Comparison of Phenotypic Methods for the Detection of Carbapenem-Resistant Enterobacteriaceae and Molecular Detection of OXA-181 Gene

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ABSTRACT

Objectives: To compare different phenotypic methods used for detecting carbapenem-resistant Enterobacteriaceae and to detect the presence of the *OXA-181* gene among them.

Methods: A hospital-based cross-sectional prospective study was conducted at Shahid Gangalal National Heart Center, Kathmandu, from September 2021 to March 2022. Altogether, 143 Enterobacteriaceae were isolated from 1042 clinical specimens and subjected to antibiotic susceptibility tests following CLSI guidelines. Several phenotypic methods (Modified Hodge Test (MHT), modified carbapenem inactivation method (mCIM), EDTA-CIM (eCIM) and CarbaNP test were performed for the detection of CRE and the results were compared. In addition, MIC of imipenem was carried out against MDR Enterobacteriaceae using agar dilution method and resistant isolates were also tested by PCR for presence of *OXA-181* gene.

Results: Among all the Enterobacteriaceae isolates, *Klebsiella pneumoniae* was the predominant isolate. Gentamycin, nitrofurantoin and carbapenem were found to be most effective, whereas the majority of the isolates were highly resistant to ß-lactam antibiotics along with ciprofloxacin. In total, 43.36% (62/143) isolates were carbapenem-resistant, showing 48.39% (30/62) positivity by MHT, 72.58% (45/62) by mCIM and 61.29% (38/62) by CarbaNP method. The *OXA-181* gene was detected in 12 carbapenem-resistant isolates (MIC ≥4 µg/ml), predominantly in *K. pneumoniae* and *E. coli* isolates.

Conclusion: mCIM is the most effective phenotypic method for detecting carbapenemase production. Presence of *OXA-181* gene among clinical isolates is a serious concern, especially in the hospital setting.

Keywords: Antibiotic susceptibility testing, carbapenem-resistant, MHT, mCIM, CarbaNP test, OXA-181

INTRODUCTION

A wide range of bacterial species are resistant to the majority of antibiotics, carbapenem-resistant Enterobacteriaceae (CRE) is a family of microorganisms that can cause both nosocomial and community-acquired infections, including bloodstream infections, wound infections, UTIs, pneumonia, cystitis, bacteremia, meningitis, and so forth (Bartolini et al 2014). Carbapenemases, an enzyme that hydrolyze carbapenem class antimicrobials and other β-lactam antibiotic drugs, but not all carbapenemase-producing isolates are carbapenem-resistant (Daikos et al 2007).

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Carbapenems including meropenem, imipenem, ertapenem and doripenem, are routinely employed as last-resort therapeutic alternatives. Meropenem has greater activity against Gram-negative bacilli, while imipenem has greater activity against Gram-positive cocci (Armstrong et al 2021). Carbapenemase enzymes are classified by their molecular structures and belong to 3 classes of β-lactamases: class A carbapenemase (*KPC* types), class B or Metallo-beta-lactamases (MBLs) (*VIM, IMP*, and *NDM* types), and class D oxacillinases (*OXA-48* and its derivatives). Class A and D carbapenemases require serine at their active site, while class B, the metallo-β-lactamases (MBLs) require zinc for β-lactam hydrolysis (Bush and Fisher 2011).

In general, two main pathways lead to carbapenem phenotypic resistance: (1) β-lactamases activity in conjunction with structural alterations, and (2) formation of carbapenemases, which hydrolyze carbapenem antibiotics. Penicillin-binding protein changes and drug efflux pumps are two additional pathways linked to carbapenem resistance in GNB (Bush and Jacoby 2010; Patel and Bonomo 2013). In addition, decreased susceptibility to carbapenem in Enterobacteriaceae may be caused by either extended spectrum beta-lactamases (ESBLs) or AmpC enzymes combined with drug decreased permeability, due to loss of porins (Suay-Garcia and Perez-Gracia 2019). To identify carbapenemase production in CRE-infected patients, many phenotypic and genotypic screening assays are available. Clinical and Laboratory Standard Institute (CLSI) suggested a ranged of straightforward and reasonably priced phenotypic assays to address these issues and enable precise identification of carbapenem positive organism. The available phenotypic tests include: (a) The Modified Hodge test (MHT), the first CLSI recommended growth-based carbapenemase detection test in 2009 with high level of sensitivity and specificity in detecting carbapenemases (CLSI 2009; Bialvaei et al 2016); (b) modified Carbapenem Inactivation method (mCIM) (c) EDTA- CIM (eCIM) which is a further modification of mCIM with the addition of EDTA (eCIM) to identify metallo-carbapenemases specifically has been recommended in the CLSI M100-S28 supplement in 2018. (d) Carba NP test (CNPt), a test employing the indicator phenol red (red to orange/yellow) to identify changes in pH values caused by the in vitro hydrolysis of imipenem by a bacterial lysate (CLSI 2017). Some other tests include the Blue Carba NP test, Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS), which detects the carbapenem molecule or its degradation product (Hrabak and Chudackovae 2014).

Particularly, reports of the class D β-lactamase *OXA-48* and its variant *OXA-181* have increased globally. The class D carbapenem hydrolyzing *OXA-48* was first identified from a *Klebsiella pneumoniae* in Turkey. *OXA-181*, one of the *OXA-48* variations, differs from *OXA-48* by four amino acids and exhibits 45 nucleotide changes, although the two enzymes have the same hydrolysis spectrum. The $bla_{\text{OXA-181}}$ gene was first discovered in a *K. pneumoniae* isolate in India (Castanheira et al 2011) and was later found in other enterobacterial species in numerous other countries, including Canada, France, Norway, Sri Lanka, Romania, New Zealand, the United Kingdom, Australia, Canada, France, Norway and Singapore (Poirel et al 2011; Poirel et al 2012).

The prevalence of infections brought on by Enterobacteriaceae that are resistant to carbapenem is predicted to be high in Nepal. The main supporting evidence for this idea is the propensity of Enterobacteriaceae to acquire resistance rapidly, the spread of biofilm and carbapenemase-producing genes among people in close proximity (such as India) and the haphazard use of antibiotics in Nepal. In impoverished nations like Nepal, it is crucial to define the prevalence of *K. pneumoniae* that produces carbapenemase and other bacteria either phenotypically or genotypically, but the knowledge on this issue in Nepal is incomprehensible. Increases in the copy number of *blaOXA-181* may indicate a different route through which Enterobacteriaceae can develop increased carbapenem resistance. Additionally, In Nepal, the two copies of *OXA-181* in *K. pneumoniae* were first reported in 2020 (Serchan et al 2020). The present study contributes to the evaluation of the prevalence of Carbapenem-Resistant Enterobacteriaceae in clinical settings, which requires high priority to reduce its spread. The main aim of this study was to compare different phenotypic methods for the detection of carbapenem-resistant Enterobacteriaceae isolates from different clinical specimens along with the detection of *OXA-181* gene by PCR.

METHODS

Study design and settings

A prospective hospital based cross-sectional study was conducted in patients visiting Shaheed Gangalal National

Heart Centre (SGNHC), Bansbari, Kathmandu during the six months from September 2021 to March 2022. Data were collected using a semi-structured questionnaire.

Sample collection and identification of bacteria

A total of 1042 clinical samples from patients visiting SGNHC were collected and processed according to the standard laboratory methods. Samples with inappropriate labeling and leaked samples were also excluded from the study. For blood culture and Endotracheal (ET) secretion culture, vials with blood and ET secretion were incubated for 24 hours at 37^0C and subsequently inoculated on MacConkey agar, Blood agar and Chocolate agar plates and were further incubated. Blood culture was done up to 96 hours. Urine was inoculated on Cysteine lysine electrolyte deficient agar (CLED). MacConkey agar and Blood agar were used for pus, sputum, tissue biopsy, pericardial fluids and wound swab. All plates were incubated at 37^0C for 24 hours. The isolates were identified based on morphology, microscopic, Gram staining, and standard biochemical tests (Forbes et al. 2007). Isolates were subjected to *in vitro* antibiotic susceptibility tests by Kirby-Bauer disk diffusion method as recommended by Clinical Laboratory Standard Institute (CLSI). Isolates resistant to one or more of the carbapenem antibiotics (Imipenem 10µg and Meropenem 10 µg) were considered as carbapenem-resistant as per the guidelines (CLSI 2020).

Phenotypic confirmation of carbapenemase production

Carbapenems resistant isolates from initial screening were further processed for the detection of carbapenemase production by different phenotypic methods. The four phenotypic methods used were: MHT, mCIM, eCIM and Carba NP test.

a. Modified Hodge Test (MHT)

Firstly, *E. coli* ATCC 25922 matching 0.5 McFarland turbidity was uniformly swabbed onto Muller Hinton Agar (MHA). A 10 µg meropenem disc was placed in the center of the MHA plate. Then, test isolate was streaked as a straight line from the edge of the disc to the edge of the plate and incubated at 37°C for 24 hrs. An indentation in the growth of the negative control towards the meropenem disc on either side of the test isolate or the formation of clover leaf like structure was considered as positive for the production of carbapenemase by the test isolate. MHT Positive *K. pneumoniae* ATCC BAA-1705 and MHT Negative *K. pneumoniae* ATCC BAA-1706 was used as control for the test (CLSI 2017).

b. modified Carbapenem Inactivation (mCIM)

A loopful of bacterial isolate from an overnight blood agar plate was emulsified in 2 mL of Tryptone Soy Broth (TSB) and vortexed for 10-15 sec. A 10µg meropenem disc was immersed in each broth using sterile forceps and incubated at 37°C for four hours. Immediately following completion of the TSB-meropenem disc suspension incubation, a 0.5 McFarland suspension (using the direct colony suspension method) of *E. coli* ATCC 25922 prepared in nutrient broth and inoculated on a Muller Hinton Agar (MHA) plate. *E. coli* ATCC 25922 acts as susceptible strain for potent meropenem disc. Plates were allowed to dry for 3-10 minutes. Meropenem disc from TSB-meropenem disc suspension was removed from the tube and placed on the MHA plate previously inoculated with the meropenem-susceptible *E. coli* ATCC 25922 indicator strains. MHA plates were incubated at 37°C for 18-24 hours. To validate the results, *K. pneumoniae* ATCC BAA-1705 and *E. coli* ATCC 25922 was used as positive and negative controls. Following incubation, zones of inhibition (ZOI) was measured and result was interpreted. ZoI of 6-15 mm or presence of colonies within a 16-18 mm zone was considered as positive for the production of carbapenemase (CLSI 2020).

c. EDTA-CIM (eCIM)

mCIM and eCIM tubes were processed parallelly. For each isolate, another 2ml TSB tube was labeled. Then 20µl of the 0.5M EDTA was added to obtain a final concentration of 5mM EDTA. Then the steps of mCIM were followed up to last incubation. Meropenem disc from eCIM and mCIM tubes was placed on the same MHA plate. Zone of inhibition was measured and result was interpreted. An increase in (≥5mm) diameter for the eCIM zone diameter compared with the mCIM zone diameter was considered as a metallo-B-lactamases. And no growth or marginal (≤4mm) increase in zone diameter in the presence of EDTA compared with mCIM zone diameter was considered as serine carbapenemase. eCIM results were interpreted only when mCIM test turns out to be positive as per the guidelines (CLSI 2020).

d. Carba NP test

For each isolate, QC organism and uninoculated control, two microfuge tubes (one “a” and one “b”) were labeled.

100µl of bacterial protein extraction reagent i.e. Tris HCL lysis buffer was added to each tube. For each isolate to be tested, 1µl loopful of bacteria from an overnight blood agar plate was emulsified in both tubes "a" and "b". Each tube was vortex for 5 seconds. (Uninoculated reagent control tubes contain only bacterial protein extraction reagent, no organism). Then, 100µl of carbaNP solution A was added to tube "a" and 100µl of solution B (solution A + imipenem) was added to tube "b". Each tube was vortex and incubated at 37^0C for up to 2 hours. Following incubation change in color was observed. If the tube "a" and tube "b" changes its color into red and yellow respectively, then it was considered as positive results. Isolate that demonstrate positive results within 2 hours was considered as carbapenemase producers following the standard guidelines (CLSI 2020).

Determination of MIC for imipenem

The *in vitro* activity of imipenem against the given MDR isolates was done using agar-dilution method. This test was performed by using MHA plates with various imipenem concentrations (ranged from 1-64µg/ml). The inoculums of the test organism were transferred to the agar surface. Plates were incubated at 37°C for 24 hours and result was interpreted using breakpoint approved by CLSI guidelines. According to CLSI recommendations, isolates that demonstrate growth at a concentration of more than or equal to 4 µg/mL were considered as resistant, whereas growth at a concentration of less than or equal to 1 µg/mL were considered as sensitive to imipenem in clinical settings (CLSI 2020).

Molecular assay

DNA from the positive carbapenem resistant bacterial isolates was extracted by phenol chloroform method followed by quantitation and purity assessment. Carbapenem resistant *OXA-181* gene was detected by polymerase chain reaction (PCR) using specific primer (Table 1) (Shanthi et al 2013).

Data Analysis

All the data of the results were entered in the Microsoft Office Excel spreadsheets, analyzed for proportions and frequencies,. and the results were presented in the form of tables and figures.

Ethical approval

The ethical approval of this research was obtained from the Institutional Review Committee (IRC), Institute of Science and Technology (IOST), Tribhuvan University (TU) (Regd. No IRCIOST-22-0006).

The study was conducted following the standards set by Nepal Health Research Council (NHRC).

Table 1: Primer sequence of *OXA-181* gene

Carbapenemase Gene Primers			
Target Gene	**Forward/ Reverse**	**Primer Sequences**	**PCR conditions/ Amplicon size**
OXA-181	Forward	5'-ATGCGTGTATTAGCCTTATCG-3'	Initial denaturation 94^0C for 3 min.
	Reverse	5'- AACTACAAGCGCATCGAGCA-3'	Denaturation at 94^0C for 30 sec, annealing at 60^0C for 30 sec, elongation at 72^0C for 30 sec, for a total of 29 cycles
			Final extension at 72^0C for 5min.
			Amplicon size 888 bp

RESULTS

Growth patterns in various clinical specimens

A total of 1042 clinical specimens were collected and processed comprising 452 (43.37%) urine samples, 272 (26.10%) blood specimens, 127 (12.18%) sputum, 58 (5.5%) endotracheal secretions, 62 (5.9%) pus, 27 (2.59%) pericardial fluids, 34 (3.26%) wound swabs, five (0.4%) tissue, three (0.28%) stool, two (0.19%) eye discharge. Of these 1042 samples, 282 (27.06%) showed bacterial growth whereas remaining 760 (72.93%) showed no growth (Figure 1).

Distribution of bacterial isolates

Out of 282 isolates, 172 (60.99%) were Gram negative bacteria, rest belonging to GPC. Among all isolates, predominant organism was *K. pneumoniae* 65 (23.04%) followed by *E. coli* 58 (20.56%), *Staphylococcus aureus* 47 (16.67%), *Staphylococcus epidermidis* 32 (11.34%), *Pseudomonas aeruginosa* 18 (6.38%), *Streptococcus pyogenes* 17 (6.02%), *Acinetobacter* spp. 11(3.90%), *Staphylococcus saprophyticus* 9 (3.19%), *Klebsiella oxytoca* 8 (2.83%), *Enterobacter* 8 (2.83%), *Enterococcus* 5 (1.77%), *Citrobacter* spp. 2 (0.70%), *Providencia* spp. 1 (0.35%) and *Proteus* spp. 1 (0.35%) (Figure 2)

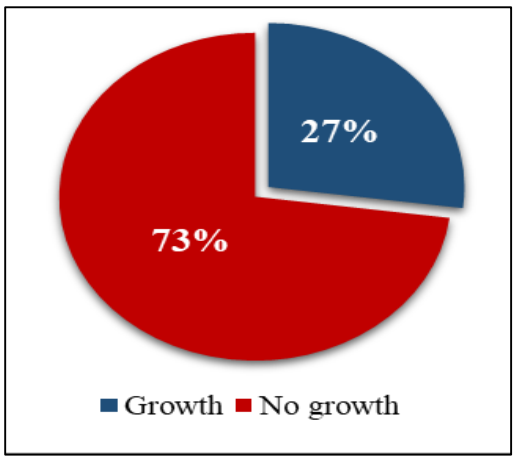

Figure **1: Growth patterns in clinical specimens**

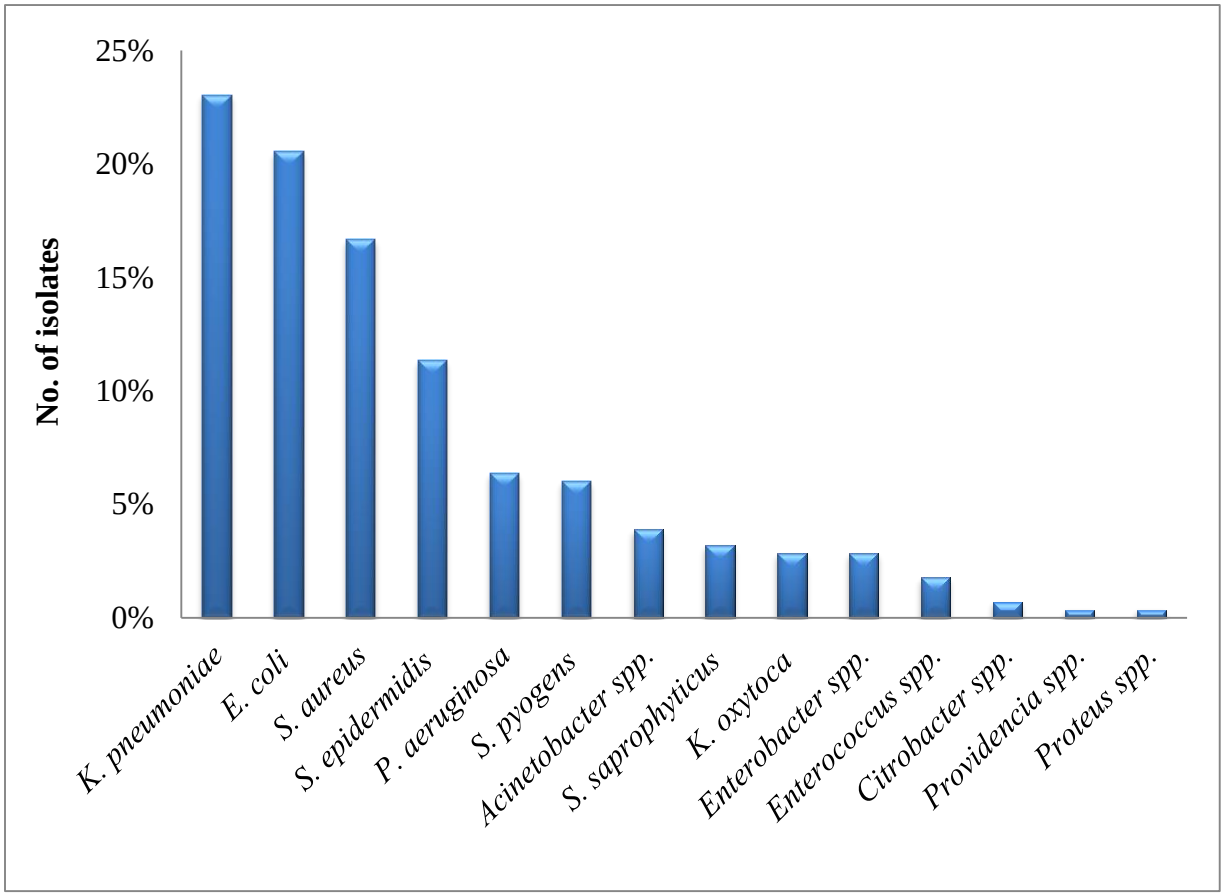

Figure 2: Patterns of bacterial isolates from clinical samples included in the study

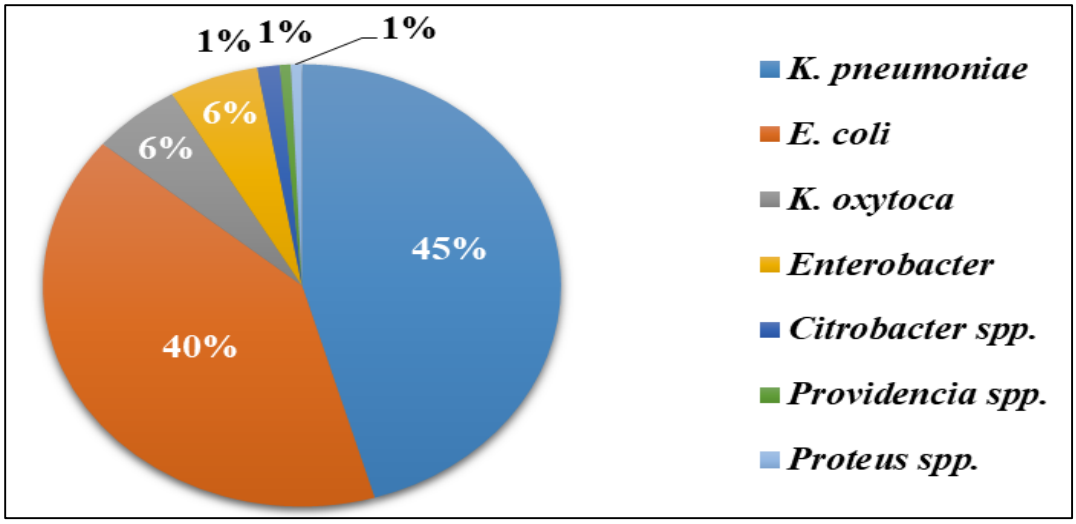

Figure 3: Pattern of the Enterobacteriaceae among the bacterial isolates

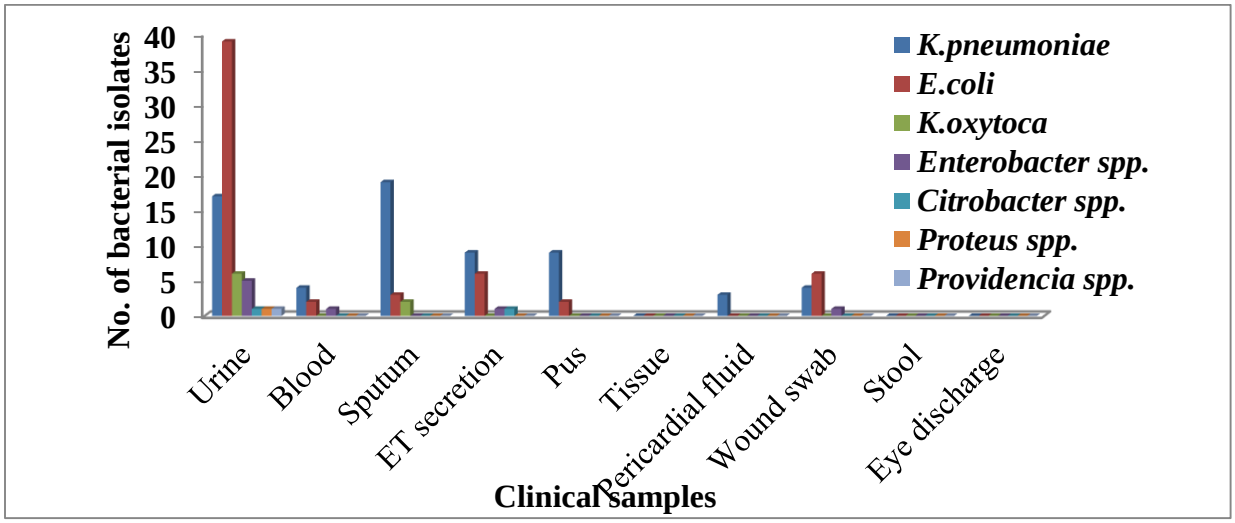

Figure 4: Distribution of Enterobacteriaceae in different clinical sample

Table 2: Incidence of Bacillus cereus in the ice- cream sample

Antibiotics	*K. pneumoniae* n=65 R (%)	*E. coli* n=58 R (%)	*K. oxytoca* n=8 R (%)	*Enterobacter* spp. n=8 R (%)	*Citrobacter* spp. n=2 R (%)	*Proteus* spp. n=1 R (%)	*Providencia* spp. n=1 R (%)
Amikacin	39 (60)	29 (50)	5 (62.50)	3 (37.50)	-	1 (100)	-
Ciprofloxacin	57 (87.69)	48 (82.76)	7 (87.50)	7 (87.50)	2 (100)	1 (100)	1 (100)
Norfloxacin	12 (18.46)	31 (53.45)	2 (25)	4 (50)	1 (50)	1 (100)	1 (100)
Nalidixic acid	48 (73.85)	30 (51.72)	2 (25)	4 (50)	1 (50)	-	1 (100)
Nitrofurantoin	13 (20)	9 (15.52)	1 (12.50)	4 (50)	-	1 (100)	1 (100)

Cotrimoxazole	55 (84.62)	42 (72.41)	4 (50)	7 (87.50)	2 (100)	1 (100)	1 (100)
Cefixime	59 (90.77)	49 (84.48)	5 (62.50)	6 (75)	2 (100)	1 (100)	1 (100)
Gentamycin	25 (38.46)	8 (13.79)	2 (25)	2 (25)	-	-	-
Cefepime	57 (87.69)	48 (82.76)	5 (62.50)	6 (75)	2 (100)	1 (100)	1 (100)
Cefotaxime	57 (87.69)	50 (86.21)	5 (62.50)	6 (75)	2 (100)	1 (100)	1 (100)
Piperacillin / Tazobactam	39 (60)	29 (50)	4 (50)	6 (75)	-	-	-
Imipenem	36 (55.38)	14 (24.13)	3 (37.50)	5 (62.50)	-	-	-
Meropenem	28 (43.08)	12 (20.69)	2 (25)	3 (37.50)	-	-	-
Ampicillin/ Salbactam	55 (84.62)	38 (65.52)	4 (50)	6 (75)	2 (100)	-	1 (100)

Table 3: MDR status of Enterobacteriaceae isolates

Bacterial isolates	Total	MDR	Non MDR
K. pneumoniae	65	59 (90.77%)	6 (9.23%)
E. coli	58	51 (87.93%)	7 (12.07%)
K. oxytoca	8	6 (75.00%)	2 (25.00%)
Enterobacter spp.	8	7 (87.50%)	1 (12.5%)
Citrobacter spp.	2	2 (100%)	-
Proteus spp.	1	1 (100%)	-
Providencia spp.	1	1 (100%)	-
Total	**143**	**127 (88.81%)**	**16 (11.19%)**

Genaral profile of Enterobacteriaceae

Out of 172 Gram-negative isolates, 143 (83.14%) isolates were of Enterobacteriaceae family. Of these, *K. pneumoniae* was predominant isolates comprising 65 (45.45%) isolates followed by *E. coli* 58 (40.55%) whereas 13.98% belong to rest of the isolates (Figure 3).

Enterobacteriaceae among clinical specimens

Bacterial growth was encountered highest from urine 70 (48.95%) followed by sputum 24 (16.78%), ET secretion 17 (11.88%), wound swab 11 (7.69%), pus 11 (7.69%), blood 7 (4.8%) and pericardial fluid 3 (2.09%). Majority of isolates i.e. 48.95% (70/143) isolates were obtained from urine followed by sputum 16.78% (24/143), Urine and sputum has the highest number of *E. coli* 67.24% (39/58) and *K. pneumoniae* 29.23% (19/65) respectively (Figure 4).

Antibiotic susceptibility of Enterobacteriaceae

Among the Enterobacteriaceae, high rate of antibiotic resistant was observed in *K. pneumoniae*, followed by *E. coli*. Out of 13 antibiotics used, gentamycin (25.87%) followed by meropenem (31.47%) and imipenem (40.56%) were found to be least resisted and thus could be the drug of choice (Table 2).

MDR status of Enterobacteriaceae isolates

Out of 143 Enterobacteriaceae isolates, 127 (88.81%) were found to be multidrug resistant. The principal MDR isolates were *K. pneumoniae, E. coli, Enterobacter* spp., and *Klebsiella oxytoca* (Table 3).

Carbapenem resistance among Enterobacteriaceae

Among 143 Enterobacteriaceae, 62 were carbapenem

resistant, and the prevalence of carbapenem resistance Enterobacteriaceae was (43.36%). Comparatively, a high percentage of carbapenem resistance was documented among K. pneumoniae 62.90% (39/62), followed by *E. coli* 24.19% (15/62), *Enterobacter* spp. 8.06% (8/62) and *K. oxytoca* 4.84% (3/62). While all *Citrobacter, Providencia* and *Proteus* isolates were susceptible against carbapenem (Table 4).

Comparison of MHT, mCIM/eCIM, Carba NP test

The table 5 shows the comparison of the different phenotypic tests done for detection of carbapenemase production. mCIM method yielded 72.58% positive result of carbapenemase production followed by CarbaNP method (61.29%) and MHT (48.39%).

Minimum Inhibitory Concentration (MIC) of imipenem against MDR Enterobacteriaceae

Out of 127 MDR Enterobacteriaceae, Imipenem's MICs were discovered to vary from 1µg/ml to 64µg/ml. Altogether, 47 isolates showed MIC equal or greater than 4 µg/ml. Of these, 30 were *K. pneumoniae*, 16 were *E. coli* and single*K. oxytoca* (Figure 5)

Detection of *OXA-181* gene among Enterobacteriaceae

Only 47 MDR isolates having MIC ≥4 µg/ml for imipenem were further processed for PCR. Of these, 12 (25.53%) isolates were found to be harboring the OXA-181 gene, among which 11 (91.67%) were carbapenem-resistant K. *pneumoniae* isolates and only one (8.33%) was carbapenem-resistant *E. coli* **(**Figure 6**)**.

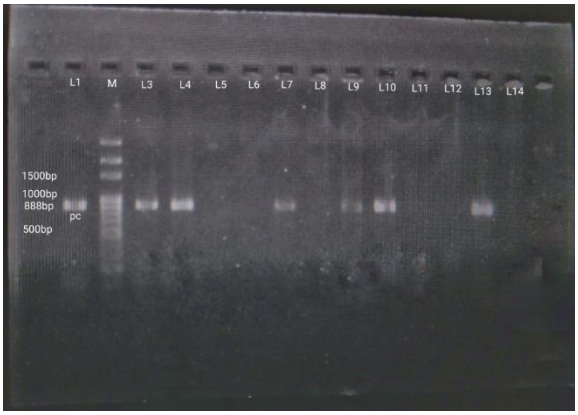

Figure 6: Agarose gel electrophoresis of PCR amplified *OXA-181* gene in carbapenem resistant Enterobacteriaceae, LaneM: DNA size marker (100-3000 bp); Lane L1: positive control; Lane L3/4/7/9/10/13: *OXA-181* positive isolates, Lane L5/6/8/11/12: *OXA-181* negative isolates and L14: negative control

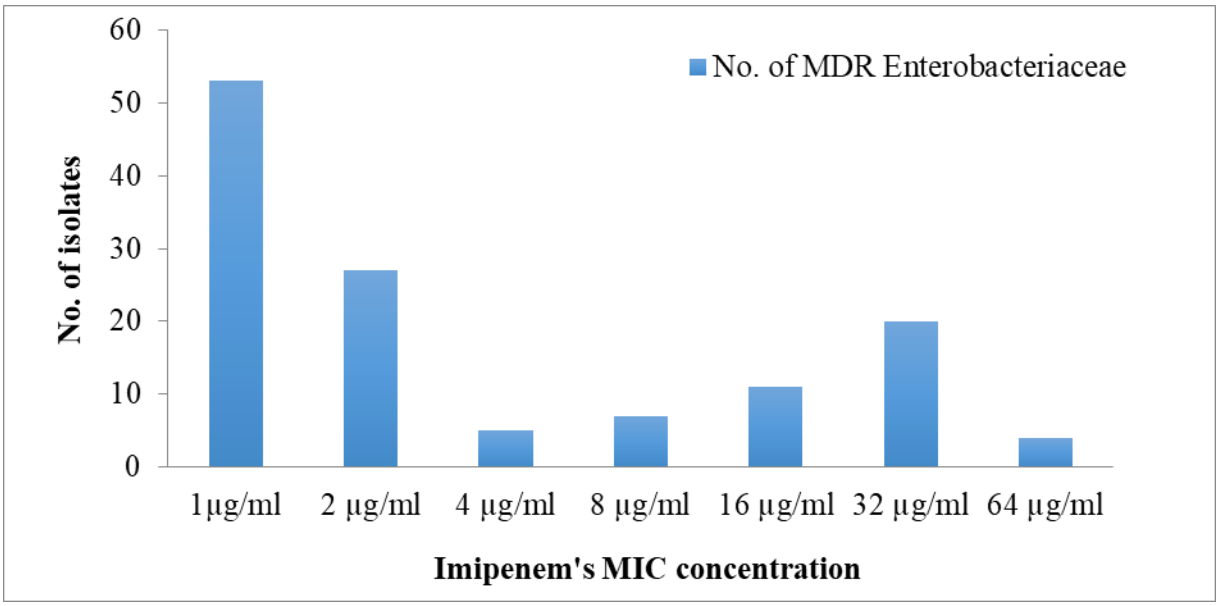

Figure 5: MIC of imipenem against MDR Enterobacteriaceae

Table 4: Prevalence of carbapenem resistant Enterobacteriaceae

Bacterial isolates	Total isolates	Carbapenem		
		Resistant (%)	Intermediate (%)	Sensitive (%)
K. pneumoniae	65	39 (60.00%)	-	26 (40.00%)
E. coli	58	15 (25.86%)		43 (74.13%)
K. oxytoca	8	3 (37.50%)	-	5 (62.50%)
Enterobacter spp.	8	5 (62.50%)	-	3 (37.50%)
Citrobacter spp.	2	-	-	2 (100%)
Proteus spp.	1	-	-	1 (100%)
Providencia spp.	1	-	-	1 (100%)
Total	**143**	**62 (43.36%)**	**0**	**81 (56.64%)**

Antibiotic susceptibility pattern of *B. cereus*

Out of 18 isolated *B. cereus* all were susceptible toward Chloramphenicol, Ciprofloxacin, Co-Trimoxazole, Erythromycin, and Levofloxacin were resistant toward Piperacillin/Tazobactum. Out of 18 isolates none of the isolate was multidrug resistant (Figure 5).

DISCUSSION

This study was aimed to evaluate different phenotypic methods used for determining carbapenemase production among Enterobacteriaceae isolates and finally detection of the presence of the plasmid-mediated *OXA-181* gene amongst carbapenem resistant isolates. In the present study, a total of 1042 different clinical specimens were investigated of which only 27.06% showed the bacterial growth. Previous researches conducted in Nepal have reported similar growth rate of 27.3% and 27.41 % by Sharma et al (2013) and Karn et al (2016) respectively. But contrary to our result, previous studies from Shahid Gangalal National Heart Center, shows lower growth rate of 11.9% and 17.8% by Shilpakar et al (2021) and by Sah et al (2021) respectively. Lower growth occurrence has also been reported by Awasthi et al 2015 (25.5%), GC et al 2018 (17.96%), Adhikari et al 2019 (16.97%), Aryal et al 2020 (22%), Sherchan et al 2020 (23.4%) and Ansari et al 2021 (22%). The varying rates of bacterial growth in various studies might be the result of variations in individual's physiological and medical conditions, research methods, population's demographics and infection epidemiology differences and prior antibiotic usage which interferes with the growth of organism (Shrestha et al 2020; Pandit et al 2020).

In the current study, the prevalence of Gram-negative bacteria was found higher as compared to f Gram-positive. Among the Gram negative bacteria, Enterobacteriaceae family (83.14%) was found to be in high number. Different studies in Nepal have reported *E. coli* as the most common isolate followed by *Klebsiella* (GC et al 2018; Aryal et al 2020; Karki et al 2021). While in this study, *K. pneumoniae* was found as predominant Enterobacteriaceae member followed by *E. coli, Enterobacter* spp., *Citrobacter* spp., *Proteus* spp. *and Providencia* spp. Other studies conducted at the same hospital previously by Pathak et al (2017) and Shilpakar et al (2021) have also reported *Klebsiella* as the chief isolate followed by *E. coli.* The frequency of various sample types, the period of the investigation and the specific area, weakened immune system, nosocominal infection may be the cause of the higher isolation rate of *K. pneumoniae* (Pathak et al 2017).

Drug-resistant microorganisms are increasingly important public health concern. Majority of the Enterobacteriaceae including *Klebsiella* spp., *E. coli, Enterobacter* spp., *Citrobacter* spp., *Proteus* spp. *and Providencia* spp. were

highly resistant to ciprofloxacin (86.01%), with all generations of cephalosporins (cefixime (86.01%), cefepime (83.91%), cefotaxine (85.31%) and cotrimoxazole (76.22%). *Klebsiella* spp. was resistant to ciprofloxacin (87.67%), cephalosporins (~90%), and cotrimoxazole (80%). Almost similar rates of resistance were observed in *E. coli*. This result resembles to the finding of Parajuli et al (2017) and Gurung et al (2020) who reported similar resistance patterns. This resistance pattern of Enterobacteriaceae towards ciprofloxacin, cephalosporins and cotrimoxazole was considerably higher when compared to earlier studies carried out by Pathak et al (2017) and GC et al (2018). In Enterobacteriaceae, resistance to β-lactams antibiotics, including cephalosporins, is mainly due to the production of β-lactamases, which may be encoded either chromosomally or on plasmids (Kattel et al 2008). High level of drug resistance against fluoroquinolones (ciprofloxacin) may be due to frequent prescription for the treatment especially for complicated cystitis in female patients (Wagenlehner 2007).

This study reported nitrofurantoin higher resistant towards isolates recovered from urine. The narrow spectrum of activity, limited tissue distribution (low or undetectable serum levels), and limited contact with germs outside the urinary tract may have contributed to the higher vulnerability from uropathogens to nitrofurantoin (Karlowsky 2002). Aminoglycosides (amikacin and gentamycin) usually have better activity against clinically important Gram-negative bacteria. However, this study exhibited the sensitivity rate of amikacin and gentamycin as 29.37% and 57.34% respectively against Enterobacteriaceae. In contrast to our study, Singh and Madhup (2013) and Shilpakar et al (2021) showed higher sensitivity against aminoglycosides whereas Parajuli et al (2017) reported 45-100% resistance against aminoglycosides. This study showed a greater susceptibility against gentamycin which may be due to the minimal use of such drugs in treatment.

In this study, 88.81% of Enterobacteriaceae were found to be multi-drug resistant. Similar to this, Yadav et al (2015) and Ghimire et al (2018) reported more than 90% Enterobacteriaceae isolates (mostly *E. coli* and *Klebsiella* spp.) as multi-drug resistant. But contrary to this, Ansari et al (2021) reported only 44.1%, isolates as MDR. In this study the prevalence of MDR in *K. pneumoniae* and *E. coli* was found to be 90.77% and 87.93% respectively. A study by Panta et al (2016) has shown that all of the *Klebsiella* isolates were MDR. Similar study has reported MDR status in the *K. pneumoniae* to be 64% (Odari and Dawadi 2021). In addition, a study by Shrestha et al (2020) and Raya et al (2020) reported low prevalence of MDR in *E. coli.* This rising occurrence of MDR may be ascribed to antibiotic abuse in Nepal, which includes non-empirical use, taking medicines insufficiently dosed, and the simple accessibility to antibiotics without a doctor's prescription.

The overall prevalence rate of carbapenem resistant in this study was 43.36%. Previous studies conducted in Nepal showed the prevalence of carbapenem resistant was between 5 to 25% (Bora et al 2014; Devkota et al 2020; Pokhrel et al 2018). A study by Adhikari et al (2019) reported similar prevalence rate of carbapenem resistant as 40.7% isolates resisted carbapenems. The overall prevalence of CRE (43.36 %) is quite alarming for a drug which is normally reserved as the drug of last resort for treatment. A study by Shahid et al (2012) reported the prevalence of CRE as 1.8% (16/893) at a tertiary hospital in North India which is quite lower as compared to the present study. Globally, slightly lower resistant rate of carbapenem in Enterobacteriaceae have been reported (Cai et al 2017; Kostyanev et al 2019; Mendes et al 2013). Contrary to these, a study conducted in Brazil by Lorenzoni et al (2017) showed high level of resistance to carbapenems (>98%). Parimala (2017) reported that prevalence of CRE in South-India was 44.3% which is quite similar to present study. Qin et al (2014) in China have reported the prevalence of CRE to be 33.3%The prevalence of CRE reported in developing countries so far is higher than those reported in developed countries. This might be due to the well-regulated use of antibiotics in developed countries compared to developing countries. Long term hospitalization, frequent and inappropriate use of antibiotics in the nation might be contributing factors to the higher resistance noted (Pyakurel et al 2021).

The resistance observed against different carbapenem was found to be 31.47% for meropenem and 40.56% for imipenem concluding meropenem as the effective drug than imipenem. This finding is in tune with an earlier finding by Ansari et al (2021) who reported higher sensitivity of meropenem (28.95%) than imipenem (32.41%). In contrast, the present finding is in contradiction with the report by Mishra et al (2012). This might be due to the reason that meropenem are more active against Gram negative bacilli, while imipenem is more active against Gram positive cocci (CLSI (2020). Following gentamycin, carbapenem antibiotics were found

to be high sensitivity in this study among the 13 different antibiotics used.

In the present study, among total carbapenem resistant isolates, higher prevalence of carbapenem resistant was observed in *K. pneumoniae* 62.90% followed by *E. coli* 24.19%, *Enterobacter* spp. 8.06% and *K. oxytoca* 4.84%. None of the isolates of *Citrobacter* spp., *Providencia* spp., and *Proteus* spp. were resistant to the carbapenems. Similar studies in Nepal by Pokhrel et al (2018), Bora et al (2014), Karn et al (2016) and Gurung et al (2020) also reported the high prevalence of carbapenem resistance *K. pneumoniae* followed by the *E. coli*.

The Enterobacteriaceae isolates resistant to carbapenem by Kirby-Bauer Disc Diffusion method were further evaluated by four different phenotypic tests. Sixty-two isolates were examined for the detection of carbapenemase production by MHT, mCIM, eCIM and Carba NP methods. In this study, MHT showed the positivity of 48.39% (30/62) with higher positivity rate in *K. pneumoniae* 60% followed by *E. coli* 30%, *Enterobacter* spp. 6.67% and *K. oxytoca* 3.33%. In contrast Gurung et al (2020) reported MHT's positive rate as 63.6% with higher positivity rate among *K. pneumoniae* (71.4%) followed by *E. coli* (28.6%). Similarly, higher rate (69.1%) has been reported by Ansari et al (2021) for MHT method. Compared to present study, Darnal et al (2021) reported similar positivity (58.33%) in *K. pneumoniae*. In Nepal with little resources, MHT is an effective tool for the detection of carbapenemase since it is straightforward, affordable, and uses supplies that are typically found in clinical laboratories (Tamma and Simner 2018). MHT was once considered as the gold standard methods (Nordmann et al 2012). But, nowadays the widely distributed MBL and *OXA-48* like enzymes along with Ambler class A carbapenemases makes it less trustworthy (Girlich et al 2012). In this study, MHT showed low positivity rates in comparison to mCIM and Carba NP method.

The mCIM approach is straightforward, affordable, less subjective, reproducible, and most sensitive (Aktas et al 2017; Pierce et al 2017; Pragasam et al 2017). Similarly, this study also shows mCIM positivity of 72.58% for carbapenem. In contrast, Giri et al (2021), Khare et al (2022) and Pawar et al (2018) all reported mCIM with higher positivity of 98.66%, 96.67% and 98.48% respectively. According to CLSI guidelines 2020, mCIM in combination with eCIM distinguishes the metallo-beta lactamase (both mCIM and eCIM positive) from serine carbapenemase (mCIM positive and eCIM negative). Following these standards, this study shows 51.61% positivity for metallo-beta lactamase while 20.97% positivity for serine carbapenemase. In comparison to MHT and Carba NP methods, mCIM reported improved sensitivity in this study.

The Carba NP test is another important and recent development for the accurate identification of CRE. This rapid (incubation upto 2hrs.), simple, and affordable method can be used in any laboratory anywhere in the globe. This test is considered as 100% sensitive and specific as molecular techniques (Bouslah Z, 2020). In this study, Carba NP test showed the positivity of 61.29% for carbapenem. In contrast, Khare et al (2022) and Walthall et al (2018) reported 98.48% and 94.4% Carba NP positivity which was quite higher than the present study. The rate was higher in comparison with MHT but significantly lower when compared with mCIM method.

The present study shows a comparative efficacy of different phenotypic tests. mCIM method showed higher positive rate (72.58%) for detection of carbapenemase production followed by CarbaNP method (61.29%) and MHT (48.39%). This finding is in line with a study by Bialvaei et al (2021) in Iran who also reported mCIM (71.3%) methods to have a high positivity compared to MHT (57.3%) and Carba NP (68.8%) methods Musawi et al (2021) reports from Gulf region also reported mCIM as the reliable assay for the detection of CRE with sensitivity of 94%. Similarly, the finding of this study is in tune with the finding by Walthall et al (2018) and Zhang et al (2022) who reported a sensitivity of 100% and 97% respectively for mCIM than the other phenotypic methods. Different studies have shown the sensitivity of mCIM ranging from 97% to 100% (Kumudunie et al 2021; Lutgring et al 2018; Zhong et al 2020). Based on these reports, mCIM can be considered as the most effective phenotypic method for the detection of carbapenem resistant.

Although the mCIM test takes longer time than the Carba NP test (24h vs. 2h), this study shows mCIM to be a highly effective, simple, and cost-effective method for earlier detection of carbapenemase that can be used in clinical laboratories, especially those with limited resources. This study has the limitation of not comparing the results with the genetic analysis to see the presence of different classes of carbapenemases. As a result, it could affect the outcomes of the present phenotypic study comparative to other previous studies.

OXA-181 is a variant of *OXA-48* which now becomes the second most prevalent carbapenemase worldwide

(Peirano et al 2019). In this study, imipenem's MICs for MDR Enterobacteriaceae were tested which were found to vary from 1µg/ml to 64µg/ml. Forty-seven isolates showed MIC equal to or greater than 4µg/ml and six isolates were highly resistant to imipenem with MIC of 32µg/ml. Of these only 25.53% isolates possessed *OXA-181* gene. Prevalence of *OXA-181* among Enterobacteriaceae in this study was Similar prevalence (25.64%) of *OXA-181* among the CRE was reported by Castanheira et al (2011) in India which is considered as the first instance of *OXA-181* being reported. In this study, of the 12 *OXA-181* positive isolates, 11 were *Klebsiella pneumonia*, and one was *E. coli*. A study conducted by Serchan et al (2020) revealed only two *OXA-181* harboring isolate out of 6 carbapenem resistant *K. pneumoniae* which is considered as the first report from Nepal. One study done by Balm et al (2013) in Singapore also reported almost similar prevalence of *OXA-181* as 24.5% considering *OXA-181* as the second most common carbapenemase encoding gene. The low prevalence of *OXA-181* was reported by Shanthi et al (2013) in India (1.8%) among the CRE. In Nepal, no any studies so far had encompassed the detection of *OXA-181* in *E. coli* prior to this. The absence of *OXA-181* gene in remaining carbapenem resistant Enterobacteriaceae indicates there might be the presence of other carbapenem genes. Though the research on *OXA-181* is limited, it should not be overlooked as its increasing copy number might be an alternative route for acquiring more carbapenem resistance in Enterobacteriaceae such as *K. pneumoniae* and *E. coli*. As this study's *OXA-181* prevalence results are greater than those of other earlier studies, it has a comparable prevalence rate with Indian reports which may be quite alarming for Nepal. The source behind this might be due to open border and close ties between Nepal and India in terms of patient interchange.

The present study and other studies on the prevalence of drug-resistant Enterobacteriaceae and comparison of phenotypic results have several notable discrepancies. Firstly, as contrary to GNB in general, the current study particularly focused on Enterobacteriaceae species. Secondly, it did not compare the findings of phenotypic methods with gold standard molecular methods for calculating sensitivity and specificity. Thirdly, as the scope of this study was limited to a single hospital in Kathmandu with specific patients and for a brief period of time, it doesn't provide a national overview of the carbapenem resistance in Enterobacteriaceae because their incidence and distribution may differ by topological features and geographic location.

Conclusion

The study reported higher rate of MDR and CRE prevalence which is an alarming situation for concerned authorities. Most of the Enterobacteriaceae were resistant to all cephalosporins used, whereas gentamycin, nitrofurantoin, carbapenems can be most effective drugs for treatment in accordance to this study. Among various method tested in this study, mCIM has high positivity rate as compared to other methods. But the findings should be supported by more studies at different level of healthcare facility to unravel the actual burden of CRE in Nepal and studies including a larger cohort of isolates and various types of carbapenemases should be utilized to analyse and determine effective method for carbapenemases detection.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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