



## Phenotypic Detection of $\beta$ Lactamases Production Among Enterobacteriaceae Isolates By Novel Disc Placement Method

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### ABSTRACT

**Background:** Increasing occurrence of  $\beta$ -lactamases producing *Enterobacteriaceae* pose great risk to morbidity and mortality of patients. Since many genotypic and automated systems detect only Extended spectrum  $\beta$ -lactamase (ESBL) coupled with the fact that diagnostic guidelines are only given for ESBL producing *E.coli*, *Klebsiella* and *Proteus* in CLSI guidelines, it raises the need for cost effective and resourceful phenotypic methods to detect all common types  $\beta$  lactamases expressed by *Enterobacteriaceae* in routine practice.

**Aim:** The present study was designed to estimate the prevalence of  $\beta$  lactamase production among *Enterobacteriaceae* on a routine basis using simple cost effective phenotypic methods.

**Material and methods:** All samples received in the laboratory from March 2015 to August 2016 were included. *Enterobacteriaceae* were isolated and identified by standard laboratory methods. Production of three major  $\beta$ -lactamase i.e. ESBL, AmpC and metallo  $\beta$  lactamase (MBL) were detected by novel disc placement method.

**Results:** A total of 530 *Enterobacteriaceae* were isolated. *E.coli* was the most common isolate (61.5%). 42.6% isolates were found to be  $\beta$  lactamase producers. ESBL was found to be most common  $\beta$  lactamase produced by 38.11% of isolates followed by MBL (3.20%) and AmpC  $\beta$  lactamase (0.56%). Combined  $\beta$  lactamase production was seen in 0.75% isolates.

**Conclusion:** Increasing occurrence of  $\beta$  lactamase producing *Enterobacteriaceae* is a matter of concern. Being affordable and easy to perform, novel disc placement method can be used on a routine basis for screening of  $\beta$  lactamase production. This will also be helpful to notify an outbreak or any change in resistance trend in an institution.

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**INTRODUCTION:**

According to the World Health Organization (WHO), every year approx. 4.5 billion cases of gram negative infections gets reported of which 1.9 million ends in death. <sup>[1]</sup> Among gram negative infections, *Enterobacteriaceae* family members have been identified as important nosocomial pathogens; infection can lead to severe morbidity and mortality, particularly in intensive care units (ICU), internal medicine and surgical units, and pediatric units. <sup>[2]</sup> Indian hospitals have reported very high gram-negative resistance rates, with very high prevalence of Extended spectrum  $\beta$  lactamase (ESBL) producers and also high carbapenem resistance rates. <sup>[3]</sup>

The marked increase in  $\beta$ -lactamase production, including the high level constitutive producers (derepressed mutants) with ESBL, leave us with few alternatives in combating serious infections.

As these enzymes mutate to make different classes of antibiotics inactive, the number of identified  $\beta$ -lactamases has multiplied, and now there are hundreds. The selective pressures which are generated by the indiscriminate use of the beta-lactam antibiotics have led to the selection of a variety of mutated forms of  $\beta$ -lactamases such as the ESBLs, AmpC  $\beta$ -lactamases and metallo  $\beta$  lactamase (MBL) which have emerged as the most worrisome resistance mechanism which poses a therapeutic challenge to the health care settings. <sup>[4]</sup> Because of the evolving and continuing antibiotic resistance phenomenon, regular monitoring of resistance patterns is necessary to improve guidelines for empirical antibiotic therapy.

Several studies have been done for phenotypic detection of ESBL, AmpC and metallo  $\beta$ -lactamases. <sup>[5-8]</sup> The coexistence of different classes of  $\beta$ -lactamases in a single bacterial isolate often pose diagnostic and treatment challenges. The detection of the coproduction of various  $\beta$ -lactamases singly or in combinations is essential for enhanced infection control and effective antimicrobial therapy. Although ESBL detection and reporting is recommended routinely by CLSI, it lacks guidelines for the AmpC or MBL or combination of various  $\beta$ -lactamases. Although genotypic methods provide epidemiological data but being expensive they are restricted to research laboratories only. However, phenotypic methods are easy to perform and interpret and feasible in resource limited settings. In view of this, the present study was planned to estimate prevalence of

different  $\beta$  lactamases production among *Enterobacteriaceae* isolates by phenotypic novel disc placement method.

**MATERIALS AND METHODS**

The study was approved by the Institutional Ethics Committee (LNMC/Dean/2015/2146 dated 12/02/2015).

**Study Design:** Observational Cross-Sectional Study

**Place of Study:** Department of Microbiology of our institute.

**Study Period:** One and a half year (March 2015-August 2016).

**Specimen collection:** All the samples received during the study period in the microbiology laboratory were included in the study

**Inclusion criteria:**

- All non-repetitive and biochemically confirmed isolates of *Enterobacteriaceae* with single type of growth were included.
- *Salmonella spp*, *Shigella spp* and *Yersinia enterocolitica* isolated from stool samples were also included.

**Exclusion criteria:**

- *Enterobacteriaceae* occurring as mixed cultures were excluded.
- Other *Enterobacteriaceae* isolated from stool samples were excluded.

**Specimen Processing**

All samples received were processed for isolation and identification by Standard laboratory methods. <sup>[9]</sup>

**Antimicrobial susceptibility testing**

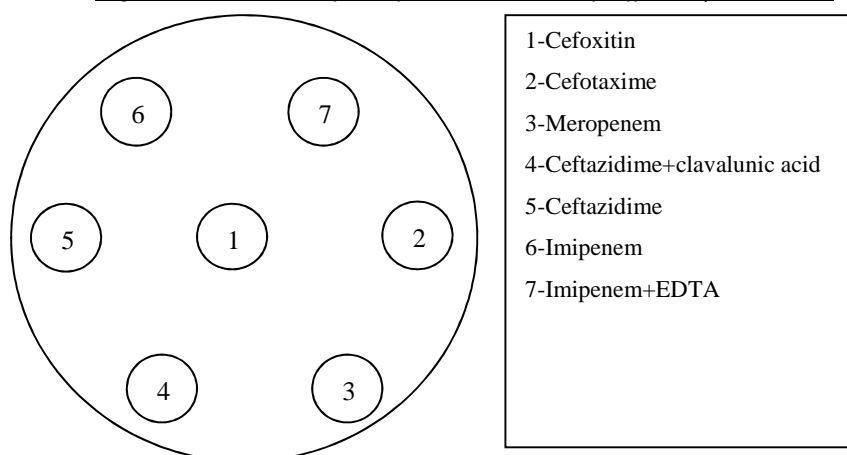
Antibiotic sensitivity test of the isolates were performed by Kirby Bauer Disc Diffusion method <sup>[10]</sup> using Mueller Hinton agar and antibiotic discs (Himedia, Mumbai), as described by Clinical Laboratory Standard Institute (CLSI) guidelines. <sup>[11]</sup>

**Detection of  $\beta$  lactamases by novel disc placement method**

Each of *Enterobacteriaceae* isolate were tested for  $\beta$  lactamases (ESBL, AmpC  $\beta$  lactamases, MBL) production by Novel disc placement method. The lawn culture of test organism was made on Muller Hinton Agar (MHA) as done for disc diffusion antimicrobial susceptibility test. In the centre of the plate, cefoxitin (30 $\mu$ g) (inducer) disc was applied. At the distance of 20mm, the disc of cefotaxime (30 $\mu$ g) was placed. From this disc in circular manner, clockwise, the discs of meropenem (10 $\mu$ g), ceftazidime + clavulanic acid (30/10 $\mu$ g), ceftazidime (30 $\mu$ g), imipenem (10 $\mu$ g), imipenem+EDTA

(10/750 µg) were placed such that any two adjacent discs will be 20mm apart from centre to centre [Figure 1].

Figure 1: Placement of disc for the detection of different β lactamase



### Detection and interpretation of different β lactamase

#### a. Extended spectrum beta lactamase(ESBL) <sup>[11]</sup>

##### Phenotypic confirmatory test as described by CLSI

ESBL production by the isolates were detected phenotypically (standard disc diffusion method) by using ceftazidime disc(30µg) and ceftazidime/clavulanic acid (30 µg/10 µg) combination disc.. Increase in zone diameter of 5 mm or greater of the combination disc when compared with the ceftazidime disc alone indicates the ESBL production by the isolates.

#### b. AmpC β lactamase Disk approximation test <sup>[12]</sup>

Cefotaxime (30µg), ceftazidime(30µgm) and cefoxitin (30µg) disks were placed 20 mm apart from centre to centre. Isolates showing blunting of the cefotaxime and ceftazidime zone of inhibition adjacent to the cefoxitin disk were identified as positive for AmpC β-lactamase production.

#### c. Metallo β-lactamase (MBL) EDTA double disc synergy test (EDTA –DDST) <sup>[13]</sup>

Imipenem and imipenem-EDTA disc (Himedia, Mumbai) were placed 20mm apart from each other centre to centre of the disc, incubated for 18-20 hrs and observed. A >7mm increase in zone size of imipenem-EDTA disc from plain imipenem disc was indicative of MBL production.

### RESULTS

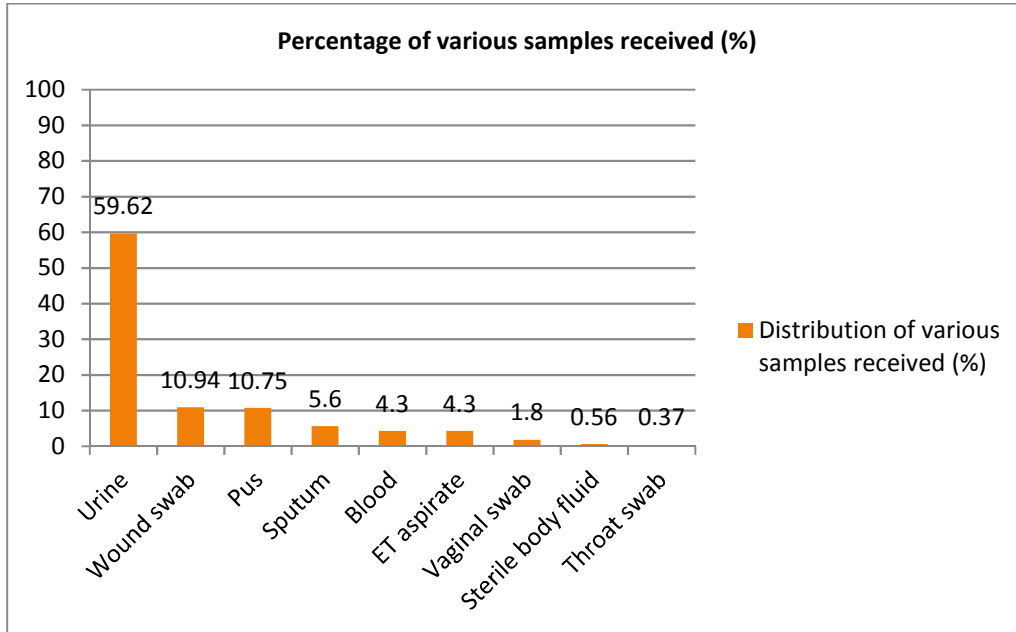
A total of 530 biochemically confirmed *Enterobacteriaceae* isolates were received in the Microbiology laboratory [Table 1].The prevalence of *Enterobacteriaceae* was found to be higher in IPD patients (53.01%).Majority i.e. 22.07% of *Enterobacteriaceae* isolates were commonly obtained from 21-30 years age group. *Enterobacteriaceae* were more commonly isolated from males (52.26%) than in females (47.7%) with male:female ratio of 1.09:1[ Table 1].

Table 1: Demographic data

Variables	Number (n)	Percentage(%)
<b>Ward wise</b>		
IPD	281	53.01
OPD	131	24.71
ICU	118	22.26
<b>Gender</b>		
Male	277	52.26
Female	253	47.7
<b>Age wise (yrs)</b>		
0-20	52	9.8
21-40	174	32.83
41-60	164	30.94
61-80	121	22.83
81-100	19	3.58

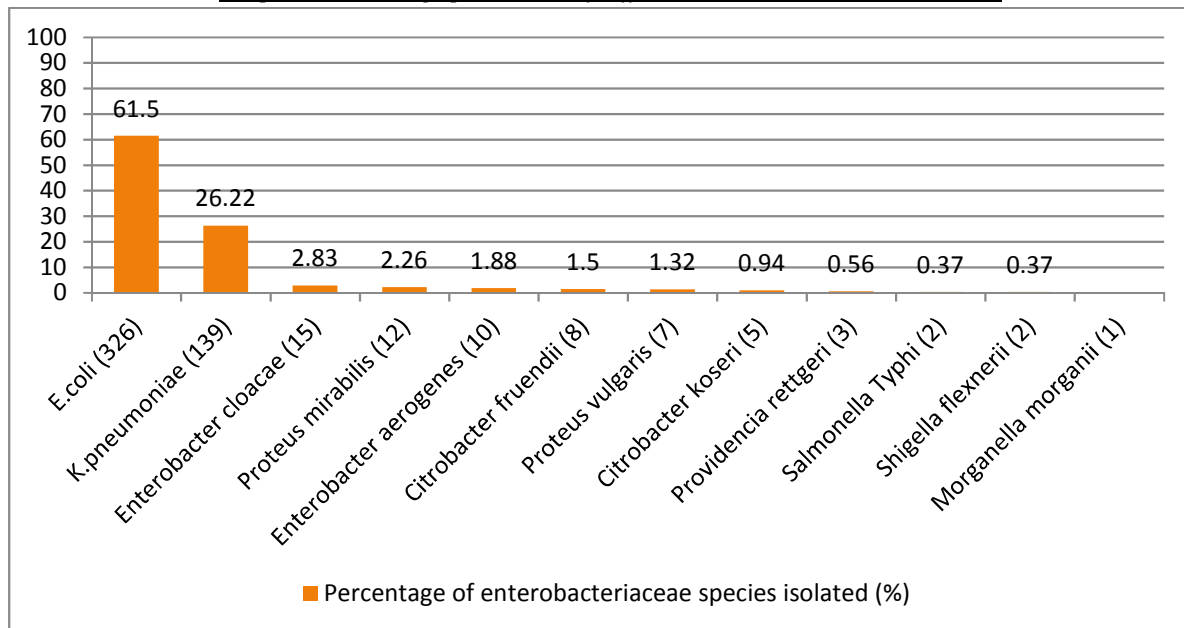
Urine samples (59.8%) comprised a major share of all the clinical samples received in the laboratory [Graph 1].

Graph 1: Distribution pattern of samples received in the lab



Among all *Enterobacteriaceae* isolates *E.coli* (61.5%) was the most common species obtained [Graph 2].

Graph 2: Percentage prevalence of different *Enterobacteriaceae* isolated



42.6% *Enterobacteriaceae* isolates were found to produce  $\beta$  lactamase. ESBL was found to be most common  $\beta$  lactamase produced by 202(38.11%) of

isolates followed by MBL 17(3.20%) and AmpC  $\beta$  lactamase 3(0.56%). Combined  $\beta$  lactamase production was seen in 4(0.75%) isolates [Table 2].

Table 2: Distribution of  $\beta$  lactamases among *Enterobacteriaceae*

Species	ESBL	MBL	AmpC	ESBL+AmpC	ESBL+MBL
<i>E.coli</i>	150	6	1		3
<i>K.pneumoniae</i>	26	7	2	1	
<i>Enterobacter aerogenes</i>	6	1			
<i>Enterobacter cloacae</i>	5				
<i>Proteus mirabilis</i>	7	1			
<i>Proteus vulgaris</i>	3	1			
<i>Citrobacter freundii</i>	2	1			

<i>Citrobacter koseri</i>	2				
<i>Morganella morgani</i>	1				
Total (n/%)	202(38.11)	17(3.7)	3(0.56)	1(0.18)	3(0.56)

## DISCUSSION

Dissemination and acquisition of *Enterobacteriaceae* infections may be silent and pose significant challenges for infection control. In our study, a total of 530 *Enterobacteriaceae* isolates were obtained during the study period. These infections are associated with increased mortality and economic costs. 53.01% of the *Enterobacteriaceae* received were from the In-Patient-Department (IPD), as compared to 24.71% from the Out-Patient-Department (OPD) and ICU (22.26%) [Table 1] highlighting the fact that most of *Enterobacteriaceae* carriage was from hospital environment and less from community. This could be due to various factors such as chronic infection, long hospital stay and invasive devices put in patients which increase the colonization susceptibility of patient. Patients at high risk for developing colonisation or infection with ESBL-producing organisms are often seriously ill patients with prolonged hospital stays and in whom invasive medical devices are present for a prolonged duration. <sup>[14]</sup>22.07% of *Enterobacteriaceae* isolates were obtained from young individuals in the age group of 21-30 years [Table 1] demonstrating the fact that young adults even though with good immunity are susceptible to acute infections by *Enterobacteriaceae*. J B Sarma et al <sup>[15]</sup> observed that young patients with no serious co-morbidities in general wards are also at risk of colonization if exposed to 'high risk' antimicrobials.

*Enterobacteriaceae* were more commonly isolated from males (52.26%) than in females (47.7%) with male:female ratio of 1.09:1 [Table 1]. Shah et al <sup>[16]</sup> studied the relation of ESBL-producing *Enterobacteriaceae* with respect to age and gender and reported more ESBL-positive isolates in males (65.33%) than females (34.67%). It has been established earlier that many Asian countries show lower disease incidence in women to be a statistical artifact related to lower reporting and care seeking for women. Urine was the most common clinical sample received in our laboratory (56.6%) followed by wound swab (11.13%), pus (10.75%) blood (3.96%) and aspirates (3.01%) [Graph 1]. Urinary samples were found to be the most common sample received in various other studies as well with distribution ranging from 29.71%-62.83%. Rudresh

et al <sup>[17]</sup>, A K Praharaj et al <sup>[18]</sup>, P N Sridhar Rao et al <sup>[19]</sup>, Nema S et al <sup>[20]</sup>. This finding confirms that most *Enterobacteriaceae* are isolated from urinary samples showing the greater prevalence of urinary tract infection as one of the top infections caused by *Enterobacteriaceae*.

Out of total 530 isolates *E.coli* (61.5%) and *K. pneumoniae* (26.2%) are the most common *Enterobacteriaceae* species that were isolated followed by other members such as *Enterobacter cloacae* (2.83%), and *Proteus mirabilis* (2.26%) [Graph 2]. Anuradha et al <sup>[21]</sup> also reported similar finding where out of total of 1252 *Enterobacteriaceae* isolates identified 61.42% were *E.coli*, 18.84% *K. pneumoniae*, 8.06% *E.cloacae* and 2.87% *Proteus mirabilis* isolates. In a study by Kargar M et al <sup>[22]</sup>, among the total *Enterobacteriaceae* detected *E. coli* (69.1%) was found to be the most common species followed by *Klebsiella* sp. (12.1%), *Enterobacter* sp. (8.4%), *Proteus* sp. (4.4%), *Citrobacter* sp. (4%) . An indiscriminate administration of  $\beta$  lactams also increases the risk of colonization of hospitalized patients with ESBL-producing *Enterobacteriaceae*. Such organisms are usually derived from colonized healthcare settings. Recent studies showed an increased prevalence of community-acquired infections with ESBL-producing organisms. <sup>[11]</sup> In the present study, ESBL was found to be the most common  $\beta$  lactamase produced by *Enterobacteriaceae* with 38.11% isolates testing positive for it followed by MBL (3.20%) and AmpC  $\beta$  lactamase (0.56%) [Table 2]. In Yulia et al.'s (2013) study <sup>[23]</sup>, the prevalence of ESBL, MBL and AmpC  $\beta$  producing *Enterobacteriaceae* isolated from intensive care unit (ICU) patients were 58.42%, 27.59% and 1.98% respectively. 35.16% ESBL, 10.98% MBL, 5.4% AmpC producers were reported by Loveena Oberoi et al <sup>[24]</sup> The reason for low prevalence of the AmpC producers in the present study could be due to the differences in the geographical distribution, which may have produced variations in the prevalence of the  $\beta$ -lactamases in the different organisms, thus giving rise to the varied resistance patterns. Resistance due to multiple mechanism in single bacterial isolate was also seen; AmpC and ESBL (0.44%) and MBL and ESBL (1.3%) [Table 2]. This observation was also noted by

Mohanty <sup>[25]</sup> in India (2010) who found 58.4% isolates of the *Enterobacteriaceae* family were carriers of both enzymes. The coexistence of ESBL and MBL was reported in 8.79% isolates, whereas the AmpC and the MBL co production was shown by 3.67% isolates and the AmpC and the ESBL co production was shown in 6.59% isolates. <sup>[24]</sup> The coexistence of different classes of  $\beta$ -lactamases in a single bacterial isolate pose diagnostic and treatment challenges. The AmpC producing organisms can act as a hidden reservoir for the ESBLs. Also, the high-level expression of the AmpC  $\beta$ -lactamases may mask the recognition of the ESBLs and it may result in a fatal and an inappropriate antimicrobial therapy. The increase in the prevalence of the AmpC, MBL and the ESBL producing isolates may be indicative of the emerging trend of more and more isolates acquiring multiple resistance mechanisms, thus rendering the antimicrobials agents ineffective. There are certain limitations of the present study. Since it is a hospital based study, it may represent only the tip of the iceberg in the overall pattern of  $\beta$  lactamases production among *Enterobacteriaceae* isolates and their resistance trends. In addition, results were not confirmed by genotypic methods.

#### CONCLUSION

High prevalence of  $\beta$  lactamase production either singly or in combination among the members of *Enterobacteriaceae* is matter of concern. The innovative disc placement method can reliably detect ESBL, MBL and AmpC  $\beta$  lactamases production. Being affordable and easy to perform this method can be used on a routine basis for screening of  $\beta$  lactamase production for all *Enterobacteriaceae* isolates. This will be helpful to notify an outbreak or any change in resistance trend in an institution. A large-scale multicentric study for detection of  $\beta$ -lactamases is need of the hour so as to know the exact burden of MDR *Enterobacteriaceae* in the community and also for initiation of evidence-based antibiotic policy. Studies should also be undertaken to compare the results with genotypic and automated methods to substantiate the findings of this simple phenotypic method.

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