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Multidrug-Resistance and Biofilm Formation among Acinetobacter baumannii Isolated from Clinical Specimens

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ABSTRACT

Background: *Acinetobacter baumannii* has emerged as a problematic pathogen due to its ability to become resistant to antibiotics and form biofilms. The aim of this study was to explore antibiotic resistance and biofilm formation, and examine any correlation between these in *Acinetobacter baumannii* isolates.

Methods: This was a cross-sectional study conducted at the 750-bed Tribhuvan University Teaching Hospital in Nepal. Identification and antibiotic sensitivity of *Acinetobacter baumannii* isolates were performed following American Society for Microbiology guidelines. Different β-lactamases were detected by standard phenotypic tests. The microtiter plate method was used to screen strains of their ability to form biofilms.

Results: Out of total 18,343 clinical samples processed, 4,249 (23.1%) showed bacterial growth. *A. baumannii* comprised of 4.7% of the total bacterial growth. Multidrug-resistant (MDR) was exhibited by 97.5% of *Acinetobacter baumannii* isolates. All multidrug-resistant *Acinetobacter baumannii* isolates were resistant to cephalosporins and carbapenems; however, they were sensitive to polymyxins. Only few isolates showed sensitivity to sulbactam-containing antibiotics (15.4-29.2%), fluoroquinolones (1.0-7.2%), aminoglycosides (2.6-5.6%), and cotrimoxazole (4.1%). Extended-spectrum-beta-lactamase (ESBL), metallo-beta-lactamase (MBL), *Klebsiella pneumoniae* carbapenemase (KPC) and AmpC production were found in 54.9%, 73.3%, 41.5% and 14.9% isolates, respectively. Among all tested isolates, 192 were able to produce biofilms, with 83.1% being classified as strong biofilm producers. Those strains that were resistant to gentamicin were more likely to produce biofilms ($P<0.05$). ESBL, MBL, KPC and AmpC were seen in 51.8%, 71.6%, 43.8% and 16.0% of strong biofilm producers respectively.

Conclusions: Only polymyxins were effective against *Acinetobacter baumannii*. Carbapenemase producers were generally strong biofilm producers, and gentamicin resistant strains were more likely to produce biofilms. The findings of this study may help to understand antibiotic-resistance mechanisms and provide valuable information in the treatment of MDR *Acinetobacter baumannii* infections.

Keywords: Acinetobacter baumannii, biofilm, carbapenemase; multidrug-resistant.

INTRODUCTION

Acinetobacter baumannii are glucose-non-fermentative, non-motile, non-fastidious, catalase-positive, oxidase negative, aerobic gram-negative coccobacilli.[1][1] This bacterium is often associated with multidrug resistance. The World Health Organization (WHO) has classified carbapenem-resistant *A. baumannii* as one of its major "Priority 1: critical group" microbes[2] owing to their capacity to produce hard to treat nosocomial infections in admitted patients.[3] The mortality rate associated with *A. baumannii* ranges from 26% to 68%.[4]

A biofilm is a community of multiple bacterial cells associated with a surface and encased in an extracellular matrix that can be composed of carbohydrates, nucleic

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acids, proteins and other macromolecules.[5] Biofilm formation helps microbes to cause device-related infections.[6] These biofilms are also involved in resistance and tolerance to antibiotics.[7] The aim of the current study was to examine the relationship between antibiotic resistance and biofilm formation in the isolates of *A. baumannii*.

METHODS

This hospital based cross-sectional study was conducted in the Department of Microbiology, Tribhuvan University Teaching Hospital (TUTH), Kathmandu from March to December 2021. Different clinical specimens (blood, urine, pus, cerebrospinal fluid, endotracheal tube, tracheal aspirate, fluids, lesion swab, genital swab, catheter tips, and sputum) received from various inpatient departments of the hospital were processed according to the American Society for Microbiology (ASM) guidelines.[8] Specimens not fulfilling the ASM criteria were excluded.

The clinical specimens were inoculated onto suitable culture media (5% Human blood agar, MacConkey agar, Chocolate agar, Brain Heart Infusion broth- HiMedia, India) according to their specific requirements. Identification of isolates was performed following standard microbiological techniques. Assurance of pure culture inoculum was done by setting purity plate along with the biochemical tests.

After identification of *A. baumannii* isolates, their susceptibility to different antibiotics was determined by the Kirby–Bauer disk diffusion method on Mueller-Hinton agar and interpreted following standard procedures recommended by the Clinical and Laboratory Standards Institute (CLSI), USA.[9] For disk susceptibility testing ampicillin-sulbactam (10/10 µg), ceftazidime (30 µg), cefepime (30 µg), piperacillin-tazobactam (100/10 µg), cefoperazone-sulbactam (75/30 µg), doxycycline (30 µg), imipenem (10 µg), meropenem (10 µg), gentamicin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), trimethoprim-sulfamethoxazole (co-trimoxazole) (25 µg), polymyxin B (300 unit), and colistin sulphate (10 µg) from HiMedia,, India were used. The control strains used were strains of *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, for which their susceptibilities were known. Isolates resistant to at least one antibiotic from three different groups of first line drugs tested were regarded as multidrug-resistant (MDR), and extensively drug-resistant (XDR) was defined as resistant to at least one agent in all antimicrobial categories but bacterial isolates remain sensitive to only one or two categories.[10]

Ceftazidime resistant isolates were subjected to extended-spectrum ß-lactamase (ESBL) detection.[11] The test for ESBL production was performed by the combination disc method. This test used the disk diffusion method with two discs, one containing ceftazidime (30 µg) and the other with ceftazidime in combination with clavulanic acid (30/10 µg). After incubation of the plate at 37˚C for 24 hours, an increase in zone of inhibition (ZOI) > 5 mm around the disc containing clavulanic acid compared to disc of ceftazidime alone indicated ESBL production.

Meropenem resistant isolates were subjected to metallo-ß-lactamase (MBL) detection.[12] The test for MBL production was performed using a combination disc method. This test used the disk diffusion methods with two meropenem discs (10 µg), with one supplemented with 10 µl of 0.1 M (292 µg) anhydrous EDTA (Sigma Chemicals, St. Louis, MO). The plate was incubated at 37˚C for 24 hours. After incubation, an increase in ZOI > 5 mm around the EDTA disc compared to the meropenem disc alone indicated MBL production.

The test for *Klebsiella pneumoniae* carbapenemase (KPC) detection was performed using a combination disk method.[12] In this test, meropenem (10 µg) disks alone or containing 10 µl (300 µg/ml) 3-AminoPhenylBoronic Acid (3-APBA) (Tokyo Chemical Co. Ltd., Japan) were placed 20 mm apart on the agar plates. An increase in zone diameter > 5 mm around the meropenem-PBA disk compared to that of the meropenem disk alone indicated KPC production.

Screening for AmpC ß-lactamase production was carried out using cefoxitin disk diffusion.[13] Isolates that yielded a ZOI < 18 mm around the cefoxitin disk were further subjected to confirmatory testing. Cefoxitin (30 µg) disks and cefoxitin (30 µg) disks supplemented with 10 µl of 300 µg/ml Phenyl Boronic Acid (PBA) (Tokyo Chemical Co. Ltd., Japan) were placed at 20 mm distance apart on lawns of *A. baumannii*. An increase in zone of inhibition by at least 5 mm around the cefoxitin disk containing PBA after overnight incubation at 37 °C were considered as positive for AmpC production by the isolates.

Biofilm formation was detected as previously described.[14] Briefly, a 0.5 McFarland adjusted standard of the bacterial suspension was diluted 100 times in BHI broth with 1% glucose. Then, 200µl of the diluted bacterial suspension was placed into wells of microtiter plates. The test was run in triplicate. The negative controls were wells containing sterile BHI broth only. The microtiter plates were incubated in static conditions for 24 hours at 37°C. After incubation, the microtiter plate was vigorously washed in physiological saline three times to remove planktonic and

loosely adhered cells. The remaining adherent bacteria were fixed with 200µl 99% (v/v) methanol then emptied after 15 minutes and left to dry. Plates were stained with a 2% Hucker's crystal violet for 5 minutes and rinsed with tap water. After complete drying, 200µl of 33% glacial acetic acid was added to dissolve the crystal violet and the OD of the resulting solution was measured at 550nm using an automated ELISA reader (A.D.Touch, apDia$_{nv,}$Belgium).

Data processing and analysis

The data was finally entered in Excel 10 version as well as recorded manually. Then data was analyzed using SPSS 20. Chi-square test was applied to test the significance of the relation between categorical values, *P* value < 0.05 was considered statistically significant.

RESULTS

From a total of 18,343 specimens, 4,249 (23.1%) showed bacterial growth among which 200 (4.7%) were *A. baumannii*. Out of the total *A. baumannii* isolates, 195 (97.5%) were identified as MDR. The patients were most commonly of 50-60 years of age, followed by 30-50 years and above 60 years.

Among the 195 clinical isolates of MDR *A. baumannii*, most were from patients admitted to the intensive care units (Table 1). The majority of *A. baumannii* were obtained from the lower respiratory tract specimens (52.3%) (Figure 1).

Table 1. Distribution of MDR *A. baumannii* isolates from different wards.

Wards	Frequency	Percentage
Intensive Care Units	90	46.2
COVID ICU	18	9.2
Surgical	15	7.7
Orthopedic	15	7.7
Medicine	15	7.7
Post-Operative	11	5.6
Nephrology	10	5.1
Ear Nose Throat	5	2.6
Surgery	6	3.0
Maternity	3	1.5
Hemodialysis Unit	3	1.5
Eye	2	1.0
Burn	2	1.0
Total	195	100.0

Specimens	Frequency	Percentage
Sputum	75	38.5
Pus	40	20.5
Blood	17	8.7
Endotracheal aspirate	17	8.7
Body Fluid	14	7.2
Urine	9	4.6
Bronchoalveolar lavage	9	4.6
Central venous catheter line	8	4.1
Urinary catheter tip	5	2.6
Cerebrospinal fluid shunt	1	0.5

Figure 1. Heat map of distribution of MDR *Acinetobacter baumannii* in various clinical specimens.

Highest value
Percentile
Lowest value

Patients infected with MDR *A. baumannii* in the patients were having different underlying health conditions, viz., Chronic Obstructive Pulmonary Disease (COPD) (10.3%), COVID-19 infection (9.2%), hypertension (6.2%), diabetes (5.6%), hemorrhage (2.6%), pleural edema (2.6%), neurogenic tumor (2.1%), tracheostomy (1.5%), disseminated tuberculosis (1.0%), liver cirrhosis (1.0%), pyelonephritis (1.0%), renal transplant (0.5%), and use of mechanical ventilation (7.7%) and any catheter (6.2%).

MDR *A. baumannii* infections generally occurred in anatomical sites with a high fluid content and manifested as pneumonia, bacteremia, urinary tract infection, meningitis and wound infection (Figure 2).

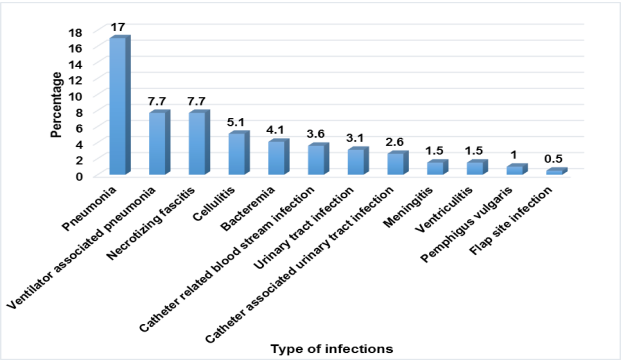

Figure 2. Frequency of infection caused by MDR *A. baumannii*

Among the 195 MDR *A. baumannii* isolates, 180 (92.3%) were XDR. Polymyxin B and colistin sulphate were the only antibiotics to which all the isolates were sensitive. (Figure 3).

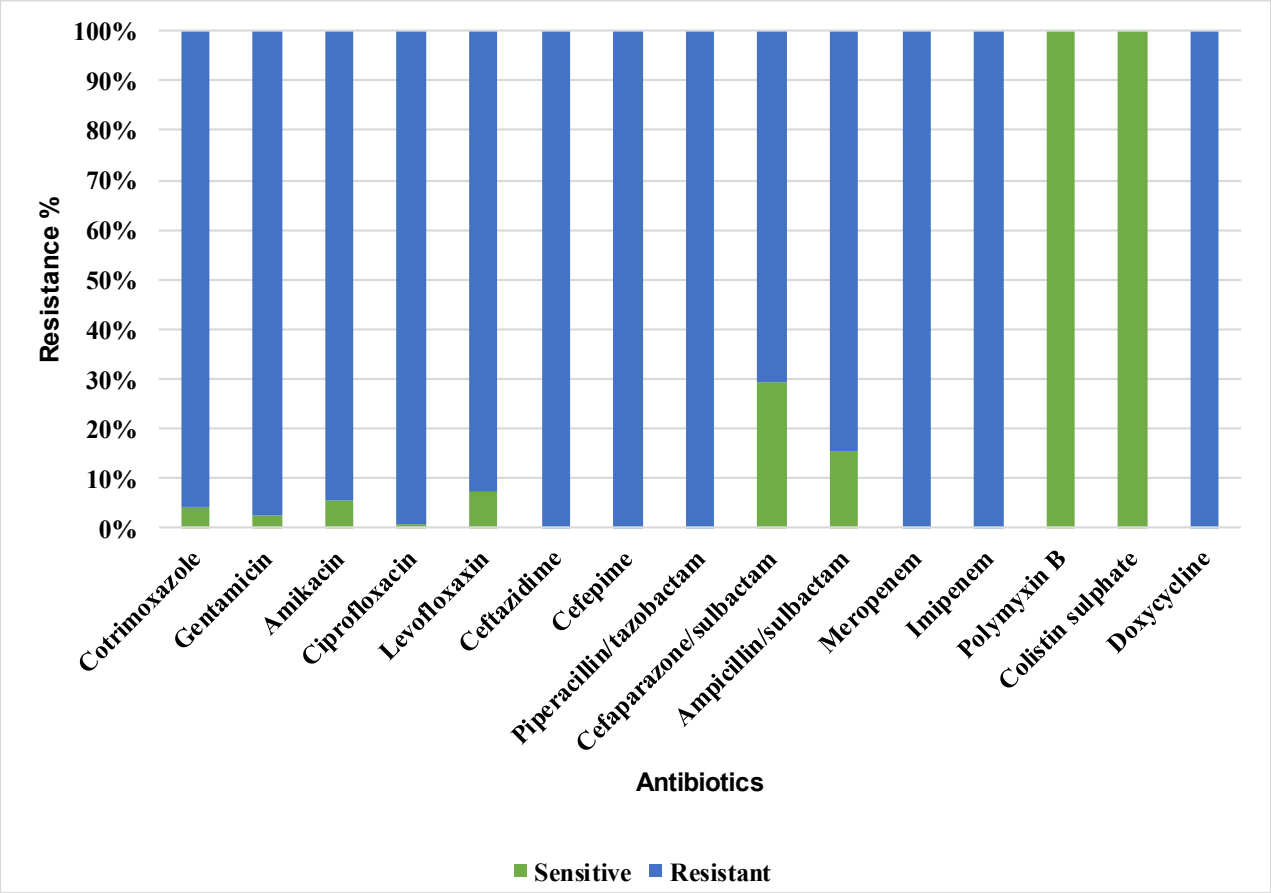

Figure 3. Percentage of antimicrobial resistance and sensitivity of MDR *A. baumannii* (N=195)

The rate of ß-lactamase producing MDR *A. baumannii* isolates was high. Over half the isolates produced MBL (73.3%) and ESBL (54.9%), followed by KPC (41.5%), then AmpC (14.9%).

Among all 195 MDR *A. baumannii* isolates, 98.5% (192) were biofilm producers. Out of these biofilm-producing strains, 83.1% were classified as strong biofilm producers, followed by 14.4% of moderate biofilm producers and 1.0% of weak producers.

The resistance rates to most antibiotics were similar in biofilm-forming and non-biofilm forming strains. When comparing non or weak biofilm producers to strong biofilm producers, the only difference was found for gentamicin (Table 2), where biofilm producers were more likely to be resistant to this antibiotic.

Table 2. Resistance pattern of biofilm producing and non-producing MDR *A. baumannii*.

Antibiotics	Biofilm		p-value
	Non+Weak producer (N=5)	Strong Producer (N=162)	
	Resistant	Resistant	
Cotrimoxazole	5 (100.0%)	156 (96.3%)	0.661
Ciprofloxacin	5 (100.0%)	160 (98.8%)	0.803
Levofloxacin	5(100.0%)	149 (92.0%)	0.509

Table 2. Resistance pattern of biofilm producing and non-producing MDR *A. baumannii*.			
Gentamicin	**4 (80.0%)**	**159 (98.1%)**	**0.009**
Amikacin	4 (80.0%)	153 (94.4%)	0.180
Ceftazidime	5 (100.0%)	162 (100.0%)	1.000
Meropenem	5 (100.0%)	162 (100.0%)	1.000
Imipenem	5 (100.0%)	162 (100.0%)	1.000
Cefepime	5 (100.0%)	162 (100.0%)	1.000
Piperacillin-tazobactam	5 (100.0%)	162 (100.0%)	1.000
Cefoperazone-sulbactam	4 (80.0%)	113 (69.7%)	0.622
Ampicillin-sulbactam	4 (80.0%)	134 (82.7%)	0.875
Polymyxin B	(0.0%)	0 (0.0%)	1.000
Colistin sulphate	(0.0%)	0 (0.0%)	1.000
Doxycycline	5 (100.0%)	162 (100.0%)	1.000

Among the total isolates, 51.8%, 71.6%, 43.8% and 16.0% of ESBL, MBL, KPC and AmpC ß-lactamase producers were biofilm producer respectively. There was no statistically significant difference ($p< 0.05$) between producer and biofilm types as shown in Table 3.

Table 3. Relation between biofilm production and drug resistance by different beta-lactamases.				
Biofilm types	**ESBL producer**	**MBL producer**	**KPC producer**	**AmpC producer**
Non+Weak producer (N=5)	3 (60.0%)	4 (80.0%)	2 (40.0%)	1(20.0%)
Strong producer (N=162)	84 (51.8%)	116 (71.6%)	71(43.8%)	26 (16.0%)
***p*-value**	0.719	0.681	0.865	0.813

DISCUSSION

In this study, *A. baumannii* were isolated and identified from various clinical specimen, from hospitalized patients, their antimicrobial susceptibility pattern along with ESBL, MBL, KPC and AmpC producers and also their relation with biofilm production were determined.

This study revealed, out of total 195 MDR *A. baumannii*, most were of respiratory origin. An extremely high rate of MDR and XDR *A. baumannii* was found in the current study complying with the results of previous studies done in 2013 and 2020.[15, 16] These high prevalence rates of MDR *A. baumannii* shows real burden of these pathogens in the hospital setting.

The high levels of resistance of *A. baumannii* to cephalosporins, was similar to previous studies at the same hospital in Nepal.[15, 17] This indicates that, at least in this hospital, cephalosporins should not be prescribed to treat *A. baumannii* infections empirically. This higher cephalosporin resistance among these organisms might be due to production of ESBL. Indeed, 54.9% of isolates produced ESBL, which is similar to studies from Lebanon, Turkey, the Middle East, Africa and India.[18-20] However, previous studies from 2013 and 2020 from the same hospital in Nepal found only 19.9% and 12.9% of *Acinetobacter* spp. were ESBL producers.[15, 16] This increase may be ascribed to antibiotic prescribing habits and the presence of other pathogens harboring the genes for ESBL production and those genes being transferred to *A. baumannii*. In the present study, the prevalence of AmpC producing MDR *A. baumannii* was 14.9% which is lower than reports from 2017 and 2020 from Nepal[15, 21] or India[22], but higher than a study from Iran.[18]

In the current study 97.5% of isolates were resistant to carbapenems. This high rate of resistance to carbapenems is similar to studies from Nepal reported in 2017.[15] However, a lower rates of carbapenem resistance of 47% to 50% have been reported in studies from Nepal from 2013 and 2015.[16, 23] This may indicate that resistance rates are increasing in the local Nepalese population. Numerous mechanisms, including decreased permeability, efflux pump over-expression and carbapenemase production (e.g. KPCs, MBLs), can be responsible for the carbapenem resistance.[24] In the present study, the prevalence of KPC producing MDR *A. baumannii* was 41.5% which was substantially higher than previous studies from Nepal

which reported prevalence of KPC of 9.5% and 5.7%.[15, 21]

The emergence of MBLs in *A. baumannii* is becoming a therapeutic challenge as these enzymes possess high hydrolytic activity that leads to degradation of carbapenems. Furthermore, plasmid-mediated MBL genes spread rapidly to other species of gram-negative bacilli.[25] Therefore, rapid detection of MBL production is necessary to modify therapy and to initiate effective infection control to prevent their dissemination. In the current study, MBL producing isolates (73%) were more common than ESBL and AmpC producers. In Nepal, only a few other studies have examined the prevalence MBL, and these rates range from 47.2% to 78.8%.[15,21,26] Further, in-depth studies on the frequency of genes for cephalosporin and carbapenem resistance, and change over time are needed.

Due to the resistance to carbapenems as well as XDR among these isolates, patients can be treated with colistin or polymyxin B as none of the MDR *A. baumannii* isolates were resistant to these. However, these antibiotics have many adverse effects, including nephrotoxicity.[27]

The resistance rate to gentamicin and amikacin was 97.4% and 94.4% of isolates in the current study, which is higher than in the previous study from the same hospital reported in 2013, 2017 and 2020.[15,16,21] This apparent emergence of highly aminoglycosides resistant strains is also a cause of concern for successful therapy.

In the current study, > 92.8% of MDR *A. baumannii* were resistant to fluoroquinolones. This high level of fluoroquinolone resistance in clinical isolates may be due to their wide use in clinical medicine as broad-spectrum antimicrobial agents. All the current isolates showed similar high levels of resistance to cotrimoxazole, doxycycline, cefoperazone-sulbactam and ampicillin-sulbactam as previous studies.[15, 21]

In the current study, 98.5% (n=192) of isolates were biofilm producers. Of the total biofilm producers, 83.1% (n=162) were strong producers, 14.4% (n=28) were moderate producers while 1.0% (n=2) were weak producers. This finding concurs with that of another study in a tertiary care hospital of Nepal in which 99.6% of *Acinetobacter* species were biofilm producers and 89%, were strong biofilm producers.[28] Taken together, the data indicate that *A. baumannii* isolates from nosocomial infections are usually strong biofilm producers, and this may contribute to their pathogenicity and ability to withstand antibiotic therapy.

The current study found no association between biofilm production and antimicrobial resistance of planktonic cells of *A. baumannii* except for gentamicin, where gentamicin resistant strains were more likely to produce biofilms. This has been previously shown in strains isolated from Thailand where 76.9% of MDR strains were biofilm producers and there was an association between biofilm formation and gentamicin resistance ($p < 0.05$).[29] The current study and the previous Thailand study have not investigated whether this correlation is causative and if so the mechanism behind the association, and this should be followed up in future experiments.

CONCLUSION

Most of the *A. baumannii* strains isolated were resistant to many of the commonly used antibiotics, and all strains were only sensitive to polymyxins. This has important implications for the treatment of these infections. The majority of MDR *A. baumannii* isolates were biofilm producers. There was a correlation between resistance to gentamicin and biofilm production, but not for the other antibiotics.

ETHICAL CONSIDERATION

The study was conducted after taking written approval from the Institutional Review Committee of Institute of Medicine (Ref: 338(6-11)E^2/077/078), and written consent was taken from patient's local guardian for participation in the study before enrolment.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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