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Research Article

**Susceptibility Trend of Drugs Among Metallo-
-lactamase Producing Strains of *Escherichia coli*,
Klebsiella pneumoniae, *Acinetobacter baumannii* and
Pseudomonas aeruginosa in India**

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ABSTRACT

The current study was conducted to observe the prevalence of different types of metallo- -lactamases (MBLs) and their variants among 849 clinical isolates obtained from various parts of India. Further, antibiotic susceptibility behaviour of these isolates to different antibiotics was analysed. Identification of isolates according to the standard microbiological techniques and VITEK-2. Susceptibility studies were carried out according to Clinical and Laboratory Standards Institute guidelines. Phenotypic screening confirmed that out of 1361 isolates, 849 (62.4%) isolates were MBL producers. The highest number of MBL producers was from *Pseudomonas aeruginosa* (235/347; 67.7%) followed by *Klebsiella pneumoniae* (88/131; 67.2%), *Escherichia coli* (151/254; 59.4%) and *Acinetobacter baumannii* (371/629; 58.9%). PCR results revealed the occurrence of NDM type MBLs were the predominant (n= 412), followed by IMP type (n= 245) and VIM type (n= 192). Susceptibility results demonstrated that approximately 94.1 %, 93.0 %, 93.2 and 91.0% of *E. coli*, *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* isolates were susceptible to CSE1034. The susceptibilities of penems (meropenem, imipenem+cilastatin) and piperacillin+tazobactam were <45 % and <16 %. Interestingly, none of the isolates were found to be susceptible to amoxicillin+clavulanic acid and cefoperazone+sulbactam. Results of the present study indicate that CSE1034 appeared to be the most efficacious and majority of the isolates were susceptible to CSE1034 and can be a potent antibacterial agent for the treatment of severe bacterial infections caused by MBL producing organisms.

Keywords: Clinical isolates, CSE1034, carbapenem, metallo- -lactamases.

INTRODUCTION

Carbapenems, particularly imipenem and meropenem are unique -lactam antibiotics because they are relatively resistant to hydrolysis by most -lactamases, their high affinity towards penicillin-binding proteins, and their permeability of the bacterial outer membranes¹. They have been recognised as the last resort drugs across the globe for the treatment of infections caused by multi-drug resistant (MDR) Gram negative and Gram positive organisms². However, in past few years, resistance to this last line antibiotics has been increasing worldwide and thus putting clinical application of this antibiotic under challenge³.

Resistance to carbapenem is mainly mediated by carbapenemases such as Ambler class B metallo- -lactamases (MBL), including IMP, VIM, and NDM-1 which have been increasing throughout the world in *Enterobacteriaceae* and *Pseudomonas* spp. and *Acinetobacter baumannii* varying from 7.5 to 71 %³⁻¹¹. Interestingly, a study by Hu et al¹² demonstrated the least susceptible of *Enterobacteriaceae* family to imipenem and meropenem, with only 6.5 and 1.3 %, respectively.

The occurrence of carbapenemases particularly NDM-1 has also been reported in seepage and drinking water samples¹³. According to a study conducted in

the Melbourne, Australia ICU reported that wet area locations, including ICU sinks, water fountains, and ice machines are the reservoir for carbapenem-resistant enterobacteriaceae (CRE) and inappropriate cleaning accounted for the primary method of transmission of CRE bacteria. Furthermore, the environmental strains of the CRE bacteria were the same strains infecting the patients in the ICU, as determined from genetic analysis suggesting that at risk patients were being infected in the hospital setting¹⁴.

Infections due to carbapenemases producing strains are associated with higher morbidity and mortality rates¹⁵. The increasing rate of resistance of drugs to MBL producing isolates encouraged us to study newly reported concept of antibiotic adjuvant entity by which the increasing failure rate of antibiotics in the treatment of the infections caused by such organisms can be controlled. Information regarding the prevalence of antimicrobial resistance in pathogens can be used for selecting an optional treatment.

Data on the prevalence of IMP, VIM and NDM variants in Enterobacteriaceae (*E. coli* and *K. pneumoniae*) and non-fermenting Gram-negative bacilli (*A. baumannii* and *P. aeruginosa*) from Indian hospitals are lacking. Therefore, the current study was undertaken to study the molecular characterisation of the selected isolates. The study also analysed the susceptibility behaviour of the isolates to different antibiotics including CSE1034, a new antibiotic adjuvant entity (AAE) which is a combination of β -lactam and β -lactamase inhibitor altogether termed as ceftriaxone plus sulbactam, with other antibiotics against these isolates.

MATERIALS AND METHODS

Clinical specimen collection and their identification

This study was conducted in the Department of Microbiology, Venus medicine Research Centre, Baddi, Himanchal Pradesh India, from the period of January 2013 to March 2014. All the specimens were collected from different hospitals of India. The name of hospitals can not be disclosed due to confidentiality agreement. All the specimens including blood, urine, sputum and pus received were processed for clinical isolate isolation and identification of these isolates were done with VITEK-2 (BioMérieux, Marcy l'Etoile, France)¹⁶. Prior to use, all the specimens were inoculated onto Soyabean Casein Digest Agar (SCDA; Hi-Media, Mumbai, India), incubated at 37 °C for overnight. At least three to five colonies of each specimen were selected from the SCDA plate and were transferred into a tube containing 10 ml of sterile Mueller-Hinton

broth (MHB; Hi-Media, Mumbai, India) to produce a suspension which matched the turbidity standard of 0.5 McFarland standard.

Detection of MBL production

Phenotypic evidence of MBL production was tested using imipenem (10 µg) and imipenem (10 µg) + EDTA (750 µg) discs as described elsewhere¹⁷. The test organism was inoculated onto Mueller-Hinton agar (MHA, Himedia, Mumbai, India) and an increase of 5 mm or more in zone diameter in the presence of EDTA compared to imipenem alone was considered to be a positive test for the presence of an MBL. The strains *K. pneumoniae* BAA-2146 (NDM-1 positive), *K. pneumoniae* NCTC 13439 (VIM-1 positive) and *E. coli* NCTC 13476 (IMP-1 positive) were used as control throughout study and were procured from LCGC, Bangalore, India. The results are explained in frequency and percentage.

DNA isolation

Five ml of each bacterial culture was centrifuged at 5000 rpm for 5 min at 4 °C and pellet was washed once in Tris-EDTA (TE) buffer (Tris-HCl 1.0 M, pH 8.0; EDTA 0.5 M, pH 8.0). After addition of 300 µl of TE buffer, 40 µl of 10% SDS (sodium dodecyl sulfate), 3 µl of 0.5 M EDTA (pH 8.0) were added and incubated for 5 min at 65 °C. After incubation, 750 µl of isopropanol was added. This was followed by centrifugation at 14,000 rpm for 5 min at 15°C. The resulting pellet was re suspended in 500 µl of TE and 2 µl of RNase A (10 mg/ml) and incubated at 65 °C for 30 min, then added 2 µl of proteinase K (20 mg/ml) and incubated at 37 °C for 15 min. Following incubation, 1 ml of phenol:chloroform (1:1) was added. The upper phase was transferred to another tube and added equal amount of chloroform, shaken well and centrifuged at 14,000 rpm for 5 min at 15°C. The supernatant was provided with 40 µl of 5 M Na-Acetate (pH 5.2) and 1 ml of ethanol and left at room temperature for 1 h, centrifuged at 7000 rpm for 5 min at 4 °C. The DNA pellet was washed with 70% ethanol and suspended in 50 µl of TE buffer.

Detection of MBLs and their variants

All the MBL positive isolates were subjected for the detection of MBL genes and their variants, *NDM-1*, *NDM-2*, *NDM-5*, *IMP-1*, *IMP-12*, *IMP-20*, *IMP-29*, *VIM-1*, *VIM-2*, *VIM-4*, and *VIM-7* as described previously [18-26]. All of the respective primers were obtained from Sigma Aldrich Chemicals Pvt. Ltd., Bangalore, India. For PCR amplifications, about 200 pg of DNA was added to 20 µl mixture containing 0.5 mM of dNTPs, 1.25 µM of each primer and 0.5 µl/unit of Taq polymerase (Bangalore Genei) in 1x PCR buffer. Amplification was

performed in a Eppendorf thermal cycler (Germany). The amplified products were separated in 1.5 % agarose gel containing 2.5µl of 10 mg/ml ethidium bromide. The gel was run at 70 volt for 1 h. The gel images were taken under ultraviolet light using gel documentation system (Bio-Rad, USA). A 100 bp ladder (Banglore Genie) was used to measure the molecular weights of amplified products. The images of ethidium bromide stained DNA bands were visualized using a gel documentation system (Bio-Rad, USA).

Antimicrobial susceptibility study

Susceptibility studies were done by broth dilution and disc diffusion methods according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) [27]. *K. pneumoniae* BAA-2146 (NDM-1 positive), *K. pneumoniae* NCTC 13439 (VIM-1 positive) and *E. coli* NCTC 13476 (IMP-1 positive) which were used as positive controls. *E. coli* ATCC 25922 was used as quality control strain. Drugs, CSE1034 (ceftriaxone sodium plus sulbactam sodium along with adjuvant disodium edetate) (1.5 g), amoxicillin plus potassium clavulanate (1.2 g), piperacillin plus tazobactam (4.5 g), meropenem (1 g), imipenem plus cilastatin (0.5 g) and cefoperazone plus sulbactam (2 g) were used in the study were used for minimum inhibitory concentration (MIC) study. The discs of CSE1034 (45 µg), piperacillin + tazobactam (110µg), amoxicillin + clavulanic acid (30 µg), cefoperazone + sulbactam (105 µg), imipenem + cilastatin (10 µg) and meropenem (10 µg) were obtained from Hi-Media (Mumbai, India). All the experiments were repeated 3 times and results were expressed as mean±SD values. Positive controls were the same which were used in MIC study. Results were interpreted according to CLSI guidelines.

RESULTS

Isolation of clinical isolate from specimens

Among 3593 clinical specimens, only 1361 (37.8%) showed the presence of targeted species which includes *A. baumannii* (629/1361; 46.2%) followed by *P. aeruginosa* (347/1361; 25.5%), *E. coli* (254/1361; 18.6%) and *K. pneumoniae* (131/1361; 9.6%) (Figure 1), while 1257 specimens did not show the presence of growth and were considered as sterile and 975 specimens showed the growth other organisms such as *Haemophilus influenzae*, *Salmonella typhi*, *Staphylococcus epidermidis*, *Sterptococcus pyogenes*, *Nisseria spp.* which were not part of study.

Detection of MBL production

Of 1361 isolates, 849 (62.4%) isolates were MBL producers as confirmed by phenotypic method and these isolates were used for further study. The highest

number of MBL producers was from *P. aeruginosa* (235/347; 67.7%) followed by *K. pneumoniae* (88/131; 67.2%), *E. coli* (151/254; 59.4%) and *A. baumannii* (371/629; 58.9%). The distribution of MBL producers in clinical samples is shown in Table 1. Of *A. baumannii*, the most MBL producers were from pus (61.3%) followed by blood (59.2%), sputum (58.9%) and urine (55.5%). For *E. coli* the greatest number of MBL positive isolates were identified from sputum (71.8%), pus (65.2%), urine (58.2%) and blood (53.8%). Among *K. pneumoniae*, the maximum number of MBL producers were from urine (70.0%) followed by sputum (68.2%), pus (63.1%) and blood (62.5%). In *P. aeruginosa*, the MBL producers were prevalent in urine (68.3%) followed by pus (68.1%), blood (67.4%) and sputum (64.5%).

Diversity of MBLs

PCR analyses using specific primers for class B - lactamase genes revealed the following frequencies of MBLs in this study: NDM-1 type MBLs (*NDM-1*, *NDM-2*, *NDM-5*) were the most frequently detected, followed by IMP type MBLs (*IMP-1*, *IMP-12*, *IMP-20*, *IMP-29*) and VIM type MBLs (*VIM-1*, *VIM-2*, *VIM-4*, *VIM-7*) (Figure 2).

Antimicrobial susceptibilities of clinical isolates

MIC

The MIC values of CSE1034, piperacillin plus tazobactam, amoxicillin plus clavulanic acid, cefoperazone plus sulbactam, imipenem plus cilastatin and meropenem were determined against all of the selected clinical isolates and presented in Table 2. MIC values of CSE1034 were between 0.125-4.0, 1-4, 1-8 and 2-8 µg/ml for *E. coli*, *A. baumannii*, *K. pneumoniae* and *P. aeruginosa*, respectively. On the other hand, penems (meropenem and imipenem+cilastatin) MIC against the <45% of the same isolate was between 0.25 to 1.0 µg/ml. MICs for amoxicillin+clavulanic acid and cefoperazone+sulbactam were > 1024 and 32 to > 1024 µg/ml, respectively. For Piperacillin + tazobactam, MIC varied from 16 to 512 µg/ml for *E. coli*, *A. baumannii* and *K. pneumoniae* whereas it was 16 to 1024 µg/ml for *P. aeruginosa*. The detailed results are shown in the Table 2 and 2a.

AST

The antibiotic susceptibility profile was determined by disc diffusion method and results are presented in Table 3. Among the drugs used in the study, CSE1034 was found to be more efficacious. Majority of the isolates of *E. coli* (94.1%) were found to be susceptible to CSE1034 followed by *P. aeruginosa* (91.06%), *K. pneumoniae* (93.2%) and *A. baumannii*

(93%). Imipenem+cilastatin was found second most active agent. Our results showed that approximately 42.0 %, 36.1%, 44.3% and 38.7 isolates of *E. coli*, *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* were susceptible to Imipenem+cilastatin whereas 25.8, 27, 33 and 28.1 % isolates of *E. coli*, *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* were susceptible to meropenem.

Piperacilline+tazobactam was susceptible to 12.9 %, 13.5%, 13.7 and 15.7 % isolates of *E. coli*, *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* respectively. None of the isolates were noted to be susceptible to Amoxicillin+clavulanic acid and cefoperazone+sulbactam. The detailed results are shown in the Table 3.

DISCUSSION

In recent years, microbial resistance to nearly all classes of antimicrobials continue to rise worldwide against MBL positive bacteria as a threat to favorable outcome in the treatment of common infections in community and hospital settings^{28,29}. As far as our knowledge this is the first report from India evaluating the antibiotic resistance among MBL positive (*E. coli* and *K. pneumoniae*) and non-fermenting Gram-negative bacilli (*A. baumannii* and *P. aeruginosa*) at a length.

In this study we isolated 849 (62.4%) MBL positive isolates out of 1361 clinical isolates among 3593 clinical specimens obtained from various parts of India. The prevalence of *A. baumannii* was 58.9 % which was similar to previous studies³⁰⁻³². In this study the prevalence of MBL in *P. aeruginosa* was 67.7% which is in agreement with other studies which noted 7 to 65 % prevalence of MBL in *P. aeruginosa*^{5,33,34}. In the present study, MBL was detected in 59.4% and 67.1% of *E. coli* and *K. pneumoniae*. This finding is in agreement with other reports which suggest the increasing prevalence of MBL in Indian subcontinent in Enterobacteriaceae^{4,13,29,35}. Increasing prevalence of MBLs in our community is suggested to be due to intense prescription of 3rd generation cephalosporins and penems in hospitals and the dissemination of these organisms by inappropriate hygienic measures as well as spread of resistant genes through plasmid transfer interspecies.

When these MBL positive isolates were subjected for study the prevalence of variant of different MBLs, NDM-1 type MBLs was most prevalent (48.5%) followed by IMP types (28.8 %) and VIM types (22.6 %). In the current investigation, CSE1034 was the most efficacious antibiotic against all the MBL producing organisms about 91 to 93 % of the isolates were susceptible to CSE1034. This is likely due to

the CSE1034 altered the outer membrane permeability which in turn increased penetration of drug inside the bacterial cells. Furthermore, CSE1034 components acts synergistically and binds with the divalent ions required for the activity of MBLs thus enhancing the susceptibility of CSE1034 towards MBLs producing organisms. Previous studies also supports the higher susceptibilities of CSE1034 against these isolates^{36,37}. The carbapenem drugs are thought to be the most active drugs³⁸. In this study 48 to 64 % of isolates were noted to be resistant to penems (meropenem and imipenem+cilastatin). It has been observed that increasing prevalence of carbapenems resistance varying from 13 to 51% in *E. coli* and *Klebsiella spp.* in New Delhi, India hospitals³⁹. Similarly, it has also been demonstrated high prevalence of resistance to various carbapenems among *Enterobacteriaceae* strains⁴⁰. A study reported a high rate of 50% carbapenem resistance among *Acinetobacter* isolates in New York⁴¹. Picao et al. reported 82 and 95 % resistance of imipenem and meropenem in *P. aeruginosa* isolates, suggested that the presence of nonenzymatic mechanism of carbapenem resistance such as porin loss and/or overexpression of efflux pumps⁴². Less susceptibility of piperacillin + tazobactam in Enterobacteriaceae (*E. coli* and *K. pneumoniae*) and non-Enterobacteriaceae (*A. baumannii* and *P. aeruginosa*) has been reported in India^{40,43,44}. In the current study, only 13 to 15 % of isolates were found to be susceptible to piperacillin+tazobactam. None of the isolates were found to be susceptible to amoxicillin+clavulanic and cefoperazone+sulbactam.

CONCLUSIONS

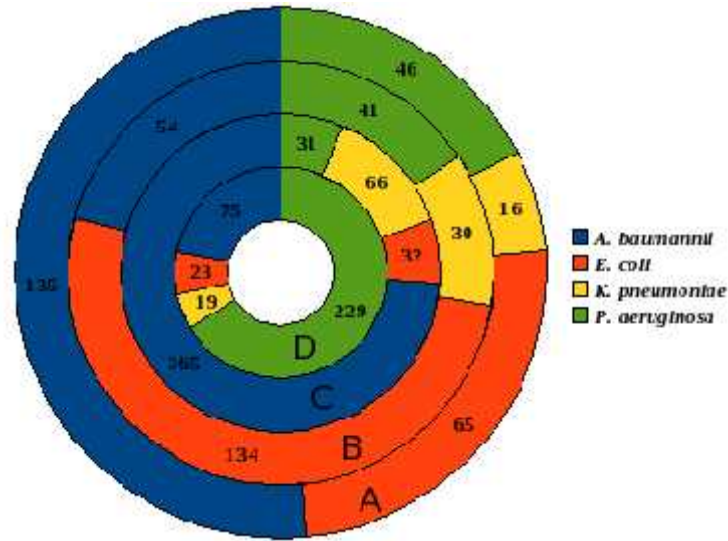
Results of this study demonstrate that the frequency of MBL mediated resistance among the clinical isolates has been increasing and the drugs commonly used for the treatment of infections caused by MBL producing organisms are getting Data obtained this investigation clearly demonstrate the potent *in-vitro* activity of CSE1034 against MBL producing organisms. Hence, in case of infection with MBL producing organisms, CSE1034 can be of drug of choice for the treatment.

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Conflict of interest statement

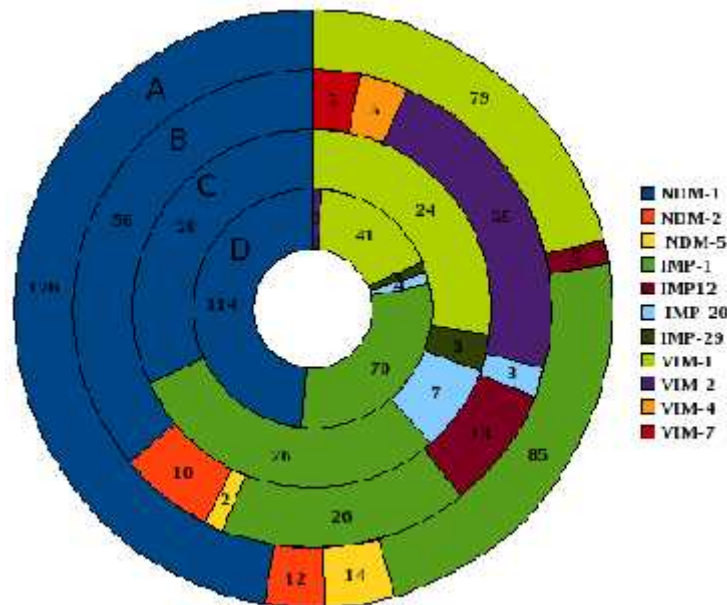
We declare that we have no conflict of interest.



Where A= blood samples; B=urine samples; C=sputum samples; D= pus samples.

Figure 1

Distribution of clinical isolates among all specimens (n=1361).



Where A= *A. baumannii*; B= *E. coli*; C= *K. pneumoniae*; D= *P. aeruginosa*.

Note: Most of the isolates contained more than one MBL genes.

Figure 2

Distribution of MBL enzymes in different bacterial species.

Table 1
Distribution of metallo β -lactamases in clinical isolates.

Name of the specimens	<i>A. baumannii</i>	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>
	No of isolates resistant to carbapenem (%)	No of isolates resistant to carbapenem (%)	No of isolates resistant to carbapenem (%)	No of isolates resistant to carbapenem (%)
Blood	80 (59.2)	35 (53.8)	10 (62.5)	31 (67.4)
Urine	30 (55.5)	78 (58.2)	21 (70.0)	28 (68.3)
Sputum	215 (58.9)	23 (71.8)	45 (68.2)	20 (64.5)
Pus	46 (61.3)	15 (65.2)	12 (63.1)	156 (68.1)
Total	371 (58.9)	151 (59.4)	88 (67.2)	235 (67.7)

Table 2
Comparative MIC values of drugs against MBL producing strains.

Name of micro-organisms	Total no of strains (801 clinical isolates)	Name of the drug. (μ g/ml)					
		Ceftriaxone +sulbactam (CSE1034)			Cefoperazone+ sulbactam		
		S	I	R	S	I	R
<i>E. coli</i>	155	0.125 - 2	16-32	64-128	-	32-64	>1024
<i>A. baumannii</i>	371	1-4	16-32	64-128	-	32-64	>1024
<i>K. pneumoniae</i>	88	1-8	16-32	64-128	-	32-64	>1024
<i>P. aeruginosa</i>	235	2-8	16-32	64-128	-	32-64	>1024
<i>Klebsiella pneumoniae</i> ATCC BAA-2146 (NDM-1 positive)	1	4.0			256		
<i>Klebsiella pneumoniae</i> NCTC 13439 (VIM-1 positive)	1	2.0			256		
<i>Escherichia coli</i> NCTC 13476 (IMP-1 positive)	1	2.0			256		
<i>E. coli</i> ATCC 25922	1	0.06			0.5		

Where S=susceptible; I=intermediate; R=resistance.

Table 2a
Comparative MIC values of drugs against MBL producing strains.

Name of micro-organisms	Total no of strains (801 clinical isolates)	Name of the drug. (µg/ml)											
		Piperacillin+ tazobactam			Amoxicillin+ clavulanic acid			Imipenem+ cilastatin			Meropenem		
		S	I	R	S	I	R	S	I	R	S	I	R
<i>E. coli</i>	155	16	32-64	128-512	-	-	>1024	0.5-1.0	2	4-32	0.5-1.0	2	4-32
<i>A. baumannii</i>	371	16	32-64	128-512	-	-	>1024	0.5-1.0	2	4-16	0.5-1.0	2	4-64
<i>K. pneumoniae</i>	88	16	32-64	128-512	-	-	>1024	0.5-1.0	2	4-16	0.25-1.0	2	4-32
<i>P. aeruginosa</i>	235	16	32-64	128-1024	-	-	>1024	0.5-1.0	2	4-16	0.5-1.0	2	8-64
<i>Klebsiella pneumoniae</i> ATCC BAA-2146 (NDM-1 positive)	1	32			128			2			8		
<i>Klebsiella pneumoniae</i> NCTC 13439 (VIM-1 positive)	1	64			128			2			8		
<i>Escherichia coli</i> NCTC 13476 (IMP-1 positive)	1	64			128			2			4		
<i>E. coli</i> ATCC 25922	1	2			8			0.03			0.15		

Where S=susceptible; I=intermediate; R=resistance.

Table 3
Comparative anti microbial susceptibility of drugs against MBL producing strains.

Name of micro-organisms	Total No of strains	Name of drugs (µg/ml)																	
		Ceftriaxone +Sulbactam (CSE1034)			Piperacillin+ Tazobactam			Amoxicillin+ clavulanic acid			Cefoperazone +Sulbactam			Imipenem+ Cilastatin			Meropenem		
		Susceptible (%)																	
		S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
<i>E. coli</i>	155	97	1	2	12.9	5.8	81.3	-	-	100	-	8.4	91.6	42	9.1	48.9	25.8	10.3	63.9
<i>A. baumannii</i>	371	93	1.9	5.1	13.5	3.8	82.7	-	-	100	-	5.4	94.6	36.1	6.2	57.7	27	7	66
<i>K. pneumoniae</i>	88	93.2	1.1	5.7	13.7	4.6	81.7	-	-	100	-	5.7	94.2	44.3	4.5	51.2	33	10.3	56.7
<i>P. aeruginosa</i>	235	95.7	2.1	2.2	15.7	2.1	82.2	-	-	100	-	7.6	92.3	38.7	7.6	53.6	28.1	7.7	64.2
<i>Klebsiella pneumoniae</i> ATCC BAA-2146 (NDM-1 positive)	1	100					100			100			100		100				100
<i>Klebsiella pneumoniae</i> NCTC 13439 (VIM-1 positive)	1	100					100			100			100		100				100
<i>Escherichia coli</i> NCTC 13476 (IMP-1 positive)	1	100					100			100			100		100				100
<i>E. coli</i> ATCC 25922	1	100			100					100			100						100

Where S=susceptible; I=intermediate; R=resistance.

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