PREVALENCE OF MULTIDRUG-RESISTANT AND CARBAPENEMASE-PRODUCING *KLEBSIELLA PNEUMONIAE* AND *PSEUDOMONAS AERUGINOSA* ISOLATES IN TERTIARY CARE HOSPITAL IN KATHMANDU, NEPAL

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ABSTRACT

Carbapenemases are the enzymes that catalyze β–lactam groups of antibiotics. The carbapenemase producers are resistant to β–lactam antibiotics and are usually multidrug-resistant bacteria challenging widely used therapeutics and treatment options. Therefore, the detection of carbapenemase activity among clinical isolates is of great therapeutic importance. We aimed to study the MDR and carbapenemase-producing *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* isolated from various clinical samples at a tertiary care hospital in Nepal. A total of 3,579 clinical samples were collected from the patients visiting the Department of Microbiology, B&B Hospital, Gwarko, Lalitpur. The samples were processed to isolate *K. pneumonia*e and *P. aeruginosa* and then subjected to antibiotic susceptibility testing (AST) by the Kirby-Bauer disk diffusion method. Phenotypic detection of carbapenemase activity was performed in the imipenem-resistant isolates by the modified Hodge test (MHT). Of the total samples, 1,067 (29.8%) samples showed significant growth positivity, out of which 190 (17.3%) isolates were *K. pneumoniae* and 121 (11.3%) were *P. aeruginosa.* Multidrug resistance was seen in 70.5% of the *K. pneumoniae* isolates and 65.3% of the *P. aeruginosa* isolates. Carbapenemase production was confirmed in 11.9%, and 12.2% of the imipenem-resistant *K. pneumoniae* and *P. aeruginosa* isolates, respectively, by the MHT. This study determined the higher prevalence of MDR among *K. pneumoniae* and *P. aeruginosa*; however, carbapenemase production was relatively low.

KEYWORDS

Carbapenems, carbapenemase, antibiotics, modified Hodge test

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INTRODUCTION

Carbapenems are antibiotics widely used to treat infections caused by bacterial pathogens suspected to be multidrug-resistant (MDR).[1] Among the β- lactams, carbapenems possess the broadest spectrum of antimicrobial activity and are more effective even against general antibiotic resistance mechanisms; therefore, they are used as "last-line antibacterial agents".[2,3] However, bacterial pathogens are ever-evolving and often evolve into carbapenemase-producing virulent strains. Carbapenemases are β-lactamases with versatile hydrolytic capacities, providing resistance against the existing carbapenem antibiotics and are the significant contributors of multidrug resistance.[2,4] Infections caused by carbapenemase-producing bacteria have been associated with higher mortality rates and severe outbreaks.[5] Concern has arisen in recent years over the increasing trend of carbapenem resistance, as the therapeutic options for treating infections caused by carbapenem-resistant bacteria are limited.[6] The rapid transmission and the lack of alternative antimicrobial drugs against carbapenem-resistant organisms have become a worrying public health issue worldwide.[7,8] It is mainly because these enzymes hydrolyze virtually all types of antimicrobial β-lactamases and are often resistant to commercially available β-lactamases inhibitors.[9]

An early screening and recognition system is required to prevent the occurrence and further dissemination of these carbapenemase-producing bacteria. Various phenotypic methods for detecting carbapenemase-producing organisms (CPO) have been mentioned in different studies.[11-13] However, each method currently recommended by the Clinical and Laboratory Standards Institute (CLSI) has certain limitations.[14,15] Due to their high sensitivity and specificity, molecular techniques remain the reference standard for identifying and differentiating carbapenemases.[10] But their high cost, the requirement for trained technicians, and the inability to detect novel carbapenemase genes limit their use in developing countries like Nepal.[10] MHT is one of the phenotypic tools for the detection of carbapenemase producers.[16] It is the first CLSI recommended growth-based carbapenemase detection test.[15] It is based on the ability of carbapenemase producers to form a cloverleaf appearance around a streak link near the carbapenem disk placed on an agar plate inoculated with a lawn of carbapenem-susceptible *Escherichia coli* ATCC 25922.[12] MHT is simple, inexpensive, and uses reagents readily available in most clinical laboratories, which makes it a valuable tool for carbapenemase detection in resource-limited countries like Nepal.[12]

Different studies have been carried out worldwide, which show the increasing prevalence of carbapenem resistance. The prevalence has been reported from 0.5% to as high as 100% in different studies in different places and at different times.[17-20] In Nepal, the prevalence of carbapenemase production in *K. pneumoniae* and *P. aeruginosa* has been documented from 4% to 55%.[21-23] This study aims to study the antibiotic resistance pattern of the *K. pneumoniae* and *P. aeruginosa* isolated from clinical samples in the tertiary care hospital in Nepal and identify the carbapenemase-producing isolates using a low-cost phenotypic method.

MATERIALS AND METHODS

Study design

A cross-sectional study was carried out in the Department of Microbiology, B&B Hospital, Gwarko, Lalitpur, for eight months from June 2018 to Janaury 2019. The ethical approval was taken from the joint Institutional Review Committee of the Shi-Gan Health Foundation and National Institute of Tropical Medicine and Public Health Research, Maharajgunj, Kathmandu, Nepal. The specimens were collected from participants who had a suspicion of infections during doctor checkups. An inappropriately collected clinical specimen and specimens suspected of contamination by the normal flora or any other external source were not included in the study. The sample size (n=3, 607) was calculated using Fischer's formula. The samples included in this study were urine, stool, pus, blood, CSF, vaginal swab, wound swab, and catheter tip.

Sample collection and processing

The clinical samples were processed and cultured following the standard microbiological techniques.[24] The specimens were cultured on nutrient agar, BHI broth (for blood samples), blood agar, and Mac-Conkey agar. The isolates were identified based on colony morphology, Gram stain, and conventional biochemical methods.[24] Only the *K. pneumoniae* and *P. aeruginosa* isolates were further processed for antimicrobial susceptibility testing (AST) and

tested for carbapenemase production using MHT.

Antibiotic susceptibility testing

AST was performed by the Kirby-Bauer disk diffusion method as recommended by the Clinical Laboratory Standard Institute.[15] The antibiotics used were amikacin (30µg), ampicillin (10µg), cefixime (5µg), ceftriaxone (30µg), imipenem (10µg), chloramphenicol (30µg), ciprofloxacin (5µg), ofloxacin (5µg), nitrofurantoin (300µg), . The bacterial isolates which showed resistance towards three or more different antibiotic classes were reported as multidrug-resistant (MDR). Screening of suspected carbapenemase-producing isolates was performed according to screening guidelines issued by CLSI (Table 1).[25]

Confirmation of carbapenemase production

The isolates resistant to imipenem (zone size ≤10mm) were subjected to phenotypic detection for carbapenemase. The isolates resistant to imipenem were subjected to phenotypic detection for carbapenemase. After complete identification, isolates were preserved on trypticase soy broth with 25% glycerol at -70°C. Confirmation of carbapenemase activity was done by MHT.[25] After 16-24 hours of incubation, the plates were examined for a cloverleaf–type indentation at the intersection of the test organism *E. coli* ATCC 25922, within the zone of inhibition of the carbapenem susceptibility disk.

Quality control

For the quality control of culture and biochemical tests, purity plates and ATCC control strains were used. For the standardization of the antimicrobial testing and phenotypic confirmation of carbapenemase production, the control strain of *E. coli* ATCC 25922 was used.

Statistical analysis

Statistical analysis was done by using SPSS version 16.0. The Chi-square test was applied at 95% CI to check the significance of association between the variables.

RESULTS

A total of 3,579, samples (1,896 from males and 1,683 from females) were received, of which 1,067 (29.8%) samples showed bacterial growth. Growth positivity was the highest in the catheter tip (74.5%) and the least in the stool (3.8%). Gram-positive and gram-negative isolates accounted for 23.8% and 76.2% of the total isolates, respectively. The growth positivity among the males and the females was 32.1% and 27.2%, respectively. Among the gram-negative isolates, 190 (23.4%) were identified as *K. pneumoniae* and 121 (14.9%) as *P. aeruginosa*. The highest number of *K. pneumoniae* and *P. aeruginosa* isolates were obtained from wound pus samples followed by urine (Table 1).

Table 1: Sample-wise growth pattern of bacteria

Sample	Total growth n (%)	Gram-negative n (%)	*K. pneumoniae* n (%)	*P. aeruginosa* n (%)
Urine (n=2096)	492 (23.5)	403 (19.2)	62 (15.9)	26 (6.5)
Wound/pus (n=825)	401 (48.6)	268 (32.5)	75 (28.0)	68 (25.4)
Sputum (n=449)	120 (26.7)	105 (23.4)	42 (40.0)	19 (18.1)
Indwelling catheter tip (n-=51)	38 (74.5)	28 (54.9)	8 (28.6)	8 (28.6)
CVP tip (n=17)	7 (41.2)	4 (23.5)	2 (50.0)	0 (0.0)
Suction tip (n=5)	2 (40.0)	2 (40.0)	1 (50.0)	0 (0.0)
Throat swab (n=83)	5 (6.0)	1 (1.2)	0 (0.0)	0 (0.0)
Stool (n=53)	2 (3.8)	2 (3.8)	0 (0.0)	0 (0.0)
Total (n=3579)	**1,067 (29.8)**	**813 (76.2)**	**190 (23.4)**	**121 (14.9)**

Note: % of *K. pneumoniae* and *P. aeruginosa* are calculated out of total gram-negative isolates

Table 2: Antibiotic susceptibility pattern of *P. aeruginosa* and *K. pneumoniae*

Antibiotics used	*P. aeruginosa* (n = 121)			*K. pneumoniae* (n = 190)		
	Sensitive n (%)	Resistant n (%)	Intermediate n (%)	Sensitive n (%)	Resistant n (%)	Intermediate n (%)
Amikacin	55 (45.5)	56 (45.5)	10 (8.3)	94 (49.5)	75 (39.5)	21 (11.1)
Amoxicillin	0 (0.0)	121 (100.0)	1 (0.0)	0 (0.0)	190 (100.0)	0 (0.0)
Imipenem	77 (63.6)	41 (33.9)	3 (2.5)	107 (56.3)	59 (31.1)	24 (12.6)
Cefepime	48 (39.7)	69 (57.0)	4 (3.3)	60 (31.6)	125 (65.8)	5 (2.6)
Ceftrioxone	38 (31.4)	81 (66.9)	2 (1.7)	45 (23.7)	138 (72.6)	7 (3.7)
Ciprofloxacin	46 (38.0)	73 (60.3)	2 (1.7)	49 (25.8)	120 (63.2)	21 (11.1)
Ofloxacin	45 (37.2)	76 (62.8)	0 (0.0)	74 (38.9)	111 (58.4)	5 (2.6)
Chloramphenicol	42 (34.7)	79 (65.3)	0 (0.0)	106 (55.8)	76 (40.0)	8 (4.2)
Colistin sulphate	120 (99.2)	1 (0.8)	0 (0.0)	187 (98.4)	3 (1.6)	0 (0.0)
Nitrofurantion	42 (34.7)	76 (62.8)	3 (2.5)	47 (24.7)	138 (72.6)	5 (2.6)

Antibiotic susceptibility pattern of *K. pneumoniae* and *P. aeruginosa*

Colistin sulphate was found to be the most effective whereas amoxicillin was found to be the least effective anibiotic againt *K. pneumoniae* and *P. aeruginosa*. A total of 59 (31.1%) *K. pneumonia* and 41 (33.9%) *P. aeruginosa* isolates were resistant to imipenem and were subjected to MHT. Beside these, all other antibiotics showed sub-optimal effectivess against both the isolates (Table 2).

Table 3: MDR distribution of *K. pneumoniae* and *P. aeruginosa*

Organism	MDR		p-value
	MDR (%)	non-MDR (%)	
K. pneumoniae	134 (70.5)	56 (29.5)	0.3
P. aeruginosa	79 (65.3)	42 (34.7)	
Total	**213 (68.3)**	**98 (31.5)**	

MDR distribution of *K. pneumoniae* and *P. aeruginosa*

Out of 190 *K. pneumoniae* isolates, 134 (70.5%) were MDR, whereas out of 121 *P. aeruginosa* isolates, 79 (65.3%) were MDR (Table 3). There was no significant association between the organisms and MDR.

Carbapenemase production pattern of *K. pneumoniae* and *P. aeruginosa*

Out of the imipenem-resistant *K. pneumoniae* (n=61) and *P. aeruginosa* (n=42) isolates, 13.1% (n=8) *K. pneumoniae* isolates and 11.9% (n=5) *P. aeruginosa* isolates were confirmed as carbapenemase producers by MHT. There was no significant association between organisms and carbapenemase activity (Table 4).

DISCUSSION

Out of 3,579 samples, 1,067 (29.8%) samples showed bacterial growth in which 76.2% (813) were Gram-negative isolates and 23.8%

Table 4: Carbapenemase production in *K. pneumonia* and *P. aeruginosa*

Organism	Screening positive isolates (n)	MHT Test		p-value
		Positive n (%)	Negative n (%)	
K. pneumoniae	59	7 (11.9)	52 (88.1)	0.6
P. aeruginosa	41	5 (12.2)	37 (87.8)	
Total	**100**	**12 (12.0)**	**88 (88.0)**	

(254) were Gram-positive isolates. Karn *et al*[22] reported similar growth positivity, whereas GC *et al*[26] and Aryal *et al*[27] reported slightly lower growth positivity in similar settings. The growth positivity reported by Pokhrel *et al*[28] was much higher than ours (48.2%). The highest growth positivity was seen in indwelling catheter tip samples (74.5%) which is discordant with the findings of Gurung *et al*[23] and GC *et al*[26]. Among the growth positive isolates, 190 (17.8%) were identified as *K. pneumoniae* and 121 (11.3%) as *P. aeruginosa.* Similar culture positivity of *K. pneumoniae* and *P. aeruginosa* was reported by Mishra *et al*[29] and Aryal *et al*[27]. In contrast, GC *et al*[26] reported slightly lower, and Karn *et al*[22] reported a much lower culture positivity for both *K. pneumoniae* and *P. aeruginosa*. The differences in the prevalences might be due to the difference in location of the study and the type of samples processed.

In our study, nearly one-third of the *K. pneumoniae,* and one-third of the *P. aeruginosa* isolates were resistant to imipenem. GC *et al*[26] reported a much lower resistance to imipenem in *K. pneumoniae* isolates, while Shanmugam *et al*[30] reported a much higher prevalence of imipenem-resistant *K. pneumoniae*. In this study, sensitivity was observed more to amikacin than to fluoroquinolone. Low sensitivity was observed against third and fourth-generation cephalosporins, which is similar to the reports given by Ganguly *et al.*[31] The increased resistance to the currently used antibiotics has led to an interest in old antibiotics, such as colistin.[32] Unfortunately, the current increase in the use of colistin as the last–resort treatment has led to the increase of resistance to colistin in these bacteria, which is believed to be the next major challenge in the context of antibiotic resistance in the coming year.[32]

There has been a rise in infections caused by *K. pneumoniae* and *P. aeruginosa.* The increasing scarcity of effective treatments makes it even worse.[33] In our study, nearly three-fourth of the *K. pneumoniae* isolates and two-third of the *P. aeruginosa* isolates were MDR. Khanal *et al,*[21] Karn *et al,*[22] and Aryal *et al*[27] reported slightly lower MDR strains of *K. pneumoniae* and *P. aeruginosa,* whereas GC *et al*[26] reported much lower prevalence. In contrast, Gautam *et al*[34] and Gurung *et al*[23] reported a slightly higher percentage (81.6%) of MDR in *K. pneumoniae.* The highest level of drug resistance seen in *K. pneumoniae* and *P. aeruginosa* is due to the production of various types of β-lactamases, primarily AmpC, ESBL, and metallo-β-lactamases, along with drug efflux.[35,36] High MDR in countries like Nepal can be attributed to the irrational use of antibiotics, self-medication, expired or counterfeit drugs.[37,38] Similarly, the lack of proper infection control measures in the hospital and the community can further promote MDR strains among bacteria.[39]

The global prevalence of carbapenemase has been documented from 2.3% - 67.7%.[8] In this study, 31.1% of *K. pneumoniae* and 33.9% of *P. aeruginosa* isolates were screened positive (imipenem resistant) for carbapenemase production, of which 7 (11.9%) *K. pneumoniae* and 5 (12.2%) *P. aeruginosa* isolates were found to be MHT positive. Gurung *et al*[23] reported a similar (33.3%), and Gautam *et al*[34] reported a much lower (8.4%) imipenem resistance in *K. pneumoniae* than ours. However, MHT positivity in *K. pneumoniae* was much higher in both of the studies; 62.5% and 86.8%, respectively.[23,34] Bora *et al*[40] reported a much lower carbapenem resistance in *K. pneumoniae*; however, all of them were confirmed as carbapenemase producers by MHT. Ramana *et al*[11] and Shanmugam[30] from India also reported higher carbapenemase production in *Klebsiella* spp. However, GC *et al*[26] reported almost similar carbapenem resistance (51.4%) and MHT positivity (16.7%) in *K. pneumoniae*. Noyal *et al*[41] reported 31.1% resistance to carbapenem (meropenem) in *P. aeruginosa,* of which 28.1% were MHT positive. Similarly, Karn *et al*[22] reported a slightly higher carbapenemase production in *Klebsiella* spp. and *P. aeruginosa* by the combined-disk method. Amudhan *et al*[42] reported MHT positivity in 29.5% of the *P. aeruginosa* isolates. We found no significant association between organisms and carbapenemase activity.

Detection methods for carbapenemase production include MHT, double-disc test, blood agar combined disc assay, PCR amplification, and DNA sequencing.[11] We used MHT as it is a cost-effective phenotypic method and has also been recommended by CLSI.[16] Although the low sensitivity in detecting NDM and possibilities of false-negative results, mainly when the isolate tested is mucoid or when the carbapenemase production is low, should be kept in mind.[43] However, incorporation of other phenotypic tests along with MHT increases the sensitivity and specificity. Our findings will be helpful to access the drug resistance pattern and MDR prevalence in hospital isolates of *K. pneumoniae* and *P. aeruginosa*, which will be beneficial in optimizing treatment therapies for infections caused by such isolates.

High culture positivity and high MDR among *K. pneumoniae* and *P. aeruginosa* were seen in this study. However, carbapenemase production was lower than other studies performed in similar settings. Colistin could be a choice of drug for the treatment of infections caused by MDR strains of *K. pneumoniae* and *P. aeruginosa*. MHT is a cheap and easy method for screening carbapenemase production, particularly for clinical laboratories from low-income regions like Nepal.

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