Beta-Lactamases Production in Multi-drug Resistant *Acinetobacter* species Isolated from Different Clinical Specimens

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ABSTRACT

Objectives: To determine the prevalence of *Acinetobacter* spp. from different clinical specimens and detect different types of β-lactamase enzymes.

Methods: Different clinical samples were collected and 125 *Acinetobacter* spp. were isolated. Various biochemical tests were carried out to speciate the *Acinetobacter* spp. The antibiotic susceptibility pattern and β-lactamase enzymes like Extended spectrum β-lactamase (ESBL), Metallo β-lactamase (MBL) and AmpC β-lactamase were determined.

Results: Of the total 125 isolates, the most predominant species was *Acinetobacter calcoaceticus-A. baumannii* (Acb) complex (80%). Highest rate of isolation of *Acinetobacter* species were from in-patients (neonates' blood sample). Among all, 44.8% isolates were found to be MDR with the majority being resistant to aminoglycosides, carbapenems and fluoroquinolones but not to colistin. ESBL, MBL and AmpC beta-lactamase was detected in 43.2%, 15.2% and 1.6% of the isolates respectively.

Conclusion: *Acinetobacter calcoaceticus-A. baumannii* complex should be considered for detection in hospitalized patients. The analysis of antibiotic susceptibility pattern and β-lactamases would be helpful to establish network surveillance in order to maintain and control the spread of these resistant strains.

Key words: *Acinetobacter* species, Acb complex, ESBL, MBL, AmpC beta-lactamase.

INTRODUCTION

Gram-negative bacteria cause different infections, which are becoming increasingly prevalent and constitute a serious threat to public health worldwide. Systemic infections from these organisms are difficult to treat and carry unacceptably high mortality, as high as 50% because of lack of efficacious treatment regimens (Kaye and Pogue 2015).

Genus *Acinetobacter* comprises more than 50 validly named species. The most significant among them is *A. baumannii* (Kolk et al. 2019) that commonly infects immuno-compromised patients (Park et al. 2017). They are ubiquitous organisms and prevail in natural environments (Kolk et al. 2019). They also represent the normal flora in humans (Almasaudi 2016).

Acinetobacter have emerged as one of the most troublesome classes of pathogen in health care-associated infections (Silveira et al. 2019). They cause various infections like hospital-acquired pneumonia, community-acquired pneumonia, bacteremia, trauma and wound infection, urinary tract infection, meningitis and other manifestations like endocarditis, peritonitis, opthalmitis or keratitis associated with contact lens use following eye surgery (Almasaudi 2016).

Acinetobacter species are well suited for genetic exchange and have the remarkable capacity for acquisition of foreign genetic material, which helps in obtaining resistance to the antibiotics (Kolk et al. 2019). *AbaR1* resistance cluster, which is an 86-kb region, have been identified in *Acinetobacter* spp. that contains 45

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resistance genes in MDR isolates (Perez et al. 2007).

Bacterial resistance against β-lactam antibiotics is now becoming threat in the interventions of antibiotics due to the production of β-lactamase enzymes. Khanal et al. (2013) reported the prevalence of ESBL, MBL and AmpC β-lactamase producing *Acinetobacter* to be 9.09%, 10.90% and 46.80% respectively. In another study conducted by Bhandari et al. (2015), 12.5% ESBL, 63.8% MBL and 31.37% AmpC β-lactamase producing *Acinetobacter* were reported. OXA-51 of *A. baumannii* is involved in cephalosporin resistance (AmpC) (Gordon and Wareham 2009).

The spread of multi drug resistant *Acinetobacter* infection has been increasing and is creating a problem in the treatment. The early detection of MDR isolates and their ability to produce β-lactamase enzyme is necessary to neutralize the serious threat. So this study was conducted with the objective to identify different *Acinetobacter* species and to detect various types of β-lactamases (ESBL, MBL and AmpC β-lactamase) produced by it that could be helpful for the treatment and analysis of resistance mechanism of this bacterium and to search the alternative therapeutic options.

MATERIALS AND METHODS

Sample size and study population

The study was conducted in Paropakar Maternity and Women's Hospital, Kathmandu, Nepal over a period of six months from May to November 2018. *Acinetobacter* spp. were isolated from various clinical specimens received in Microbiology laboratory of the hospital. A total of 10,265 samples were investigated which included blood sample, ear swab, Eustachian tube, high vaginal swab (HVS), pus and urine. The samples were collected from females and neonates.

Ethical issues

The informed consent was taken from adult females. In case of neonates, the informed consent was taken from their guardians. Permission to conduct the study was obtained from the participating hospital.

Isolation and identification of *Acinetobacter* species

The specimens were directly inoculated on Blood agar and MacConkey agar plates and incubated at 37°C for 24 hours. The species of *Acinetobacter* was identified phenotypically in the laboratory by series of biochemical tests (Table 1) (Gupta et al. 2015).

Table 1: Phenotypic characteristics of *Acinetobacter* spp.

Name of test	Acinetobacter species				
	Acb. complex	*A. lwoffii*	*A. Haemolyticus*	*A. junnii*	*A. radioresistens*
Gram staining	Gram negative cocci or coccobacilli				
Catalase	+	+	+	+	+
Oxidase	–	–	–	–	–
Motility	–	–	–	–	–
Urease	V	V	–	–	–
Citrate	+	–	+	+	–
OF glucose	+	–	V	–	–
Nitrate reduction test	–	–	–	–	–
Hemolysis	–	–	+	–	–
Gelatin Hydrolysis	–	–	+	–	–
Growth at 42	+	–	–	–	–
Chloramphenicol sensitivity	R	S	R	R	R
Arginine hydrolysis	+	–	+	+	+

+: Positive, - : Negative, V: Variable, S: Sensitivity, R: Resistant, OF: Oxidative-fermentative.

Antibiotic susceptibility testing

The antibiotic susceptibility tests were performed on Muller-Hinton agar (MHA) via modified Kirby-Bauer method of disk diffusion following guidelines of CLSI (2018). In this study the antibiotics used were Amikacin (30 µg), Ampicillin (10 µg), Azithromycin (15µg), Cefixime (5µg), Cefotaxime (30 µg), Cefalexin (30 µg), Ciprofloxacin (5µg), Colistin (10 µg), Gentmicin (10 µg), Meropenem (10 µg), Nitrofurantoin (300 µg), Norfloxacin (10 µg), Piperacillin (100 µg), Piperacillin-tazobactam (100/10 µg), Tetracycline (30 µg), and Trimethoprim-sulfamethoxazole (1.25/23.75 µg).

Criterion for multidrug resistance

The defining criterion for an isolate to be multidrug resistant (MDR) was set as resistance to three or more drugs belonging to different structural classes (Magiorakos et al. 2012).

Tests for ESBL

The ESBL production in bacterial isolates was screened by using Cefotaxime disc (30µg) and Ceftazidime disc (30 µg). If the zone of inhibition was less than or equal to 25 mm for Cefotaxime and if it was less than or equal to 22mm for Ceftazidime, the isolate was considered as potential ESBL producer on the basis of guidelines of CLSI (2018). The screened isolates were further confirmed by combined disc method. Cefotaxime (30 µg), Cefotaxime-clavulanate (30/10µg) and Ceftazidime (30 µg), Ceftazidime-clavulanate (30/10 µg) were used for confirmation of ESBL producing strains. After overnight incubation at 37°C, greater than or equal to 5 mm increase in a zone of diameter for either Cefotaxime/clavulanate (30/10 µg) or Ceftazidime/clavulanate (30/10 µg) vs the zone diameter of Cefotxime (30µg) or Ceftazidime (30µg) was interpreted as ESBL producer as recommended by CLSI (2018).

Tests for MBL

The screening test for the MBL production was performed by using Imipenem disc (10µg). If the zone of inhibition was less than or equal to 18 mm for Imipenem, the isolate was considered as potential MBL producer as stated by CLSI (2018). The screened isolates were further confirmed by combined disc method using Imipenem (10 µg) alone and in combination with EDTA. After overnight incubation at 37°C, if the increase in inhibition zone with Imipenem-EDTA disc was greater than or equal to 7 mm than the Imipenem (10 µg) alone, it was interpreted as MBL producer as stated by Anwar et al. (2016) and Sujatha and Goyal (2017).

Tests for AmpC β-lactamase

AmpC-lactamase production was screened by using Cefoxitin (30 µg) disc. If the zone of inhibition was less than or equal to 14 mm for Cefoxitin, the isolate was considered as potential AmpC β-lactamase producer (Saad et al. 2016). The screened isolates were further confirmed by disc approximation test. Imipenem (10µg), Ceftazidime (30 µg), Cefoxitin (30 µg) and Amoxicillin-clavulanate (20/10µg) were used for the confirmation of AmpC β-lactamase producing strains. After overnight incubation at 37°C, the plate was examined for any blunting or flattening of the zone of inhibition between the ceftazidime disc and the imipenem, cefoxitin and amoxicillin-clavulanate discs. The presence of any blunting or flattening of the zone was interpreted as AmpC β-lactamase producer (Saad et al. 2016).

RESULTS

Out of 10,265 clinical specimens, 807 (7.86%) were found to be culture positive and the occurrence of *Acinetobacter* was found to be 125 (15.48%). 113 (23.01%) of *Acinetobacter* species were isolated from in-patients and 12 (3.78%) from out-patients (Table 2).

Table 2: Status of bacterial infections in suspected patients

Category	Culture		Total N (%)	Acinetobacter spp. N (%)
	Positive N (%)	Negative N (%)		
In-patients	491 (13.96)	3025 (86.04)	3516 (34.25)	113 (23.01)
Out-patients	316 (4.68)	6433 (95.32)	6749 (65.74)	12 (3.78)
Total	**807 (7.86)**	**9458 (92.16)**	**10265**	**125 (15.48)**

Amidst the *Acinetobacter* isolates, 65 (52%) were obtained from neonates' blood whereas only 1 (0.8%) was obtained from ear swab (Figure 1).

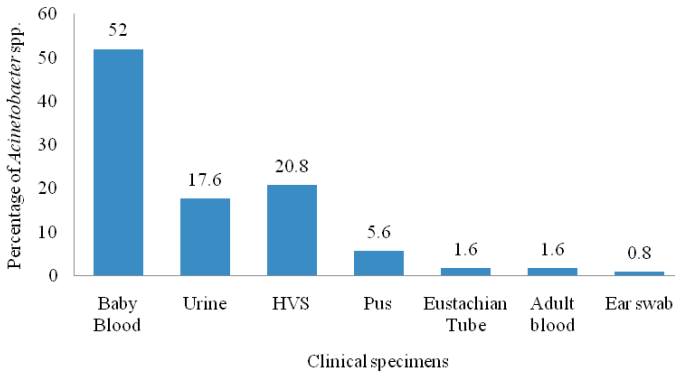

Figure 1: Distribution of *Acinetobacter* spp. in clinical specimens

Out of 125 *Acinetobacter* spp., the most predominant species was *Acinetobacter calcoaceticus-baumannii (Acb* complex*)* (80%) followed by *A. lowffii* (10.4%) (Figure 2).

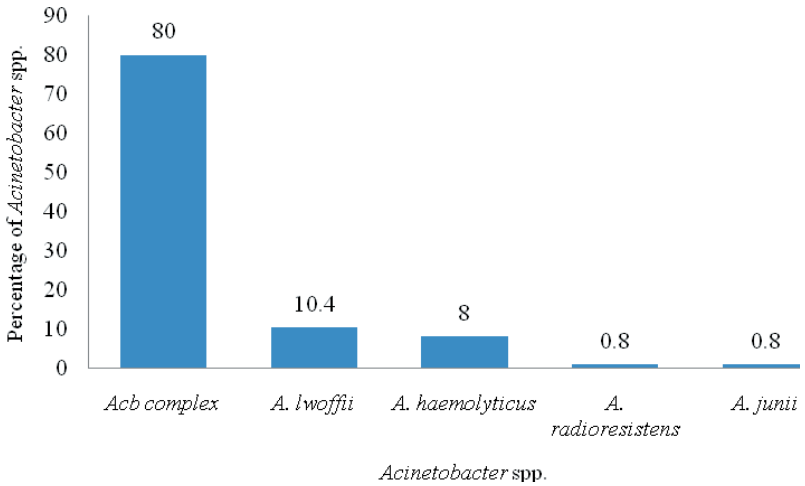

Figure 2: Distribution of various species of *Acinetobacter*

Amongst total 125 *Acinetobacter* spp., 67 (53.6%) of the isolates were resistant towards Cefotaxime and 55 (44%) towards Piperacillin while all the isolates were sensitive towards Colistin followed by Tetracycline (85.6%) (Table 3).

Table 3: Antibiotic susceptibility profile of *Acinetobacter* spp. (n=125).

Antibiotics	Sensitive		Resistant	
	No.	%	No.	%
Amikacin	93	74.4	32	25.6
Cefotaxime	58	46.4	67	53.6
Ciprofloxacin	104	83.2	21	16.8
Colistin	125	100	0	0
Gentamicin	92	73.6	33	26.4
Meropenem	90	72	35	28
Piperacillin	70	56	55	44
Piperacillin-Tazobactam	100	80	25	20
Tetracycline	107	85.6	18	14.4
Trimethoprim-Sulfamethoxazole	79	63.2	46	36.8

Of the total *Acinetobacter* spp. 56 (44.8%) were MDR, 32 (57.14%) were ESBL producer, 18 (32.14%) were MBL producer and 2 (3.57%) were AmpC β-lactamase producer. The ESBL production and MBL production in MDR isolates were found to be statistically significant while the AmpC β-lactamase production in MDR isolates was found to be statistically insignificant (Table 4).

Table 4: Profile of β-lactamase producing *Acinetobacter* species

Tests	ESBL		MBL		AmpC ß-lactamase	
	Positive N (%)	Negative N (%)	Positive N (%)	Negative N (%)	Positive N (%)	Negative N (%)
MDR	32 (57.14)	24 (42.86)	18 (32.14)	38 (67.86)	2 (3.57)	54 (96.43)
Non MDR	22 (31.88)	47 (68.12)	1 (1.45)	68 (98.55)	0 (0)	69 (100)
Total	54	71	19	106	2	123
p-value	**0.005**		**0.001**		**0.114**	

Among 125 *Acinetobacter* spp. 57.14% of the isolates were MDR and ESBL producer, 32.14% were MDR and MBL producer, 3.57% of the isolates were MDR and AmpC producer, 17.85% of the isolates were MDR and both ESBL as well as MBL producer while 1.78% of the isolates produced all the three beta lactamase enzymes along with being MDR isolate (Table 5).

Table 5: Relationship between MDR, ESBL, MBL and AmpC β-lactamase production in *Acinetobacter* spp.

Characteristics	*Acinetobacter spp.*	
	No.	%
MDR + ESBL	32	57.14
MDR + MBL	18	32.14
MDR + AmpC	2	3.57
MDR + ESBL + MBL	10	17.85
MDR + ESBL + AmpC	1	1.78
MDR + MBL + AmpC	1	1.78
MDR + ESBL + MBL + AmpC	1	1.78

DISCUSSION

Acinetobacter species are ubiquitous organisms and prevail in natural environments (Kolk et al. 2019). They also represent the normal flora in humans (Almasaudi 2016). Transmission of isolate is usually through the hands of staff, contaminated equipment or overall hospital environment. Moreover, the virulence factors of *Acinetobacter* spp. are porins, surface structures such as capsular polysaccaharide and lipopolysaccaharide (LPS), phospholipases, iron acquisition systems, outer membrane vesicles, protein secretion systems, regulatory proteins, biofilm associated proteins, different types of binding proteins. They are also well suited for genetic exchange and have the remarkable capacity for acquisition of foreign genetic material, which helps in obtaining resistance to the antibiotics (Kolk et al. 2019).

The incidence of *Acinetobacter* spp. from in-patients was found to be 90.4%, which is in accordance with previous studies carried out by Gupta et al. (2015) and Joshi et al. (2017). The incidence of *Acinetobacter* infection was highest in in-patients and highest number of bacteria was isolated from neonates' blood as also stated by Gupta et al. (2015). It is because *Acinetobacter* spp. is low virulence organism responsible for opportunistic infections in immuno-compromised patients, which increases the incidence of nosocomial infections. One of the reasons for the increased nosocomial infections by *Acinetobacter* spp. might be their endurance in dry conditions for long period of time and survival in a hospital environment and on the surface of healthcare worker hands (Park et al. 2017). The immune system of neonates is immature when they are born and it takes time to fully develop this immunity and thus they are easily attacked by various bacterial pathogens (Park et al. 2017).

The predominantly isolated species was *Acinetobacter calcoaceticus baumanii (Acb complex)* as also reported by Raina et al. (2015) and Gupta et al. (2015). Almost half of the isolates were multi-drug resistant which is consistent with previous reports by Pathak et al. (2017) and Shrestha et al. (2015). The development of resistance in *Acinetobacter* spp. may be due to the presence of wide array β-lactamases that hydrolyze and confer resistance to penicillins, cephalosporins and carbapenems, presence of efflux pumps and loss of porin proteins. Also the inappropriate use of antibiotics and lack of hygiene practices are also the factors that help in the spread of antibiotic resistant bacteria (Awad et al. 2016; Khanal et al. 2013).

Of the total *Acinetobacter* spp., 54 (43.2%) were ESBL producer, 19 (15.2%) were MBL producer and 2 (1.6%) were AmpC β-lactamase producer while 1.78% of the isolates (*Acinetobacter calcoaceticus baumanii*) produced all the three beta lactamase enzymes along with being MDR isolate. ESBL production and MBL production in MDR *Acinetobacter* spp. was found to be statistically significant while AmpC production in MDR *Acinetobacter* spp. was found to be statistically insignificant.

ESBL production might be due to the presence of ESBL producing genes like bla_{OXA-23} and antibiotic genes that can be transferred to other bacteria horizontally through conjugation and due to excessive use of broad spectrum antibiotics (Joshi et al. 2017; Shrestha et al. 2017). The acquisition of MBL-encoding genes such as *vim1, vim2, imp1* and *imp2* is one of the ways to acquire resistance to carbapenems like imipenem, meropenem and ertapenem (Davoodi et al. 2015). Phenotypic detection of AmpC β-lactamase enzyme is generally considered inappropriate because there are no standardized screening methods and also there are no CLSI recommended guidelines (Saad et al. 2016). Molecular methods are the most reliable and appropriate methods for the detection of AmpC β-lactamase enzyme (Delgado et al. 2016).

Acinetobacter spp. are becoming the troublesome pathogen with multiple antibiotic resistance mechanisms, especially in hospital settings. Thus, infection prevention and control measures are required to minimize or prevent the transmission of infections and antibiotic stewardship programs can be implemented effectively in hospitals for optimizing the treatment of infections and reducing adverse events associated with antibiotic use.

CONCLUSION

Acinetobacter calcoaceticus-baumannii was the most common bacterial isolate which was mostly recovered from neonates' blood. All isolates were sensitive to Colistin while more than half of the isolates were resistant towards Cefotaxime and Piperacillin. Significant proportions of ESBL, MBL and AmpC beta lactamase producers were MDR. This suggests for regular monitoring of these resistant pathogens for their control.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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