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Detection of Carbapenemases - Metallo Beta-Lacatamase and *Klebsiella pneumoniae* Carbapenemase Production in Gram-negative bacteria causing Urinary Tract Infections isolated in a tertiary care Hospital.

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ABSTRACT

Gram-negative bacteria are considered as the important cause to different types of infections related to urinary system. The aim of this study is to detect Carbapenemases (Metallo-beta-lactamases MBL and *Klebsiella pneumonia* KPC) production in Gram-negative bacteria causing UTIs. Among total samples received, 100 samples were isolates as Gram negative bacilli. *E. coli* (n=75) was the dominant isolate. Out of total gram negative bacilli, 28 isolates were found to be MBL and 9 to be KPC producers. All KPC producers were MBL producers but all MBL producers were not KPC producers. Enterobacteria were mostly susceptible to imipenem, meropenem, piperacillin/tazobactam, ampicillin/sulbactam whereas in case of *Pseudomonas* spp., piperacillin/tazobactam (100%) was the most effective; for *Acinetobacter* spp., polymixin B and tigecycline (100%) were effective. This study demonstrates the high prevalence of Crabapenemase producers among *Klebsiella* spp followed by *E. coli*. Prevalence of Carbapenemase producing bacteria are increasing in alarming rate which is also problematic burden to the world. Therefore, regular monitoring of infections by those bacteria is necessary and rational use of antibiotics would limit the spread of carbapenemase producers.

Keywords: Urinary tract infection, MBL, KPC

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INTRODUCTION

Urinary Tract Infections (UTIs) are one of the most common infectious diseases ranking next to upper respiratory tract infection which is more common [4]. UTI is most common bacterial infections occurring in both males and females of all ages. It is expected that about 35% of healthy women experiences warning signs of UTIs [15]. The dominance of this disease is additional in developing countries owed to deprived sanitation, living method, undernourishment, and ecological stipulation. Mostly, neonates, girls, young women, infants, young children and older men are mainly vulnerable to UTIs [15].

UTIs are caused by both Gram-negative and Gram-positive bacteria, as well as by certain fungi. The most common causative agent is uropathogenic *Escherichia coli* (UPEC) predominant both in community and hospital settings accounting for 70-90% of UTI [21]. UPEC is followed by *Klebsiella pneumoniae*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, group B *Streptococcus* (GBS), *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida* spp. [3, 17].

Their microbial spectrum and susceptibility pattern against different antibiotic vary with different geographical regions and the previous use of antibiotic is important predictor of resistance. The high incidence of UTI and need of starting treatment before availability of microbiological results leads to adoption empirical therapy which is based on the local susceptibility pattern [18].

Bacteria are being resistance to antibiotics by various mode of action such as biochemical aspects and genetic aspects. Biochemical aspects include antibiotic inactivation, efflux pumps, peptidoglycan structure alteration and target modification and genetic aspects include mutation and horizontal gene transfer [14].

Carbapenems are a class of highly effective antimicrobial agents commonly used for the treatment of severe or high-risk bacterial infections. Carbapenems play a critically important role in antibiotic armamentarium. Of the many hundreds of different beta-lactams, carbapenems possess the broadest spectrum of activity and greatest potency against Gram-positive and Gram-negative bacteria. As a result, they are often used as “last-line agents” or “antibiotics of last resort” when patients with infections become gravely ill or are suspected of harboring resistant bacteria [10]. Unfortunately, the recent emergence of multidrug-resistant pathogens seriously threatens this class of life saving drugs [11]. Several recent studies clearly show that resistance to carbapenems is increasing throughout the world.

The production of enzymes that degrade antimicrobial agents is the way that bacteria resist the inhibitory effects of antimicrobial agents. Beta-lactamases are enzymes that cleave the beta-lactam ring, opening up structure and destroying the ability of beta-lactam to bind to its target [7].

Metallo beta-lactamases (MBLs) producers have ability to hydrolyze all beta-lactam except aztreonum. MBLs require zinc for their activity which can be inhibited by ethyl diamine tetraacetic acid (EDTA) and thiol-based compound but cannot be inhibited by clavulanate, sulbactam or tazobactam that are effective against serine-based, chromosome encoded Nmc-A, Sme, IMI-1 and SFC-1 and plasmid mediated genes like KPC, IMI-2 and GES beta-lactamases. Among these, the most common enzyme is KPC which has drawn public health interest [20]. MBLs have a broad substrate spectrum and can catalyze the hydrolysis of virtually all beta-lactam antibiotics with the exception of monobactams [9]. Antibiotic reistance is the growing concern nowadays which is increasing significantly because of the unwanted use of antibiotics which helps bacteria to be more resistance, producing carbapenemases in the near future. Therefore, to detect MBL and KPC production in Gram-negative uropathogens this study was carried out.

MATERIALS AND METHODS

The study was hospital based prospective on Urine sample of urinary tract infection cases in Manmohan Memorial Community Hospital, Kathmandu from May 2018- November 2018. A total of 454 non repeat samples were collected from patients clinically suspected of UTI referred for urine culture and antibiotic susceptibility by physicians.

A freshly void midstream urine samples (10-20 ml) were collected in a sterile wide mouth container by the patients. Semi-quantitative culture technique was used to culture urine sample. Urine specimens were

mixed well and aseptically inoculated on Blood and Mac-Conkey agar using a standard calibrated nichrome loop. The culture plates were incubated aerobically at 37°C for 24 hours. A single bacterial species from the urine sample with a colony count of > 10^5 CFU/ml was considered significant bacteriuria and reported as significant growth. Uropathogens were identified based on standard laboratory procedures including, morphological characteristics, Gram's stain, rapid tests and biochemical tests.

The antibiotic sensitivity test (AST) was performed on Mueller-Hinton agar media by modified Kirby Bauer's disc diffusion method as described in the guidelines of CLSI (2018). In this study, resistances to two or more than two antibiotics of different structural classes were considered to be multidrug resistance.

Screening for carbapenemase production

By combined disc diffusion, each of the isolates that were resistant to third generation cephalosporin and produce ≤19 mm zone with imipenem (10 µg) in AST were screened as carbapenemase producer according to the CLSI (2018) guidelines.

Phenotypic confirmation for MBL production

For the phenotypic confirmation, EDTA was used as the inhibitor of the carbapenemase on the imipenem disc. To the lawn culture of the test isolate that matches 0.5 MacFarland turbidity standard, discs containing imipenem alone and combination of imipenem plus EDTA (750 µg) were placed and incubated at 37°C for 18-24 hours. An increase in zone diameter greater than 4mm around imipenem disc plus EDTA compared to the inhibition zone around imipenem discs alone were confirmed MBL producer.

Phenotypic confirmation for KPC production

The phenotypic detection of the carbapenemase production was performed by the Modified Hodge Test (MHT) as described by CLSI. Briefly, a 0.5 McFarland standard suspension of *E. coli* ATCC 25922 was prepared in 5 ml peptone water and diluted 1:10 by adding 0.5 ml of the 0.5 McFarland to 4.5 ml of peptone water. A lawn of the 1:10 dilution of *E. coli* ATCC 25922 was prepared on a MHA plate as for the routine disc diffusion procedure. The plate was allowed to dry 3 to 10 minutes. A 10 µg meropenem disc was placed in the centre of the test plate and the test organism was streaked in a straight line from the edge of the disc to the edge of the plate. Three organisms were tested on the same plate with one drug. The plate was incubated at 37°C in ambient air for 18–24 hours. After incubation, a positive MHT test was indicated by a clover leaf-like indentation of the *E. coli* ATCC 25922 growing along the test organism growth streak within the disc diffusion zone and a negative MHT test was indicated by no growth of the *E. coli* ATCC 25922 along the test organism growth streak within the disc diffusion zone.

Statistical analysis

All the study data were entered into computer using standard format, checked for errors and verified. Statistical programmed statistical package for social science (SPSS 20.0) and Microsoft word were used to analyze all the obtained data. A value of P≤0.05 was assumed statistically significant and 95% confidence intervals along with the exact p-values were presented.

RESULTS

Out of 454 urine samples processed, 115 (25.0%) showed significant growth of organism. Out of 115 bacterial isolates, 100 were Gram-negative bacteria. The most predominant isolates were *E. coli* followed by *Klebsiella* spp, *Citrobacter* spp. (Table 1).

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Isolated Organisms	No (%)
Escherichia coli	75 (75.0)
Klebsiella spp.	7 (7.0)
Citrobacter spp.	6 (6.0)
Enterobacter spp.	3 (3.0)
Proteus spp.	2 (2.0)
Providencia spp.	1 (1.0)
Pseudomonas spp.	4 (4.1)
Acinetobacter spp.	1 (1.0)
Alcaligenes spp.	1 (1.0)
Total	100 (100)

Table 1: Distribution of bacterial isolates from urine samples

Antibiotic resistance profiles revealed that the majority of bacterial isolates were resistant to multiple antibiotics. These isolates exhibited the highest resistance to amoxicillin (72.8-100%) followed by cotrimoxazole (28.6-83.3%), cefixime (40.0-100%), ceftriaxone (28.6-83.3%), norfloxacin (28.6-83.5%) and so on. They were most susceptible to Nitrofurantoin (33.3-89.3%), gentamicin (33.3-84.6%), levofloxacin and ofloxacin (33.3-71.4%) (Table 2).

Organism	***E. coli***		***Klebsiella* spp.**		***Citrobacter* spp.**	
Antibiotics	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)
Amoxycillin	16(27.2)	59(72.8)	-	-	0(0)	6(100)
Levofloxacin	49(65.3)	26(34.7)	5(71.4)	2(28.6)	2(33.3)	4(66.7)
Ceftriaxone	50(66.7)	25(33.3)	5(71.4)	2(28.6)	1(16.7)	5(83.3)
Cefixime	45(60.0)	30(40.0)	4(57.3)	3(42.7)	0(0)	6(100)
Cotrimoxazole	47(62.7)	28(37.3)	5(71.4)	2(28.6)	1(16.7)	5(83.3)
Nitrofurantoin	68(90.6)	7(9.4)	5(71.4)	2(28.6)	1(16.7)	5(83.3)
Norfloxacin	49(65.3)	26(34.7)	5(71.4)	2(28.6)	1(16.7)	5(83.3)
Ofloxacin	48(64.0)	27(36.0)	5(71.4)	2(28.6)	2(33.3)	4(66.7)
Gentamicin	63(84.0)	12(16.0)	4(57.3)	3(42.7)	2(33.3)	4(66.7)
Amikacin	71(94.6)	4(11.8)	7(100)	0(0)	4(66.6)	2(33.4)
Imipenem	49(65.3)	26(34.6)	4(57.2)	3(42.8)	1(16.6)	5(83.4)
Meropenem	73(97.3)	2(2.7)	5(71.5)	2(28.5)	1(16.6)	5(83.4)
Ampicillin/sulbactam	23(85.4)	11(14.6)	5(71.5)	2(28.5)	3(50.0)	3(50.0)
Piperacillin/Tazobactam	72(96.0)	3(4.0)	5(71.5)	1(28.5)	5(83.4)	1(16.6)

Table 2: Antibiogram of pattern of uropathogens

All the Gram-negative isolates were screened for the production of possible carbapenemase producers, among them 42 (42.0%) isolates showed carbapenemase screening test positive of which 28 (28.0%) isolates were confirmed to be MBL producers with 9 (9.0%) isolates to be both MBL and KPC producers (Table 3).

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Organisms	Screening test (%)	Confirmatory test		MBL+KPC
		MBL (%)	KPC (%)	
E. coli (75)	26 (34.6)	17 (22.6)	2	2
Klebsiella spp. (7)	3 (42.8)	2 (28.5)	1 (14.3)	1
Citrobacter spp. (6)	5 (83.3)	4 (66.7)	3 (50.0)	3
Proteus spp. (2)	2 (100)	2 (100)	2 (100)	2
Enterobacter spp. (3)	1 (33.4)	1 (33.4)	0	0
Pseudomonas spp. (4)	2 (50.0)	0 (0)	0 (0)	0
Acinetobacter spp. (1)	1 (100)	1 (100)	1 (100)	1
Alcaligenes spp. (1)	1 (100)	1 (100)	0 (0)	0
Providencia spp. (1)	1 (100)	0 (0)	0 (0)	0
Total (100)	42 (42.0)	28 (28.0)	9 (9.0)	9

Table 3: Carbapenemase production among different gram negative bacteria

Higher percentage of MBL and KPC producers were isolated from *Acinetobacter* spp. (100%) of OPD patient. Similarly, in case of *Proteus* spp. same figure of MBL and KPC producers were isolated from OPD (100%) and IPD (100%) patients (Table 4).

Organisms	OPD(%) IPD(%)	MBL (%)	KPC (%)	MBL+KPC
E. coli (75)	67 (89.4) 8 (10.6)	15 (22.3) 2 (25.0)	2 (2.9) 0 (0)	2 0
Klebsiella spp. (7)	6 (85.8) 1 (14.2)	2 (33.4) 0 (0)	1 (16.7) 0 (0)	1 0
Citrobacter spp. (6)	5 (83.3) 1 (16.7)	3 (60.0) 1 (100)	2 (40.0) 1 (100)	2 1
Proteus spp. (2)	1 (50.0) 1 (50.0)	1 (100) 1 (100)	1 (100) 1 (100)	1 1
Enterobacter spp. (3)	2 (66.7) 1 (33.3)	1 (50.0) 0 (0)	0 (0) 0 (0)	0 0
Pseudomonas spp. (4)	4 (100) 0 (0)	1 (25.0) 0 (0)	0 (0) 0 (0)	0 0
Acinetobacter spp. (1)	1 (100) 0 (0)	1 (100) 0 (0)	1 (100) 0 (0)	1 0
Total		28 (28.0)	9 (9.0)	9

Table 4: Frequency of carbapenemase producers in OPD and IPD patients

MBL were detected mostly in male and females of age group 21-40 years and in males of age group 61-80 years. KPC production was high among female in age group 41-60 years and among male in age group 61-80 years (Table 5).

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Age	Gender	Carbapenemase producer	
		MBL	KPC
≤20	Male	0	0
	Female	1	0
21-40	Male	3	1
	Female	10	1
41-60	Male	2	0
	Female	3	2
61-80	Male	3	2
	Female	3	1
≥81	Male	2	1
	Female	1	1

Table 5: Age and sex wise distribution pattern of Carbapenemase producers

DISCUSSION

Out of 454 urine samples processed, 115 (25%) samples showed significant bacterial growth. Similar occurrence of growth has been shown in Nepal by various researchers [12, 13]. Occurrence of Gram negative bacteria among the total uropathogen was found to be 86.9% in this study which is in agreement with the results reported [13]. The possible cause of low rate of growth positivity might be due to urine samples obtained from patients under treatment, infection due to slow growing organisms or due to those organisms that were not able to grow on the routine media we used. Gram negative bacteria were predominant causing UTI with the percentage of 86.9%. In this study the prevalence of Gram negative bacteria was more than that of Gram positive bacteria causing urinary tract infection. Eleven different genera of bacteria were isolated as uropathogens, of which 9 genera were Gram-negative bacteria. *E. coli*, *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp., *Proteus* spp., *Providencia* spp. (all members of Enterobacteriaceae family) and *Alcaligenes* spp., *Pseudomonas* spp., *Acinetobacter* spp., were the isolated Gram-negative bacteria.

E. coli (65.2%) was the prominent uropathogen isolated in accordance with other studies carried out in Nepal [6]. *Pseudomonas* spp. (3.4%) was the common opportunistic uropathogen which is in lower occurrence than observed in other studies [16]. *Alcaligenes* spp. was isolated which showed complete resistance to amikacin, imipenem, meropenem and piperacillin where as polymixin-B and tigeycline found to be effective drug which is similar to the finding reported for the treatment of complicated UTIs [14].

The main objective of this study is the detection of carbapenemase production in Gram-negative bacteria causing UTI. The most common mechanism for carbapenem resistance in Gram-negative bacteria are the production of carbapenemases belonging to Amber class A, especially KPC, or Amber class B, MBLs such as IMP and VIM types [2]. The early detection of MBL-producing isolates would be important for the reduction of mortality rates and also to avoid the intra-hospital dissemination of such strains.

All the Gram-negative isolates were screened for carbapenemase production according to the CLSI 2018 using screening agents, among them 42% were found to be resistant to imipenem and 13% were resistant to both imipenem and meropenem in screening test. For the confirmatory test, imipenem resistant (possible MBL producers) isolates were applied to combined disc technique and isolate resistant to both imipenem and meropenem (possible KPC producers) to Modified Hodge Test (MHT) for MBLs and KPC production. In this study, only 28 (28.0%) isolates were confirmed to be MBL producers and of these 28 isolates, 9 (9.0%) isolates were confirmed to be MBL and KPC producers. Therefore, the prevalence rate of carbapenemase production in the present study was found to be 28% MBLs (total) and 9.0% both MBL and KPC which is in accordance with the research reported in USA for carbapenemase production [19].

Out of 75 *E. coli*, 17(22.6%) was found to be MBL producers and only 2 isolates to be both MBL and KPC producers. *Proteus* spp. (2) and *Acinetobacter* spp. (1) were both MBL and KPC producers where as *Alcaligenes* spp. (1) was only MBL producer. MBL producers were reported from OPD (27.2%) and IPD (33.3%) isolates and 28.5% OPD and 50% IPD isolates were KPC producers among the confirmed carbapenemase producers [17]. In present study, more of carbapenemase producers (MBL in majority) were detected from the

isolates of female patient of age group 21-40 years however male patient isolates were from the age group 61-80 years. From the above discussion we can say that KPC were detected in all the age groups of male and females except for the ≤20 age group.

Although CLSI guideline recommends MHT only for Enterobacteria, this MHT was done in all Gram negative isolates which could be useful for determining the efficiency of MHT and also could help in comparing the results obtained from other tests done in the same hospital. Similarly, MHT was also done for the non-fermenters in the study carried out along with other tests, so detection of MBL and KPC is of utmost importance in deciding the most appropriate therapeutic regimen for treatment of carbapenem resistant non-fermenters [8]. Other study carried out reported 7.8% of carbapenemase producers were Enterobacreiaceae of which only 5.7% were MBL producers [2]. Hence, confirmatory test should be carried out to confirm carbapenemase productions as has been recommended by CLSI.

Prevalence of antibiotic resistance observed in this study may be due to the irrational and haphazard use of antibiotics. More or less people in communities intake antibiotics by their own choice without any prescription by clinicians which should be stopped to reduce emergence of resistance and preserve some antibiotics for next generation.

The limitations in this study included the lack of molecular test for the identification of uropathogens as well as for the confirmation carbapenemase producers. Though these tests are costly, it is necessary to be carried out as third and above generation of cephalosporin and carbapenem resistances have been an ever increasing problematic burden to the world.

CONCLUSION

From this study, 25% samples show significant growth where female experience comparatively high UTI. Out of total Gram-negative bacteria isolated, 42.0% isolates were possible carbapenemase producer by the screening test where as only 28.0% were later confirmed to be MBL producers by Imipenem-EDTA and of these MBL producer, 9.0% isolates to be MBL and KPC producers by Imipenem-EDTA and MHT.

Therefore, immediate infection control coupled with antibiotic guidance programs in order to limit the spread of carbapenemase producing organism is mandatory, since carbapenemase producers confer a high resistance to carbapenem antibiotics and traits being transferred through genes and their emergence among bacterial isolates warrents detection in routine laboratory.

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