http://jmscr.igmpublication.org/home/
ISSN (e)-2347-176x ISSN (p) 2455-0450
crossref DOI: https://dx.doi.org/10.18535/jmscr/v7i12.91

IGM Publication
Journal Of Medical Science And Clinical Research
An Official Publication Of IGM Publication

Original Article

Extended and Pan-drug resistance in *Klebsiella pneumoniae* due to Carbapenemase and Extended Spectrum β-lactamase enzymes

Authors
Lokhande Suman R.[1], Pawar Sunil T.[*2], Karad Dilip. D.[3]
[1,3]Ph.D., Associate Professor, [2]M.Sc., Associate Professor
[1,3]Department of Microbiology, Shri Shivaji Mahavidyalaya, Barshi – 413 411
[2]Department of Microbiology, Tuljaram Chaturchand College of Arts, Science & Commerce, Baramati – 413102
*Corresponding Author
Prof. Sunil T Pawar
Department of Microbiology, Tuljaram Chaturchand College of Arts, Science & Commerce, Baramati – 413102, India

Abstract
Purpose: *Emergence of resistance to multiple antimicrobial agents in pathogenic bacteria has become a significant public health threat as there are fewer, or even sometimes no effective antimicrobial agents available for infections caused by these bacterial pathogens. Infections caused by carbapenem-resistant Klebsiella pneumoniae (CRKP) and Extended Spectrum β-lactamase (ESBL) enzyme is a worldwide problem associated with high rates of treatment failure and mortality. Objective of the present investigation is to study molecular characterization of K. pneumoniae resistant due to ESBL and carbapenemase enzymes and other mechanism of antibiotic resistant.*
Methods: *Three hundred and eleven K. pneumoniae cultures were isolated and identified from clinical samples from hospitals in Barshi-Solapur, Maharashtra, India. Antibiotic susceptibility was determined by manually as well as by Vitek-2 machine. The enzymatic β-lactam resistance mechanism was confirmed through specific β-lactamase gene PCR genotype determination.*
Results: *ESBL resistance was observed in 310 (88.57%) isolates and carbapenemase in 181 (51.71%) isolates were the main mechanism of antibiotic resistance. We found 29 (8.28%) isolates K. pneumoniae pan-drug resistant meaning resistant to all antibiotics and 52 (14.85%) isolates susceptible to colistin only. We found extreme drug resistance in 135 (38.57%) of the K. pneumoniae isolates. Majority of K. pneumoniae isolated were resistant to all antimicrobial agents denoting prevalence of high antibiotic resistance.*
Conclusion: *Carbapenemase and Extended Spectrum β-lactamase enzymes are responsible for extended and pan-drug resistance in K. pneumoniae isolated from clinical samples. PCR assay for genes in six isolates were detected and responsible for the drug resistance.*
Keywords: *Klebsiella pneumoniae, Vitek-2, Drug resistance, ESBL, Carbapenemase.*

Introduction

Klebsiella pneumoniae is a common opportunistic and nosocomial organism capable of causing serious infection. Bacterial resistance to antibiotics has become a major public health issue worldwide. The reality of threat of antibiotic resistance was

acknowledged in the WHO 2014 report (www.who.int/drug resistance/en). Resistance to carbapenems in these species is related either to combined mechanisms of resistance (over-expression of broad-spectrum ß-lactamases together with efflux pumps and impermeability) or expression of carbapenem-hydrolyzing ß-lactamases, known as carbapenemases. In *Enterobacteriaceae*, carbapenemases represent the most important mechanism of resistance, since the carbapenemase genes are mostly plasmid-encoded, associated with multi- or pan-drug resistance and are highly transferable, at least within the enterobacterial species, making them potentially responsible for outbreaks [1]. Infections caused by the ESBL results in multi-drug resistance (MDR) in *Enterobacteriaceae* especially *K. pneumoniae* are now resistant to a broad range of β-lactams, including third generation cephalosporins. The nosocomial infections caused by these ESBL producing MDR *K. pneumoniae* has complicated the therapy treatment options.

The global increased prevalence of ESBL-producing bacteria creates an urgent need for laboratory diagnostic methods that will accurately and rapidly identify the presence of ESBL phenotypes in clinical isolates. The VITEK 2 AES System appears a reliable tool for the detection and interpretive reading of clinically important mechanisms of resistance and can be recommended for routine work[2]. The VITEK 2 Advanced Expert System (AES) is an automated system that uses the antimicrobial susceptibility data generated to suggest the phenotype of the tested isolate. It has been used successfully to determine presence of ESBL in *Klebsiella* spp. The Vitek 2 System (bio Merieux, France) is a rapid automated microbiological system used for bacteria, antimicrobial susceptibility testing (AST), resistance mechanism detection and epidemiologic trending and reporting using its advanced expert system. Consequently, Vitek 2 system is a reliable semi-automated microbiology system which may be used for routine, accurate and rapid detection of ESBL strains of clinical importance.

K. pneumoniae is becoming resistance for the β-lactams and cephalosporins in the western part of India. The proportions of resistant *K. pneumoniae* isolates were above 84.9% during 2004-2014. *Klebsiella* spp. isolates exhibited carbapenem resistance levels as high as 84.1% [3]. Hence there is need to study non-susceptibility of *K. pneumoniae* in the area of the investigation, Barshi Dist-Solapur, a rural part of Maharashtra.

Objectives of present investigation are to find out resistance to β-lactam antibiotics in clinical samples and to find out mechanism of antibiotic resistance by molecular methods and also by using Vitek-2 machine and to assess antibiotic resistance towards second, third and fourth generation cephalosporins and carbapenems and other antibiotics in the area of investigation.

Material and Methods

Patients

All the samples included in this study were collected from Dr. Jagdale Mama Hospital, Barshi, Dist. Solapur, Maharashtra State, India. The isolates were collected during March to December 2017. In all, 311 K. *pneumoniae* were isolated from clinical samples viz. urine (121), pus (117), sputum (40), stool (15), Blood (12), Tracheal secretion (4), and ascitic fluid (2).

Susceptibility Test Determination

The isolates were grown on blood agar and Mac Conkey's agar and preserved in glycerol for further analysis. Identification of the clinical isolates was established by Vitek-2 (bioMérieux) system [4]. The average analysis time by Vitek-2 was 9.56 minutes with maximum 18 minutes and minimum of 3.75 minutes. Antimicrobial susceptibility testing was done on Mueller-Hinton agar using the disc diffusion method and interpretation of results were done according to the recommendation of Clinical and Laboratory Standards Institute [5].The choice of antibiotics was based on routine antimicrobials used for gram negative bacteria. The β-lactams and carbapenem antibiotics tested were at the concentrations indicated: Ampicillin, ticaracillin, piperacillin, cefoperazone / sulbactum, cefazolin,

ceftriaxone, cefuroxime axetil, ceftazidime, cefuroxime, aztreonam, ampicillin / sulbactam, cefepime, amoxicillin/clavulanic acid, ciperacillin / tazobactam, and carbapenems imipenem, meropenem, doripenem and ertapenem antibiotics/drugs were tested. All the antibiotic discs were obtained from HI Media Ltd, Mumbai, India.

Susceptibilities of all the isolates were done according to CLSI (M07-A10) recommended broth micro-dilution method (Table 1). Briefly, minimum inhibitory concentrations (MIC) were determined for the commonly prescribed antibacterial agents as well as against certain novel combination agents like amoxicillin-clavulanic acid, ampicillin-sulbactam etc. The drugs used for MIC determination were ceftazidime alone and in combination with clavulanic acid or avibactam at 4 mg/L, cefepime alone and in combination with tazobactam at 8 mg/L, piperacillin in combination with tazobactam at 4 mg/L imipenem alone and in combination with salbactam at 4 mg/L, meropenem alone and in combination with EDTA-200 mg/L, amikacin, levofloxacin, tigecycline, colistin and sulfamethoxazole-trimethoprim (Table 3). All the MIC's were determined in triplicate.

Antimicrobial Susceptibility Testing (AST) and Microbial Identification by Vitek-2

All clinical isolates were subjected to Antibiotic Susceptibility Testing (AST) using gram-negative (GN) AST and identification cards for the Vitek 2 Compact system (bioMerieux, France) following the manufacturer's protocol. AST-GN69 and AST- XN06 Vitek 2 Gram negative cards were used for AST, and for microbial identification, Vitek 2 GN ID card was used. Briefly, the clinical isolates were sub-cultured onto nutrient agar and incubated for 18-24 hours at 37°C in incubator. A cell suspension of each sample with optical density of 0.5 – 0.63 McFarland Standard was prepared. The suspension was loaded onto the ID and AST cards in the biological safety cabinet, and then transferred to the Vitek 2 Compact machine for analysis. The results of the susceptibility profile were analyzed on the Vitek 2 Advanced Expert system (AES) version 5.04 (bioMerieux) according to the U.S. Food and Drug Administration (FDA) (previous CLSI breakpoints) and the Current CLSI carbapenem susceptibility breakpoints. Furthermore, the AES was applied to our analysis to determine the phenotype of ESBL and carbapenem resistance implicated in our isolates. AES uses the knowledge base of the Vitek 2, to determine resistance profile, resistance phenotype, and therapeutic interpretation of the results. AES uses all information available rather than MIC values alone to determine resistance [4].

Genotype Determination

The enzymatic β-lactam resistance mechanism was confirmed through specific β-lactamase gene PCR. The primers used were as reported in the literature and shown in Table 2. Primers used for PCR assay were as per Table 2 [6-8].

Results

Of the total 311 K. pneumoniae isolates 17 *K. pneumoniae* isolates were wild type and sensitive to most of the antibiotics and no antibiotic resistance mechanism was detected by Vitek-2. Mechanisms of antibiotic resistance predominantly observed were ESBL in 288 (92.60%) isolates. Other β-lactam antibiotic resistance mechanisms found are listed in Figure 1. Clavulanate, sulbactam, and tazobactam have been used extensively for the last 30 years, together with β-lactam antibiotics, to inhibit the effect of β-lactamases. Mechanism of drug resistance observed was for Impermeability (cephamycins) in 130 isolates (41.80%), ESBL CTX-M like in 105 (33.76%), carbapenemase (metallo or KPC) in 75 (24.12%), resistant carbapenems (impermeability) in 75 (24.12%), HL cephalosporinase (AmpC) in 49 (15.76%), acq. cephalosporinase except ACC-1) in 37 (11.90%), New Delhi Metalloprotease (NDM-1) in 23 (7.40%), Acq. Penicillinase in 21 (6.75%), SHV1 hyperproduction in 9 (2.89%), Penicillinase

in 9 (2.89%), Inhibitor Resistant PASE (IRT or OXA) in 9 (2.89%) and ESBL OXA-30 like in 2 (0.64%) (Figure 1).

Ticarcillin is a carboxypenicillin, a semisynthetic broad-spectrum penicillin antibiotic. All the *K. pneumoniae* isolates tested were resistant to ticaracillin 10 (100%) and piperacillin 13 (100%). Piperacillin is a broad-spectrum β-lactam antibiotic and it is most commonly used in combination with the β-lactamase inhibitor tazobactam. Isolates tested in combination of piperacillin and tazobactam resistant were in 154 (57.46%). Ticarcillin is also often paired with a β-lactamase inhibitor such as clavulanic acid. Ticarcillin is a penicillin-type antibiotic and clavulanic acid is an antibiotic that prevents bacteria from inactivating the ticarcillin resistance of the combination ticarcillin and clavulanic acid was found in 2 (33.33%) isolates tested (Table 1 and Figure 2).

Ceftazidime and ceftriaxone are third generation cephalosporins and ceftazidime resistance found was in 91 isolates (94.79%) and Ceftriaxone in 179 (93.75%) isolates. Resistance for cefazolin which is first generation cephalosporin was found in 60 (93.79%) isolates. Aztreonam is the β-lactam antibiotics for which antibiotic resistance was found in 137 isolates (89.54%). For cefuroxime-Axetil, a second-generation cephalosporin β-lactam, the antibiotic resistance was observed in 121 (85.21%) *K. pneumoniae* isolates. Cefuroxime is also an second-generation cephalosporin β-lactam antibiotic, the resistance was observed in 124 (84.93%) isolates. Antibiotic resistance for Cefepime, a fourth-generation cephalosporin was found in 202 (68.24%) isolates. Ampicillin is a semisynthetic penicillin and antibiotic resistance observed was in 151 (48.40%) isolates. Cefoperazone, a third generation β-lactam antibiotic and Sulbactum exhibits a marked synergistic action and its resistance in combination was observed in only 4 (22.22%) isolates. Cefaperazone, is a semi synthetic broad spectrum cephalosporin antibiotic. Sulbactum nullifies the action of any resistance by β-lactamase (Table 1 and Figure 2).

Ampicillin/sulbactam is a combination of the common penicillin-derived antibiotic. The ampicillin and sulbactam, an inhibitor of bacterial β-lactamase showed resistance in 64 (84.21%) isolates. Amoxicillin/ clavulanic acid are used in combination for treatment. Amoxicillin is a semisynthetic penicillin and clavulanic acid is a β-lactam drug that stops bacterium from inactivating the amoxicillin. Clavulanic acid is not effective by itself as an antibiotic. The antibiotic resistance for this combination was observed in 94 (71.76%) isolates. Tazobactam is a type of antibiotic called a β-lactamase inhibitor which stops the bacteria from inactivating the piperacillin, which increases bactericidal activity that the piperacillin can kill. The resistance for Tazobactam and piperacillin was found in 154 (57.46%) isolates (Table 1 and Figure 2).

Antibiotic resistance for fluoroquinolone antibiotic moxifloxacin was in 54 (88.52%), for ciprofloxacin in 231 (75.74%) and levofloxacin antibiotic was found in 74 (74.75%) isolates. Nalidixic acid a quinolone antibiotic for which the antibiotic resistance observed was in 112 (78.32%) isolates. The antibiotic resistance found for aminoglycosides Gentamicin was found in 172 (56.46%), Amikacin 85 (28.91%) and for tobramycin antibiotic resistance was found in 46 (63.01%) isolates. Amongst the carbapenems, the antibiotic resistance found was in 49 (54.44%), for meropenem in 137 (45.21%), imipenem in 127 (42.47%), and ertapenem in 74 (40.22%) isolates. Trimethoprim (TMP) and sulfamethoxazole (SMZ) used in conjunction with each other due to their synergistic action. TMP-SMZ combination for which the drug resistance was observed in 188 (62.05%) isolates. Nitrofurantoin is the quinolone antibiotics. Nitrofurantoin is concentrated in the urine, leading to higher and more effective levels in the urinary tract than in other body fluids is used for treatment in Urinary tract infection showed resistance in 80 (39.02%) isolates (Table 1).

Many a time tigecycline and colistin was the only alternatives available for the treatment in case of multidrug resistant K. pneumoniae isolates. Tigecycline is the drug of glycylcycline class of antibiotics showed antibiotic resistance in 47 (16.15%) isolates. Colistin which belongs to polymyxins class of antibiotic showed resistance in 37 (15.74%) isolates (Table 1 and Figure 2).

Table 1: Antibiotic susceptibility/resistance observed in *K. pneumoniae* to various antibiotics (n=311) by Vitek-2.

Sr. No.	Antibiotic	Isolates tested	Sensitive		Intermediate		Resistant	
			No of isolates	%	No. of isolates	%	No. of isolates	%
1.	Ticaracillin	10	0	0.00	0	0.00	10	100.00
2.	Piperacillin	13	0	0.00	0	0.00	13	100.00
3.	Ceftazidime	96	5	5.21	0	0.00	91	94.79
4.	Cefazolin	64	4	6.25	0	0.00	60	93.75
5.	Ceftriaxone	195	14	7.18	2	1.03	179	91.79
6.	Aztreonam	153	15	9.80	1	0.65	137	89.54
7.	Moxifloxacin	61	7	11.48	0	0.00	54	88.52
8.	Cefuroxime Axetil	142	17	11.97	4	2.82	121	85.21
9.	Cefuroxime	146	20	13.70	2	1.37	124	84.93
10.	Ampicillin / Sulbactam	76	10	13.16	2	2.63	64	84.21
11.	Nalidixic acid	143	31	21.68	0	0.00	112	78.32
12.	Ciprofloxacin	305	55	18.03	19	6.23	231	75.74
13.	Levofloxacin	99	19	19.19	6	6.06	74	74.75
14.	Amoxicillin/ Clavulanic acid	131	20	15.27	17	12.98	94	71.76
15.	Cefepime	296	94	31.76	0	0.00	202	68.24
16.	Tobramycin	73	21	28.77	6	8.22	46	63.01
17.	TMP/SMZ	303	115	37.95	0	0.00	188	62.05
18.	Piperacillin / Tazobactam	268	83	30.97	31	11.57	154	57.46
19.	Gentamicin	307	130	42.35	5	1.63	172	56.03
20.	Doripenem	90	41	45.56	0	0.00	49	54.44
21.	Ampicillin	312	161	51.60	0	0.00	151	48.40
22.	Meropenem	303	165	54.46	1	0.33	137	45.21
23.	Imipenem	299	165	55.18	7	2.34	127	42.47
24.	Ertapenem	184	107	58.15	3	1.63	74	40.22
25.	Nitrofurantoin	205	63	30.73	62	30.24	80	39.02
26.	Ticarcillin/ Clevvulanic acid	6	4	66.67	0	0.00	2	33.33
27.	Amikacin	294	201	68.37	8	2.72	85	28.91
28.	Cefoperazone / Sulbactum	18	14	77.78	0	0.00	4	22.22
29.	Tigecycline	291	230	79.04	14	4.81	47	16.15
30.	Colistin	235	198	84.26	0	0.00	37	15.74

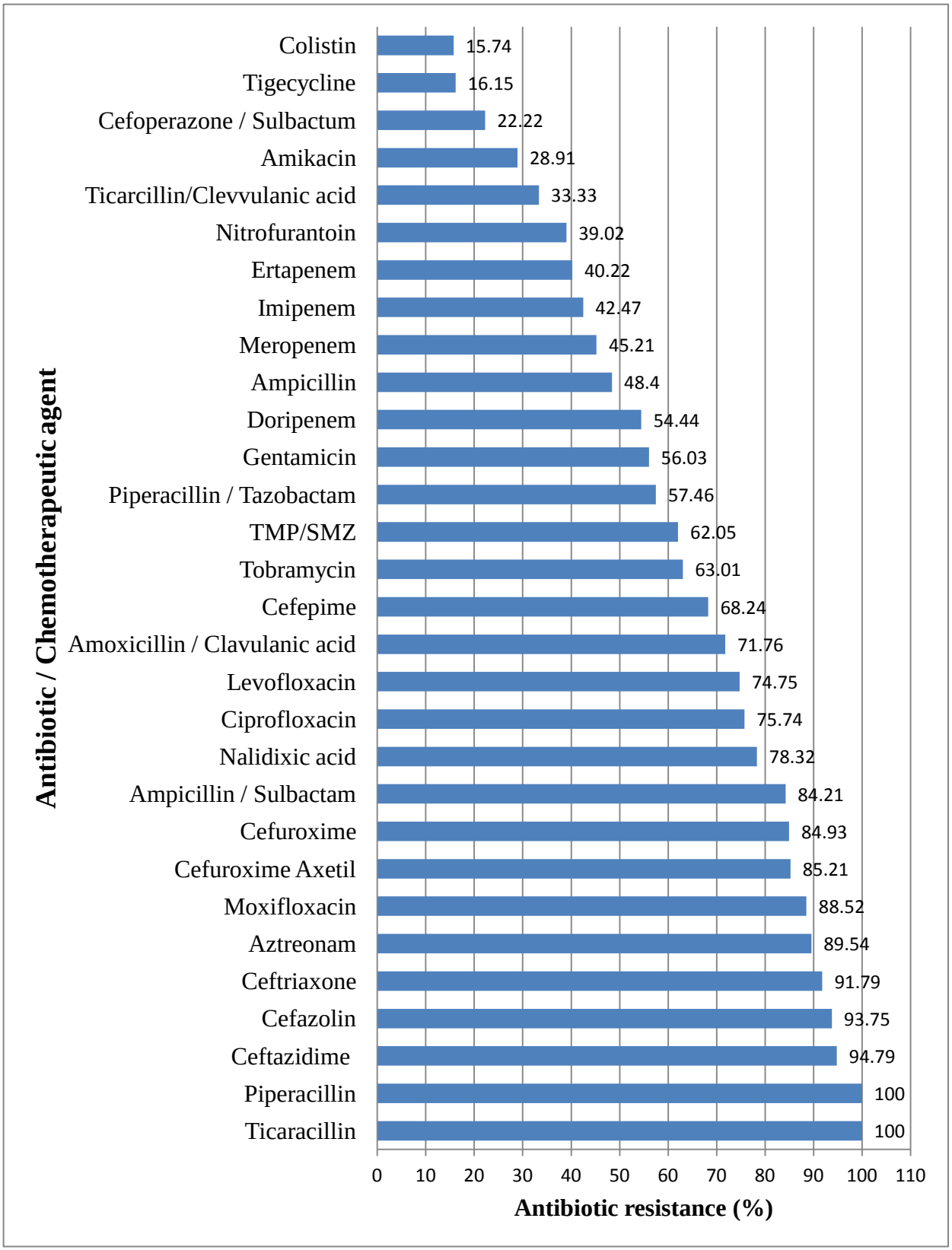

Figure 2: Antibiotic resistance observed for *K. pnaumoniae* (n=311).

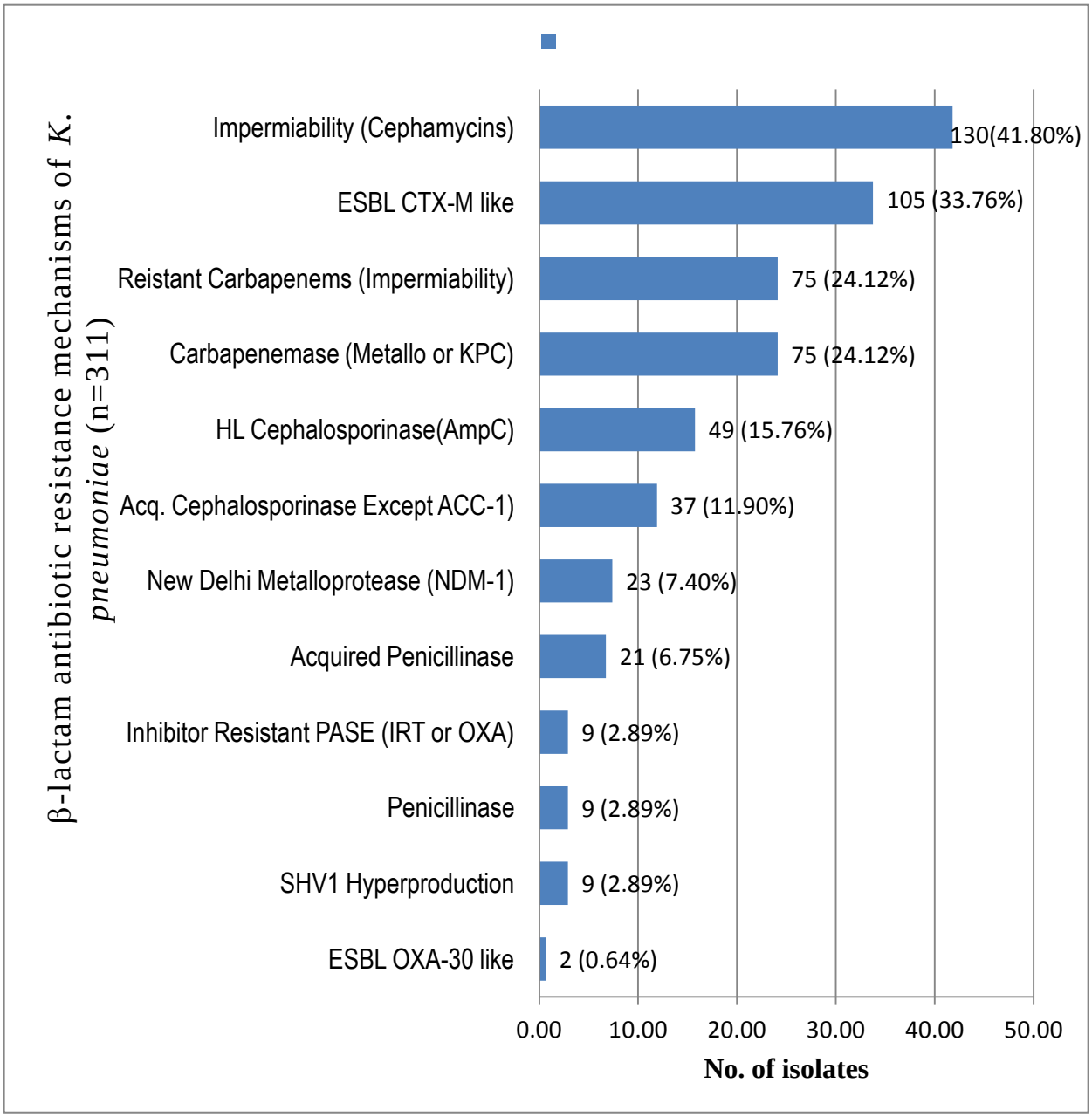

Figure 1: β-lactam antibiotic resistant phenotypes observed in 288 isolates of *Klebsiella pneumoniae* by Vitek-2.

Table 3: Phenotype, genotype and MIC obtained for the *Klebsiella pneumoniae* isolates

Organism	Phenotype	Genotype	MIC (µg/mL)												
			CAZ	CLV	FEP	PTZ	MEM	MED	IPM	CST	TIG	AMK	LEV	SXT	NIT
K. pneumoniae SL-1	Class C	TEM, CTX-M2, 3,	>64	16	>64	64	0.03	0.03	0.25	0.25	0.25	2	64	>128	16
K. pneumoniae SL-2	Class A	TEM, CTX-M2, 3, NDM	>64	1	64	8	0.03	0.03	0.25	0.12	0.25	2	64	>128	16
K. pneumoniae SL-3	Class C	TEM, CTX-M-2,	>64	>64	>64	128	0.06	0.03	0.5	0.25	0.5	2	16	0.12	8
K. pneumoniae SL-4	Class C	TEM, CTX-M-2,	>64	>64	>64	128	0.03	0.03	0.5	0.25	0.25	2	16	0.12	8
K. pneumoniae SL-5	Class B	TEM, CTX-M-2, CMY, NDM	>64	>64	>64	>128	64	0.12	16	0.25	0.25	>256	32	>128	64
K. pneumoniae SL-6	Class A	TEM, CTX-M2, 3, CMY, NDM	>64	>64	>64	16	<0.03	4	0.5	0.25	0.25	128	32	>128	16

CAZ: ceftazidime,CAV: ceftazidime in combination with clavulanic acid at 4 mg/L, FEP: cefepime, PTZ: piperacillin in combination with tazobactam at 4 mg/L, MEM: meropenem, MED: meropenem in combination with EDTA at 200 mg/L, IPM: imipenem, CST: colistin, TIG: tigecycline, AMK: amikacin, LEV: levofloxacin, SXT: trimethoprime/sulfamethoxazole, NIT: nitrofurantoin

Determination of genotype by PCR assay

The PCR assay was done for the genes CMY-2, CTXM-2, NDM-1 and TEM-1 responsible for extended spectrum β-lactamase (Figure 3-6). PCR products were separated in a 1.5% agarose gel Lanes at right: molecular size marker (in bp) (Fig 3-6). The PCR assay for ESBL *bla*$_{CMY-2}$-like gene was observed in isolates *K. pneumoniae* SL5 and SL6 (Fig 3), for bla$_{CTXM-2}$ like gene was found in all *K. pneumoniae* isolates (Figure 4), for NDM-1 we found *bla*$_{NDM-1}$-like gene in isolate no. *K. pneumoniae* SL2, SL5 and SL6 isolates (Fig 5) and PCR assay for *bla*$_{TEM}$-like gene was found in all six *K.*

Table 2: Primers used for amplification

B-lactamase gene	Primer Sequence	Amplicon Size (bp)	Annealing temp (°C)	Annealing location	Reference
TEM	CATTTCCGTGTCGCCCTTATTC	800	55	13-34	[6-8]
	CGTTCATCCATAGTTGCCTGAC			812-791	
NDM	GGTTTGGCGATCTGGTTTTC	621	55	133-153	[6-8]
	CGGAATGGCTCATCACGATC			734-754	
OXA-48	GCGTGGTTAAGGATGAACAC	438	52	251-271	[6-8]
	CATCAAGTTCAACCCAACCG			689-669	
CMY-2	GCCGTTGCCGTTATCTAC	511	56	145-163	[6-8]
	AATCTTTTTGTTCGTTCTGCG			656-635	
CTX-M group I	GACGATGTCACTGGCTGAGC	499	55	416–435	[6-8]
	AGCCG CCGACGCTAATACA			914-896	
CTX-M group II	GCGACCTGGTTAACTACAATCC	351	55	313-334	
	CGGTAGTATTGCCCTTAAGCC			663-643	
CTX-M group III	CGCTTTGCCATGTGCAGCACC	307	55	475-495	
	GCTCAGTACGATCGAGCC			781-764	
CTX-M group IV	GCTGGAGAAAAGCAGCGGAG	474	62	1857-1876	
	GTAAGCTGACGCAACGTCTG			2330-2311	

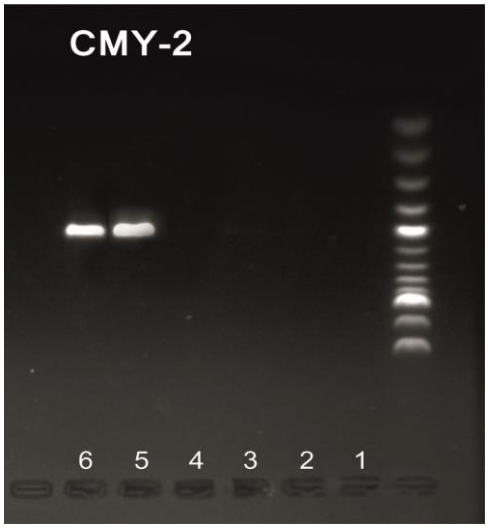

Figure 3: PCR assay for *bla*$_{CMY-2}$-like gene.

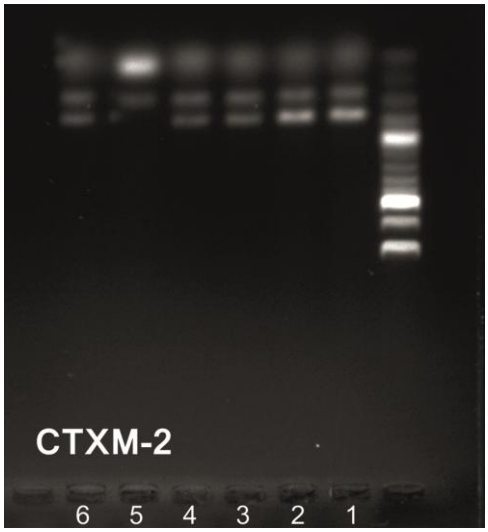

Figure 4: PCR assay for *bla*$_{CTXM-2}$-like gene.

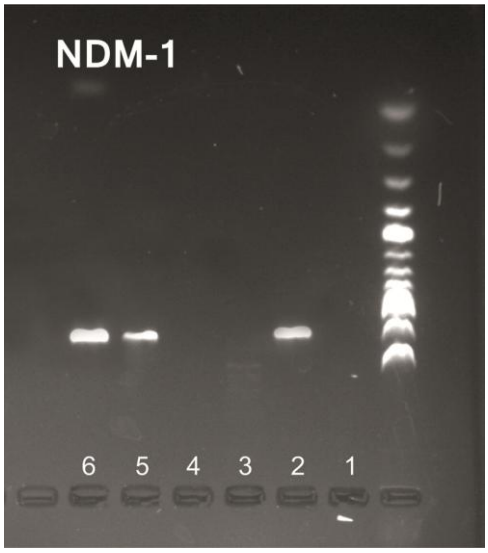

Figure 5: PCR assay for *bla*$_{NDM-1}$-like gene

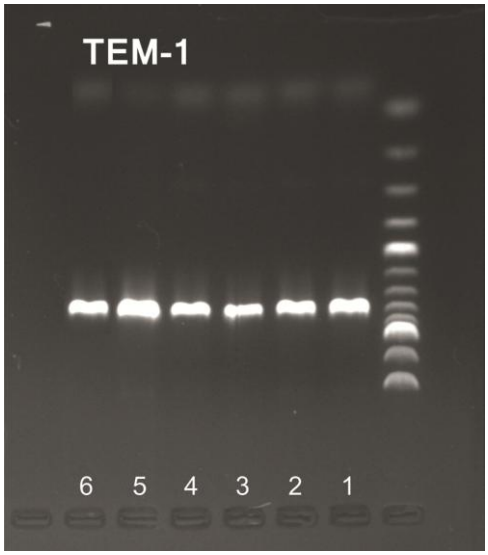

Figure 6: TEM-1 PCR assay for *bla*$_{TEM}$-like gene.

PCR products were separated in a 1.5% agarose gel Lanes at right: molecular size marker (in bp); 1. K. pneumoniae SL1, 2, *K. pneumoniae* SL2 3, *K. pneumoniae* SL3 4, *K. pneumoniae* SL4 5, *K. pneumoniae* SL5; *K. pneumoniae* SL6.

Discussion

β-Lactam antibiotics, including penicillins, cephalosporins and carbapenems, inhibit growth of bacteria by inactivating peptidoglycan transpeptidases irreversibly. β-Lactamases are enzymes of entirely bacterial origin that degrade β-lactam antibiotics into microbiologically inert compounds. The enzymes thus protect the organisms against the lethal actions of β-lactam antibiotics and the enzymes are the primary cause of bacterial resistance to these drugs. The worldwide proliferation of life-threatening metallo-β-lactamase (MBL)-producing gram-negative bacteria is a serious concern to public health. MBLs are compromising the therapeutic efficacies of β-lactams, particularly carbapenems, which are last-resort antibiotics indicated for various multidrug-resistant bacterial infections. Antibiotic resistance caused by β-lactamase production continues to present a growing challenge to the efficacy of β-lactams and their role as the most important class of clinically used antibiotics. In response to this threat, only a handful of β-lactamase inhibitors have been introduced in the market over the past thirty years. The production of β-lactamases is the predominant cause of resistance to β-lactam antibiotics in gram-negative bacteria. These enzymes cleave the amide bond in the β-lactam ring, rendering β-lactam antibiotics harmless to bacteria[9].

The tigecycline antibiotic was the most effective antimicrobial agent. The spread of carbapenem-resistant *K. pneumoniae* especially bla$_{NDM-1}$-carrying isolates is a great concern worldwide. Tracking and urgent intervention is necessary for control and prevention of these resistant isolates [10]. Results the study showed that the prevalence rate of ESBL-producing K. pneumoniae isolates is increasing in MDR strains, which raises concerns regarding the treatment of *K. pneumoniae*. Therefore, molecular research in the field of antimicrobial resistance of bacteria is essential to prevent the spread of resistant strains[11].

Resistance in bacteria to carbapenems is due to the production of carbapenem hydrolyzing enzymes called carbapenemases. In the present investigation the main mechanism of β-lactam antibiotic resistance detected was impermeability to cephamycins was found in 130 isolates (41.80%), followed by CTX-M like ESBL in 105 (33.76%). Carbapenemase either metallo or KPC type and resistant carbapenems due to impermeability was found in 75 (24.12%) isolates

each. High level cephalosporinase enzyme production in 49 (15.76%), acquired cephalosporinase in 37 (11.90%) and acquired penicillinase was identified in 21 isolates (6.75%). SHV1 hyper-production, inhibitor resistant PASE (IRT or OXA) and Penicillinase was detected in 9 isolates each (2.89%) and the lowest was OXA-30 like ESBL in 2 isolates 0.64%). Life threatening throughout the world New Delhi Metalloprotease (NDM-1) was detected in 23 (7.40%) isolates (Figure 1). In the past, 3rd and 4th generation cephalosporins were first choice in the treatment of *Enterobacteriaceae* infections. However, resistance of *Enterobacteriaceae* to these antibiotics has been well documented in recent times. KPC spread worldwide and have been identified in many gram-negative species, even though KPC enzymes are still mostly identified in *K. pneumoniae*[12].

The ß-lactamases-metallo-β-lactamases the second group of enzymes is that of the metallo-ß-lactamases (MBLs), including NDM ß-lactamases. MBLs hydrolyze all ß-lactams except aztreonam. One of the most clinically significant carbapenemases is NDM-1 identified coincidentally in 2009 in *K. pneumoniae* and *E. coli* isolates from a patient in Sweden previously hospitalized in India [13]. The main identified reservoir of NDM-producing *Enterobacteriaceae* is the Indian subcontinent especially Pakistan, India and Sri Lanka [14]. These countries are experiencing multiple on-going outbreaks of different NDM-1 producers. Significant spread of NDM-1 producers has also been identified in the United Kingdom (UK) due to its close connections with India and Pakistan. NDM-1 carbapenemase has been reported from 40 countries worldwide, encompassing all continents except South America and Antarctica. The spread of NDM has a complex epidemiology involving the spread of a variety of species of NDM-1-positive bacteria and the inter-strain, inter-species and inter-genus transmission of diverse plasmids containing bla_{NDM} with this mechanism having played a more prominent role to date. The spread of NDM-1 illustrates that antibiotic resistance is a public health problem that transcends national borders and will require international cooperation between health authorities if it is to be controlled[15]. Subsequently, NDM producers in *Enterobacteriaceae* have been reported almost worldwide, including many countries in Asia, Africa, Australia, America, and Europe NDM-1-producing bacteria. *K. pneumoniae* and *Escherichia coli* were the most commonly reported bacteria producing NDM-1 enzyme [16]. We found 23 (7.40%) NDM-1 type of β -lactam antibiotic resistance in present investigation by Vitek-2 (Figure 1). Originally described from New Delhi in 2009, this gene is now widespread in *Escherichia coli* and *K. pneumoniae* from India and United Kingdom[16]. Of the six *K. pneumoniae* isolates we found 3 showing blaNDM-1 like genes (Fig 5) by PCR genotypic assay.

The review[17] aims to summarize current knowledge regarding the detection of NDM-1 producers, the mechanism of action of NDM-1 and to highlight recent attempts toward the development of clinically useful inhibitors. No inhibitors for NDM-1 are available in therapy, nor are promising compounds in the pipeline for future NDM-1 inhibitors. Despite the valuable progress in terms of structural and mechanistic information, the design of a potent NDM-1 inhibitor to be introduced in therapy remains challenging. Certainly, only the deep knowledge of NDM-1 architecture and of the variable mechanism of action that NDM-1 employs against different classes of substrates could orient a successful drug discovery campaign [18].

Carbapenems are β-lactamase inhibitor antibiotics and are reserved for the treatment of severe microbial infections, especially those targeting the *Enterobacteriaceae*. Introduced in the 1980s, carbapenems have been used successfully and in the 1990s resistance was discovered. Carbapenem resistance is conferred through the production of carbapenemases. Carbapenem-resistant *Enterobacteriaceae* (CRE) are among the most difficult to treat emerging multidrug-resistant

organisms. Carbapenem-resistant *E. coli* or *Klebsiella* were identified in 22 of 31 African countries[19]. Carbapenem resistance in *Enterobacteriaceae* is related either to a combination of decreased outer-membrane permeability with over-expression of ß-lactamases possessing limited carbapenemase activity (cephalosporinase [AmpC] or clavulanic-acid inhibited extended-spectrum ß-lactamase (ESBLs, mostly CTX-M) or to expression of true carbapenemases. Currently, the spread of carbapenemase producers is the most important clinical issue in antibiotic resistance in gram negatives, particularly in *Enterobacteriaceae.* Over the last decade, CRE has emerged as a significant public health threat. The goal for the next 3 decades will be to design inhibitors that will be effective for more than a single class of beta-lactamases. CRE is a major concern for emerging drug resistance during the last decade because of significantly compromising the efficacy of carbapenem agents, has currently become an important focus of infection control.

Although rarely reported a decade ago, carbapenem-resistant gram-negative bacilli are increasingly identified worldwide. The future threat is the evolution of these gram-negative organisms from multi-drug resistance to pan-drug resistance. Taking into account the size of the reservoir of carbapenem-resistant bacteria and their worldwide location, reversion of carbapenemase-resistant to susceptible isolates will not occur, at least in *Enterobacteriaceae*. It is therefore essential to screen both carriers and infected patients with carbapenem-resistant bacteria. We feel that Vitek-2 ESBL test system is a reliable time saving tool for routine identification of ESBL-producing isolates. It provides results in 5 to 18 hours (median 8.2 hrs). None of the currently available phenotypic methods have proved to be full specific and sensitive[20]. In conclusion, carbapenemase and ESBL enzymes are responsible for extended and pan-drug resistance in *K. pneumoniae* isolated from clinical samples. PCR assay for genes in six isolates were detected and responsible for the drug resistance. ESBL and carbapenemase are most prevalent mechanism of antibiotic resistance in present investigation. Bla_{CTX-M2}, bla_{CMY2}, bla_{TEM} and bla_{NDM-1} are the genes responsible for MDR, XDR and Pan-drug resistance in *K. pneumoniae*.

References

1. Nordmann P. Carbapenemase-producing *Enterobacteriaceae*: overview of a major public health challenge. Med Mal Infect 2014; 44:51-56. PMID: 24360201
2. Stefanuk E, Mrowka A, Hryniewicz W. Susceptibility testing and resistance phenotypes detection in bacterial pathogens using the VITEK 2 System. Pol J. Microbiol 2005; 54 (4):311-6. PMID: 16599303
3. Odsbu I, Khedkar S, Lind F, Khedkar U, Nerkar SS, Orsini N *et al*. Trends in Resistance to Extended-Spectrum Cephalosporins and Carbapenems among Escherichia coli and Klebsiella spp. Isolates in a District in Western India during 2004-2014. Int J Environ Res Public Health. 2018 19;15(1). pii: E155. PMID: 29351236
4. Livermore DM, Struelens M, Amorim J, Baquero F, Bille J, Canton R, *et al*. Multicentre evaluation of the VITEK 2 Advanced Expert System for interpretive reading of antimicrobial resistance tests. J Antimicrob Chemother. 2002, *49(2)*,289-300. PMID: 11815570
5. Performance Standards for Antimicrobial Disk Susceptibility Tests. 13th ed. CLSI standard M02. Clinical and Laboratory Standards Institute 2018.
6. Mlynarcik Patrik, Roderova M, Kolar M. Primer Evaluation for PCR and its Application for Detection of Carbapenemases in Enterobacteriaceae. Jundishapur J. Microbiol. 2016 Jan 2;9(1):e29314. PMID: 27099689
7. Savitha Rani, Jahnavi I, Nagamani K. Phenotypic and Molecular Characterization of ESBLs producing Enterobacteriaceae in

A Tertiary Care Hospital OSR. Journal of Dental and Medical Sciences. 2016; 15 (8): Ver. IX: PP 27 – 34 DOI: 10.9790/0853-1508092734

8. Dallenne C, Da Costa A, Decre D, Favier C, Arlet G. Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases in Enterobacteriaceae. J. Antimicrob. Chemother. 2010 Mar; 65 (3):490-5. PMID: 20071363
9. Tehrani KHME, Martin NI. β-lactam/β-lactamase inhibitor combinations: an update. Medchemcomm. 2018**.** 17; 9(9):1439-1456. PMID: 30288219
10. Shoja S, Ansari M, Faridi F, Azad M, Davoodian P, Javadpour S, *et al.*, Identification of Carbapenem-Resistant Klebsiella pneumoniae with Emphasis on New Delhi Metallo-Beta-Lactamase-1 (bla_{NDM-1}) in Bandar Abbas, South of Iran. Microb Drug Resist. 2018; 24(4): 447-454. PMID: 28972857 DOI: 10.1089/mdr.2017.0058
11. Shakib P, Ramazanzadeh R, Taherikalani M, Nouri B. Detection of Extended-Spectrum Beta-Lactamases (ESBLs) and Antibiotic Susceptibility Patterns in *Klebsiella pneumoniae* in Western Iran. Infect Disord Drug Targets. 2018;18(2):156-163. PMID: 28707597
12. Cuzon G, Naas T, Truong H, Villegas MV, Wisell KT, Carmeli Y. *et al*. Worldwide diversity of *Klebsiella pneumoniae* that produce ß-lactamase blaKPC-2 gene. Emerg Infect Dis 2010; 16:1349-1356. PMID: 20735917
13. Kumarasamy KK, Toleman MA, Walsh TR, Bagaria J, Balakrishnan R. et al. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. Lancet Infect Dis. 2010; 10(9):597-602. PMID: 20705517
14. Dortet L, Poirel L, Nordmann P. Worldwide dissemination of the NDM-type carbapenemses in Gram-negative bacteria. Biomed Res Int. 2014:249856. PMID: 24790993
15. Johnson AP, Woodford N. Global spread of antibiotic resistance; the example of New Delhi metallo-ß-lactamase (NDM)-mediated carbapenem resistance type. J Med Microbiol 2013; 62: 499-513. PMID: 23329317
16. Berrazeg M, Diene S, Medjahed L, Parola P, Drissi M., Raoult D, Rolain J. New Delhi Metallo-beta-lactamase around the world: an eReview using Google Maps.Euro Surveill. 2014;19 (20): pii: 20809. PMID: 24871756
17. Groundwater PW, Xu S, Lai F, Varadi L, Tan J, Perry JD, Hibbs DE. New Delhi metallo-β-lactamase-1: structure, inhibitors and detection of producers. Future Med Chem. 2016 Jun; 8(9):993-1012. PMID: 27253479
18. Linciano P, Cendron L, Gianquinto E, Spyrakis F, Tondi D. Ten Years with New Delhi Metallo-β-lactamase-1 (NDM-1): From Structural Insights to Inhibitor Design. ACS Infect Dis. 2018**;** 28. PMID: 30421910
19. Mitgang EA, Hartley DM, Malchione MD, Koch M, Goodman JL, Review and mapping of carbapenem-resistant Enterobacteriaceae in Africa: Using diverse data to inform surveillance gaps. Int J Antimicrob Agents. 2018; 52 (3):372-384. PMID: 29864500
20. Aguirre-Quinonero A, Martinez-Martinez L. Non-molecular detection of carbapenemases in Enterobacteriaceae clinical isolates. J Infect Chemother 2017**,** 23(1*):* 1-11.