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Phenotypic Detection of Different Classes of Beta-lactamases among the Isolates of Carbapenem Resistant 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 specimens of urine, blood, pus, sputum, stool and body fluids 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 typed into different molecular classes 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 carbapenemresistants 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 beta-lactamases of CRE, to reduce the risk of severe calamity effective detection procedures are mandatory in all the clinical laboratories.

Keywords: Enterobacteriaceae, Carbapenem Resistant, β-lactamase, *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 (Nikaido, 2009; Pereira *et al*., 2011 and Savard*et al*., 2011).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 (Nordman*et al.*, 2012).

A common mechanism of bacterial resistance to β-lactams is the production of β-lactamases that hydrolyze the drugs (Forbes *et al*., 2007).After ESBLs and AmpCβ-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) (Hildron*et al.*, 2008).

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 (Giske*et al*., 2011).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 (Tsakris*et al*., 2009).

In recent years, carbapenemases have been widely detected among the members of Enterobacteriaceae including *Escherichia coli, Klebsiella pneumoniae and Citrobacterfreundii*(Arnold *et al*., 2011 and Peleg& Hooper, 2010).Though various cases of multidrug resistance are repeatedly being encountered in Nepal(Baral, 2008; Pokhrel*et al*.,2006and Poudel, 2010), very limited data are available for the resistance mechanisms of CRE.Present study focuses on theassessmentof the burden of carbapenem resistant isolates of Enterobacteriaceaeand determination of the different classes of β-lactamases produced by them.

MATERIALS AND METHODS

Study Site and Population

Present 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 (Brenner *et al*., 2005).

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was performed forAmoxycillin, Amikacin, Aztreonam, Cotrimoxazole, Ciprofloxacin, Ceftriaxone, Cefotaxime, Ceftazidime, Cefexime, Doxycycline, Gentamycin, Imipenem and Meropenemby modified Kirby-Bauer disk diffusion method as recommended by Clinical and Laboratory Standards Institute (CLSI, 2011). Finally, β-lactamase producers were screened as described by Livermore and Brown (2001)and multi drug resistannt (MDR) isolates were selected as described by Magiorakos*et al.,* (2012).

Phenotypic detection of ESBL and Carbapenemase production

The ESBL producers and carbapenemase producers were screened (CLSI, 2011) among the enterobacterial isolates. The ESBL producing phenotypes were detected by combine disc diffusion method using 'CTX/CTX+CV' (Cefotaxime versus Cefotaxime plus Clavulanate) and 'CAZ/CAZ+CV' (Ceftazidime versus Ceftazidime plus Clavulanate) combinations(CLSI, 2011). The confirmation of carbapenemase production was determined by Modified Hodge Test (CDC, 2009).

Phenotypic differentiation of typeof β-lactamases

Differentiation of types was done with minor optimization of Combination disc test: CDT (Ambretti*et al*., 2013 and Birgy*et al*., 2012).Meropenem, Meropenem-PBA, Meropenem-EDTA, and Meropenem-Cloxacillinwere placed on a cation-adjusted MHA with a lawn of 0.5 McFarland test bacteria.Augmentation of the inhibition zone by ≥5 mm around the meropenem disc with PBA was taken as indicative for production of Class-A or KPC carbapenemase, with EDTA as indicative of production of Class-B (MBL)carbapenemaseand with both PBA and EDTA as indicative for the co-production of KPC and MBL enzymes. However, an increase in zone size with both PBA and cloxacillin was considered as the presence of Class C (AmpC β-lactamase) enzyme with porin loss.

Quality Control

Quality of each test was standardized by using *Escherichia coli* ATCC 25922 as a control strain.

Data Processing and Analysis

All the study data were entered into a computer database and verified. Data maintained in the computer sheets 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. The growth was found significantly high in number i.e. 237(76.5%) in urine sample while least number of pathogens i.e. 4 (1.3%) were encountered in each of body fluids and other samples (sputum, stool, throat swabs, endotracheal tube sample). *E. coli* was the most predominant bacteria constituting 198 (63.9%) and the least count of 2(0.7%) growths was found in each of the*Citrobacter*sp.and *Enterobacter*sp. among all the 10 species isolated.

Number of bacteria isolated from different clinical specimens

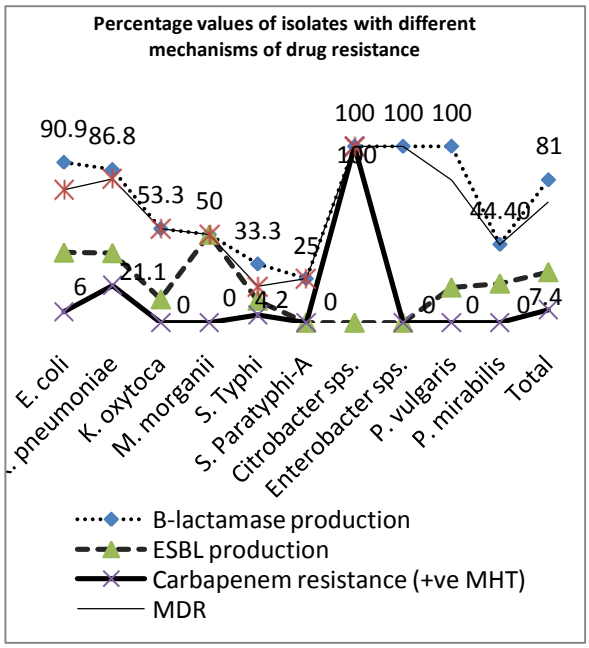
Organisms	Urine	Blood	Pus	Body fluids	Other	Total
E. coli	182	2	14	0	0	198
K. pneumoniae	21	6	5	2	4	38
K. oxytoca	10	0	3	2	0	15
M. morganii	4	0	0	0	0	4
*S.*Typhi	3	21	0	0	0	24
S. Paratyphi-A	0	8	0	0	0	8
Citrobactersps.	0	2	0	0	0	2
Enterobactersps.	2	0	0	0	0	2
P. vulgaris	6	0	4	0	0	10
P. mirabilis	9	0	0	0	0	9
Total	237	39	26	4	4	310

Of the13 antibiotic used,imipenemwas the most effective drugand was resistant only with 8.4% islates. Meropenemwas found resistant to 13.2% and the configuration was followed by other antibiotics such as amikacin (17.1.0%), azteronam (22.9.1%), doxycycline (24.2%) and gentamycin (30.7%) while the lower class β-lactams like amoxicillin (79.7%) and cephalosporins were least sensitive.

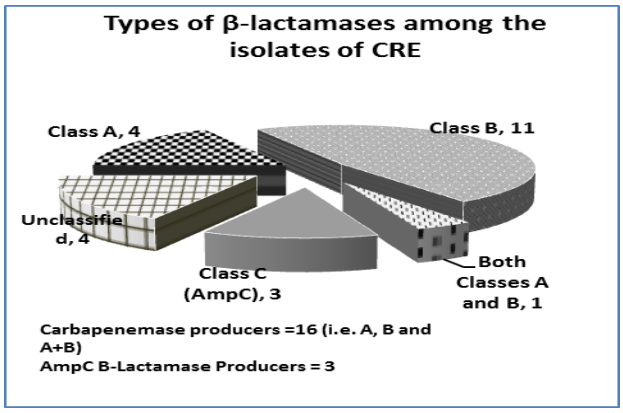
Antibiotics	AK	AMX	AZM	CFM	CTX	CAZ	CTR	CIP	COT	DOX	GEN	IMP	MRP
Resistants (%)	17.1	79.7	22.9	69.7	63.2	65.8	67.8	63.2	61.0	24.2	30.7	8.4	13.2

Among all,251 (81%) were β-lactamase producers, 213 (68.7%) were identified as MDR strains, 89 (28.7%) were ESBL producers and 23 (7.4%) of them were confirmed to be resistant to carbapenem antibiotics. Highest percentage i.e., 100%of β-lactamase producers were found in *Citrobacter*sp.,*Enterobacter*sp. and *P. vulgaris* followed by *E. coli* (90.9%) and *K. pneumoniae* (86.8%). After *Citrobacter*sp. and *Enterobacter*sp. greater percentage of MDR was found in *K. pneumoniae*(81.6%), *P. vulgaris* (80.0%) and *E. coli*(75.3%) 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 isolates of*Citrobacter* sp. were Carbapenem resistance. Among others, 21.1% *K. pneumoniae*, 6.0% *E. coli* and 4.2% *S.*Typhi were found resistant to carbapenems.

carbapenems.



Distribution of β-lactamase producers among 23 MHT positive isolates was found as 4 in Class-A, 11 in Class-B, 3 Class-C and 1 both A and B while 4 were undefined.



Among the total CRE 52.1% were *E. coli*, 34.8%*K. pneumoniae*, 8.7% *Citrobacter*sp. and rest was *S.* Typhi.

Organism	CRE	
	Number	Percentage
E. coli	12	52.1%
K. pneumoniae	8	34.8%
S. Typhi	1	4.3%
Citrobacter sp.	2	8.7%

Among 23 Carbapenem resistant isolates of Enterobacteriaceae13 were resistant to both 'CTX/CTX+CV' and 'CAZ/CAZ+CV' combinations. These isolates were found to be distributed to all the enzyme classes except Class-Ci.e 4 in A, 6inB, 1 in A+B and 2 non-classified. Among 3 bacteria conveying positive results with both the combinations of ESBL tests 2were carrying Class B and 1 was with Class C enzyme. Similarly, 7 CRE with CTX-M ESBLwere distributed as 3 in Class-B, 2 in Class C and 2non-classified whereas none of them were found to produce TEM/SHV type ESBL.

ESBL test	**CRE**	**Classes of 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 foundonly in 20.7% of the specimens. *E. coli*was the most frequently isolated organism with 63.9% of the total isolates. *E. coli* is a normal flora of human body and is highly opportunistic if patient is immunocompromised (Todar, 2012). It is one of the most diverse bacterial species that only 20% of the genome is common to all the strains and about 2% of *E. coli* DNA consists of mobile genetic elements, which are responsible for their continuous evolution (Lukjancenko*et al*., 2010).

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%), Ceftazidime (65.8%); in comparision with 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 (Zhanel*et al*., 1988; Piller*et al*., 2008). 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 similar study in a tertiary care hospital in India, reported 22.2% resistance to meropenem and 17.3% resistance to imipenem (Padmini and Appalaraju, 2004) which seems in harmony with our result. In a study from 2004 to 2008, Rhomborg and Jones (2009) have explained the gradual increase meropenem resistance than imipenem in later years than before.

Among the total, 81.0% were β-lactamase producers and 68.7% were MDR isolates. Within the species, 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. In similar studies, Baral (2008) and Poudel (2010) found 41.1% and 61.3% MDR from clinical specimens, which indicate a gradual increase in the number of cases with MDR in Nepal. Bush (1997) has explained the increasing level of drug resistance as a consequence of evolution of new β-lactamases.

In our study we found 28.7% of the total isolates were ESBL positive, however, the global prevalence of ESBL positive isolates presently varies from 1%-74% (Thokar*et al*., 2010). Similar previous studies conducted by Ashrafian*et al*. (2012) and Srisangkaew and Vorachit (2003) found 32.7% and 40% ESBL producers respectively. Susic, (2004) has mentioned various mutants of CTX-M, TEM and SHV beta-lactamases, as responsible for the increasing cases in species of Enterobacteriaceae.

We found 23 of the isolates as carbapenemase producers by MHT. Among these, 4 of them were *Klebsiella pneumoniae*carbapenemaseproducers, 11 were Metallo β-lactamasecarbapenemaseproducers while an isolate of *E. coli* was found to produce both enzymes in combination. According to Nordmann*et al*., (2009) higher proportions of Class-Aenzymes,providing resistance towards carbapenems, are of KPC (*Klebsiella pneumoniae*carbapenemase) type. Rhomberg and Jones, (2009) mentioned that there is an increasing prevalance of KPC producing *Klebsiella pneumoniae* worldwide.

In this study, among the 11 isolates producing Class-B carbapenemases 4 were sensitive towards aztreonam. Class-B enzymes are MBLs (metallo β-lactamases) requiring zinc ion on their active site and are inhibited by divalent cationchelators such as EDTA which can typically hydrolyze extended spectrum cephalosporins and carbapenems efficiently but not aztreonam(Giske*et al*. 2011). IMP and VIM are the most predominant carbapenemases of this group(Yong et al., 2009); nonetheless, in recent years a new type of enzyme called New Delhi Metallo β-lactamase is gaining a great concern due to its capability to spread rapidly (Savard*et al*., 2011). Walsh (2010) and Zhao and Hu (2011) have explained additional genes in bacteria carrying MBL carbapenemase that render them resistanteven withaztreonam.

Class-C enzymes or AmpC β-lactamase are not considered as carbapenemase (Giske*et al*. 2011); however, 3 isolates in our study producing Class-C enzymes capable of conferring resistance to carbapenams were falsely detected as carbapenemases by modified Hodge test. Previous study of Bradford *et al*., (1997) reported the carbapenem resistance in *K. pneumoniae*from New York due to the over production of ESBLs along with AmpC β-lactamase combined with the porin loss. Philippon*et al*., (2002) described it as, decreased outer membrane permeability in AmpC β-lactamase producers that is capable of providing resistance for *E. coli* and *K. pneumoniae* isolates towards cephalosporins, monobactams and carbapenems even in the absence of carbapenemases.

We have observed wide variation in mechanisms of resistance of enterobacterialpathogens and a huge dynamic pattern of antibacterial sensitivity of carbapenemase producing bacteria.Higher classes of antimicrobials with greater potency are in use due to the resistance developed in earlier antimicrobials. Due to this there is a growing rate of resistance to the pinnacle of antibiotic, carbapenem, and is emerging as a great threat in recent

years (Upadhayay*et al*., 2012).The results from our study have contributed to a better understanding of the subtleties of multidrug-resistant β-lactamase producers along with carbapenemase producers, their defense mechanisms, selection and susceptibility to antibiotic therapy.We have observed that many strains of CRE were susceptible to many other common antibiotics. This result is signifying the possibility of treatment with relevant combination-therapies of monobatam, tetracyclines, aminoglycosides, fluoroquinolones and carbapenems if they have synergistic and bactericidal effects against emerging resistant pathogens.

CONCLUSION

The number of β-lactamase producersand the MDR isolates in Enterobacteriaceaewere found increasing. With this study, Carbapenem resistance in Enterobacteriaceae was detected to have emerged in Nepal. Distribution of carbapenemase and β-lactamase was varied and several were found in combination with ESBL production displaying the diverse pattern among Enterobacterial pathogens. Both KPC and MBLcarbapenemases were identifiedeither existing as single or in combination. In addition to carbapenemases, hyper-productions of AmpC β-lactamases along with ESBLs were also noticed as the cause of resistance towards carbapenems.

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