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Detection of *bla*$_{oxa-23}$ Gene from Carbapenem-resistant *Acinetobacter Baumannii*

Laxmi Neupane,[1] Anil Kumar Sah,[2] Binod Rayamajhee,[3,4] Anil Pokhrel,[1] Anjana Singh[1]

[1]Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal, [2]Annapurna Research Center, Maitighar, Kathmandu, Nepal, [3]Department of Infection and Immunology, Kathmandu Research Institute for Biological Sciences (KRIBS), Lalitpur, Nepal, [4]School of Optometry and Vision Science, Faculty of Medicine and Health, UNSW, Sydney, Australia.

ABSTRACT

Background: Antibiotic resistance is a great concern for public health and *Acinetobacter baumannii*-associated infections are increasing in many parts of the world, including Nepal. However, limited data is available on the prevalence of *A. baumannii* harboring *blaOXA*-23 from Nepal.

Methods: A hospital-based cross-sectional study was designed to detect the *blaOXA-23* gene from carbapenem-resistant *A. baumannii* isolates in Nepal. A total of 380 clinical specimens were collected and processed following standard microbiological procedures. Antibiotic susceptibility test was performed as per the protocol of the Kirby-Bauer disk diffusion technique and the CLSI guidelines, while screening of carbapenemase production was assessed by the Modified Hodge Test using meropenem (10µg) disc. The presence of the *blaOXA-23* gene in carbapenemase-positive *A. baumannii* was confirmed by PCR.

Results: Among 380 specimens analyzed, 210 (55.3%) samples were positive for bacterial growth, where 33(15.7% of total growth) of the isolates were *A. baumannii,* and most of them were isolated from the ICU patients (20/33, 60.6%) and sputum (16/33, 48.5%). Thirty-two isolates (97%) were colistin sensitive, while only four (12.1%) isolates were sensitive to meropenem and imipenem. Twenty-three (69.7%) of *A. baumannii* were carbapenemase positive as revealed by the Modified Hodge Test test, and 19 of them (57.6% of total *A. baumannii*) harbored the *blaOXA*-23 gene.

Conclusions: A high prevalence of the *blaOXA*-23 gene among carbapenem-resistant *A. baumannii* isolates were found. Systematic network surveillance should be established to check the spread of such isolates, especially in the intensive care units of tertiary care hospitals in Nepal.

Keywords: *Acinetobacter baumannii*; antibiotic-resistant; *bla*$_{OXA-23}$; carbapenemase; Nepal

INTRODUCTION

Antibiotics of the carbapenem group are considered a reserve drug for the treatment of multidrug-resistant *Acinetobacter baumannii*-caused infections, but carbapenem-resistant strains of *A. baumannii* have been reported from multiple places.[1] *A. baumannii* can develop carbapenem resistance through a variety of mechanisms, but the main one is antibiotic hydrolysis by bacterial enzymes, particularly the carbapenem-hydrolyzing -lactamases group of enzymes such as oxacillinases.[2-4]we developed a loop-mediated isothermal amplification (LAMP The increased distribution of the antibiotic resistance *blaOXA*-23-like gene has increased the hospital stay of patients with severe illness, resulting in significant health costs. *A. baumannii* is often the most prevalent bacterial pathogen isolated from hospital settings in Nepal, and a high number of *A. baumannii* are isolated during late summer and early winter. [5]Despite frequent antibiotic resistance cases being reported in Nepal, there is very limited data on the prevalence of *blaOXA*-23-carrying *A. baumannii*. Furthermore, very few researchers have reported the use of the modified Hodge test (MHT) as a screening test for carbapenemase-resistant *A. baumannii*, so this study was designed to determine the antibiotic susceptibility patterns and report the data on the *blaOXA*-23 gene among the carbapenem-resistant *A. baumannii* isolates

Correspondence: Anjana Singh, Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal. Email: anjanas67@gmail.com.

screened by MHT from a tertiary care hospital in central Nepal.

METHODS

This hospital-based cross-sectional study was conducted at Annapurna Neurological Institute and Allied Science (ANIAS) Hospital and Annapurna Research Centre (ARC), Kathmandu, Nepal over six months from February to August 2018.

A total of 380 different clinical specimens, including urine, blood, sputum, catheter tips, cerebrospinal fluid (CSF), tracheal aspirates, and central venous catheter (CVP) tips, were collected from the general ward, post-operative ward, and intensive care unit (ICU) of the hospital. A pre-formed questionnaire was used to record the patient's clinical and demographic data.

Each specimen was separately collected in a sterile container and was processed and analyzed in the hospital laboratory. Cerebrospinal fluid, urine, tracheal aspirates, sputum, and other samples were inoculated on MacConkey agar (MA) and Blood agar (BA) plates and incubated for 24 hours at 37°C. Blood specimens were incubated aerobically for at least 7 days at 37 °C in BHI (Brain Heart Infusion) broth. Each day, one loopful of the inoculated broth was inoculated into MA and BA plates, which were incubated at 37°C overnight. All the inoculation and media preparation work were strictly performed under the laminar flow cabinet to prevent contamination.

A. baumannii was identified through a series of biochemical tests such as positive catalase and citrate tests, negative oxidase and urease tests, non-motile, indole negative, oxidative in Hugh and Leifson's medium, negative gelatin hydrolysis test, acid production from glucose, lactose, xylose, galactose, mannose but not from sucrose and mannitol, and the ability to grow at both 37°C and 44°C, alkaline slant/alkaline butt i.e. glucose, lactose, and sucrose non-fermenter, H_2S, and gas negative in the triple sugar iron (TSI) test [6]

A. baumannii isolates were inoculated on separate Mueller–Hinton agar (MHA) plates and their susceptibilities to carbapenem were tested by the Kirby-Bauer disc diffusion method (Supplementary file Figure 4) according to the guidelines of the Clinical and Laboratory Standard Institutes (CLSI-M100-S25, 2015).[7] This study used 12 antibiotic discs of different classes for the antibiotic susceptibility test and three carbapenems (Ertapenem-10 µg, Meropenem-10 µg, Imipenem-10 µg) to check the carbapenemase resistance among the *A. baumannii* isolates. The minimum inhibitory concentration (MIC) of the tested antibiotics was not determined due to limited research funds and resources. After the AST, the confirmed carbapenem-resistant *A. baumannii* isolates in pure culture were preserved in 20% glycerol-containing Tryptic Soya broth and kept at -70°C until further processing.

A. baumannii isolates which have shown susceptible or intermediate zones on AST for imipenem disc (16–21 mm) were further tested by the MHT for phenotypic detection of carbapenemase production.[8]carbapenem-resistant isolates are emerging at an alarming rate. This study aimed at phenotypically and molecularly characterizing seventy four carbapenem-unsusceptible A. baumannii isolates from Egypt to detect the different enzymes responsible for carbapenem resistance. Methods: Carbapenemase production was assessed by a number of phenotypic methods: modified Hodge test (MHT For the MHT, a 0.5 McFarland dilution of *Escherichia coli* ATCC 25922 in 5 ml of broth was prepared and diluted by adding 0.5 ml of the preparation to 4.5 ml of saline. A lawn of the diluent was streaked on MHA and left to dry for 3-5 minutes. Then 10µg meropenem/ertapenem antibiotic disc was placed at the center of the plate. Then, *A. baumannii* isolates were streaked straight from one edge of the disc to the edge of the plate at 3 different places, keeping an equal gap between them, and the plates were incubated for 24 hours at 35°C in the presence of ambient air. After the incubation period, clover leaf-type depression at the intersection of *E. coli* 25922 and *A. baumannii* was considered MHT positive, while there was no growth of *E. coli* 25922 along the test isolates growth streak on the antibiotic disc diffusion area.[9]a carbapenem inactivation assay, has shown poor sensitivity in detecting the worldwide spread of New Delhi metallo-Β-lactamase (NDM

DNA was extracted by the alkaline hydrolysis method, in which the *A. baumannii* strain was cultured in LB (Luria Bertani) broth at 37 °C overnight, as described previously.[10] The amount of extracted DNA was examined by spectroscopy at 260 nm. A PCR reaction to identify the *blaOXA*-23 gene was performed using a specific primer pair (forward: 5′-GATCGGATTGGAGAACCAGA-3′, reverse: 5′-ATTTCTGACCGCATTTCCAT-3′).[11,12]an emerging pathogen, is less commonly reported from Nepal. In this study we determined the antibiotic susceptibility profile and genetic mechanism of carbapenem resistance in clinical isolates of A. baumannii. Methods: A. baumannii were isolated from various clinical specimens and identified based on Gram staining, biochemical tests, and PCR amplification of organism specific 16S rRNA and

bla OXA-51 genes. The antibiotic susceptibility testing was performed using disc diffusion and E-test method. Multiplex PCR assays were used to detect the following ß-lactamase genes: four class D carbapenem hydrolyzing oxacillinases (bla OXA-51, bla OXA-23, bla OXA-24 and bla OXA-58 The amplification was performed with an initial denaturation at 94 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 40 s, and a final extension at 72 °C for 10 minutes. PCR products were examined using 1% agarose gel electrophoresis containing 0.5 µg/ml ethidium bromide.[2]

All the results were entered into and analyzed with the Statistical Package of Social Sciences (IBM SPSS, USA V:16.0). The Chi-square test was used to determine the association of independent variables. A p-value of ≤0.05 was considered significant at a 95% confidence interval.

RESULTS

Among 380 analyzed specimens, 55.3% (210/380) showed aerobic bacterial growth, while 44.7% (170/380) of the specimens were negative for bacterial growth. Out of 210 culture-positive, 15.7% (33/210) were confirmed as *A. baumannii*. The majority of identified *A. baumannii* strains were isolated from sputum (48.48%, 16/33) specimens, while no *A. baumannii* isolates were reported from CVP tips or CSF specimens (Supplementary file **Table 1**). Most *A. baumannii* isolates were isolated from the ICU (60.6%, 20/33) (Supplementary file **Figure 2**). The highest prevalence of *A. baumannii* was found in male patients (19/33, 57.6%) and patients in the age group of 51–60 years (Supplementary file **Table 2**).

Table 1. Antibiotic susceptibility profile of *A. baumannii* against different classes of antibiotics.

Antibiotics	Concentration (µg)	Sensitivity		
		R	I	S
		N (%)	N (%)	N (%)
AMP	10	31 (94)	0	2 (6.1)
AK	30	33 (100)	0	0
AMC	30 (20/10)	30 (91)	1 (3)	2 (6.1)
CTX	30	32 (97)	1 (3)	0
CIP	5	30 (91)	1 (3)	2 (6.1)
COT	25 (23.75/1.25)	32 (97)	0	1 (3)
C	30	29 (87.9)	2 (6.1)	2 (6.1)
MRP	10	29 (87.9)	0	4 (12.1)
IMP	10	29 (87.9)	0	4 (12.1)
PIT	100/10	30 (91)	1 (3)	2 (6.1)
CPM	30	27 (81.9)	3	3 (9.1)
CL	10	1 (3)	0	32 (97)

Keys: S = Sensitive, R = Resistant, AMP = Ampicillin, AK = Amikacin, AMC = Amoxicillin/Clavulanic Acid, CTX = Cephotaxime, CIP = Ciprofloxacin, COT = trimethoprim + sulfamethoxazole (cotrimoxazole), C = Chloramphenicol, MRP = Meropenem, IMP = Imipenem, PIT = Piperacillin-Tazobactam, CPM = Cefepime, CL = Colistin (Methane Sulphonate)

Thirty-two of *A. baumannii* isolates were sensitive to colistin, while only 12.1% (4/33) of isolates were sensitive to meropenem and imipenem antibiotics. A high rate of resistance was seen against amikacin (100%), cefotaxime (97%), and cotrimoxazole (97%) (**Table 1**).

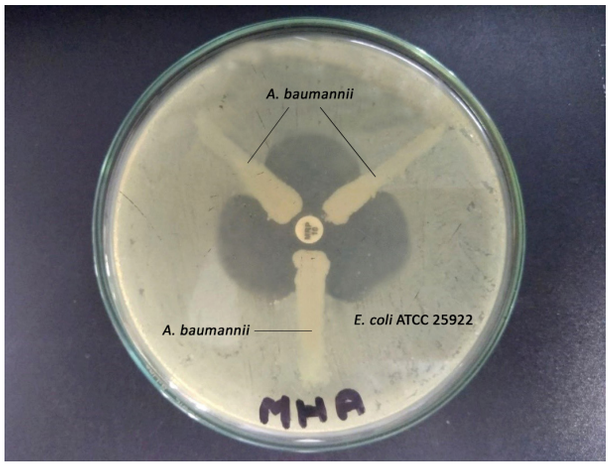

Figure 1. Positive Modified Hodge Test (MHT with Meropenem Disc, sample code: 2873).

While 29 isolates were positive for carbapenemase production by Kirby-Bauer disc diffusion (Supplementary file **Figure 3**), only 23 isolates were confirmed as carbapenemase producers by the Modified Hodge Test. Out of 29 carbapenem-resistant *A. baumannii* isolates by AST, significantly higher isolates were found to be MHT-positive (Table 2, Figure 1).

Table 2.Comparative evaluation of carbapenemase production by phenotypic tests

Type of phenotypic test	Total no. of isolates	Carbapenemase producers n (%)
AST by Kirby-Bauer	33	29 (87.9)
MHT	33	23 (69.8)
	Modified Hodge Test	

Carbapenemase	Positive (%)	Negative (%)	Total (%)	p value
R	23 (69.7)	6 (18.2)	29 (87.9)	0.001
S	0	4 (12.1)	4 (12.1)	
Total	23 (69.7)	10 (30.3)	33 (100)	

Key: S = Sensitive, R = Resistant, AST = Antibiotic Susceptibility Test, MHT = Modified Hodge Test

Of the 23 MHT-positive *A. baumannii*, 19 of them harbored the *blaOXA*-23 gene. This could imply that the MHT can be used to detect carbapenemase-producing *Acinetobacter baumannii*,[13]an increase in carbapenem resistance among A. baumannii due to Ambler class B metallo-betaß-lactamases or class D OXA carbapenamases has been reported. In this study we detected the presence of OXA carbapenamases and coproduction of metallo-betaß-lactamases (bla VIM and blaIMP because the presence of the carbapenemase gene cluster corresponds to the presence of the *blaOXA*-23 gene, [14] and MHT detected most of (19/23) carbapenemase- producing *A. baumannii*. Although the *blaOXA*-23 gene was found highest in the isolates from the sputum sample, there was no statistically significant association between the specimens' type and the presence of the *blaOXA*-23 gene (p>0.05) **(Table 3, Figure 2)**.

Table 3. Distribution of *bla*OXA-23 gene as per processed specimens and age of patients.

	*bla*OXA-23 gene			
MHT	**Positive (%)**	**Negative (%)**	**Total (%)**	**p value**
Positive	19 (57.6)	4 (12.1)	23 (69.7)	<0.05
Negative	0	10 (30.3)	10 (30.3)	
Total	**19 (57.6)**	**14 (42.4)**	**33 (100)**	
Age Group	**Total no. of isolates**	***bla*OXA-23 gene**		**p value**
		Positive	**Negative**	
10-20	5 (15.2)	1 (3.0)	4 (12.1)	0.657
20-30	5 (15.2)	3 (9.1)	2 (6.1)	
30-40	4 (12.1)	3 (9.1)	1 (3.0)	
40-50	6 (18.2)	4 (12.1)	2 (6.1)	
50-60	7 (21.2)	4 (12.1)	3 (9.1)	
60-70	4 (12.1)	3 (9.1)	1(3.0)	
70-80	2 (6.1)	1 (3.0)	1 (3.0)	
Total	**33 (100)**	**19 (57.6)**	**14 (42.4)**	

Type of specimens	*blaOXA-23* gene		Total	p value
	Positive	Negative		
Urine	2 (6.1)	1 (3.0)	3 (9.1)	0.310
Sputum	9 (27.3)	7 (21.2)	16 (48.5)	
Swab	4 (12.1)	4 (12.1)	8 (24.2)	
Blood	0	1 (3.0)	1 (3.0)	
Tracheal Aspirates	4 (12.1)	0	4 (12.1)	
Foleys Tips	0	1 (3.0)	1 (3.0)	
Total	**19 (57.6)**	**14 (42.4)**	**33 (100)**	

Keys: MHT = Modified Hodge Test

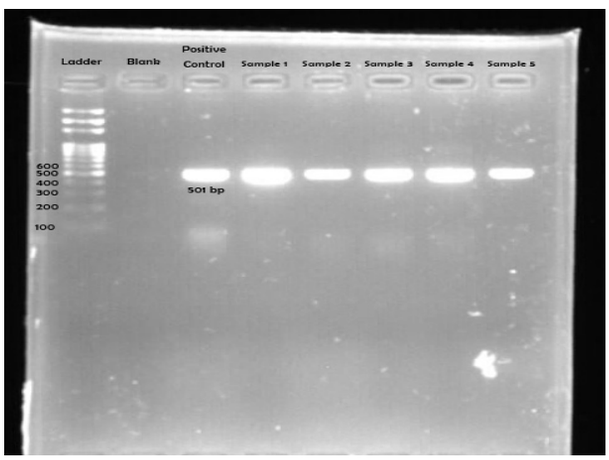

Figure 2. Gel electrophoresis of PCR amplicons of bla_{OXA-23} gene.

DISCUSSION

The emergence and spread of drug-resistant *A. baumannii* have been a great public health concern, which is highly associated with HAIs.[15] Identification of carbapenemases among multidrug-resistant (MDR) strains of *A. baumannii* (MDR-AB) is crucial in planning the therapeutic regimen for the clinician because carbapenemase-resistant *A. baumannii* (CRAB) limits the treatment options and can cause more deaths of infected patients. Owing to this, the WHO has designated CRAB as a global priority pathogen in the critical group, requiring immediate efforts to develop new antibiotics for CRAB treatment.[16]

Among 210 bacterial growth recorded from 380 non-duplicate clinical specimens analyzed in this study, 33(15.7%) of the isolates were confirmed as *A. baumannii*, this isolation rate is lower than that reported by Ghimire et al. (2021). [17] Earlier studies have also reported *A. baumannii* as a major cause of HAIs in different countries[18-20] and Nepal.[5,11] In our study, the highest

number of *A. baumannii* isolates was found in sputum, but there was no significant association (P = 0.310) between isolation and specimen type. In agreement with this finding, the highest isolates were reported from respiratory tract specimens including sputum [5,11,21,22] previously in Nepal. This study observed the higher prevalence of *A. baumannii* among ICU patients, suggesting the role of *A. baumannii* as a major cause of ventilator-associated pneumonia (VAP), especially among critically ill patients, and recent studies show the mortality rate of hospital patients from MDR-AB ranged from 52–66%, and the high rate of MDR-AB transmission among ICU patients is very common, mainly in resource-limited settings.[5,23] Contrary to the results of this study, some have reported a higher rate of MDR-AB in females and adults in Nepal.[5]

This study found a high rate of resistance to amikacin (100%) and cefotaxime (97%) by disk diffusion technique, which suggests amikacin and cefotaxime might no longer be effective for the treatment of *A. baumannii*-associated infections, while 97% of isolates were sensitive to colistin, so colistin could be the drug of choice for the treatment of *A. baumannii* caused infections in Nepalese patients which is also supported by earlier studies from Nepal.[5,11] However, a lower rate of amikacin resistance (86.3%) was reported by Yadav et al. (2020),[21] where *A. baumannii* isolates were 100% sensitive to colistin.[21] The presence of extended-spectrum ß-lactamase (ESBL) and AmpC-lactamase are often associated with the low rate of *A. baumannii* susceptibility to the third and fourth generations of cephalosporin antibiotics.[24,25] In essence, OXA-type carbapenemases are common in *A. baumannii*, and the acquired *blaOXA*-23 gene is the main genetic element in Asian countries. A plasmid carrying the *blaOXA*-23 gene can be transported within the strains of *A. baumannii* via conjunction so that MDR and CRAB are speedily growing globally.[26] Other OXA-type genes are more common in isolates of *A. baumannii* from the European and American regions.[27-29] In agreement with the finding of Raut et al. (2020),[5] this study also documented a high resistance to ciprofloxacin, which could be attributed to absurd use of antibiotics[5] and resistance transfer mediated by R-plasmid.[30]

The MHT was used as a phenotypic test for the detection of carbapenemase enzyme among isolated *A. baumannii* and has shown that 23 isolates were positive for the MHT test, while AST has screened 29 isolates as carbapenem-resistant. PCR analysis confirmed that all MHT-positive isolates were positive for the *blaOXA*-23 gene. As supported by earlier studies,[8,11] PCR was convincing in assessing carbapenemase production. The most common mechanisms that play a positive role in carbapenem resistance are the production of carbapenem-hydrolyzing class D -lactamase (CHDLs) and metallo--lactamase (MBL).[31] As per CLSI 2018 guidelines, MHT is not recommended for carbapenemase detection as a phenotypic test, which could be due to the poor specificity of the MHT when confirming some ESBL production happening with porin loss.[32] Nevertheless, 82.6% of MHT specificity in our study could be due to the detection of only one carbapenemase gene, *blaOXA*-23, by PCR. Nevertheless, some argue that MHT can be effective in screening the CRAB. [13]

We cannot reveal the exact prevalence of CRAB harboring the *blaOXA*-23 gene without conducting a nationwide study with a large study population along with a collection of data on possible risk factors. Due to time and fund limitations, 16s rRNA sequencing was not done in this study. To generalize the results, more studies at the molecular level with a longer study duration and a larger number of participants from different parts of the country are required.

CONCLUSIONS

This study reveals the high prevalence of the *blaOXA*-23 gene among carbapenem-resistant *A. baumannii* in Nepalese patients, which suggests an urgent need to plan the control and treatment strategies for CRAB-infected patients in our hospital setting. Systematic network surveillance and organized infection control strategies should be established for monitoring drug resistance patterns and controlling the surplus use of broad-spectrum antibiotics to win the race against drug-resistant *A. baumannii*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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