage of 49.09% of MHT positive *E. coli* isolates and 40% *K. pneumoniae* isolates which varies from other studies such as 30.9% isolates in India in 2011 [32] and 86.8% isolates reported in Madagascar [33]. Romana *et al*. reported 28.7% *K. pneumoniae* and 20.3% *E. coli* as MHT positive isolates [34]. Difference in this ratio could be due to the inclusion of carbapenem resistant isolates only in the above-mentioned studies, while we used clinical isolates specifically from patients with urinary tract infections, followed by checking their resistant pattern.

In our study, 29% *E. coli* and 22% *K. pneumoniae* isolates were reported as MBL producers. China reported 23.3% MBL producing *K. pneumoniae* in 2012 that had significantly increased from 2005 (0.91%) to 2009 (12.8%) [35]. Some studies vary from our study such as a study conducted in 2010 reported 76.8% *E. coli* as MBL producers [36]. Another study in 2011 showed 61.11% *K. pneumoniae* and 57.69% of *E. coli* as MBL producers [37]. Ranjan *et al*. in 2016 showed 8% of *E. coli* as MBL producers [38].

In this study 37% MBL producing *E. coli* and 40% MBL producing *K. pneumoniae* were positive for bla_{NDM-1} gene. A study in Pakistan conducted by Nahid *et al*. in 2013 reported 41% *K. pneumoniae* isolates with only 9.0 harboring NDM-1 gene [8]. Recently, a five-year surveillance report of 2010-2014 indicates increased predominance of NDM-1 gene in *K. pneumoniae*, this percentage being 7.1% in 2010, 10.8% in 2011, 39.3% in 2012, 47.5% in 2013 and 63.0% in 2014 [39]. This high increase in the percentages could be due to the difference in sample size or origin of samples.

Conclusions

In the present study, the emergence of NDM-1 gene producing *E. coli* and *K. pneumoniae* highlights an urgent need to overcome the over-the-counter availability and the inappropriate use of antibiotics both in public and private health care settings. The drug regularity authority of Pakistan (DRAP) should develop an implementable monitoring and evaluation system to tackle this issue. Besides, alternative therapies should be explored and assessed for treating and preventing carbapenem resistant infections.

Acknowledgements

The authors are thankful to the Institute of Basic Medical Sciences, Khyber Medical University Peshawar, Khyber Pakhtunkhwa, Pakistan, for supporting and facilitating this work.

Authors' Contributions

SN, TUR: study concept and design; SN: acquisition of data; SN, HB: analysis and interpretation of data; SN, MAK, FH: drafting of the manuscript; TUR, HM, SB: critical revision of the manuscript for important intellectual content; TUR: study supervision.

References

1. Bharadwaj R, Joshi S, Dohe V, Gaikwad V, Kulkarni G (2012) Prevalence of New Delhi metallo-β-lactamase (NDM-1)-positive bacteria in a tertiary care centre in Pune, India. Int J Antimicrob Agents 39: 265–266.
2. Cantón R, Akóva M, Carmeli Y, Giske CG, Glupczynski Y, Gniadkowski M, Livermore DM, Miriagou V, Naas T, Rossolini GM (2012) Rapid evolution and spread of carbapenemases among Enterobacteriaceae in Europe. Clin Microbiol Infect 18: 413–431.
3. Zenati F, Barguigua A, Nayme K, Benbelaïd F, Khadir A, Bellahsene C, Bendahou M, Hafida H, Timinouni M (2019) Characterization of uropathogenic ESBL-producing *Escherichia coli* isolated from hospitalized patients in western Algeria. J Infect Dev Ctries 13: 291–302. doi: 10.3855/jidc.10702.
4. Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, Walsh TR (2009) Characterization of a new metallo-β-lactamase gene, *bla*$_{NDM-1}$, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. Antimicrob Agents Chemother 53: 5046–5054.
5. Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Butt F, Balakrishnan R, Chaudhary U, Doumith M, Giske CG, Irfan S, Krishnan P, Kumar AV, Maharjan S, Mushtaq S, Noorie T, Paterson DL, Pearson A, Perry C, Pike R, Rao B, Ray U, Sarma JB, Sharma M, Sheridan E, Thirunarayan MA, Turton J, Upadhyay S, Warner M, Welfare W, Livermore DM, Woodford N (2010) Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. Lancet Infect Dis 10: 597–602.
6. Monaco M, Giani T, Raffone M, Arena F, Garcia-Fernandez A, Pollini S, Network EuSCAPE-Italy, Grundmann H, Pantosti A, Rossolini GM (2014) Colistin resistance superimposed to endemic carbapenem-resistant *Klebsiella pneumoniae*: a rapidly evolving problem in Italy, November 2013 to April 2014. Euro Surveill 19: 20939.
7. Zhang X, Lou D, Xu Y, Shang Y, Li D, Huang X, Li Y, Hu L, Wang L, Yu F (2013) First identification of coexistence of *bla*$_{NDM-1}$ and *bla*$_{CMY-42}$ among *Escherichia coli* ST167 clinical isolates. BMC Microbiol 13: 282.
8. Nahid F, Khan AA, Rehman S, Zahra R (2013) Prevalence of metallo-β-lactamase NDM-1-producing multi-drug resistant bacteria at two Pakistani hospitals and implications for public health. J Infect Public Health 6: 487–493.
9. Ejaz H, Wang N, Wilksch JJ, Page AJ, Cao H, Gujaran S, Keane JA, Lithgow T (2017) Phylogenetic analysis of *Klebsiella pneumoniae* from hospitalized children, Pakistan. Emerg Infect Dis 23: 1872.
10. Codjoe FS, Donkor ES (2018) Carbapenem resistance: a review. Med Sci 6: 1.
11. Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ (2015) Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol 13: 42–51.
12. Ejaz H (2013) Detection of extended-spectrum β-lactamases in Klebsiella pneumoniae: Comparison of phenotypic characterization methods. Pak J Med Sci 29: 768.
13. Clinical and Laboratory standard institute (CLSI) (2018) Performance Standards for Antimicrobial Susceptibility Testing 28th Edition.CLSI document M100-S28 (ISBN: 1-56238-838-X).
14. Rawat D, Nair D (2010) Extended-spectrum β-lactamases in Gram Negative Bacteria. J Glob Infect Dis 2: 263–274.
15. Ejaz H, Alzahrani B, Hamad MF, Abosalif KO, Junaid K, Abdalla AE, Elamir MY, Aljaber NJ, Hamam SS, Younas S (2020) Molecular Analysis of the Antibiotic Resistant NDM-1 Gene in Clinical Isolates of Enterobacteriaceae. Clin Lab 66: 190727.
16. Lee K, Lim YS, Yong D, Yum JH, Chong Y (2003) Evaluation of the Hodge test and the imipenem-EDTA double-disk synergy test for differentiating metallo-beta-lactamase-producing isolates of *Pseudomonas* spp. and *Acinetobacter* spp. J Clin Microbiol 41: 4623–4629.
17. Pasteran F, Mendez T, Rapoport M, Guerriero L, Corso A (2010) Controlling false-positive results obtained with the Hodge and Masuda assays for detection of class a carbapenemase in species of enterobacteriaceae by incorporating boronic. Acid. J Clin Microbiol 48: 1323–1332.
18. Feliciello I, Chinali G (1993) A modified alkaline lysis method for the preparation of highly purified plasmid DNA from *Escherichia coli*. Anal Biochem 212: 394–401.
19. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403–410.
20. Lee JH, Park KS, Karim AM, Lee C-R, Lee SH (2016) How to minimise antibiotic resistance. Lancet Infect Dis 16: 17–18.
21. Seid M, Dekebo A, Babu N (2018) Phytochemical Investigation and Antimicrobial Evaluation of Foeniculum vulgare Leaves Extract Ingredient of Ethiopian Local Liquor. J Pharm Nutr Sci 8: 20–28.
22. N G M, Math GC, Nagshetty K, Patil SA, Gaddad SM, Shivannavar CT (2014) Antibiotic Susceptibility Pattern of ESβL Producing *Klebsiella pneumoniae* Isolated from Urine Samples of Pregnant Women in Karnataka. J Clin Diagn Res 8: DC08-11.
23. Ullah F, Malik SA, Ahmed J (2009) Antimicrobial susceptibility pattern and ESBL prevalence in *Klebsiella pneumoniae* from urinary tract infections in the North-West of Pakistan. Afr J Microbiol Res 3: 676–680.
24. Tahanasab Z, Mobasherizadeh S, Moghadampour M, Rezaei A, Maleki N, Faghri J (2016) High Prevalence of Multiple Drug Resistance among ESBLs-Producing *Klebsiella pneumoniae* Isolated from Hospitalized Patients in Isfahan, Iran. J Med Bacteriol 5: 29–38.
25. Cantón R, Novais A, Valverde A, Machado E, Peixe L, Baquero F, Coque TM (2008) Prevalence and spread of extended-spectrum β-lactamase-producing Enterobacteriaceae in Europe. Clin Microbiol Infect 14: 144–153.
26. Heinz E, Ejaz H, Scott JB, Wang N, Gujaran S, Pickard D, Wilksch J, Cao H, Haq I, Dougan G (2019) Resistance mechanisms and population structure of highly drug resistant *Klebsiella* in Pakistan during the introduction of the carbapenemase NDM-1. Sci Rep 9: 1–13.
27. Paterson DL, Bonomo RA (2005) Extended-spectrum beta-lactamases: a clinical update. Clin Microbiol Rev 18: 657–686.

28. Sharma M, Pathak S, Srivastava P (2013) Prevalence and antibiogram of Extended Spectrum β-Lactamase (ESBL) producing Gram negative bacilli and further molecular characterization of ESBL producing *Escherichia coli* and *Klebsiella* spp. J Clin Diagn Res7: 2173–2177.
29. Mshana SE, Falgenhauer L, Mirambo MM, Mushi MF, Moremi N, Julius R, Seni J, Imirzalioglu C, Matee M, Chakraborty T (2016) Predictors of $bla_{CTX-M-15}$ in varieties of *Escherichia coli* genotypes from humans in community settings in Mwanza, Tanzania. BMC Infect Dis 16: 187.
30. Shakya P, Shrestha D, Maharjan E, Sharma VK, Paudyal R (2017) ESBL production among *E. coli* and *Klebsiella* spp. causing urinary tract infection: a hospital based study. Open Microbiol J 11: 23-30.
31. Ahmed SF, Ali MMM, Mohamed ZK, Moussa TA, Klena JD (2014) Fecal carriage of extended-spectrum β-lactamases and AmpC-producing *Escherichia coli* in a Libyan community. Ann Clin Microbiol Antimicrob 13: 22.
32. Mulla S, Charan J, Panvala T (2011) Antibiotic sensitivity of Enterobacteriaceae at a tertiary care center in India. Chron Young Sci 2: 214.
33. Andriamanantena TS, Ratsima E, Rakotonirina HC, Randrianirina F, Ramparany L, Carod J-F, Richard V, Talarmin A (2010) Dissemination of multidrug resistant *Acinetobacter baumannii* in various hospitals of Antananarivo Madagascar. Ann Clin Microbiol Antimicrob 9: 17.
34. Ramana KV, Rao R, Sharada CV, Kareem M, Reddy LR, Ratna Mani M (2013) Modified Hodge test: A useful and the low-cost phenotypic method for detection of carbapenemase producers in Enterobacteriaceae members. J Nat Sci Biol Med 4: 346–348.
35. Hu F, Chen S, Xu X, Guo Y, Liu Y, Zhu D, Zhang Y (2012) Emergence of carbapenem-resistant clinical Enterobacteriaceae isolates from a teaching hospital in Shanghai, China. J Med Microbiol 61: 132-136.
36. Franco MRG, Caiaffa-Filho HH, Burattini MN, Rossi F (2010) Metallo-beta-lactamases among imipenem-resistant *Pseudomonas aeruginosa* in a Brazilian university hospital. Clinics (Sao Paulo) 65: 825-829.
37. Vipin K, Rohit KM, Avantika C, Pramila G (2011) Incidence of β-lactamase producing gram-negative clinical isolates and their antibiotic susceptibility pattern: A case study in Allahabad. Int J Res Pure Appl Microbiol 1: 36–39.
38. Ranjan A, Ahmed N (2016) Genotypic, phenotypic and functional profiles of NDM harboring Carbapenem resistant *Escherichia coli* from India. Int J Infect Dis 45: 241.
39. Fang D, Jie LU, Yan W, Jin SHI, Zhen JH, Ping CHU, Yang Z, Han SJ, Guo YL, Song WQ (2017) A five-year surveillance of carbapenemase-producing Klebsiella pneumoniae in a pediatric hospital in China reveals increased predominance of NDM-1. Biomed Environ Sci 30: 562–569.

Corresponding author

Tayyab Ur Rehman, Dr.
Institute of Basic Medical Sciences, Khyber Medical University, Phase 5 Hayatabad, Peshawar, Khyber Pakhtunkhwa, Pakistan.
Phone: 0092-91-5892873
Email: tayyab.ibms@kmu.edu.pk

Conflict of interests: No conflict of interests is declared.