Journal of Scientific Research & Reports
8(1): 1-9, 2015; Article no.JSRR.16933
ISSN: 2320-0227

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Susceptibility Pattern of Extended-Spectrum β-Lactamase (ESBL) Producing *Escherichia coli, Klebsiella* spp. and *Enterobacter* spp. to Ciprofloxacin, Amikacin and Imipenem

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Authors' contributions

This work was carried out in collaboration between all authors. Author JNS designed and did the study, collected the data and drafted the manuscript. Authors SA and AAS supervised the study. Authors SMAB and SAS drafted and revised the manuscript. Authors RB and HS helped to design the study and to collect data. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JSRR/2015/16933
Editor(s):
(1) Karl Kingsley, University of Nevada, Las Vegas - School of Dental Medicine, USA.
Reviewers:
(1) Anonymous, Baskent University, Turkey.
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(3) Anonymous, University for Development Studies, Ghana.
(4) Anonymous, National Institute of Health Dr Ricardo Jorge, Portugal.
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(6) Anonymous, Federal University of Ceará, Brazil.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=1264&id=22&aid=9689

Original Research Article

Received 19th February 2015
Accepted 25th May 2015
Published 10th June 2015

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ABSTRACT

This study was carried out to determine the susceptibility pattern of Extended-Spectrum β-lactamase (ESBL) producing *Escherichia coli, Klebsiella* spp. and *Enterobacter* spp. to ciprofloxacin, amikacin and imipenem. A total of 100 ESBL producing *Escherichia coli, Klebsiella* spp. and *Enterobacter* spp. were studied and identified by double disc synergy test (DDST) and were confirmed phenotypically as ESBL producer by phenotypic confirmatory disc diffusion test (PCDDT). Minimum inhibitory concentrations of ciprofloxacin, amikacin and imipenem among ESBL-producing strains were determined using agar dilution method. Out of 75 DDST positive ESBL-producing *E. coli*, 71 (94.67%) were also positive by PCDDT. All DDST positive *Klebsiella* spp. and *Enterobacter* spp. were also positive by PCDDT. All ESBL-producing *E. coli, Klebsiella* spp. and *Enterobacter* spp. were 100% susceptible to imipenem by both agar dilution and disc diffusion method. About 7.04% *Escherichia coli,* 21.05% *Klebsiella* spp. were resistant but 100% *Enterobacter* spp. were susceptible to amikacin by both methods. About 85.92% ESBL-producing *Escherichia coli,* 73.68% *Klebsiella* spp. and 33.33% *Enterobacter* spp. were resistant to ciprofloxacin by agar dilution method but 87.32% *Escherichia coli,* 78.95% *Klebsiella* spp. and 50% *Enterobacter* spp. were resistant to ciprofloxacin by disc diffusion method. ESBL-producing *Escherichia coli*, *Klebsiella* spp. and *Enterobacter* spp. showed high resistance to ciprofloxacin. Imipenem and amikacin were most effective against ESBL positive strains.

Keywords: Extended-spectrum β-lactamase; Escherichia coli; Enterobacter spp; Klebsiella spp; minimum inhibitory concentrations.

1. INTRODUCTION

Extended-Spectrum β-lactamases (ESBLs) are enzymes that mediate resistance to extended-spectrum (third generation) cephalosporins (e.g., ceftazidime, cefotaxime and ceftriaxone) and monobactams (e.g., aztreonam) but do not affect cephamycins (e.g., cefoxitin and cefotetan) or carbapenems (e.g., meropenem or imipenem) [1]. The majority of ESBL-producing organisms are *Klebsiella* spp. and *Escherichia coli*. Other organisms reported to harbour ESBL include *Enterobacter* spp., *Proteus mirabilis, Serratia marcescens, Salmonella* spp., *Morganella morganii* and *Pseudomonas aeruginosa* [2].

The first ESBL-producing isolates were discovered in Western Europe in 1983 and subsequently in the United States in 1988 [3]. In the United States, occurrence of ESBL production in *Enterobacteriaceae* ranges from 0% to 25%. In Asia, the percentage of ESBL production in *E. coli* and *K. pneumoniae* varies, from 4.8% in Korea to 8.5% in Taiwan and up to 12% in Hong Kong [4]. In India prevalence rate varies in different institutions from 28% to 84% [5].

Several phenotypic methods for detection of ESBL have been proposed including; Double disc synergy test (DDST), Phenotypic confirmatory disc diffusion test (PCDDT), E-test Extended-Spectrum β-lactamase strips, Three dimensional test, Vitek system and the Cica Beta Test 1. Phenotypic methods are based upon the resistance that ESBL confer to oxyimino-beta-lactams (*e.g.* ceftriaxone, cefotaxime, ceftazidime and aztreonam) and the ability of a beta-lactamase inhibitor, usually clavulanate to block this resistance [6]. Till now there is no gold standard test for detection of ESBL [3].

ESBL-positive isolates show false susceptibility to extended-spectrum cephalosporin in standard disc diffusion method, rendering it difficult to reliably detect ESBL production by the routine DDST [7]. PCDDT is a sensitive procedure for detection of ESBL [8].

ESBL-producing organisms are a breed of multidrug-resistant pathogens. Infections caused by these organisms are associated with higher rate of mortality, morbidity as well as health care costs [9]. Antibiotic options in the treatment of these organisms are extremely limited including carbapenem, fluoroquinolone and aminoglycoside [10]. Treatment of these infections with cephalosporins has been associated with poor clinical outcomes, even if the causative organisms appeared to be susceptible to the antibiotics in vitro. Carbapenems (e.g., imipenem and meropenem) are regarded as the drug of choice in treating

infections caused by ESBL-producing organisms. Carbapenems are stable against hydrolytic activity of ESBL and treatment with carbapenems showed a significantly better clinical outcome [11]. Fluoroquinolones and aminoglycosides may be used if they show in vitro susceptibility [12].

The purpose of this study was to determine susceptibility patterns of ESBL producing *E. coli, Klebsiella* spp. and *Enterobacter* spp. to ciprofloxacin, amikacin, and imipenem.

2. MATERIALS AND METHODS

2.1 Study Samples

The study group comprised of a total of 100 ESBL-producing *E. coli* (75), *Klebsiella* spp. (19) and *Enterobacter* spp. (06) obtained from urine, pus, wound swab, blood, sputum and bile that were received in the Department of Microbiology & Immunology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh during the period of October, 2010 to December, 2011.

2.2 Test for Presence of Extended-Spectrum β-lactamase (ESBL)

Screening for ESBL was carried out by double disc synergy test (DDST) as described by Jarlier et al. [13] and were confirmed phenotypically as ESBL-producer by phenotypic confirmatory disc diffusion test (PCDDT).

2.2.1 Double disc synergy test (DDST)

Mueller-Hinton agar plates were inoculated with standardized inoculum of the test organism (corresponding to 0.5 McFarland tube) with sterile cotton swab. A disc of augmentin (20 µg of amoxicillin and 10 µg of clavulanic acid) was placed in the middle of the inoculated plate. 3rd generation cephalosporin discs of cefotaxime (30 µg), ceftriaxone (30 µg) and ceftazidime (30 µg) were then placed 20 mm distance from augmentin disc. Extension of the edge of the inhibition zone of cefotaxime, ceftriaxone and ceftazidime disc on the side exposed to the disc containing amoxicillin and clavulanic acid is positive for ESBL (Fig. 1).

2.2.2 Phenotypic confirmatory disc diffusion test (PCDDT) for ESBL production

ESBL detection was performed as recommended by Clinical Laboratory Standards Institute (CLSI) confirmatory procedure Phenotypic confirmatory disc diffusion test using cefotaxime (30 µg) and ceftazidime (30 µg) discs alone and in combination with clavulanic acid (10 µg). A ≥ 5 mm increase in zone diameter for cefotaxime and ceftazidime in combination with clavulanic acid versus its zone when tested alone, confirmed an ESBL-producing organism [14]. *E. coli* ATCC 25922 was used as the negative control and in house ESBL-producer *E. coli* was used as the positive control (Fig. 2).

2.3 Antimicrobial Susceptibility Test

All the ESBL-producing isolates were tested for antimicrobial susceptibility by both Kirby-Bauer disc diffusion and agar dilution method against ciprofloxacin, amikacin and imipenem. *Escherichia coli* ATCC 25922 was used as quality control strain.

2.3.1 Disc diffusion method

Antimicrobial susceptibility testing of ESBL-producing isolates was done by disc diffusion method using Kirby-Bauer technique [15]. Amikacin (30 µg), ciprofloxacin (5 µg) and imipenem (10 µg) discs were obtained from Oxoid Ltd., Basingstoke, Hampshire, UK. Antibiotic potency of the discs were standardized against the reference strain, *E. coli* ATCC 25922.

The inoculum of the organism was adjusted to the turbidity of a 0.5 McFarland standard corresponding to 1.5×10^8 organisms/ml and swabbed onto the surface of a Muller-Hinton agar plate. After placing the antimicrobial disc onto the inoculated plates, the plates were incubated at 37ºC for 18-24 hours. All susceptibility results were interpreted as per recommendations of CLSI [14].

2.3.2 Agar dilution method

Minimum inhibitory concentrations (MICs) of ciprofloxacin, amikacin and imipenem were done by the standard agar dilution method. *E. coli* ATCC 25922 was used as control. The values of range of concentrations of tested antimicrobial agents were used as follows: ciprofloxacin 0.004-8 µg/ml, amikacin 0.0625-128 µg/ml and imipenem 0.125-32 µg/ml. MICs were recorded as lowest concentration of the antimicrobial agents that yielded no growth. MICs breakpoints were used in the interpretation of the results into three categories namely sensitive (S), intermediate (I) and resistant (R) as per recommendations of CLSI [14].

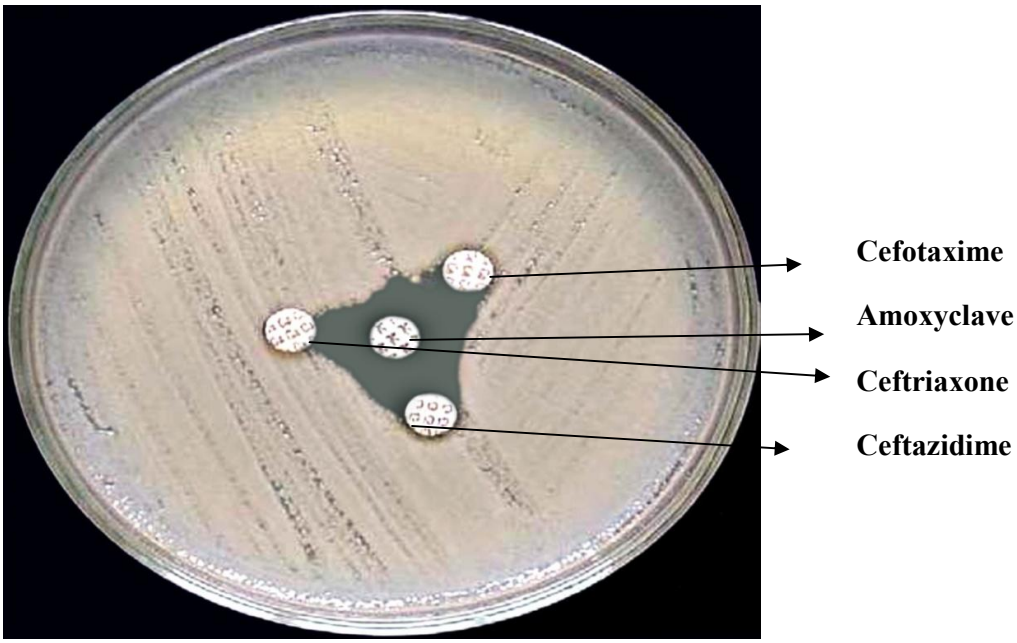

Fig. 1. Double disc synergy test (ESBL positive strain)
DDST: Double disc synergy test

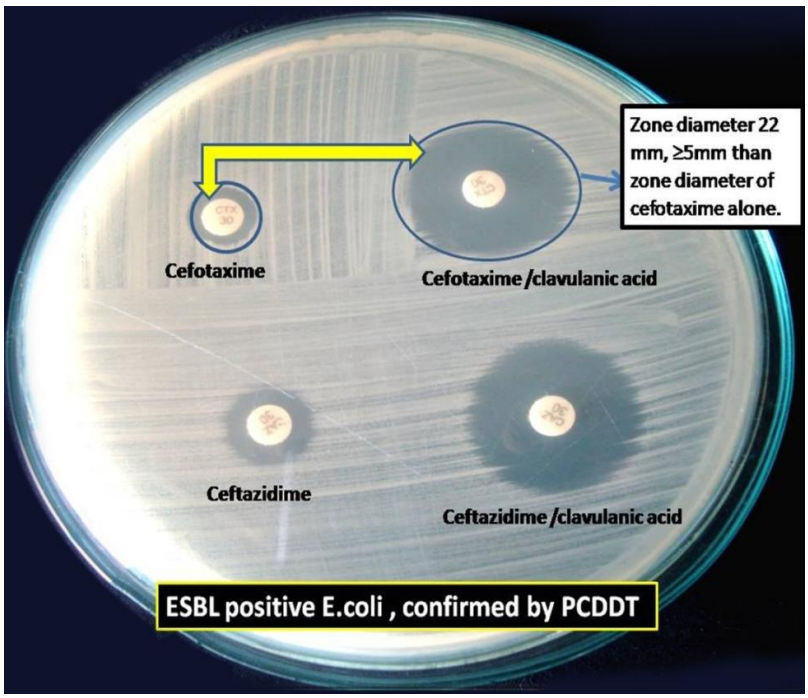

Fig. 2. Phenotypic confirmatory disc diffusion test (ESBL positive strain)
PCDDT: Phenotypic confirmatory disc diffusion test

3. RESULTS AND DISCUSSION

3.1 Results

A total of 100 ESBL-producing isolates were studied, of which 87 were from urine samples, 06 from pus, 04 from wound swab, 01 from blood, 01 from bile, 01 from sputum (Table-1). Out of 100 ESBLs which were detected by DDST, were also tested by PCDDT. Out of 75 DDST positive *E. coli*, 71 (94.67%) were also found positive by PCDDT. All DDST positive *Klebsiella* spp. (19) & *Enterobacter* spp., (06), were also positive by PCDDT (Fig. 3).

Table-2, Table-3, Table-4 and Table-5 shows the correlation of susceptibility pattern of ESBL-producing *E. coli, Klebsiella* spp. and *Enterobacter* spp. against ciprofloxacin, amikacin and imipenem by agar dilution and disc diffusion method. It was found that 85.92% ESBL-producing *E. coli* were resistant by agar dilution method in comparison 87.32% were resistant to ciprofloxacin by disc diffusion method. 4.22% ESBL-producing *E. coli* were resistant to amikacin in comparison 7.04% were resistant to amikacin by disc diffusion method. 73.68% ESBL-producing *Klebsiella* spp., were resistant to ciprofloxacin by agar dilution method in comparison 78.95% were resistant to ciprofloxacin by disc diffusion method. 21.05% ESBL-producing *klebsiella* spp., were resistant to amikacin by both disc diffusion and agar dilution method. 33.33% ESBL-producing *Enterobacter* spp. were resistant to ciprofloxacin by agar dilution method in comparison 50.0% by disc diffusion method. 100% ESBL- producing *E. coli, Klebsiella* spp. and *Enterobacter* spp. were sensitive to imipenem by both agar dilution and disc diffusion method.

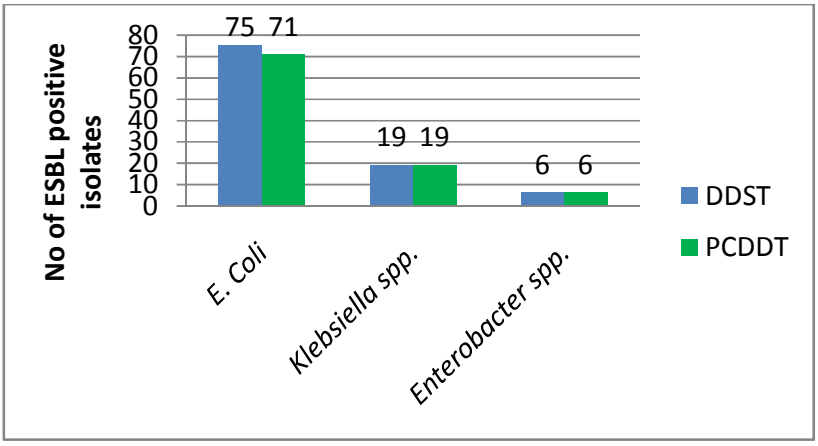

Fig. 3. Confirmation of ESBL positive isolates by PCDDT (phenotypic confirmatory disc diffusion test) among DDST (double disc synergy test) positive ESBL-producing isolates (n= 100)

PCDDT: Phenotypic confirmatory disc diffusion test. DDST: Double disc synergy test

Table 1. Distribution of ESBL-producing *E. coli, Klebsiella* spp. and *Enterobacter* spp. among study samples (n=100)

Study samples (n = 100)	ESBL-producing bacteria		
	E. coli **(n = 75)**	***Klebsiella*** **spp. (n = 19)**	***Enterobacter*** **spp. (n = 06)**
Urine (n = 87)	67	15	05
Pus (n=06)	03	02	01
Wound swab (n=04)	04	-	-
Blood (n=01)	01	-	-
Bile (n=01)	-	01	-
Sputum (n=01)	-	01	-

n = number of study samples

Table 2. Susceptibility pattern of ESBL-producing *E. coli, Klebsiella* spp. and *Enterobacter* spp. against ciprofloxacin by agar dilution and disc diffusion method

ESBL producing isolates (n = 96)	Susceptibility pattern of ESBL-producing isolates against ciprofloxacin by					
	Agar dilution method			Disc diffusion method		
	S (≤1 µg/ml)	I (2 µg/ml)	R (≥4 µg/ml)	S (≥21 mm)	I (16-20mm)	R (≤15 mm)
E. coli (n = 71)	10 (14.08)	2 (2.82)	59 (83.10)	9 (12.68)	-	62 (87.32)
Klebsiella spp. (n = 19)	5 (26.32)	1 (5.26)	13 (68.42)	4 (21.05)	1 (5.26)	14 (73.69)
Enterobacter spp.(n = 6)	4 (66.67)	-	2 (33.33)	3 (50)	1 (16.67)	2 (33.33)

n= number of study samples,S= Sensitive, I= Intermediate, R =Resistant

Table 3. Susceptibility pattern of ESBL-producing *E. coli, Klebsiella* spp. and *Enterobacter* spp. against amikacin by agar dilution and disc diffusion method

ESBL producing isolates (n = 96)	Susceptibility pattern of ESBL-producing isolates against amikacin by					
	Agar dilution method			Disc diffusion method		
	S (≤ 16 µg/ml)	I (32 µg/ml)	R (≥ 64 µg/ml)	S (≥17 mm)	I (15-16 mm)	R (≤14 mm)
E. coli (n = 71)	68 (95.78)	-	3 (4.22)	66 (92.95)	2 (2.82)	3 (4.22)
Klebsiella spp. (n = 19)	15 (78.95)	-	4 (21.05)	15 (78.95)	-	4 (21.05)
Enterobacter spp. (n = 6)	6 (100)	-	-	6 (100)	-	-

n= number of study samples, S= Sensitive, I= Intermediate, R =Resistant

Table 4. Susceptibility pattern of ESBL-producing *E. coli, Klebsiella* spp. and *Enterobacter* spp. against imipenem by agar dilution and disc diffusion method

ESBL producing isolates (n = 96)	Susceptibility pattern of ESBL-producing isolates against imipenem by					
	Agar dilution method			Disc diffusion method		
	S (≤ 1 µg/ml)	I (2 µg/ml)	R (≥ 4 µg/ml)	S (≤ 19 mm)	I (20-22 mm)	R (≥ 23 mm)
E. coli (n = 71)	71 (100)	-	-	71 (100)	-	-
Klebsiella spp. (n = 19)	19 (100)	-	-	19 (100)	-	-
Enterobacter spp. (n = 6)	6 (100)	-	-	6 (100)	-	-

n= number of study samples, S= Sensitive, I= Intermediate, R =Resistant

3.2 Discussion

Extended-Spectrum β-lactamases (ESBLs) constitute a class of plasmid-mediated β-lactamases which confer resistance to broad spectrum β-lactam antibiotics. The prevalence of ESBL-producing organism is increasing worldwide [6]. In addition resistance to cephalosporins, ESBL-producing organisms are also exhibiting resistance to fluoroquinolones group of drugs limiting further therapeutic options [16].

In this study, out of 75 DDST positive *E. coli*, 71 (94.67%) were confirmed as ESBL-producer when tested by PCDDT. All DDST positive *Klebsiella* spp. (19) and *Enterobacter* spp. (06) were confirmed as ESBL-producer by PCDDT. The result of this study was consistent with the study by Ingviya et al. [7] in Thailand, who

showed that among 100 DDST positive *E. coli* and 137 DDST positive *K.pneumoniae*, 96 (96.0%) *E. coli* and 129 (94.2%) *K. pneumoniae* were proved as ESBL-producer by PCDDT. No significance difference observed between the result of DDST and PCDDT for the detection and confirmation of ESBL phenotypically in this study.

In this study, 85.92% ESBL-producing *E. coli* and 73.68% *Klebsiella* spp. showed high MICs value against ciprofloxacin (2 µg/ml to 128 µg/ml) indicating high level resistance to ciprofloxacin. This less susceptibility may be due to widespread indiscriminate use, their oral route of administration, easy availability and affordability of ciprofloxacin over the country [17]. Rising MIC values of ciprofloxacin may lead to prolonged treatment, delayed recovery or post treatment failure. Similar findings were observed by Hassan et al. [17], who found 85% ESBL-producing *E. coli* were resistant to ciprofloxacin. The result of this study was not consistent with the study by Inviya et al. [7] in Thailand, who reported 47% ESBL-producing *E. coli* and 12% *K. pneumoniae* were resistant to ciprofloxacin. In this study, 33.33% ESBL-producing *Enterobacter* spp. were resistant to ciprofloxacin. These variations could be due to fewer number of ESBL-producing *Enterobacter* spp. tested in this study against ciprofloxacin.

About 87.32% ESBL-producing *E. coli,* 78.95% *Klebsiella* spp. and 50% *Enterobacter* spp. were resistant to ciprofloxacin by disc diffusion method in this study. This result was consistent with the study by Datta et al. [5] in India, who showed 90.8% ESBL-producing *E. coli*, 74.7% *Klebsiella pneumoniae* and 50% *Enterobacter* spp. were resistant to ciprofloxacin by disc diffusion method. Chaikittisuk and Munscrichoom [10], showed that 89% ESBL-producing *E. coli* and 72% *Klebsiella* spp. were resistant to ciprofloxacin. These findings suggest that sensitivity of ESBL-producing bacteria to ciprofloxacin is gradually decreasing.

In this study, about 4.22% ESBL-producing *E. coli* and 21.05% *Klebsiella* spp. were found to be resistant to amikacin. Similar findings were described by Soriozano et al. [18] in Spain and Liao et al. [19] in Taiwan, who found 18.7% ESBL-producing *E. coli* and 27.7% *Klebsiella* spp. to be resistant to amikacin. In this study it is found that all ESBL-producing *Enterobacter* spp. were sensitive to amikacin and MIC value of amikacin against these isolates were low (0.0625 µg/ml to 4 µg/ml).

About 7.04% ESBL-producing *E. coli*, 21.05% *Klebsiella* spp. and 100% *Enterobacter* spp. were resistant to amikacin by disc diffusion method. This result was consistent with the study by Datta et al. [5], who showed 16% ESBL-producing *E. coli*, 28.5% *Klebsiella pneumoniae* & 20% *Enterobacter* spp. were resistant to amikacin by disc diffusion method. Sashirekha et al. [20] in India, showed that 5% ESBL-producing *E. coli*

Table 5. The MIC parameter of ciprofloxacin, amikacin ad imipenem against ESBL-producing *E. coli*, *Klebsiella* spp. and *Enterobacter* spp.

ESBL producing isolates / Antimicrobial agents	MIC (µg/ml)			% susceptibility
	Range	MIC_{50}	MIC_{90}	
***E. coli* (n=71)**				
Ciprofloxacin	0.004-8	16	128	14.08
Amikacin	0.0625-128	0.5	8	95.78
Imipenem	0.125-32	0.25	0.25	100
***Klebsiella* spp. (n=19)**				
Ciprofloxacin	0.004-8	16	128	26.32
Amikacin	0.0625-128	1	8	78.95
Imipenem	0.125-32	0.25	0.25	100
***Enterobacter* spp. (n=06)**				
Ciprofloxacin	0.004-8	0.5	32	66.67
Amikacin	0.0625-128	0.25	2	100
Imipenem	0.125-32	0.25	0.25	100

n= number of study samples, MIC= Minimum Inhibitory Concentration, MIC_{50} = The concentration that will inhibit 50% of the isolate of a given bacterial class, MIC_{90} = The concentration that will inhibit 90% of the isolate of a given bacterial class, CLSI = Clinical and Laboratory Standards Institute

Note: Based on susceptibility breakpoints defined by CLSI: ciprofloxacin ≤ 1 µg/ml, amikacin ≤ 16 µg/ml and imipenem ≤ 1 µg/ml

and 10% *Klebsiella* spp. were resistant to amikacin. This result indicates that amikacin can be considered as drug of choice in the treatment of infections caused by ESBL-producing organisms.

In this study it has been found that, 100% ESBL-producing *E. coli, Klebsiella* spp. and *Enterobacter* spp. were sensitive to imipenem (MIC 0.125 µg/ml to 0.25 µg/ml) by both agar dilution and disc diffusion method. Similar findings were observed by Liao et al. [19], Soriozano et al. [18], Ingviya et al. [7], Sasirekha et al. [20], who found 100% sensitivity to imipenem against ESBL-producing *E. coli*, *Klebsiella* spp. and *Enterobacter* spp. Carbapenems (e.g., imipenem) are known to be stable against ESBL enzymes and effective in the treatment caused by ESBL-producing bacteria [21].

In this study, minor difference was observed between the sensitivity result of disc diffusion method and agar dilution method for ciprofloxacin. About 14.08% ESBL-producing *E.coli,* 26.32% *Klebsiella* spp. and 66.67% *Enterobacter* spp. were susceptible to ciprofloxacin in agar dilution method but 12.68% *E. coli,* 21.05% *Klebsiella* spp. and 50% *Enterobacter* spp. were susceptible to ciprofloxacin in disc diffusion method. This difference may be due to several factors affecting disc diffusion method; medium formulation and P^H, disc content, its storage and drug diffusion, inoculums size, incubation time and temperature [22].

4. CONCLUSION

Imipenem and amikacin are the most active and reliable agents for the treatment of infections caused by ESBL-producing organism.

FINANCIAL DISCLOSURE

There was not any financial interests related to the material in the manuscript.

FUNDING/SUPPORT

The funding organizations are public institutions and had no role in the design and conduct of the study; collection, management, and analysis of the data; or preparation, review, and approval of the manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
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http://www.sciencedomain.org/review-history.php?iid=1264&id=22&aid=9689