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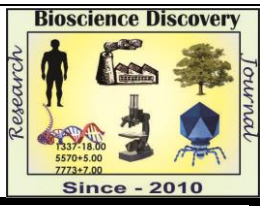

Prevalence of β-Lactamase Producing Carbapenem-Resistant Enterobacteriaceae among the Patients Attending Bharatpur Hospital

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Abstract

Carbapenems are regarded as the last drugs of choice for the treatment of the infections caused by drug-resistant bacteria. The emergence of carbapenem-resistant Enterobacteriaceae (CRE) has become a major public health concern worldwide and a new challenge in the treatment of infectious diseases. The study was conducted to determine the prevalence of CRE at a tertiary care hospital in Bharatpur Chitwan, Nepal from September 2016 to February 2017. Aseptically collected clinical specimens (urine, pus, and blood) were cultured in Mac-Conkey agar and blood agar and the Gram-negative isolates obtained were subjected to an array of biochemical tests. Antibiotic Susceptibility Test (AST) was performed against carbapenem antibiotics for Enterobacteriaceae isolates by the disc-diffusion method according to CLSI (Clinical and Laboratory Standards Institute) guidelines. Among 1720 samples, only 287 showed growth. Enterobacteriaceae isolates were recovered from 140 samples. Carbapenem resistance in *Klebsiella pneumoniae* (57.1%) was higher than in *E. coli* (41.3%). Overall prevalence of CRE was 40.7%. Among the CRE isolates, *E. coli* (78.9%) was more frequent than *K. pneumoniae* (21.0%). On phenotypic testing, the carbapenemase-producing bacteria were found to be *K. pneumoniae* (33.3%), *Enterobacter aerogenes* (33.3%) and *E. coli* (15.6%). Higher prevalence of carbapenem-resistant Enterobacteriaceae isolated from hospitalized and OPD-patients emphasize an urgent need for CRE control activities and rationalizing the use of antibiotics.

INTRODUCTION

The Enterobacteriaceae is a family of bacteria with many different genera and species. They are Gram-negative, non-spore forming and facultative anaerobes, which includes many opportunistic and pathogenic species (Brenner *et al.*, 2005). *E. coli, K. pneumoniae, Enterobacter* spp. and *Proteus mirabilis* are the common clinical isolates of the Enterobacteriaceae family. Similarly, other common genera include *Shigella* spp., *Salmonella* spp., *Citrobacter* spp., *Yersinia* spp., *Serratia* spp. (etc) a (Mahon *et al.*, 2010). In the treatment of infections caused by *K. pneumoniae* and other Enterobacteriaceae, cephalosporins and other β-lactams are common anti-microbial agents. In the past, third and fourth generation cephalosporins were the first choice in the treatment of Enterobacteriaceae infections. However, in recent times, the resistance of Enterobacteriaceae to these antibiotics has been well documented (Paterson *et al.*, 2003; Saurina *et al.*, 2000).

Carbapenem antibiotics-ertapenem, imipenem and meropenem are known as "the last line of antibiotic defense." They are capable of inhibiting both Gram-negative and Gram-positive organisms and possess the broadest spectrum of antibacterial activities and potency of all the β-lactam antibiotics (Bassetti *et al.*, 2009). Carbapenem-resistant Enterobacteriaceae (CRE) is a family of Gram-negative bacteria that are difficult to treat because they have high levels of resistance to the antibiotics which may be due to overproduction of multidrug efflux pumps by bacterial cell membranes (CDC, 2013; Nair and Vaz, 2013). Antimicrobial activity of carbapenem antibiotics is exceptionally high. Many multidrug-resistant, hospital-acquired bacteria are often sensitive to carbapenems. Nevertheless, extended use of carbapenems has resulted in some carbapenem resistance in Gram-negative organisms such as certain Enterobacteriaceae and *Pseudomonas* (Murray et al., 2009). Increasing prevalence of Carbapenem-Resistant Organisms (CROs) in health-care facilities has been reported not only from Asia, but also from the USA, Africa and Europe (Gupta *et al.*, 2011; Cantón *et al.*, 2012; Hawkey, 2008; Brink *et al.*, 2012; Nordmann *et al.*, 2011).

Presence of carbapenemases among the members of Enterobacteriaceae such as *K. pneumoniae*, *Enterobacter*, *E.coli* and *Citrobacter freundii* predominantly contribute to the emergence of carbapenem resistance among them (Peleg and Hooper, 2010). Bacteria are mainly resistant to β-lactam antibiotics by producing enzymes, such as extended-spectrum beta-lactamase (ESBL), AmpC beta-lactamase and carbapenem-hydrolyzing enzymes (carbapenemases) (Jacoby *et al.*, 2004). Carbapenemases including *K. pneumoniae* Carbapenemase (KPC), Verona Integron-encoded Metallo-beta-lactamase (VIM), Imipenem-hydrolysing lactamase (IPM) and Oxacillinase (OXA) belong to different families of beta-lactamases (Queenan and Bush, 2007).

Infections caused by carbapenem-resistant Enterobacteriaceae (CRE) are a great challenge to physicians (CDC, 2009; Munoz-prince and Quinn, 2009). In Shanghai, China, *K. pneumoniae* is the second most commonly isolated bacterial pathogen among Gram-negative bacilli according to the Shanghai Bacterial Resistance Surveillance Program (Zhu and Zhang, 2006; Zhu and Zhang, 2007). Similarly, one of the studies in tertiary care hospital of Kathmandu, Nepal showed the incidence of carbapenem resistance among the bacteria belonging to Enterobacteriaceae ranged from 4.5% to 20.0% (Karn *et al.*,2016). Even though in recent years, increased rates of carbapenem resistance have been reported from all around the world, in the context of Nepal, very limited studies investigating the emergence of CRE have been reported. In light of this background, the study was aimed at assessing the prevalence of β-lactamase producing carbapenem-resistant Enterobacteriaceae at a tertiary hospital in Bharatpur, Nepal.

MATERIALS AND METHODS

Study area and research design

This cross-sectional study was carried out from September 2016 to February 2017 in Bharatpur hospital, Chitwan, which is a tertiary care government hospital in the central part of Nepal situated in the province no 3 and the microbial analyses were carried out at the Microbiology laboratory of the hospital. Over this period, a total of 1720 non-duplicated clinical specimens (pus, blood, and urine) were collected aseptically from both the in and out-patients. The samples were collected in clean, sterile and leak-proof plastic containers. Verbal consent was sought from each patient prior to sample collection.

Isolation and identification of bacteria

The aforementioned clinical specimens were cultured on blood agar and Mac-Conkey agar and incubated at 37°C for 24 hours. The isolates were Gram stained, and Gram-negative rods were further subjected to various biochemical tests to ensure that they belonged to Enterobacteriaceae. Different biochemical tests performed were-indole, MR-VP (Methyl Red-Voges Proskaeur), oxidase, catalase, citrate utilization, SIM (Sulfide Indole Motility), TSI (Triple Sugar Iron), nitrate reduction, oxidative-fermentative and urease (Forbes *et al.*, 2007).

Antibiotic susceptibility test

Susceptibility tests were performed by the disc diffusion method (CLSI, 2011). The turbidity of inoculum was adjusted to the equivalent turbidity of 0.5 McFarland standards. Eighteen hours cultures of test organisms incubated at 37°C were standardized by diluting to 0.5 McFarland turbidity standards before spreading over the surface of Mueller-Hinton agar (Titan Biotech Ltd., Bhiwadi-301019, Rajasthan, India) plates using a sterile cotton swab and allowed to dry for 2 to 5 minutes. Using sterile tweezers, antimicrobial discs-ertapenem (10 µg), imipenem (10 µg) and meropenem (10 µg) [Hi-Media Laboratories Pvt. Ltd.,

Mumbai, India] were placed widely spaced aseptically at three corners on the surface of the plate. Tweezers were re-flamed after application of each disc. The plates were incubated at 37°C for 24 hours. Following incubation, the diameter of inhibition zone was measured with a transparent ruler and expressed in millimeters (mm) as sensitive, intermediate and resistant based on CLSI guidelines (CLSI, 2011).

Phenotypic confirmation of carbapenemase and AmpC beta-lactamase production

Only Enterobacteriaceae showing resistant to carbapenem antibiotics were subjected to phenotypic confirmation of carbapenemase and AmpC beta-lactamase production by combined disc diffusion method. Metallo-beta-lactamase production was confirmed by using imipenem and imipenem+ ethylenediaminetetraacetic acid (EDTA) discs (Bhandari *et al.,* 2015). Likewise, *K. pneumoniae* carbapenemase (KPC) production was detected by using imipenem and imipenem+ phenylboronic acid discs (Tsakris *et al.,* 2011). Furthermore, cefoxitin and cefoxitin+phenylboronic acid discs were used for the confirmation of AmpC beta-lactamase production (Bhandari *et al.,* 2015).

Quality control for test

For quality control of biochemical tests, purity plate was used. Similarly, for the standardization of the culture and antimicrobial susceptibility testing, *E. coli* (ATCC 25922) was used as a control strain.

Statistical analysis

Data collected were summarized, tabulated and analyzed using Statistical Package for Social Sciences (SPSS 20.0) software. The results were presented through tables, pie-chart, and bar-diagram.

RESULTS

Out of 1720 samples examined, only 287(16.7%) showed growth of organisms while rest of 1433(83.3%) samples didn't show any growth. Enterobacteriaceae isolates were recovered from 140 samples. *E. coli* was obtained from the majority of the samples (77.9%), followed by *K. pneumoniae* (15.0%) and *E. aerogenes* (4.3%) whereas *Proteus* spp. was the least frequent (2.9%) isolate. Non-Enterobacteriaceae isolates were recovered from 147 samples. *Staphylococcus* spp. was the most common isolate present in 78.9% samples, followed by *Acinetobacter* spp. (10.9%) and *Pseudomonas* spp. (8.2%) whereas *Enterococcus* spp. was the least frequent (2.0%) isolate (Table 1). Majority of the CRE isolates were recovered from urine sample (54.0%) followed by pus (44.0%) and blood (2.0%) (Figure 1).

Table 1: Sample distribution by type of isolates

Family	Isolates	No. of sample	%
Enterobacteriaceae	*E. coli*	109	77.9
	K. pneumoniae	21	15.0
	E. aerogenes	6	4.3
	Proteus spp.	4	2.9
Non-Enterobacteriaceae	*Staphylococcus* spp.	116	78.9
	Acinetobacter spp.	16	10.9
	Pseudomonas spp.	12	8. 2
	Enterococcus spp.	3	2.0

Out of 109 *E. coli* isolates, 8 (7.2%) were resistant to meropenem, 9 (8.2%) were resistant to imipenem, and 28 (25.7%) were resistant to ertapenem. Out of 21 *K. pneumoniae* isolates, only one (4.8%) was resistant to meropenem, 3 (14.3%) were resistant to imipenem, and 8 (38.0%) were resistant to ertapenem. Out of 6 *E. aerogenes* isolates, all (100.0%) were sensitive to meropenem and imipenem whereas 4 (66.7%) were sensitive and 2 (33.3%) were intermediate to ertapenem. Out of 4 *Proteus* spp. isolated, all were sensitive to both meropenem and imipenem and 3 (75.0%) were sensitive and 1 (25.0%) was intermediate to ertapenem (Table 2).

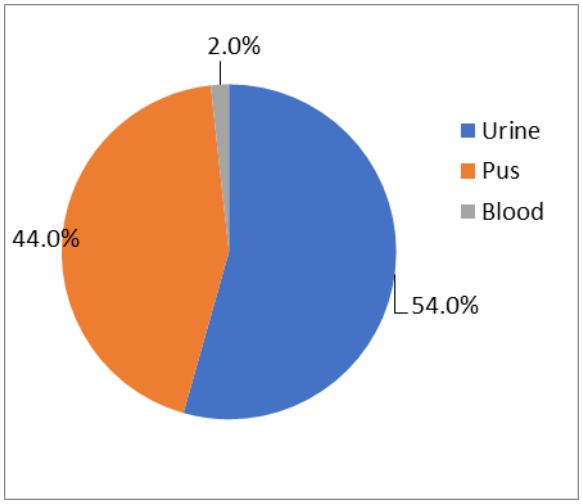

Figure 1. Sample-wise distribution of CRE

Table 2: Antibiotic susceptibility patterns of Enterobacteriaceae against carbapenems

Enterobacteriaceae isolates (N=140)	**Antibiotics susceptibility pattern (By disc diffusion method)**								
	Meropenem			**Imipenem**			**Ertapenem**		
	S(%)	**I(%)**	**R(%)**	**S(%)**	**I(%)**	**R(%)**	**S(%)**	**I(%)**	**R(%)**
E. coli (n=109)	99(90.9)	2(1.90)	8(7.2)	92(84.4)	8(7.3)	9(8.2)	69(63.3)	12(11.0)	28(25.7)
K. pneumoniae (n=21)	19(90.5)	1(4.8)	1(4.8)	15(71.4)	3(14.3)	3(14.3)	11(52.4)	2(9.5)	8(38.0)
E. aerogenes (n=6)	6(100.0)	0(0.0)	0(0.0)	6(100.0)	0(0.0)	0(0.0)	4(66.7)	2(33.3)	0(00.0)
Proteus spp. (n=4)	4(100.0)	0(0.0)	0(0.0)	4(100.0)	0(0.0)	0(0.0)	3(75.0)	1(25.0)	0(0.0)
Total	128	3	9	117	11	12	87	17	36

Note: S=Sensitive, I=Intermediate and R=Resistant

Out of 140 isolates of Enterobacteriaceae, 57(40.7%) showed resistance to carbapenems. Among them, 45(78.9%) were *E. coli*, and 12(21.0%) were *K. pneumoniae*, whereas *E. aerogenes* and *Proteus* spp. did not show resistance to carbapenems. On phenotypic testing, the most common carbapenemase-producing bacteria were found to be *K. pneumoniae* (33.3%) and *E. aerogenes* (33.3%). *E. coli* (15.6%) produced carbapenemase whereas no *Proteus* spp. was found to produce carbapenemase (Table 3).

Table 3. Carbapenemase production among Enterobacteriaceae

Enterobacteriaceae isolates (N=140)	MBL		AmpC		MBL+KPC		MBL + AmpC		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%
E. coli (n=109)	9	8.2	4	3.7	2	1.8	2	1.8	17	15.6
K.pneumoniae(n=21)	6	28.6	-	-	1	4.8	-	-	7	33.3
E. aerogenes(n=6)	1	16.7	-	-	1	16.7	-	-	2	33.3
Proteus spp. (n=4)	-	-	-	-	-	-	-	-	-	-

Note: MBL: Metallo-β-lactamase, KPC: *K. pneumoniae* carbapenemase, AmpC: AmpC β-lactamase

DISCUSSION

In the present study, among 1720 clinical samples examined, only 287 (16.7 %) showed growth of organisms while the remaining 1433 (83.3 %) didn't show any growth.

Enterobacteriaceae isolates were recovered from 140 samples only (111 urine, 25 pus, and 4 blood). Majority of the Enterobacteriaceae isolates were recovered from urine (79.3%) followed by pus (17.8%) and blood (2.8%). *E. coli* (77.9%) was the most predominant Enterobacteriaceae isolate followed by *K. pneumoniae* (15.0 %), *E. aerogenes* (4.3%) and *Proteus* spp. (2.9%). In the study performed at an Urban Community of Meerut city, India by Prakash and Saxena, 2013, *E. coli* was found to be the most predominant uropathogen with the prevalence rate of 42.6% and the second most prevalent isolate was *K. pneumoniae* (18.7%) followed by *Proteus* spp. (9.0%) and *E. aerogenes* (7.1%). In a similar type of study performed by Zaidah *et al.*, 2017, out of 477 clinical growth positive samples, the prevalence of *K. pneumoniae* was found to be 85.5% (408/477). Their study showed quite a higher frequency of *K. pneumoniae* isolates than our study (15.0%).

We observed a higher prevalence of carbapenem-resistance in *K. pneumoniae* 12/21 (57.1%) followed by *E. coli* 45/109 (41.2%). None of the isolates of *E. aerogenes* and *Proteus* spp. were resistant to the carbapenems. In the study conducted by Morfin-Otero *et al.*, 2012, both *E. aerogenes* and *Proteus* spp. were 100.0% sensitive towards meropenem and imipenem which is similar to the findings in the present study. A previous study in Nepal by Bora *et al.* (2014) highlighted that 18.9% of *E. coli* and 21.0% of *K. pneumoniae* were found to be carbapenemase producers. In the present study, altogether 57 CRE isolates were obtained that makes the overall prevalence rate of carbapenem-resistance 40.7% (57/140). Among the CRE isolates, *E. coli* 78.9% (45/57) was the most common followed by *K. pneumoniae* 21.0% (12/57%). The overall prevalence of CRE (40.7 %) is quite high for a drug which is normally reserved as the drug of last resort. Shahid *et al.* 2012 reported that the prevalence of CRE at a tertiary hospital in North India was 1.8% (16/893) that consisted of *E. coli* (10) and *K. pneumoniae* (6). Their study showed quite a lower prevalence of CRE than the present study. Prevalence of CRE in our study may be higher because no strict antibiotics policies are guiding the sale of antibiotics in the country; therefore haphazard use of antibiotics and self-medication are common practices. Parimala, 2017 reported the prevalence of CRE in South-India was 44.3% which is higher than the present study. In another study conducted by Brennan *et al.* 2014, among 97.0% of clinical culture, 102 cases of CRE were reported over 957220 patients. Among them 89 (87.0 %) were *K. pneumoniae* and 13 (13.0 %) were *E. coli*. In the study conducted by Jacoby *et al.* (2013), out of 48813 isolates 11155 were reported as *K. pneumoniae*, 31890 were reported as *E. coli*, and 5768 were reported as *E. aerogenes*. Among these isolates, 593 (5.3%) *K. pneumoniae*, 32 (0.1%) *E. coli* and 69 (1.2%) *E. aerogenes* were found to be CRE. Ling et al. 2015 conducted a similar study at a tertiary care hospital in Singapore, which reported 203 cases of CRE in 203 patients. Among them, 42.2% *K. pneumoniae* and 24.3% *E. coli* showed carbapenem resistance. In the present study, the CRE identified maximum was from urine (54.0%) followed by pus (44.0%) and blood (2.0%). In a similar study from a tertiary hospital in Mumbai, India, Nair and Vaz 2013 reported that majority of the CRE isolate was obtained from urine (46.0%) followed by pus (16.0%). In their study also, only 5.0% of the isolates belonged to blood. We observed ertapenem resistance (63.1%) was higher than imipenem resistance (21.0%) and meropenem resistance (15.8%) among the CRE isolates. Contrary to our finding, Parimala, (2017), reported that among the CRE isolates, meropenem resistance (31.3%) was higher than imipenem resistance in South India.

In our study, overall 15.6 % isolates were found to be MBL resistant which is lesser than a figure reached by Khanal *et al.* (2013), in Kathmandu, Nepal who detected 36.7% MBL producing isolates. In their study, 37.2% of isolates were found to be AmpC β-lactamase producer which is higher than the present study. Similarly, Karn *et al.* (2016), in Nepal, reported 0.16% of each MBL+KPC and MBL+AmpC producing isolates. However, our research has confirmed the presence of comparatively higher prevalence (1.8%) of each MBL+KPC and MBL+AmpC isolates. On phenotypic testing, they detected a higher rate of carbapenem resistance in *Acinetobacter baumanii* (44.0%). In contrary to their study, the present study showed a higher occurrence of carbapenem resistance in *K. pneumoniae* (33.3%) and *E. aerogenes* (33.3 %). The class B Metallo-β lactamases (MBLs) showing resistance to carbapenems are a significant problem in many

other parts of the world as multiple genera of Gram-negative bacilli including *P. aeruginosa*, *Acinetobacter* spp., and Enterobacteriaceae have been found to possess this enzyme (Walsh, 2005; Walsh, 2005). Similarly, the presence of KPC, the clinically significant molecular class A carbapenemase enzyme, was previously confined to *K. pneumoniae*; however, it has been transferred to other members of Enterobacteriaceae like *E. coli* and *E. aerogenes* (Bratu *et al.,* 2007; Bratu *et al.,* 2005). The KPC-producing isolates not only have the capability to confer resistance to all of the carbapenems, meropenem, imipenem, ertapenem, and doripenem but also are resistant to all of the β-lactam-β-lactamase inhibitor combinations, comprising ampicillin-sulbactam, amoxicillin-clavulanic acid, and piperacillin-tazobactam (Mushtaq *et al.,* 2004; Miriagou *et al.,* 2003; Villegas *et al.,* 2006). Similarly, isolates synthesizing AmpC carbapenemase enzyme show limited carbapenem-resistance; however, they typically exhibit resistance to extended-spectrum cephalosporins (Philippon *et al.,* 2002). The limited carbapenemase activity of this enzyme is enhanced by its ability to cause porin loss in bacteria which result in the decreased cellular penetration of carbapenem antibiotic and finally make the bacteria resistant to carbapenem (Cornaglia *et al.,* 1992; Poirel *et al.,* 2004). Presence of MBL, KPC, and AmpC producing Enterobacteriaceae in the present study indicates a frightening scenario which should not be overlooked.

CONCLUSION

The prevalence of CRE in the clinical samples recovered from Bharatpur hospital, Chitwan, Nepal was found to be high (40.7%). Hence proper policies to control the spread of infection by CRE should be formulated and strictly implemented. Carbapenems should be used as reserved drug in the treatment of severe Gram-negative infections. Preventive efforts such as infection control procedures, hand hygiene among healthcare workers, antibiotic stewardship as well as careful use of carbapenems should be aggressively pursued within the healthcare settings. We couldn't do the molecular characterization of the isolates due to resource scarcity. Future studies should consider this factor while addressing the prevalence of CRE.

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