Phenotypic Detection of ESBL, AmpC, MBL, and Their Co-occurrence among MDR *Enterobacteriaceae* Isolates

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Abstract

Background Emergence of extended-spectrum beta-lactamases (ESBLs), AmpC β-lactamases, and metallo-β lactamases (MBL), and their co-existence among members of *Enterobacteriaceae* pose newer diagnostic and therapeutic challenges. The present study examines the ESBL, AmpC, and MBL production by various phenotypic methods and their co-occurrence among the multidrug-resistant (MDR) *Enterobacteriaceae* clinical isolates.

Materials and Methods Four hundred non-repetitive *Enterobacteriaceae* clinical isolates were collected from the Central Referral Hospital, Sikkim. The isolates were used for identification and their antibiotic susceptibility tests were performed according to the Clinical and Laboratory Standard Institute (CLSI) guidelines. ESBL was detected by double-disc synergy test (DDST) and phenotypic confirmatory disc-diffusion test (PCDDT), AmpC detection by AmpC E-test, and boronic acid disc diffusion (BD) test. MBL was detected using the imipenem–imipenem/EDTA disc and carba-NP tests.

Results Around 76% were considered MDR. ESBL was seen in 58% and 50.4% based on DDST and phenotypic confirmation disc-diffusion test (PCDDT), respectively. AmpC was detected in 11.8% and 13.1% using a commercial E-test and boronic acid test, respectively. MBL were identified in 12.8% and 14.8% based on MBL imipenem-EDTA and carba-NP tests, respectively. Co-occurrence of ESBL and AmpC, ESBL and MBL, AmpC and MBL was seen in 5.2%, 11.5%, 1.3%, respectively, whereas a combination of these three β-lactamases was observed in only 0.3% of 304 MDR isolates.

Keywords
- enterobacteriaceae
- multidrug resistant
- beta-lactamases
- co-occurrence

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Conclusion The findings highlight a high prevalence of β-lactamases and their co-production among the *Enterobacteriaceae*, mainly in *Klebsiella pneumoniae* and *Escherichia coli* isolates. The study further highlights the necessity to identify the MDR β-lactamases stains for effective therapy in severe as well as mild bacterial infections, thereby enabling to reduce the risk of MDR in hospital and community settings.

Introduction

Infectious disease burden and antimicrobial resistance (AMR) are serious global problems related to health not only in humans but also in animals, particularly in developing countries as in the case of India. India is known for its largest antibiotic use globally, popularly known as "AMR capital of the world." Infections caused by gram-negative bacteria are considerably more worrisome than those caused by gram-positive bacterial infections as they are more commonly multidrug-resistant (MDR).[1] MDR organisms are those organisms that show resistance to one agent in any three or more antibiotics classes. Infections due to MDR organisms are consistently increasing and hence pose a challenge toward effective therapeutic options. As per the data of the World Health Organization (WHO), the mortality rate due to MDR organisms in patients is significantly much greater than that of non-MDR organisms.[2] The national pharmaceutical sales data 2000–2010 stated that more than 10 units of antibiotics consumption per person in India were highlighted in 2010 alone.[3] MDR *Enterobacteriaceae* is emerging globally as one of the most serious health problems, leading to treatment failure of both community-acquired as well as nosocomial infections.[4] One of the major causes of bacterial resistance is the inappropriate and unnecessary use of β-lactam drugs, leading to the selection of a variety of mutated forms of β-lactamases. ESBLs, AmpC, and MBL have presently emerged as the most worrisome resistance mechanisms, leading to an uncontrollable impact on antimicrobial chemotherapy. The plasmid helps in carrying these genes, facilitating the spread between microorganisms of the same family, and is often co-expressed in the same isolate.[5]

ESBLs are β-lactamases showing resistance to penicillins, cephalosporins, and aztreonam (but not to cephamycins or carbapenems) by hydrolyzing these antibiotics but inhibited by β-lactamase inhibitors such as clavulanic acid. Despite being resistant to β-lactam drugs, ESBL-producing organisms are also frequently found to show resistance to other classes of drugs such as aminoglycosides, cotrimoxazole, tetracycline, and fluoroquinolones.[6] AmpC β-lactamases are cephalosporins that have the ability to hydrolyze and inactivate cephalosporins, cephamycins, aminopenicillins, and monobactams but are less inhibited by clavulanic acid.[7] Carbapenems were known to be the only treatment for ESBL and AmpC producing infections until the emergence of carbapenem-resistant isolates. Hence, the future of antibiotics has fallen into the darkness due to the emergence of MBL producers. Adding up to this global health security threat, carbapenem-resistance *Enterobacteriaceae* (CRE) in time and again is found to be co-associated with ESBL or AmpC β-lactamase or sometimes both and can co-transferred with the plasmids.[8]

Such co-occurrence of different types of β-lactamases in a single organism may lead to diagnostic and treatment failure in crucial times, mostly in severe cases. Hence, for effective treatment of infections, it is necessary to identify the co-occurrence as antibiotic susceptibility testing alone cannot detect these resistant organisms. So, further confirmation is required by various phenotypic tests in laboratory settings. There are insufficient data regarding ESBL, AmpC, and MBL detection; also, to the best of our knowledge, no studies were found on the co-occurrence of ESBL, AmpC, and MBL β-lactamases among the members of *Enterobacteriacae* strains causing infections in Gangtok, East Sikkim, India. Sikkim is one of the northeastern states in India mainly of hilly regions having a total population of over 6 lakhs with more rural areas and fewer healthcare facilities and hospitals.[9] Detecting and analyzing these β-lactamases and their co-existence may be of great awareness in the prevention and control from further spread of such infections as well as in the treatment of severe cases. Further, MDR infections are increasing rapidly in hospital settings due to the direct use of expanded spectrum cephalosporins avoiding effective control measures. With this background, the present study has been undertaken to highlight the ESBL, AmpC, and MBL production by various phenotypic methods and their co-occurrence among the multidrug-resistant (MDR) *Enterobacteriaceae* isolates in a tertiary care hospital in Sikkim.

Materials and Methods

The present study was performed from June 2018 to May 2019 in the department of Microbiology, Sikkim Manipal Institute of Medical Sciences (SMIMS), Gangtok, Sikkim. A total of 400 non-repetitive clinical isolates of *Enterobacteriaceae* were collected from the clinical specimens (urine, sputum, pus, blood, endotracheal tip [ET], catheter tip [CT], and body fluid) sent to the microbiology laboratory of the Central Referral Hospital affiliated to SMIMS. All the isolates were stored at −80°C. The sample size was calculated using the formula, $n = z^2p(1\text{-}p)/d^2$,. where n is the sample size, z is the statistic corresponding to the level of confidence, p is the expected prevalence from studies, and d is precision (corresponding to effect size).[10] In the present study, the estimation of sample size was done using the prevalence value $p = 50\%$ (0.5) based on previous studies, correspondingly, the z value of 1.96 and precision of 5% (0.05) were considered.[2]

Based on this calculation, the *n* value was estimated and obtained to be 400 in the present study.

Inclusion Criteria

In this study, only members of *Enterobacteriaceae* isolated from different clinical specimens that is, urine, sputum, pus, blood, ET, CT, and body fluids were included.

Exclusion Criteria

All clinical isolates other than *Enterobacteriaceae* were excluded.

Identification of the clinical isolates

Microscopy was done for each specimen by Gram staining and was inoculated into MacConkey agar (MA) and blood agar (BA) plates (HiMedia Laboratories Pvt. Ltd, Mumbai, India) and incubated for 18 to 24 hours at 37°C aerobically. All isolates were then identified up to the species level for the members of *Enterobacteriaceae* by studying morphology, Gram staining, and by standard biochemical tests.[11]

Antimicrobial Susceptibility Testing

All the identified members of *Enterobacteriaceae* were subjected to antibiotic susceptibility testing using the Kirby–Bauer disk diffusion method,[12] Mueller–Hinton agar (MHA) following the CLSI guidelines.[13] The antibiotics used are ampicillin (10 µg), amoxicillin clavulunic acid (20/10 µg), piperacillin–tazobactam (100/10 µg), cefuroxime (30 µg), cefuroxime axetil (30/20 µg), ceftazidime (30 µg), cefoperazone–sulbactam (75/25 µg), cefepime (30 µg), ertapenem (10 µg), imipenem (10 µg), meropenem (10 µg), amikacin (30 µg), gentamicin (10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), nitrofurantoin (300 µg) and trimethoprim-sulfamethoxazole (1.25/23.75 µg). *Escherichia coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as controls in each set of susceptibility tests.

Beta-lactamases Detection by Phenotypic Methods

Three hundred four *Enterobacteriaceae* isolates were found to be MDR as they showed resistance to at least one antibiotic in the three or more antimicrobial categories. These MDR isolates were further screened using various phenotypic methods for the detection of ESBL, AmpC, and MBL production. *E. coli* ATCC 25922 (ESBL negative) and *K. pneumoniae* ATCC 700603 (ESBL positive) were used as control strains.

ESBL Detection Tests

Double-disc synergy test: The isolates were screened for ESBL production following the method reported by Kolhapura et al.[14] Phenotypic confirmatory disk-diffusion test: ESBL production was confirmed using the method and interpretation from the previous study by Shukla et al.[15]

AmpC Detection Tests

AmpC E-test: Double-sided AmpC E-test strips (AB Biomerieux, Sweden) containing cefotetan in one end and cefotetan–cloxacillin was used following as per the previous publication.[16] AmpC boronic acid disc diffusion test: Screening for AmpC was done using the phenotypic test following the previous study.[5]

MBL Phenotypic Detection Test

Detection using imipenem/imipenem EDTA disc: Phenotypic detection of MBL production was performed using a disc of imipenem (10 µg) and another combination disc of imipenem. EDTA disc (10/750 µg) was performed as per the method described by Chanu et al.[7]

Carba-NP test: The test was performed and interpreted as per the study done by Nordmann et al.[17]

Data Analysis

The statistical data analyses were performed using the computer software program Statistical Package for Social Sciences (SPSS) version 20. Chi-square test for ESBL-AmpC and ESBL-MBL was done using an online calculator open-epi version-3.0 2×2 considering *p*-value less than 0.05 as significant. Multidrug-resistance Index (MDR Index) was calculated using the reported study by Krumperman,[18] formulated as a/b where "a" is the number of antibiotics showing resistance by the isolate and "b" is the number of antibiotics used. In our study, the value of "a" is taken as the MDR isolates showing resistance to three or more antimicrobial categories and "b" is the number of antibiotics (19 in total) used. The MDR index was calculated only for the maximum isolated pathogens that are *E.coli* and *K. pneumoniae* as other organisms were significantly low. MDR index of less than 0.2 and greater than 0.2 was taken as an indicator to differentiate between low- and high-risk drug-resistant pathogens.

Results

Bacterial Isolates

Out of 400 non-duplicate members of *Enterobacteriaceae* isolates obtained from various clinical samples. *E. coli* (283) 70.8% was the mostly pathogen isolated, followed by *K. pneumoniae* (88) 22.0%, *Enterobacter cloaceae* (9) 2.25%, *Morganella morganii* (8) 2%), 1% (4) isolates each of *Serratia marcescens* and *Salmonella enteric serovar Typhi*, and 0.25% (1) isolate each of *Proteus vulgaris, Providencia rettgeri, Citrobacter fruendii*, and *Shigella sonnei*. The majority of the isolates were from clinical specimen of urine (271) 67.8% and others from sputum (49) 12.3%, pus (35) 8.8%, blood (30) 7.5%, ET (10) 2.5%, CT (4) 1.0%, body fluid (1) 0.25% isolated from various in-patient (IP) and out-patient departments (OPDs).

Antimicrobial Susceptibility Test

All 400 *Enterobacteriaceae* isolates were tested for antimicrobial susceptibility following the CLSI guidelines.[13] Out of which, 304 (76%) isolates were found to be MDR, showing resistance to at least one of the agents in three or more antimicrobial categories. *E.coli* (74.91%) and *K. pneumoniae* (73.86%) isolates showed the maximum MDR. The single isolated pathogen of *P. vulgaris*, *P. rettgeri*, *C. fruendii,* and *S. sonnei* also showed MDR (►**Table 1**). The MDR isolates

Table 1 Multidrug-resistant (MDR) pattern in different *Enterobacteriaceae* isolates

Enterobacteriaceae isolates ($n = 400$)	MDR	None MDR
Escherichia coli ($n = 283$) 70.8%	212 (74.91%)	71 (25.08%)
Klebsiella pneumoniae ($n = 88$) 22%	65 (73.86%)	23 (26.13%)
Enterobacter cloaceae ($n = 9$) 2.25%	8	1
Morganela morganii ($n = 8$) 2%	8	0
Serratia marcescens ($n = 4$) 1%	4	0
Salmonella enteric serovar Typhi ($n = 4$) 1%	3	1
Proeus vulgaris ($n = 1$) 0.25%	1	0
Providencia rettgeri ($n = 1$)	1	0
Citrobacter fruendii ($n = 1$)	1	0
Shigella sonnei ($n = 1$)	1	0
Total: 400	304 (76%)	96 (24%)

exhibited maximum resistance to antimicrobial categories of penicillins (32–98%), cephalosporins (25–94%), quinolones classes (79–86%), followed by aminoglycosides (18–36%), nitrofurantoin (41%) and sulphonamides (40%). Also, *Salmonella enteric serovar Typhi* showed higher resistance to aminoglycosides (75%) than cephalosporins (50%) and fluoroquinolones (25%). These 304 MDR *Enterobacteriaceae* were isolated from IP wards (77.6%) and OP wards (22.4%). Out of these, specifically, 10.9% were from ICUs and 9.5% were from pediatric patients.

Beta-lactamases Detection by Phenotypic Methods

All 304 MDR *Enterobacteriaceae* isolates were detected for ESBLs, AmpCs, and MBLs production by various phenotypic methods as shown in ►**Table 2**.

Around 56 (18.4%) isolates of the overall MDR isolates showed co-occurrence either with any two or all three β lactamases as represented in ►**Table 3**. The calculated value was found to be $P^{*} = 0.01073$ and $P^{**} = 0.00561$ for the co-production of ESBL-AmpC and ESBL-MBL, respectively.

Discussion

In our study, the majority (304 [76%]) of the total 400 *Enterobacteriaceae* isolates were found to be MDR, which is the main cause of worry as this could hamper the current therapeutic scenario. One possible reason could be the rise in the pharmaceutical sector in Sikkim, which could have contributed to a greater rate of antibiotic resistance due to the amount of waste reaching the various waterways that may indirectly act as a continuous source of AMR.[19] Other associated reasons could be the increasing rate of diseases, inadequate hospitals, or healthcare centers, lack of appropriate diagnostic methods, poor infection control practices, and the affinity of clinicians with the empirical treatment practices may have further supported the global crisis of AMR.[3] The increase in healthcare costs could be another main reason in developing countries such as India. Considering the male-female ratio (111:193) among the MDR isolates, females (63.48%) were much higher than males (36.51%). One of the possible reasons could be due to high-risk factors for urinary tract infections in females than males.[20]

Out of the 304 MDR isolates, the majority (77.6%) were isolated from IP compared with OP 22.4%. This could be due

Table 2 Beta-lactamase production in different *Enterobacteriaceae* isolates

MDR *Enterobacteriaceae* isolates ($n = 304$)	ESBLs		AmpCs		MBLs	
	DDST	PCDDT	E-test	BDD test	I/I-EDTA	CarbaNP
Escherichia coli (212) 69.7%	111 (52.3%)	96 (45.2%)	25 (11.8%)	25 (11.7%)	14 (6.6%)	17 (8%)
Klebsiella pneumoniae (64) 21%	49 (76.5%)	43 (67.1%)	7 (10.9%)	11 (17%)	20 (31%)	23 (35.9%)
Enterobacter cloacae (9) 2.9%	5 (55.5%)	4 (44.4%)	2 (22.2%)	2 (22.2%)	2 (22.2%)	1 (11.1%)
Morganella morganii (8) 2.6%	5 (62.5%)	5 (62.5%)		2 (25%)	1 (12.5%)	0
Serratia marcescens (4) 1.3%	2 (50%)	3 (75%)		0	1 (25%)	2 (50%)
Salmonella enteric serovar Typhi (3) 0.9%	0	0	0	0	0	0
Proeus vulgaris (1) 0.3%	0	0	0	0	0	0
Providencia rettgeri (1) 0.3%	1	1	1	0	1	1
Citrobacter fruendii (1) 0.3%	1	0	1	0	0	0
Shigella sonnei (1) 0.3%	1	1	0	0	0	0
Total	175 (58%)	153 (50.4%)	36 (11.8%)	40 (13.1%)	39 (12.8%)	44 (14.8%)

Table 3 Co-occurrence of β-lactamases in different *Enterobacteriaceae* isolates

Isolates	ESBL + AmpC	ESBL + MBL	AmpC + MBL	ESBL + AmpC + MBL
Escherichia coli	8	11	2	0
Klebsiella pneumoniae	5	19	2	1
Enterbacter cloacae	2	1	0	0
Serratia marcescens	0	2	0	0
Morganella morganii	1	1	0	0
Providentia rettgiri	0	1	0	0
Total	16 (5.2%)	35 (11.5%)	4 (1.3%)	1 (0.3%)

to prolonged hospitalization, overuse of third-generation cephalosporins in hospital settings, and the presence of invasive devices.[21] Yet, another cause of worry is that from the 68 isolates from OPDs, ESBL production was seen in 20 isolates, 8 AmpC, and 4 MBL producers, mostly observed was *E. coli* followed by *K. pneumoniae* and *E. cloacae*. The co-production was also seen in four of the isolates. This could be a risk factor favoring the community spread as members of *Enterobacteriaceae* are known to cause community as well as hospital-acquired infections.[21] *Enterobacteriaceae* family, especially *E. coli* and *K. pneumoniae* are also known to cause UTIs that may further become critical if not treated.[22] In the present study, cephalosporins and aminoglycosides resistance were seen slightly higher in *K. pneumoniae* (45–73%) and (26–30%) than in *E. coli* that showed (25–70%) and (9–25%), respectively. The majority of the isolates showed carbapenem resistance in *K. pneumoniae* (28–32%), followed by *E. coli* (7–8%). This could be possibly due to the expression of carbapenemases, plasmid AmpC, and permeability changes predominantly in *K. pneumoniae* than *E. coli*.[23] On the contrary, fluoroquinolones resistance was more in *E. coli* (65–76%) than *K. pneumoniae* (56–60%). A possible reason could be that fluoroquinolones are the drug of choice for bacterial infections such as urinary tract infections (UTIs), which are known to be mainly caused by *E. coli*.[20] Though lesser numbers of isolates of *S. enteric serover Typhi* 4 (1%) was isolated in our study, three (75%) isolates showed resistance to fluoroquinolones and one isolate (25%) was found to be ciprofloxacin-resistant. This thoroughly co-relates with the analysis done by Britto et al in India.[24] Ciprofloxacin is recommended as the antibiotic most appropriate for enteric fever as first-line cephalosporins are restricted to avoid ciprofloxacin-resistant *S. enteric* (ICMR AMRS 2016–2018). MDR index was calculated for the major isolated pathogens of *E.coli* and *K. pneumoniae* that showed a value of less than 0.2 in 74.91% of *E.coli* and 73.86% of *K. pneumoniae* isolates. Yet again, a high rise in the MDR index among commonly found hospital isolates indicates that there is a higher risk of infection by such MDR pathogens to humans. It also highlights a prompt investigation to provide a better risk assessment to patients infected by such MDR pathogens.

Based on the phenotypic test, the present study showed ESBL detection of 50.4% which is lesser than the study reported by Mirza et al,[8] but much higher than the other studies reported by Kolhapura et al,[14] Khanna et al,[25] Shivanna and Rao.[5] Higher rates of ESBL production were seen in *K. pneumoniae* (67.1%) than *E. coli* (45.2%) isolates, in which a similar rate of ESBL among *K. pneumoniae* (42%) than *E. coli* (33%) has also been reported from a multicentric study done in India earlier.[21] The ESBL detection rate in major hospitals of India highlights a range from 19% to 60%.[26] AmpC production was detected in 13.1% isolates, which is slightly lower than the study done by Shivanna and Rao,[5] but similar to Mirza et al.[8] Many studies too indicate boronic acid disc-diffusion test as a better method than other phenotypic methods to identify the producers of AmpC although no specific confirmatory phenotypic tests have been announced for the detection of AmpC enzymes by CLSI so far.[27] Our result for MBL production was found to be much higher (14.8%) than the studies done by Mirza et al,[8] but lower than that reported by Chanu et al.[7] The difference in the prevalence rate in our study could also be endorsed due to various factors such as our hospital antibiotic guidelines and practices, ethnic differences in various populations, different phenotypic methods and procedures performed in other studies.[25,28]

The present study showed the co-occurrence of ESBL and AmpC in 5.2% of isolates, which is similar to the study done by Chanu et al (5.7%)[7] and Khanna et al (5.6%),[25] but much lower than that reported by Shivanna and Rao (19%).[5] The present study reported a co-occurrence pattern of ESBL and MBL in 11.5% of isolates, which is higher than that reported in other studies.[7,8,25] The AmpC and the MBL co-production in our study was found in only four (1.3%) isolates as compared with the study done by Kolhapura et al (6.2%),[14] but similar as reported by Mirza et al (1.7%).[8] The present study also found co-occurrence of the three β-lactamases, that is, ESBL, AmpC, and MBL together in one isolate, whereas none of these studies[5,7,8,25] had shown it, except the study reported by Kolhapura et al (5.1%),[14] which reported a much higher co-occurrence than our study.

Co-production of these β lactamases in this study gives the idea of horizontal transfer of multiple resistance enzyme genes in the same isolate. This re-emphasizes the utmost need for continuous supervision, especially MDR *Enterobacteriaceae* in the hospital as well as community settings, for timely and suitable therapy.[8] The co-production of β-

lactamases in ESBL-AmpC and ESBL-MBL was statistically proven using the chi-square test that showed that such co-production in β-lactamases is statistically significant. Hence, whenever such MDR organisms are isolated, they should be screened and dealt with proper antibiotics to avoid therapeutic failure. The infections caused by various β-lactamase pathogens, especially *Enterobacteriaceae* is life-threatening as there are no specific guidelines provided to detect such β-lactamases production. This may lead to inappropriate antibiotic therapy, further worsening the present situation of antimicrobial resistance.[25] The high MDR rate detected in such a small populated and remote region highlights a peak of danger in bigger populated cities of India. With the present scenario of the pandemic crisis of COVID-19, people may consume antibiotics by themselves because of fear or ignorance; this may show a more dangerous elevated graph of antibiotic resistance pattern in India. Molecular methods are more specific and reliable but costly to be affordable by a common setting in developing countries such as India. However, these phenotypic tests can detect various β-lactamases in simple laboratory settings, are faster and easy to access on a routine basis, and are more valid and cost-effective. Such phenotypic methods can be implemented in every simple laboratory setting with a lower cost to screen, report, and record data for the presence of these β-lactamases in different rural regions of India.

Conclusion

The members of *Enterobacteriaceae* in this geographical region showed high multidrug resistance. A high prevalence of β-lactamases and their co-production were also found among the *Enterobacteriaceae* family, mainly in *K. pneumoniae* and *E. coli* isolates. The present study highlights the necessity to identify the MDR β-lactamases stains for effective therapy in severe as well as mild bacterial infections, thereby enabling to reduce the risk of MDR in hospital and community settings. Further, similar studies in specific geographical regions may be encouraged to have a brief idea of organism-based antibiotic susceptibility patterns and β-lactamase production for effective management and treatment regime.

Ethical Approval

The present study protocol was reviewed and approved by the Institutional Ethics Committee, SMIMS (SMIMS/IEC/2018–033). Informed consent was taken from the study participants. The privacy of the information taken was retained by omitting names and other personal details from the extraction sheet.

Author's Contributions

Salvia T. and Dolma K.G. contributed to conceptualization and design. Salvia T., Khandelwal B., and Dolma K.G. contributed to data acquisition. Salvia T., Khandelwal B., and Dhakal O.P. contributed to data analysis and interpretation. Salvia T. contributed to writing the original draft. Laishram S. Singh, Salvia T., and Dolma K.G. contributed to drafting and revising the manuscript. Laishram S. Singh and Dolma K.G. gave the final approval for publishing.

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Conflict of Interest

None declared.

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