Rapid Detection of Carbapenemase Genes (Bla-Vim And Bla-Imp) By Multiplex Pcr in Gram-Negative Clinical Isolates

Mehraj Ansari (✉ mrseeyon@gmail.com)

ShiGan International College of Science and Technology

Kulraj Rai

Institute of Microbiology (University of Chinese Academy of Sciences)

Ganesh Rai

ShiGan International College of Science and Technology

Subhas Aryal

Golden Gate International College

Shiba Rai

Nepal Medical College

Anil Shah

Annapurna Neurological Institute and Allied Sciences (Annapurna Research Centre)

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**RAPID DETECTION OF CARBAPENEMASE GENES (bla-VIM AND bla-IMP) BY
MULTIPLEX PCR IN GRAM-NEGATIVE CLINICAL ISOLATES**

**Mehraj Ansari¹, Subhas Chandra Aryal², Ganesh Rai¹, Kul Raj Rai⁵, Anil Kumar Shah,³
and Shiba Kumar Rai^{1,4}**

¹Shi-Gan International College of Science and Technology, Nepal, ²Golden Gate International
College, ³Annapurna Neurological Institute and Allied Sciences (Annapurna Research Centre),
Nepal, ⁴Nepal Medical College, Nepal and ⁵Institute of Microbiology (University of Chinese
Academy of Sciences), China

Corresponding author: mrseeyon@gmail.com (Mehraj Ansari)

Abstract

Background

Carbapenems have been the choice of antibiotics for the treatment of infections caused by multidrug-resistant bacteria. However, during recent years, carbapenems resistant bacteria have emerged significantly. The main objective of this study was to determine the prevalence of carbapenemase (bla-VIM and bla-IMP) producing isolates among Enterobacteriaceae, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*.

Results

Of the total 1,151 clinical samples, 253 (22.0%) showed growth positive. Of them, 226 (89.3%) were identified as members of Enterobacteriaceae, *P. aeruginosa* and *A. baumannii*. Among the 226 isolates, 106 (46.9%) were multidrug-resistant. Of the 106, 97 (91.5%) isolates showed resistance to at least one of the carbapenem used. Among the 97 isolates, 67 (69.1%) showed MHT positive results. bla-VIM and bla-IMP were detected in 40 and 38 isolates, respectively.

Conclusion

This study determined the higher prevalence of MDR and carbapenem resistance among Enterobacteriaceae, *P. aeruginosa* and *A. baumannii* as detected by the presence of bla-VIM and bla-IMP genes.

Keywords: Carbapenems, Carbapenemase, Modified Hodge Test, bla-VIM, bla-IMP

Background

Carbapenems are versatile β -lactam antibiotics having a broad-spectrum antibacterial activity. Carbapenems have been considered to be the most reliable last-resort treatment for infections caused by multidrug-resistant (MDR) pathogens [1]. However, the rapid emergence of carbapenem-resistant gram-negative bacteria, namely, carbapenem-resistant Enterobacteriaceae (CRE), *Acinetobacter baumannii* (CRAB) and *Pseudomonas aeruginosa* (CRPsA), pose a significant threat to public health [2] and has become a major public health concern worldwide

over the last decade [3,4,5]. WHO (2017) ranks carbapenem-resistant Enterobacteriaceae (CRE), carbapenem-resistant *P. aeruginosa* (CRPsA) and carbapenem-resistant *A. baumannii* (CRAB) in the highest priority category (i.e. critical) in the global priority list of pathogens. These bacteria are difficult to treat due to high levels of antibiotic resistance and are associated with high mortality [2]. They have the potential for widespread transmission of resistance via mobile genetic elements [2]. Carbapenem resistance in Gram-negative bacteria is the major contributing factor for multidrug-resistance [1]. Most importantly, various mechanisms are predicted to continue the evolution of carbapenem-resistant Gram-negative nosocomial pathogens in the future [1,6]. If it continues in this pace, untreatable infections could emerge on a large scale and the world may experience in some cases dramatic situations of the pre-antibiotic era [1].

The early recognition of CRE-CRAB-CRPsA and specific required IPC practices and procedures to effectively prevent their occurrence, appropriate antimicrobial therapy and control their spread in acute health care facilities is much required [2,7]. Identification of such carbapenemase producers can be done by both phenotypic and molecular based techniques [8]. Various non-molecular-based tests have been proposed for detection of carbapenemase activity, however each of them have certain limitations and none of them is 100% sensitive or 100% specific [8]. Modified Hodge test (MHT), is a simple and economic tool for the phenotypic detection of carbapenemase activity [9]. It is based on conventional gold standard phenotypic biochemical testing and is the first Clinical & Laboratory Standards Institute (CLSI) recommended growth-based carbapenemase detection test [10]. The MHT relies on the ability of carbapenemase producers to decrease the local concentration of carbapenem antibiotics, enabling the carbapenem-susceptible *E. coli* isolate to grow uninhibited around the streak line near the carbapenem disk, producing a cloverleaf appearance [11]. The MHT is also sensitive to examine activities of several carbapenemases, including VIM, IMP, and OXA-48-like enzymes [12]. Molecular techniques like RT-PCR on the other hand remain the reference standard for the identification and differentiation of specified set of carbapenemase genes with excellent sensitivity and specificity [8].

Variable prevalence of carbapenemase genes (from as low as 0.5% to as high as 100%) has been documented worldwide [13, 14, 15, 16, 17, 18, 19, 20]. In Nepal, studies show the prevalence of carbapenem resistance between 5% to 25% [21, 22, 23, 24]. However, the prevalence of carbapenemase genes among bacterial clinical isolates in Nepal is largely under-reported. We in this paper, therefore, report the prevalence of carbapenemase genes among bacterial clinical isolates (Enterobacteriaceae, *A. baumannii* and *P. aeruginosa*) in Nepal.

Results

Out of 1,151 samples, 253 (22.0%) were culture positive. Of them, 226 (89.3%) were identified as members of Enterobacteriaceae, *P. aeruginosa* or *A. baumannii*. Of the 226 isolates, 116 (53.1%) were from male patients and 110 (48.7%) from female patients. Among the 226 isolates, 41 (18.1%) were blood samples, 40 (17.7%) were catheter tips, 8 (3.5%) were CSF, 23 (10.2%) were pus, 17 (7.5%) were stool, 65 (28.8%) were urine, 14 (6.2%) were vaginal swab and 18 (7.9%) were wound swab. Among the total culture-positive isolates, *E. coli* (n=72, 28.5%) was the predominant organism followed by *P. aeruginosa* (n=41, 16.2%), *A. baumannii* (n=40, 15.8%), *Staphylococcus aureus* (n=27, 10.7%), *Klebsiella pneumoniae* (n=25, 9.9%), *K. oxytoca* (n=12, 4.7%), *Proteus mirabilis* and *Enterobacter cloacae* (n=9, 3.6%), *Citrobacter freundii* (n=8, 3.2%), *P. vulgaris* (n=5, 2.0%), *Citrobacter koseri* (n=4, 1.6%) and *Salmonella* Typhi (n=1, 0.4%) being the least.

AST and carbapenem resistance

The highest resistance was found against cefoxitin 66.8% (151/226) and the least resistance was observed against polymyxin B (19.5%; 8/41). Meropenem was found to be the most effective carbapenem antibiotic and ertapenem was found to be the least effective. A total of 31.0% (70/226) of the total isolates showed resistance to meropenem. Ertapenem was used only against Enterobacteriaceae isolates, 35.2% (51/145) of which showed resistance to ertapenem. Likewise, 32.4% (47/145) and 29.0% (42/145) isolates of Enterobacteriaceae were resistant to imipenem and meropenem respectively. Except for the carbapenems, amikacin, to which 38.9% (88/226) of the isolates showed resistance, was found to be the most effective second-line drug (Table 1).

Table 1: Antibiotic Susceptibility pattern of growth positive organisms

S No.	Antibiotics used	Resistant	Sensitive
		N (%)	N (%)
1	Ciprofloxacin (n = 226)	121 (53.5%)	105 (46.5%)
2	Azithromycin (n =226)	106 (46.9%)	120 (53.1%)
3	Cotrimoxazole (n =226)	109 (48.2%)	117 (51.8%)
4	Amoxycillin (n =226)	108 (47.8%)	118 (52.2%)
5	Amikacin (n =226)	88 (38.9%)	138 (61.1%)
6	Cefoxitin (n =226)	151 (66.8%)	75 (33.2%)
7	Ceftazidime (n =226)	132 (58.4%)	94 (33.6%)
8	Cefepime (n =226)	117 (51.8%)	109 (48.2%)
9	Meropenem (n =226)	70 (31.0%)	156 (69.0%)
10	Imipenem (n =226)	75 (33.2%)	151 (66.8%)
11	Ertapenem (n =145)	51 (35.17%)	94 (64.83%)
12	Piperacillin/Tazobactam (n =226)	98 (43.40%)	128 (56.60%)
13	Aztreonam (n =226)	108 (47.8%)	118 (52.2%)
14	Nitrofurantoin (n =119)	26 (21.85%)	93 (78.15%)
15	Colistin (n =41)	12 (29.27%)	29 (70.63%)
16	Polymyxin B (n =41)	8 (19.51%)	33 (80.49%)

114

115 ***Multidrug resistance (MDR) and MHT positivity among the isolates:***

116 Out of 226 isolates, 106 isolates were found to be MDR. Highest MDR was seen in *C. freundii*
117 isolates (100.0%) followed by *P. aeruginosa* (53.7%), *A. baumannii* (50.0%), *C. koseri* (50.0%),
118 *E. coli* (n = 33, 45.8%), *E. cloacae* (44.4%), *K. oxytoca* (41.7%), *K. pneumonia* (40.0%) and *P.*
119 *mirabilis* (22.2%). (Table 2). Out of the 226 isolates, 97 (42.9%) showed resistance against at
120 least one of the carbapenem used. MHT was performed on all of these 97 isolates out of which
121 67 (69.1%) showed MHT positive results. MHT positivity was predominant in *Citrobacter* spp.
122 (50.0%) and least in *Proteus* spp. (7.14%) (Table 2). Among the 97 isolates that showed
123 resistance to at least one of the carbapenem antibiotic used, 88 (90.7%) were MDR whereas 9
124 (9.3%) isolates were non-MDR (Table 3). Among the MHT positive isolates, 63 isolates were

MDR and 4 isolates were non-MDR (Table 3). In this study, there was a statistically significant relation between MDR and MHT positivity ($p < 0.001$).

bla-VIM and bla-IMP among the isolates:

Among the 67 MHT positive isolates, 40 (59.7%) isolates were found to be harbouring the bla-VIM gene. Out of them, 30.0% (12/40) were *E. coli*, 20% (8/40) were *P. aeruginosa*, 15.0% (6/40) were *Klebsiella* spp., 15.0 (6/40) were *A. baumannii* (50%), 10.0% (4/40) were *Citrobacter* spp. and 5.0% (2/40) were *E. cloacae*. Similarly, 38 (57.7%) isolates were found to be harbouring the bla-IMP gene. Out of them 31.6% (12/38) were *E. coli*, 18.4 (7/38) were *Klebsiella* spp., 18.4% (7/38) were *A. baumannii*, 15.8% (6/38) were *P. aeruginosa*, 10.5% (4/38) were *Citrobacter* spp. and 2.6% (1/38) were *E. cloacae* (33.33%) (Table 2). Out of the 67 isolates, 19 isolates showed the presence of both genes, however, 8 isolates didn't show the presence of either bla-VIM or bla-IMP gene (Table 4).

Table 2: Distribution of MDR, bla-VIM, and bla-IMP among the isolates

Organisms	MDR	MHT positive	bla-VIM	bla-IMP
<i>A. baumannii</i> (n=40)	20	12	6	7
<i>C. freundii</i> (n=8)	8	4	3	3
<i>C. koseri</i> (n=4)	2	2	1	1
<i>E. cloacae</i> (n=9)	4	3	2	1
<i>E. coli</i> (n = 72)	33	22	12	12
<i>K. oxytoca</i> (n=12)	5	3	2	2
<i>K. pneumoniae</i> (n=25)	10	8	4	5
<i>P. mirabilis</i> (n=9)	2	1	0	1
<i>P. vulgaris</i> (n=5)	0	0	0	0
<i>P. aeruginosa</i> (n=41)	22	12	8	6
<i>S. typhii</i> (n=1)	0	0	0	0
Total (n=226)	106	67	40	38

Table 3: MDR and MHT positivity among carbapenem resistant isolates (n=97)

MDR	MHT		Total
	Negative	positive	
MDR	25 (28.4%)	63 (71.6%)	88
non-MDR	5 (55.5%)	4 (44.5%)	9
Total	30 (30.9%)	67 (69.1%)	97

Table 4: Relation between MHT, MDR and carbapenemase gene

MHT	MDR	Carbapenemase gene	
		Positive	Negative
MHT positive (n = 67)	MDR (n = 63)	59	4
	non-MDR (n = 4)	0	4

Discussion

Carbapenems are β -lactam antibiotics having a broad-spectrum activity and are used for the treatment of infections known or suspected to be caused by MDR bacteria (Meletis 2016). The emergence and rapid spread of carbapenemases in *Enterobacteriaceae*, *Pseudomonas* and *Acinetobacter* (EPA) species is becoming a major public health crisis worldwide [2]. Studies have shown an increasing trend of carbapenem resistance over the past decade [3].

In this study, a total of 1,151 samples were processed out of which 253 (22.0%) samples were found to be culture positive. A slightly higher rate of growth positivity (27.4%) in the clinical samples was reported by Karn et al [21]. The prevalence of Gram-negative isolates in this study was 19.6% (n=226), while the one reported by Karn et al [21] was 23.7%. In this study, the major isolates was *E. coli* followed by *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae*. Karn et al [21] reported a much higher prevalence of *E. coli* (81.0%) among the Gram-negative isolates followed by *K. pneumoniae*, *A. baumannii* and *C. freundii* being the major isolates. In another similar type of study, Binod et al [25] reported 18.0% culture positivity which included 44.0% culture positive for *E. coli* followed by *K. pneumoniae* (16.5%) and *Acinetobacter* (8.0%). High

prevalence of *E. coli* in these clinical specimens was not surprising as it is a normal flora of the human body, and is highly opportunistic in immunocompromised patients [26].

Two-third of the isolates showed resistance to cefoxitin whereas nearly one-fifth showed resistance to polymyxin B. Carbapenems were found to be the most effective antibiotics against gram-negative organisms followed by amikacin. A total of 38.4% of Enterobacteriaceae isolates were found to be resistant to at least one of the carbapenem antibiotics used. Among the Enterobacteriaceae isolates, the resistance against carbapenem antibiotics was found to be 32.41% (imipenem), 28.96% (meropenem) and 35.17% (ertapenem). Slightly lower rates of resistance in Enterobacteriaceae have been reported worldwide [27, 28, 29]. Gupta et al [23] reported 22.16% resistance to meropenem and 17.3% to imipenem. Karn et al [21] reported the prevalence of carbapenem resistance among the bacteria belonging to Enterobacteriaceae ranging from 4.5% to 20.0%. Similarly, Gupta et al [23] reported the prevalence of carbapenem resistance among Enterobacteriaceae to be varying from 17% to 22%. In this study, 67.5% *A. baumannii* isolates and 70.7% *P. aeruginosa* isolates were resistant to carbapenem antibiotics. For *A. baumannii*, 0-100% resistance has been reported in South Asian region, 32.0-36.5% in North America and 58.1-60.1% in Europe [30]. For *P. aeruginosa*, 17.0-50.0% resistance rates have been reported in Asia-Pacific region [27], 0%–35.6% in Europe [28] and 10.3-19.4% in North America [29].

In this study, two-fifth of isolates were found to be MDR. This was similar to multidrug-resistance rate reported by [26, 31]. In this study, 44.1% of the members of Enterobacteriaceae were found to be MDR among which the highest MDR rate was seen in *Citrobacter* spp. (83.3%) followed by *E. coli* (45.8%) and others. In a similar study done by Binod et al [25], 53.3% of total Enterobacteriaceae were recorded to be MDR. The highest MDR however, was seen in *E. coli* (61.7%) and *K. pneumoniae* (60.0%). Binod et al [25] reported MDR in 58.8% *A. baumannii* and 46.6% in *P. aeruginosa* which was also similar to the findings of this study. Similarly, Awasthi et al [31] reported 48.1% of MDR in *E. coli* isolates, 19.0% of *K.*

pneumoniae, and 50.0% of *P. aeruginosa*. This study indicated a slight increase in the level of MDR bacteria in Nepal.

Out of the total 97 organisms showing resistance to at least one of the carbapenem antibiotic used, 69.1% isolates were found to be MHT positive. The highest MHT positivity was observed in *Citrobacter* spp. (50.0%). A similar rate of MHT positivity was reported by Amjad et al [9]. A slightly higher MHT positive rate (73.2%) has been reported by Gheitani and Fazeli [32]. The bla-VIM was detected in 59.7% and bla-IMP was detected 56.7% of the total MHT positive isolates. Satir et al [18] from Sudan have also reported similar findings (56.6% for bla-VIM and 50.6% for bla-IMP genes). However, these findings were higher than those reported by Adam and Wafa [19]; 38.9% for bla-VIM gene and 26.4% for bla-IMP gene. Kazeminezhad et al [20] reported much lower rate of bla-VIM (5.8%) and bla-IMP (1.9%) gene. These differences in the prevalence of the carbapenemase genes might be because of the difference in distribution and dissemination of the carbapenemase genes in the different geographical regions and the pattern of antibiotics used. This study showed a high prevalence of carbapenemase genes in the clinical isolates.

Only MHT was used as the phenotypic method for the detection of carbapenemase activity in this study. Although the MHT is inexpensive, relatively straightforward to perform, and uses readily available reagents [33], it has various limitations like requirement of an overnight incubation, difficulty in interpretation, low specificity (approximately 91%) [10] and chances of false positivity in isolates producing ESBLs or AmpC cephalosporins in conjunction with porin mutations [34, 35]. Genotypic detection of carbapenemase gene is gold standard, although it only detects a prespecified set of known carbapenemase genes and seems less suitable for epidemiology of ESBL genes [36]. Furthermore, other disadvantages like the high cost, the requirement for trained technicians, and the inability to detect novel carbapenemase genes [36] limits the use of the molecular-based technologies for detection of carbapenemase gene especially in low income countries like Nepal. Hence, the combination of phenotypic tests like MHT and genotypic detection by PCR can be a rapid and cost effective solution for screening

and detection of carbapenemase producers, developing antibiotic strategies and preventing the dissemination of the MDR pathogens [8, 9].

Conclusion

Different genes are responsible for carbapenemase activity in the bacteria. Among them, the bla-VIM and bla-IMP genes are the major ones. This study indicated a slight increase in the level of MDR bacteria in Nepal. The prevalence of carbapenemase genes (bla-VIM and bla-IMP) was also found to be high. Therefore, it is recommended to do the study to monitor the increase in the drug resistance.

Methods

Study design and Ethical Approval

A cross-sectional study was conducted during a period of six months from April 2019 to September 2019. The samples size was calculated using Fischer's formula. All patients (inpatients and outpatients) attending of Annapurna Neurological Institute of Health and Allied Sciences who submitted a properly collected and labeled clinical specimens were included and the The samples included in this study were urine, stool, pus, blood, CSF, vaginal swab, wound swab, and catheter tip.

Isolation and identification of the bacterial of isolates

A total of 1,151 samples were collected and were processed (cultured) following the standard microbiological techniques [37]. Briefly, the specimens were cultured on nutrient agar, brain heart infusion (BHI) broth (blood samples), MacConkey agar and blood agar. The isolates were identified based on colony morphology, Gram's stain result and conventional biochemical methods [38]. Only the isolates belonging to Enterobacteriaceae, *P. aeruginosa* and *A. baumannii* were further processed for antibiotic sensitivity testing (AST), MHT, and molecular typing.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) was done employing the Kirby-Bauer disk diffusion technique [39] The antibiotics used included amikacin (30µg), piperacillin/tazobactam

(100/10µg), nitrofurantoin (300µg), ciprofloxacin (5µg), azithromycin (15µg), ceftazidime (30 µg), aztreonam (30ug), amoxicillin (20ug), imipenem (10µg), meropenem (10µg), ertapenem (10ug), cotrimoxazole, cefoxitin (30ug), polymyxin B (300 units), colistin (10µg) and cefepime (30µg). The bacterial isolates showing resistance towards three or more different classes of antibiotics were reported as multidrug-resistant (MDR). *Escherichia coli* (ATCC 25922) was used as the control of the antimicrobial susceptibility testing and phenotypic confirmation of carbapenemase production by MHT.

Molecular characterization of bla-VIM and bla-IMP producers

All the MHT positive isolates were preserved in tryptic soy broth (TSB) containing 40% glycerol and stored at -20°C until used. DNA extracted by phenol-chloroform extraction technique using sodium dodecyl sulfate (SDS) and proteinase-K. A mixture of phenol:chloroform:isoamyl alcohol (25:24:1) was added leaving the isolated DNA in the aqueous phase. Following centrifugation, the aqueous phase containing the purified DNA was transferred to a clean tube for analysis. DNA was recovered and concentrated from the aqueous phase by ethanol precipitation. DNA extracts were resuspended in Tris-EDTA (10mM Tris-HCL, 0.10 mM EDTA, (pH 8.0) buffer and stored at 4°C for further analysis. Then, the DNA extracts were amplified by multiplex PCR. Final volume (25µl) of PCR mixture consisted of master mix (12.5µl), forward primer (10 picomolar) (0.5µl), backward primer (10 picomolar) (0.5µl), DNA template (4µl) and ddH₂O (7.5µl) was prepared. The master mix solution used for PCR was manufactured by Thermo Fisher Scientific (V.A. Graiciuno g.8 Vilnius, LT-02241 Lithuania). The primers used were: VIM-F, 5'-GATGGTGTTTGGTCGCATA-3'; VIM-R, 5'-CGAATGCGCAGCACCAG-3' and IMP-F, 5'- GGAATAGAGTGGCTTAAYTC; IMP-R, 5'-GGTTTAAYAAAACAACAACC-3'. The PCR was done as described by Poirel *et al* (2011) using PCR machine (Proflex, Thermo Fisher, USA) [40] (initial denaturation at 94°C for 5 minutes, denaturation at 94°C for 45 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 45 seconds with a final extension at 72°C for 10 minutes at the end of 35 cycles, followed by maintenance at 4°C). The amplified PCR products were separated by agarose gel

265 electrophoresis (1.5%) in 1x TAE buffer (0.04 Tris-acetate. 0.001 M EDTA, pH 8.0), stained
266 with ethidium bromide and visualized by using gel-doc system.

267 ***Data analysis***

268 SPSS v16.0 was used for statistical analysis. Chi-square test was applied and $p < 0.05$ was taken
269 as statistically significant.

270

271 **List of abbreviations**

272 VIM : Verona Imipenemase

273 IMP : Imipenemase

274 AST : Antimicrobial Susceptibility Testing

275 MHT : Modified Hodge Test

276 DNA : Deoxyribonucleic acid

277 PCR : Polymerase Chain Reaction

278 MDR : Multidrug resistant

279 CRE : Carbapenem-resistant Enterobacteriaceae

280 CRAB : Carbapenem-resistant *Acinetobacter baumannii*

281 CRPsA: Carbapenem-resistant *Pseudomonas aeruginosa*

282 WHO : World Health Organization

283 OXA : Oxacillinase

284 IRC : Institutional Review Committee

285 BHI : Brain Heart Infusion

286 TSB : Tryptic Soy Broth

287 SDS : Sodium dodecyl sulphate

288 EDTA : Ethylenediaminetetraacetic acid

289 TAE : Tris-acetate-EDTA

290

291 **Declarations**

292 **Ethical approval and consent to participate**

The ethical consideration was obtained from Research Review Committee of National Institute of Tropical Medicine and Public Health Research (NITMPHR) centre, Research and Ethical committee, Shi-Gan and NITMPHR Health Foundation Maharajgunj, Kathmandu, Nepal (IRC Reference No: 04/2071/08/26). Written informed consent was obtained from all the participants prior to sample collection.

All methods were carried out in accordance with relevant guidelines and regulations.

Consent for publication:

Not applicable

Availability of data and materials

The datasets the support the findings of this study are available from Annapurna Research Centre but restrictions apply to the availability of these data, which were used under license for the current study and so are not publicly available. However, the original and detailed study file from which the manuscript have been prepared is included in supplementary file folder. Any further information required will be available from the corresponding author on reasonable request and with permission of Annapurna Research Centre, Kathmandu, Nepal.

Form of the material: .pdf

Location of the material: Supplementary file repository

Competing interests

The authors declared no conflicts of interest to the research, authorship, and publication of this article.

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Author's Contribution

Ansari M designed performed experiments and wrote the manuscript. **Aryal SC** helped in the experiments. **Rai G** and **Shah AK** revised and approved the work plan and experimental design, supervised the overall work, and helped in data analysis. **Rai KR** revised the manuscript and

helped in data analysis. **Rai SK** revised the work plan, experimental design, and, manuscript. All authors read and reviewed the manuscript.

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Limitations of the study

This study was performed in a resource limited country. Only MHT was used as the method for phenotypic detection of carbapenemase activity. The inclusion of other methods too would have increased the specificity and sensitivity of the study. Furthermore, only bla-VIM and bla-IMP genes were detected in this study although, many other genes are responsible for the carbapenemase activity.

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Authors' information

Mehraj Ansari

ShiGan International College of Science and Technology Kathmandu, Nepal

Email: mrseeyon@gmail.com

Kul Raj Rai

ShiGan International College of Science and Technology, Kathmandu, Nepal

Institute of Microbiology, Chinese Academy of Sciences (CAS), Beijing, China

Email: kulrajrai701@gmail.com

Ganesh Rai

PhD Scholar (TU)

ShiGan International College of Science and Technology (SICOST), Kathmandu, Nepal

Email: ganesh.fg@gmail.com

Subhas Chandra Aryal

Golden Gate International College, Kathmandu, Nepal

Email: subasaryal98@gmail.com

Anil Kumar Shah

Research Director

Annapurna Neurological Institute and Allied Sciences (Annapurna Research Centre),
Kathmandu, Nepal

Email: anilshah4in@gmail.com

Prof. Dr. Shiba Kumar Rai

Shi-Gan International College of Science and Technology (SICOST), Kathmandu, Nepal

Nepal Medical College, Kathmandu, Nepal

Email: drshibakrai.nepal@gmail.com

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