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Research Article

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Phenotypic Detection of Carbapenemases and β-lactamases induced Carbapenem Resistance in Enterobacteriaceae

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Abstract Carbapenem-resistant Enterobacteriaceae has challenged the therapeutic efficacy and raised the most worrisome global issue of public health importance. The study was conducted to determine the burden of carbapenem resistant isolates of Enterobacteriaceae producing different types of β-lactamases. During the study period, a total of 310 enterobacterial pathogens were isolated and identified from the clinical specimens obtained from the patients visiting Kanti Children Hospital. By Kirby Bauer disc diffusion method, β-lactamase producers and multidrug resistant isolates were detected and ESBLs and carbapenemase producers were screened among them. Phenotypic detection of ESBLs was done by Combination disc test. Modified Hodge test was employed for detection of carbapenemase producers and they were typified by inhibitor based combination disk tests. A total of 251 (81.0%) isolates were β-lactamase producers and 213 (68.7%) were multidrug resistance among the Enterobacteriaceae. Typification of β-lactamases from 23 (7.4%) Modified Hodge Test positive isolates assorts 4 as KPC producers, 11 as MBL producers, 3 as AmpC β-lactamase producers and 1 with both KPC and MBL enzyme while 4 remained unclassified. The majority of carbapenem resistants were *E. coli* (52.1%) followed by *K. pneumoniae* (34.8%) and most of the CRE were resistant (56.52%) to all the combinations of ESBL test. Various classes of carbapenemases were found to have emerged among Enterobacteriaceae in Nepal. Since, the profound variation is found in β-lactamases of CRE, to reduce the risk of severe calamity effective detection procedures are mandatory in all clinical laboratories.

Keywords Carbapenem Resistant, Enterobacteriaceae, *Klebsiella pneumoniae* carbapenemase, Metallo β-lactamase, AmpC β-lactamase, Modified Hodge test

Introduction

Emergence of multidrug resistant Enterobacteriaceae and their global recirculation is one of the most worrisome public health problems worldwide [1-3]. Carbapenems, the last line of therapy, are now frequently being used to treat multidrug resistant infections, and increasing resistance of bacteria to this class of β-lactams leaves the health care system with almost no effective drugs choice [4].

A common mechanism of bacterial resistance to β-lactams is the production of β-lactamases that hydrolyze the drugs [5]. After ESBLs and AmpC β-lactamases the emergence of novel β-lactamases (*i.e.* carbapenemases) with direct carbapenem-hydrolyzing activity has contributed to an increased prevalence of carbapenem resistant Enterobacteriaceae (CRE) [6].

Classification of the β-lactamases categorizes them into 4 molecular classes A-D, among these 3 of them (Class A, B and D) comprise carbapenemases [7]. *Klebsiella pneumoniae* carbapenemase (KPC) is the most important enzyme of class-A. Class-B comprises Metallo-β-lactamase (MBL) and Class-D comprises Oxa-type β-lactamases [8]. However, Class-C β-lactamase (i.e. AmpC β-lactamase), a non-carbapenemase, in hyper-production with combined porin loss may also provide resistance towards carbapenems [9].

In recent years, carbapenemases have been widely detected among the members of Enterobacteriaceae including *Escherichia coli, Klebsiella pneumoniae and Citrobacter freundii* [10-11]. Although many cases of multidrug resistance are repeatedly being encountered in Nepal [12-14], very limited data are available for the resistance mechanisms of CRE. Present study focuses on the assessment of the burden of carbapenem resistant isolates of Enterobacteriaceae and determination of the different types of enzymes produced by them.

Materials and Methods

Study Site and Population

The study was carried out in Pathology Department of Kanti Children Hospital, Maharajgunj, Kathmandu from September 2013 to May 2014. During the period, a total of 2,688 clinical specimens from patients under the age of 12 years were collected and processed according to the standard laboratory methods.

Isolation and identification of Enterobacteriaceae

A total of 310 Enterobacterial species were isolated and identified as described in the Bergey's Manual of systemic bacteriology [15].

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing (AST) was performed for Amoxycillin (AMX), Amikacin (AK), Aztreonam (AZM), Cotrimoxazole (COT), Ciprofloxacin (CIP), Ceftriaxone (CTR), Cefotaxime (CTX), Ceftazidime (CAZ), Cefexime (CFM), Doxycycline (DOX), Gentamycin (GEN), Imipenem(IMP) and Meropenem (MRP)[16]. β-lactamase producers [17] and multi drug resistant (MDR) isolates [18] were selected.

Detection of ESBL and Carbapenemase production

The ESBL producers and carbapenemase producers were screened [15] among the enterobacterial isolates. ESBL producers were detected by combine disc diffusion method using 'CTX versus CTX+CV (Cefotaxime-Clavulanate)' and 'CAZ versus CAZ+CV (Ceftazidime-Clavulanate)' [15]. Carbapenemase production was confirmed by Modified Hodge Test (MHT) [19].

Differentiation of β-lactamase types

Combination disc test (CDT) was employed to classify the β-lactamase-types [20-21]. All the MHT positive strains were differentiated as KPC, MBL and AmpC β-lactamases producers.

Quality Control

Each test was standardized by using *Escherichia coli* ATCC 25922 as a control strain.

Data Processing and Analysis

The study data were organized and analyzed by using Statistical Package for Social Science (SPSS) software (version 19.0).

Results

A total of 310 pathogenic enterobacterial isolates were identified from 2,688 clinical specimens. *E. coli* was the most predominant bacteria, constituting 198/310 (63.9%), among the 10 species isolated.

Table 1: Isolated species of bacteria from 2,688 clinical specimens

Ec	Kp	Ko	Mm	ST	SP	Cit	Ent	Pv	Pm
198	38	15	4	24	8	2	2	10	9

Ec: *E.coli*; Kp: *K. pneumoniae*; Ko: *K. oxitoca*, Mm: *M. morganii*; ST: *S.* Typhi; SP: *S.* Paratyphi-A; Cit: *Citrobacter* sp.; Ent: *Enterobacter* sp.; Pv: *P. vulgaris*; Pm: *P. mirbilis*

Among the 13 antibiotics used, imipenem was the most effective drug and was resistant only with 8.4% of isolates. Meropenem (13.2 %), amikacin (17.1.0 %), azteronam (22.9.1 %), doxycycline (24.2 %) and gentamycin (30.7 %) were more effective than the lower-class β-lactams like amoxicillin (79.7 %) and cephalosporins.

Table 2: % Resistance of various antibiotics

Antibiotics	AK	AMX	AZM	CFM	CTX	CAZ	CTR	CIP	COT	DOX	GEN	IMP	MRP
Resistants (%)	17.1	79.7	22.9	69.7	63.2	65.8	67.8	63.2	61.0	24.2	30.7	8.4	13.2

Among all, 251 (81%) isolates were β-lactamase producers and 213 (68.7%) were MDR. Similarly, 89 (28.7%) were ESBL producers and 23 (7.4%) were confirmed as carbapenem resistant by MHT. Highest percentage (100%) of β-lactamase producers were found among *Citrobacter* sp., *Enterobacter* sp. and *P. vulgaris* followed by *E. coli* (90.9%) and *K. pneumoniae* (86.8%). *Citrobacter* sp. (100%), *Enterobacter* sp. (100%), *K. pneumoniae* (81.6%), *P. vulgaris* (80.0%) and *E. coli* (75.3%) were found to have greater percentage of MDR than any other species. Similarly, 39.8% *E. coli*, 39.5% *K. pneumoniae*, 13.3% *K. oxytoca* and 50% of *M. morganii* were ESBL producers. All the *Citrobacter* isolates, 21.1% *K. pneumoniae*, 6.0% *E. coli* and 4.2% *S.* Typhi were found resistant to carbapenems.

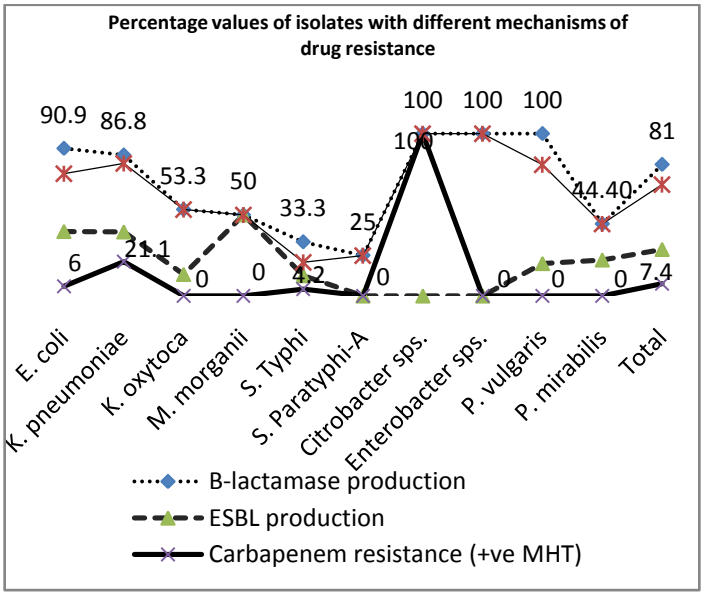

Figure 1: *Percentage values of isolates with different mechanisms of drug resistance*

Classification of 23 MHT positive isolates grouped 4 in Class-A, 11 in Class-B, 3 Class-C and 1 both A and B while 4 were undefined. Among these 23, 13 were resistant to both 'CTX/CTX+CV' and 'CAZ/CAZ+CV' combinations of ESBL test and were found to be distributed as 4 in A, 6 in B, 1 in A+B and 2 non-classified. The3 isolates were positive to both the combinations of ESBL tests in which 2 were carrying Class-B and 1 was with Class-C enzyme. Similarly, 7 CRE positive for CTX/CTX+CV combination were distributed as 3 in Class-B, 2 in Class C and 2 non-classified.

Table 3: Results of ESBL test

ESBL test	**CRE**	**Classes of β-lactamase enzymes**				
		A	B	C	A+B	Undefined
+ve for CTX combination	7	0	3	2	0	2
+ve for both combinations	3	0	2	1	0	0
Resistant to both ESBL combinations	13	4	6	0	1	2
Total	23	4	11	3	1	4

Discussion

In the present study, of the 2,688 clinical specimens, growth of Enterobacteriaceae was found only on 20.7 % of the specimens. *E. coli* was the most frequently isolated organism with 63.9 % of the total isolates.

AST result revealed higher level of resistant to lower antibiotics of β-lactam group: Amoxycillin (79.7%), Cefixime (69.7%), Ceftriazone (67.8%), Cefotaxime (63.2%) and Ceftazidime (65.8%) in comparision to Aztreonam (22.9%), Gentamycin (30.7%), Doxycycline (24.2%), Amikacin (17.1%) and carbapenems. For carbapenems, many researchers have claimed 4-16 folds greater potency of meropenem than imipenem in *E. coli* and other enterobacterial members [22-23]. By contrast, in our study imipenem with 8.4% resistance was found as more active against Enterobacteriaceae, than meropenem (13.2%). Among the 23 CPE, imipenem was sensitive against 21.7% isolates while meropenem was sensitive only on 8.7% of isolates. A harmonic result of 22.2% resistance to meropenem and 17.3% resistance to imipenem was reported in a tertiary care hospital in India [24]. Rhomborg and Jones have justified it as the gradual increase meropenem resistance in recent years [25].

Among the total, 81.0% were β-lactamase producers and 68.7% were MDR isolates. 90.9% *E. coli* and 86.8% *K. pneumoniae* were β-lactamase producers while, 75.3% *E. coli* and 81.6% *K. pneumoniae* were MDR. Similar previous studies in Kathmandu found 41.1% [12] and 63.4% [14] MDR from clinical specimens. This shows a gradual increase in the number of cases of MDR in Nepal.

In our study we found 28.7% of the total isolates as ESBL positive, however, the global prevalence of ESBL positive isolates presently varies from 1%-74% [26]. Susic described various mutants of CTX-M, TEM and SHV beta-lactamases, as responsible for the increasing cases in species of Enterobacteriaceae [27].

We found 23 of the isolates as carbapenemase producers by MHT. Among these, 4 of them were KPC producers, 11 were MBL carbapenemase producers while an isolate of *E. coli* was found to produce both enzymes in combination. Among the Class-B carbapenemases producers 4 were sensitive towards aztreonam. Such phenomenon have been frequently described and taken as an indicator of MBL production [7]. There is an increasing prevalance of KPC

worldwide [28]. Among MBL carbapenemases a new type of enzyme called New Delhi Metallo β-lactamase is gaining a great concern due to its capability to spread rapidly [29].

In our study 3 isolates producing Class-C enzymes capable of conferring resistance to carbapenams were falsely detected as carbapenemases by MHT. Over production of ESBLs along with AmpC β-lactamase combined with the porin loss may have provided resistant to carbapenems in them [30]. Decreased outer membrane permeability in AmpC β-lactamase producers is capable of providing resistance for *E. coli* and *K. pneumoniae* towards cephalosporins, monobactams and carbapenems even in the absence of carbapenemase enzymes [31].

Due to the resistance developed in lower classes of antimicrobials, antimicrobials with greater potency are in use. This favors for growing rate of resistance to the pinnacle of antibiotic; carbapenem, which is arising as a global threat in recent years [32]. We have observed different mechanisms of carbapenem resistance in enterobacterial pathogens and a huge variation in pattern of antibacterial sensitivity. The results from our study have contributed to a better understanding of the subtleties of multidrug-resistant β-lactamase producers along with carbapenemase producers and their resistant mechanisms. We observed many strains of CRE susceptible to some other common antibiotics, signifying the possibility of treatment with relevant combination-therapies. This may contribute to provide imminent direction to fight against these emerging resistant pathogens.

Conclusion

The rate of β-lactamase production and the count of MDR isolates were found higher in Enterobacteriaceae. With this study, Carbapenem resistant Enterobacteriaceae was found to have emerged in Nepal. Distribution of carbapenemases and β-lactamases was varied and several were found in combination with ESBL production displaying the diversity. Both KPC and MBL carbapenemases were identified either existing as single or in combination. In addition to carbapenemases, hyper-productions of AmpC β-lactamases along with ESBLs were also detected as the cause of resistance towards carbapenems.

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