Biofilm Production and Antibiotic Resistance in Clinical Isolates of *Acinetobacter* Species

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ABSTRACT

Objective: To study the occurrence of the biofilm producing *Acinetobacter* spp. from different clinical specimens and to assess the antibiotic susceptibility pattern of *Acinetobacter* species

Methods: This study was conducted at B&B Hospital Pvt. Ltd., Lalitpur, Nepal from February to September 2018. Various specimens including pus, sputum, urine, catheter tips, body fluids (bile, peritoneal fluid, CSF), suction tube, and blood were collected from the patients (n=5141) visiting B&B hospital. The bacterial isolates were subjected to antibiotic susceptibility testing and *Acinetobacter* spp. isolates were subjected to biofilm detection by microtiter plate method.

Results: Out of 5141 specimens, 1179 (23%) were culture positive. *Escherichia coli* (40.8%) was found to be the predominant organism. A total of 83 isolates of *Acinetobacter* spp. were isolated among which 76(91.57%) were biofilm producers. Biofilm producing isolates of *Acinetobacter* spp. were found more resistant to the tested antibiotics than non- biofilm producing *Acinetobacter* spp.

Conclusion: Most *Acinetobacter* spp. was capable of producing the biofilm. The biofilm producers were more resistant to the antibiotics under study which help to increase the resistivity nature of the bacteria. All of the isolates susceptible to colistin showed that the appropriate therapeutic option for infection caused by biofilm forming *Acinetobacter* spp.

Key words: Gram-negative bacteria, biofilm, antibiotic resistance, MDR

INTRODUCTION

Biofilms are dynamic, heterogeneous community of microorganisms enclosed within complex matrix of extra polymeric substance that have integrated metabolic activities (Sanchez et al. 2013). *Acinetobacter* spp., due to capability of biofilm formation on invasive devices, are increasingly evolved as an important nosocomial pathogen responsible for variety of infections, including bacteremia, urinary tract infection, wound infection, secondary meningitis, particularly ventilator-associated pneumonia (Doughari et al. 2011; Mirnejad et al. 2013). Bacteria in biofilms are phenotypically different from their planktonic counterparts and are more resistant in nature. The nature of biofilm and the physiological state of bacterial cells inside the biofilm makes it resistant to the different classes of antibiotics thereby making the treatment problematic (Longo et al. 2014; Sharma et al. 2008). Biofilm are also responsible for the chronic and recurrent infections which adds up the economic burden for the treatment process (Ejrnaes et al. 2011). Hence, the regular surveillance of distribution of biofilm producing *Acinetobacter* spp. is necessary to stop the spread of nosocomial infection.

MATERIALS AND METHODS

This study was conducted at B&B Hospital Pvt. Ltd., Gwarko, Lalitpur in collaboration with GoldenGate International College, Kathmandu, Nepal from February to September 2018. A total of 5141 non-

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duplicate clinical specimens including pus, sputum, urine, catheter tips, body fluids (bile, peritoneal fluid, CSF), suction tube, and blood from all the patients visiting hospital during study period were studied. The inadequate and improperly labelled samples with visible contaminations were excluded. The samples were inoculated onto Mac Conkey Agar (MA), Blood Agar (BA), Chocolate Agar and Brain Heart Infusion (BHI) broth. The MA and BA have been incubated aerobically at 37°C for 24-48 hours while CA plates were incubated in a candle jar at 37°C for 24-48 hours (Cheesebrough 2006). BHI broth was incubated at 37°C for 7 days and sub-cultured onto MA plates. The bacterial isolates were identified by standard microbiological procedures including microscopy, colony morphology and biochemical tests as described by the American Society of Microbiology (ASM). Antibiotic Susceptibility tests of the bacterial isolates were performed by Modified Kirby-Bauer Disk Diffusion technique using Mueller Hinton Agar (CLSI 2015). Among all the isolates, *Acinetobacter* spp. isolates were subjected to biofilm detection by the microtiter plate method.

Detection of biofilm by microtiter plate method

The biofilm formation by the *Acinetobacter.* spp. was studied by the microtiter plate culture method as described by Christensen (Christensen et al. 1982). The suspension of *Acinetobacter* spp. isolates was prepared in Tryptic Soya Broth (TSB) supplemented with 1% glucose. Then the suspension was diluted at 1:100 with fresh TSB. 200 µl of diluted suspension was loaded into wells of 96 well sterile flat-bottom polystyrene micro-titer plate. A set of 3 such microtiter plates were prepared. *Acinetobacter.* spp. ATCC 19606 and TSB with 1% glucose were used as the positive and negative control respectively. The micro-titer plate with bacterial suspensions was then incubated at 37°C for 24 hours. After incubation, the suspension was removed by gentle tapping and each well was washed with 200 µl of Phosphate Buffer System (pH 7.3) four times. Subsequently, 2% sodium acetate was used for fixation of biofilm formed by bacteria followed by staining with 100µl of 0.1% crystal violet. The plates were washed with de-ionized water to remove excess stain and dried. The microtiter plate was then rinsed with 0.2 ml of ethanol-acetone (80:20 v/v) to solubilize crystal violet. The ELISA reader was used to obtain the absorbance at a wavelength of 570 nm. The value of optical densities for each isolate was calculated from the average of three wells. The value was compared to the optical density of the negative control (ODc). The isolates were classified based on mean optical densities (Stepanovic et al. 2000):

OD ≤ ODc (≤0.658): non-biofilm producer

ODc < OD ≤ 2× ODc (>0.658-1.361): weak biofilm producer 2× ODc < OD ≤ 4 × ODc (>1.316-2.632): moderate biofilm producer

4× ODc < OD (>2.632): strong biofilm producer

Ethical approval and consent

Ethical approval was taken from Institutional Review Committee at B & B Hospital Pvt. Ltd. After giving brief information about this research, written informed consent was obtained from patients prior to sampling. In case of illiterate participant, information was provided by reading the consent form in presence of witness.

RESULTS

Culture of samples and frequency of bacterial isolates

A total of 5141 different clinical specimens were processed. Among them, 1179 (23%) specimens showed significant growth including 991 (84.05%) Gram negative bacteria and 188 (15.95%) Gram positive bacteria. *Escherichia coli* (481; 40.8%) was the most predominant bacteria followed by *Klebsiella pneumonia* (232; 19.7%). A total of (83; 7.04%) *Acinetobacter* spp. were isolated.

Table 1: Frequency of bacterial isolates isolated from clinical specimens

Bacterial isolates	
Gram negative bacteria	**Number of isolates (%)**
Escherichia coli	481 (40.8)
Klebsiella pneumonia	232 (19.7)
Pseudomonas aeruginosa	130 (11.02)
Acinetobacter spp.	83 (7.04)
Salmonella Typhi	17 (1.44)
Enterobacter spp.	17 (1.44)

Bacterial isolates	
Gram negative bacteria	**Number of isolates (%)**
Proteus spp	16 (1.36)
Morganella spp	5 (0.42)
Citrobacter spp.	3 (0.25)
Haemophillus influenza	3 (0.25)
Salmonella Paratyphi	2 (0.17)
Shigella flexneri	2 (0.17)
Gram positive bacteria	
Staphylococcus aureus	84 (7.12)
Enterococcus spp.	51 (4.32)
CONS	47 (4)
Streptococcus spp.	5 (0.42)
Micrococcus spp.	1 (0.08)
Total	1179

Distribution of *Acinetobacter* spp. in different clinical specimens

Out of total *Acinetobacter* spp. isolated from culture positive specimens, 59 (71.08%) and 24 (28.92%) isolates were from male and female patients respectively. Similarly, 77(92.8%) and 6 (7.20%) isolates were from inpatients and outpatients respectively. The patients of age groups 21-30 and 31-40 were found to be mostly affected by the *Acinetobacter* spp (n=16; 19.28%). The maximum number of *Acinetobacter* spp. (50; 60.24%) was isolated from pus specimen and minimum number being isolated from the blood specimen 1(1.20%).

Table 2: Distribution of *Acinetobacter* spp. based on age group

Age groups (years)	Number of *Acinetobacter (%)*
NB-10	5 (6.02)
11-20	12 (14.46)
21-30	16 (19.28)
31-40	16 (19.28)
41-50	10 (12.05)
51-60	14 (16.87)
61-70	4 (4.82)
71-80	4 (4.82)
81-90	2 (2.40)

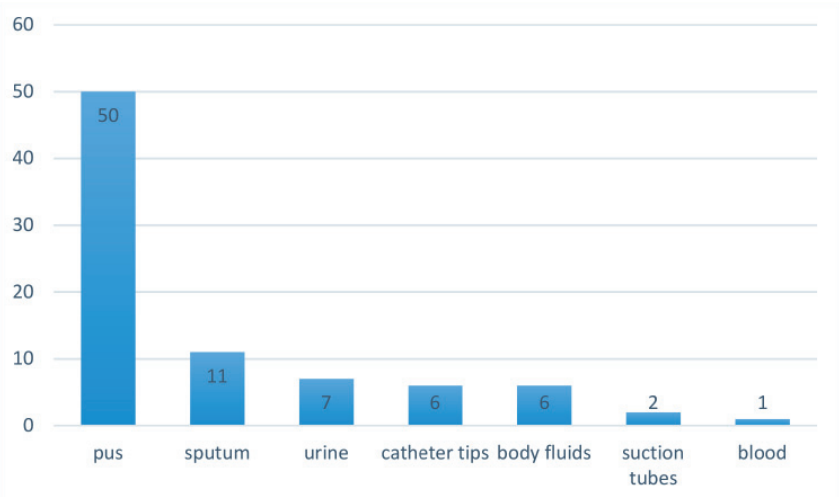

Figure 1: Bar-chart showing frequency of *Acinetobacter* spp. in different clinical specimens

Antibiotic resistance pattern of *Acinetobacter* spp.

All the isolated *Acinetobacter* spp. (100%) were resistant to amoxicillin and sensitive to colistin. Higher rate of resistance was found towards ceftriaxone (92.77%) followed by ceftazidime (80.72%). Amikacin was found to be the most sensitive antibiotics (57.83%). A total of 54 *Acinetobacter* spp. were found to be Multi-Drug Resistant (MDR) strains.

Table 3: Antibiotic Resistance Pattern of *Acinetobacter* spp.

Antibiotic category	Antibiotics used	AST pattern of *Acinetobacter* spp.	
		No. of sensitive isolates (%)	No. of resistant isolates (%)
First line drugs			
Penicillin+ ß-lactamase inhibitors	Amoxicillin	0	83 (100)
Extended spectrum cephalosporins; third generations	Ceftriaxone	6 (7.23)	77 (92.77)
	Ceftazidime	16 (19.28)	67 (80.72)
Extended spectrum cephalosporins; fourth generations	Cefepime	17 (20.48)	66 (79.52)
Aminoglycosides	Gentamicin	29 (34.94)	54 (65.06)
Second line drugs			
Carbapenems	Imipenem	26 (31.33)	57 (68.67)
	Meropenem	33 (39.76)	50 (60.24)
ß-lactamase inhibitors	Piperacillin/ Tazobactum	19 (22.89)	64 (77.11)
Extended spectrum cephalosporins third generation cephalosporins	Cefoperazone/ Sulbactum	26 (31.33)	57 (68.67)
Fluoroquinolones	Ciprofloxacin	22 (26.51)	61 (73.49)
Aminoglycosides	Amikacin	35 (42.17)	48 (57.83)
Third line drugs			
Polymixins	Colistin	83 (100)	0

Biofilm producing *Acinetobacter* spp.

Among the 83 *Acinetobacter* spp., 76 isolates were found biofilm producers; out of which 22 (28.95%) were strong, 40 (52.63%) were moderate and 14 (18.42%) were weak biofilm producers.

The highest number of biofilms producing *Acinetobacter* spp were isolated from pus specimen (n= 45) followed by sputum specimen (n=11). Similarly, 70 (92.11%) isolates from the inpatients and 6 (7.89%) isolates from outpatients were biofilm producer.

Table 4: Distribution of biofilm producing *Acinetobacter* spp in different clinical sampels

Specimen	Number of biofilm producer (n=76)			
	Strong (%)	Moderate (%)	Weak (%)	Total (%)
Pus	16	19	10	45 (59.21)
Sputum	3	8	-	11 (14.47)
Urine	2	4	1	7 (9.21)
Catheter tips	-	4	2	6 (7.89)
Body fluids	1	2	1	4 (5.26)
Suction Tubes	-	2	-	2 (2.64)
Blood	-	1	-	1 (1.32)
Total	**22 (28.95)**	**40 (52.63)**	**14 (18.42)**	**76 (100)**

Antibiotic resistance pattern of biofilm producing and non-producing *Acinetobacter* spp. and MDR strain distribution

A total of 54 *Acinetobacter* spp. were found to be MDR including 52 (68.42%) biofilm producers and 2 (28.57%) biofilm non-producers. The overall antibiotic resistance pattern of *Acinetobacter* spp. showed that the biofilm producers were more resistant to tested antibiotics than biofilm non-producers. All the biofilm producing isolates showed complete resistance towards the amoxicillin. Besides amoxicillin, the highest number of biofilms producing *Acinetobacter* spp. were resistant to ceftriaxone followed by ceftazidime. Similarly, all the biofilm non-producing *Acinetobacter* spp. showed complete resistance towards amoxicillin and ceftriaxone.

Table 5: Antibiotic resistance pattern of biofilm producing and non-producing *Acinetobacter* spp

Antibiotics used	Resistance pattern	
	Biofilm producers (%)	Biofilm non-producers (%)
Amoxicillin	76 (100)	7 (100)
Ceftriaxone	70 (92.11)	7 (100)
Ceftazidime	61 (80.26)	6 (85.71)
Cefepime	60 (78.95)	6 (85.71)
Gentamicin	49 (64.47)	5 (71.43)
Imipenem	51 (67.11)	6 (85.71)
Meropenem	45 (59.21)	5 (71.43)
Piperacillin/ Tazobactum	58 (76.32)	6 (85.71)
Cefoperazone/ Sulbactum	55 (72.37)	2 (28.57)
Ciprofloxacin	56 (73.68)	5 (71.43)
Amikacin	44 (57.9)	4 (57.14)
Colistin	0	0

DISCUSSION

Acinetobacter spp. has been an emerging nosocomial pathogen as it is capable of causing invasive device related infections. The propensity of this bacteria to form biofilm on devices causes reduced penetrability of antibiotics, thereby increasing the antibiotic resistance (Bala et al. 2016; Bernards et al. 2004). *Acinetobacter* spp. isolated in this study showed high rate of antibiotic resistance indicating a challenge in treatment of several human infections. In addition, we also reported most *Acinetobacter* spp. isolated from the patients with infections have the ability to form biofilm. The increased antibiotic resistance was observed among biofilm producing strains than biofilm non-producing strains which may add more economic burden in antimicrobial therapy to treat infections.

The prevalence of *Acinetobacter* spp. in causing human infections was found to be 7% which was similar to the study by Koripella et al. (2016). However, the prevalence of *Acinetobacter* spp. was 2.9% in another study by Saha et al. (2018). Different factors like immunosuppressed hosts, patients with severe underlying disease, previous use of antibiotics, duration of hospital stay and more frequent use of antibiotics can affect the prevalence of *Acinetobacter* spp. in the patients (Rungruanghiranya et al. 2005). The male patients (71.08%) were found to be more infected by the *Acinetobacter* spp. The result coincided with the studies by (Nirwati et al. (2018); Tripathi et al. (2014). This may be due to the fact that the male reports more frequently to the hospital compared with female (Tripathi et al. 2014). The culture positivity was higher in the specimens from the inpatients (92.80%) than outpatients (7.2%) which reflects the probability of increased health care associated infections. A similar result was obtained in the study by Rebic et al. (2018). The higher prevalence of *Acinetobacter* spp in inpatients may be due to the use of invasive diagnostic procedure, use of broad-spectrum antimicrobials and prolonged duration of hospital stay (Dash et al. 2013). The patients of age group 21-30 and 31-40 harbored high number of *Acinetobacter* spp. (19.28%). Sivaranjani et al. (2013) also found higher prevalence rate within the age group 20-40. Adults are susceptible to several infections as they have their increased exposure to various adverse environmental conditions (Saha et al. 2018). The highest number of *Acinetobacter* spp. were isolated from pus specimens (60.24%) followed by sputum specimens (13.25%) and urine specimens (8.43%) which is similar with other studies conducted by Kulkarni et al. (2017). However, the study conducted by Saha et al. (2018) reported the highest number of *Acinetobacter* spp. from the urine specimens.

Antibiotic resistance is the main cause of treatment failure of infected patients with all *Acinetobacter* species, particularly those with *A. baumannii* (Gehrlein et al. 1991; Rao et al. 2008). *Acinetobacter* spp. isolates showed the complete resistance to amoxicillin. Nahar et al. (2013) also concluded that *Acinetobacter* spp. are completely resistant to amoxicillin which may be due to constitutively expressed chromosomal class-A beta lactamases that makes the bacteria intrinsically resistant to amoxicillin. Ceftazidime, ceftriaxone, cefepime, imipenem, amikacin and quinolones can be used as the first line drugs for treatment of *Acinetobacter* infections. However, increasing resistance to first line drugs have urged the use of second line drugs. *Acinetobacter* spp. isolates showed high rate of resistance towards commonly used cephalosporins like ceftazidime (80.72%), ceftriaxone (92.77%), and cefepime (79.52%).

Saha et al. (2018) and Gales et al. (2019) also concluded similar resistance rate towards ceftazidime and cefepime. Baneerjee et al. (2018) reported even higher resistance rate towards ceftazidime (92.01%) and cefepime (89.9%). A similar resistance rate towards ceftriaxone (87.1%) was reported by Mishra et al. (2014). The high rate of resistance to cephalosporin is due to the widespread use of third-generation cephalosporins without knowing the severity of infections (Mshana et al. 2009). Meropenem was found to be comparatively more effective than imipenem which was similar to the study by Banerjee et al. (2018). However, Jaggi et al. (2012) reported 90% of resistant to carbapenem drug. Among the aminoglycosides, amikacin was found more effective than gentamicin which coincided with the study by Gales et al. (2019). Fluoroquinolones have excellent clinical activity against *Acinetobacter* spp but the frequency of ciprofloxacin- resistant *Acinetobacte*r has increased worldwide in recent years (Hamidian and Hall 2014). We found that 73.49% of *Acinetobacter* isolates were resistant to ciprofloxacin which is similar to the study conducted by Mishra et al. (2013). All isolates were susceptible to colistin. Other studies by (Shareek et al. 2012; Dash et al. 2013; Saha et al. 2018) also reported colistin as the most sensitive drug.

In this study, 91.56% *Acinetobacter* spp. were found to be biofilm producers which is similar to the study by Qi et al. (2016). The studies by Rodrıguez- Banò et al. (2008), Dheepa et al. (2011) andAbdi-Ali et al. (2014)) reported 63%, 60% and 69% of biofilm producers respectively which were lower than our findings. This may be due to the difference in the number of clinical isolates from different sources (Abdi-ali et al. 2014). Among the biofilm producers, the highest producers were isolated from the pus specimens. Similarly, the inpatients harbored the maximum number of biofilms producing *Acinetobacter* which could have been due to use of invasive diagnostic procedures and patient's association with indwelling devices (Dash et al. 2013). The biofilm producing *Acinetobacter* spp showed greater resistance to different antibiotics. A higher prevalence of MDR was seen among biofilm producers (68.42%) which was similar to the findings by (Nahar et al. 2013; Gurung et al. 2013). Higher prevalence of MDR in biofilm producing strains may be due to transfer of resistant gene to other organism that initially does not show such resistance (Gurung et al. 2013). The biofilm production not only contribute the pathogens to adopt in the different environmental niche and also help them to resist towards many antimicrobial agents. The slow growth rate and presence of protective exopolysaccharide layer alters the penetration of antimicrobial agents through biofilm thereby increasing the rate of antimicrobial resistance (Hung and Henderson 2008; Hall et al. 2014). The biofilm promotes the chronic infectious diseases by rendering the inefficient antibiotics treatment (Alves et al. 2014; Rao et al. 2008; Sanchez et al. 2013;). Irrational use of antimicrobial agents and spread of antimicrobial resistance genes among the bacteria leads to the development of MDR nature in bacteria (Ahmed et al. 2013). The growing rate of MDR among bacterial pathogens are of great concern as the infections caused by those pathogens might result into longer hospital stay with higher morbidity and mortality. Hence, it is very necessary to routinely screen the bacterial pathogens for biofilm production with their antibiogram patterns.

In this study, biofilm production was studied under in-vitro condition which may vary from in- vivo condition. The antimicrobial resistance pattern of *Acinetobacter* was determined by modified Kirby Bauer disc diffusion method, which do not exactly explain the antimicrobial resistance of the biofilm producers when they are within the biofilm. Although the phenotypic studies are simpler and cost effective, genotypic techniques are needed for deeper understanding. Molecular techniques could not be used in this study due to lack of resources. Moreover, short duration and small sample size may not generate more reliable results.

CONCLUSIONS

This study reveals the predominance of Gram-negative bacteria in various human infections. The occurrence of *Acinetobacter* spp. has been increasing in the patients with high rate of resistance to routinely used antibiotics. The study also reported most of the clinical isolates of *Acinetobacter* spp. from the patients with various infections have the ability to produce biofilm. The increased antimicrobial resistance was observed among biofilm producers. Hence, a continuous monitoring of antibiotic susceptibility tests of *Acinetobacter* isolated from different clinical sources in every region seems necessary.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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