GoldenGate Journal of Science & Technology 2017, 3: 68 - 73 ©GJST
ISSN 2505-0656 (Print)

Microbiology

ANTIBIOGRAM OF BIOFILM PRODUCER *ACINETOBACTER* ISOLATES FROM DIFFERENT CLINICAL SPECIMENS

Yadav SK[1], Lekhak B[1], Upreti MK[1], and Lekhak S[2]
[1]Department of microbiology, GoldenGate International College, Battishputali Kathmandu;
[2]Department of Pathology, ANIAS Hospital, Maitighar

Abstract

Acinetobacter calcoaceticus-Acinetobacter baumannii (ACB) complex has emerged as the most common opportunistic nosocomial pathogen. The biofilm producing ability of nosocomial strains play a significant role in health care setting and antimicrobial resistance. A prospective cross-sectional study was performed in the microbiology laboratory of Annapurna Neurological Institute and Allied Science, Kathmandu Nepal, from May to December 2015. The study was performed to determine biofilm production ability of ACB complex and to compare the antimicrobial susceptibility profile of biofilm producing strains with non-producing ACB complex. Clinical specimens were processed for the isolation of bacterial pathogens according to standard microbial technique. The bacterial isolates were identified by cultural and biochemical test. Antimicrobial susceptibility testing of the isolates was performed by modified Kirby-Bauer disk diffusion method. Detection of biofilm formation was done by both qualitative (tube method) and quantitative methods (microtitre plate method). Altogether 1150 clinical specimens were subjected to microbial analysis of which 250 showed growths positive. ACB complex was detected in 45 (17.5%) growth positive specimens, of which, 60% (27) of ACB complex were biofilm producers. ACB complex isolates showed high resistance to ceftazidime (80 %) and piperacillin (80%) and only 2.3 % showed resistance toward colistin. Out of 45 ACB complex 71.2% (32) were found to be MDR. All ACB isolates were susceptible to polymyxin B. Since, prevalence of the biofilm producing ACB complex was seen to be high, it is necessary for routine monitoring of antibiotic susceptibility of clinical ACB complex isolates.

Key words: ACB complex, Biofilm, MDR, Antimicrobial susceptibility testing.

Introduction

Acinetobacter calcoaceticus-Acinetobacter baumannii (ACB) complex comprises four species namely *A. calcoaceticus, A. baumannii, Acinetobacter* genospecies 3 (now referred to as *A. pitti*) and *Acinetobacter* genospecies 13TU (now referred to as *A. nosocomialis*), which are genotypically and phenotypically highly related and difficult to distinguish phenotypically (Dijkshoorn et al 2007; Howard et al 2012; Peleg et al 2008). *Acinetobacter calcoaceticus* is a soil organism that has very rarely been implicated in human infections which may mislead the name ACB complex (Towner 2006) because except *Acinetobacter calcoaceticus,* all are nosocomial species (Chang et al 2005). In the clinical environment, *Acinetobacter baumannii* is of great importance, as it accounts for the vast majority of nosocomial infections and hospital outbreaks involving *Acinetobacter* spp. (Towner 2009). *Acinetobacter* spp. were considered as rare causes of nosocomial infections in the intensive care unit (ICU) until 1970 but in recent years, *Acinetobacter* infections possess a greater threat to hospitalized patients around the world (Rungruanghiranya et al 2005). In a Europe, *A baumannii* appeared to be the eighth most common cause of nosocomial pneumonia (Howard et al 2012). Another study conducted in India showed *A. baumannii* apperead to be the second most common nosocomial organism after *Pseudomonas aeruginosa* among the aerobic non-fermentative, Gram negative bacilli (Rao et al 2008).

The persistent presence of *Acinetobacter* spp in the environment may be due to its ability to form biofilms on both abiotic and biologic surfaces (McQueary and Actis 2011). Biofilm formation is also a mechanism of pathogenesis in device-related infections and provides a source of repeated transmission by prolonging survival on inanimate objects (Lewis 2001). Under harsh environmental conditions, *A. baumannii* cells deep in the biofilm can undergo dormancy, becoming metabolically inactive and robust to environmental stress (Lee et al 2008).

Acinetobacter is known to show resistance to a majority of commercially available antibiotics (penicillins, aminoglycosides, cephalosporins, quinolones) and therefore raises an important therapeutic problem (Shin et al 2009). Treatment of infections caused by ACB complex has been very difficult during last few years due to increase in antimicrobial resistance, especially multidrug resistance, with some strains now resistant to almost all commonly used

antimicrobial agents (Blossom and Srinivasan 2008). Susceptibility of *Acinetobacter* spp. against antimicrobials is considerably different among countries, regions, hospitals and even among the wards of a given hospital (Chen et al 2006). It is hypothesized that its ability to persist in the hospital environment as well as its virulence is a result of its capacity to form biofilms (Gaddy et al 2009). Biofilm formation is a relevant process because of being as a mechanism for antibiotic resistance, transfer of resistance plasmids and a medium for intracellular communication (Gurung et al 2013).

Materials and method

Study site and Population

This was a descriptive cross-sectional study conducted at Annapurna Neurological Institute and Allied Science, Kathmandu, Nepal. The study period was from May to December 2015. The study population of this study includes the patients attending the hospital whose samples were sent to Microbiological laboratory with requested form. The samples include urine, pus, body fluids, sputum, Foley's/catheter tip, suction/endotracheal tip (ET), wound swab, cervical swab. Patients were made aware of their participation in the study during the sample collection time. The data collection sheet of the study has been included in Appendix I. A total of 1150 different clinical specimens were included from the patients suspected with respective related infections.

Identification of ACB complex

The identification of *Acinetobacter* spp. was done by standard laboratory procedure. After overnight incubation, typical non-fermenting colonies on MA were subcultures on NA and subjected to further processing via Gram staining and other biochemical tests. ACB complex was identified on the basis of various characteristics such as positive catalase test, negative oxidase test, non-motile, indole negative, citrate positive, urease variable, Alk/Alk $H_2S^-G^-$ in Triple Sugar Iron Agar (TSIA) medium, oxidative in Hugh and Leifson's medium and its ability to grow at 37° C and 41°C (Peleg et al 2008).

Antibiotic susceptibility testing

All ACB complex isolates were subjected to antibiotic susceptibility testing by employing the modified Kirby Bauer disk diffusion technique as per the Clinical and Laboratory Standards Institute (Version M100-S24) guidelines (CLSI 2014). In brief, bacterial cultures were grown overnight on nutrient agar and an inoculum was prepared in sterile water to a turbidity of a 0.5 McFarland Standard. The suspension was inoculated onto Mueller Hinton agar as per CLSI methodology. The antibiotic discs were placed onto the lawn and incubated at 37°C for 24 hours. The antibiotics used were: Ceftazidime (30µg), cefepime (30µg), gentamicin (10µg), amikacin (30µg), ciprofloxacin (5µg), aztreonam (30µg), meropenem (10µg), imipenem (10µg), piperacillin (100µg), piperacillin-tazobactam (100/10µg), and polymyxin B (300U) from the Hi-media laboratories limited, India. Following incubation, the diameters of the zones of inhibition for each antibiotic disc were measured and classified as resistant, susceptible or intermediately susceptible. *P. aeruginosa* ATCC 27853 was used as susceptible control. MDR ACB complex was defined as resistance to at least 3 of the following antimicrobial groups: cephalosporins (only ceftazidime or cefepime), aminoglycosides,fluoroquinolones, carbapenems, and piperacillins (Falagas et al 2006).

Biofilm detection by (Tube and Microtitre plate culture method)

This qualitative assay for biofilm formation was performed according to the method described by Christensen et al (1982). Borosilicate glass tubes containing 3 mL of tryptone soy broth (TSB) with 1% glucose were inoculated with a loopful of a pure culture of ACB complex grown overnight from nutrient agar plate. Tubes containing only tryptone soy broth (TSB) with 1% glucose were inoculated in the test as negative controls whereas tube containing *A. baumanni* ATCC 19606 as positive control. After 24 hours of incubation at 37°C, the content of each tube was decanted and then washed with phosphate buffer saline (PBS) (pH 7.3) for 2-3 times. The washed tubes were then stained with 0.1% crystal violet for 15 minutes. Then the tubes were again washed with de-ionized water to remove the excess stain and dried at 37°C for half to 1 hour. A positive result was indicated by the presence of an adherent film of stained material on the inner surface of the tube. Tubes were examined and the amount of biofilm formation were scored as 0- absent, 1-weak, 2-moderate and 3-strong. Experiment was performed in triplicate and repeated three times (Abdi-Ali et al 2014; Hassan et al 2011).

This quantitative test was performed as described by Christensen et al 1985). A loopful of test organism isolated from fresh agar plates were inoculated in 10ml of tryptone soya broth (TSB) with 1% glucose. Broths were incubated at 37° for 24 hours which were then diluted 1:100 with fresh TSB. Individuals well of sterile 96 well microtitre plate were filled with 200µl of diluted cultures broths. TSB with 1% glucose was used as the negative control and *A. baumanni* ATCC 19606 as a positive control. The plate was then incubated at 37° for 24 hours. After the incubation, contents of each well were removed by gentle tapping. The wells were then washed with 0.2 ml PBS (pH 7.3) four times to remove the free-floating bacteria. Biofilm formed by the bacteria adherent to the wells were fixed by 2% sodium acetate and then stained by 100µl of 0.1% crystal violet for 15 minutes at room temperature. Excess stained was removed by washing the plate with de-ionized water and biofilm was quantified by measuring the corresponding $OD_{630\ nm}$ of the supernatant following the solubilization of CV in 95% ethanol (Sanchez et al 2013). The experiment was performed in triplicate and repeated three times. The interpretation of biofilm production was done according to the criteria of Stepanovic et al (2000).

Interpretation of biofilm production (Stepanovic et al 2000)

OD value	Biofilm production
OD ≤ ODc/ODc< OD ≤2× ODC	Non/Weak
2×ODc< OD≤4×Odc	Moderate
4×ODc<OD	Strong

Quality control

Mueller Hinton agar and the antibiotic discs were checked for their lot number, manufacture and expiry date, and proper storage. For the standardization of Kirby-Bauer test and for performance testing of antibiotics and MHA, control strains of *P. aeruginosa* ATCC 27853 was tested primarily. Quality of the test plates were determined by incubating plates for 48 hours at 37°. Only those plates which showed no growth were utilized.

Data analysis

All the data obtained were analyzed using statistical programmed SPSS (Version 21.0).

Results

A total of 1150 different clinical specimens, 250 showed significant growth (21.7%), 145 (21%) from male and 105(22.8%) from female showed significant growth.

Table 1: Bacterial isolates in different age groups and sex

Age group	Male	Female	Significant growth	
			Male (%)	Female (%)
≤10	13	7	2	1
11-20	52	30	6	4
21-30	55	45	5	5
31-40	74	70	12	13
41-50	168	106	35	27
51-60	222	140	56	40
61-70	100	60	25	15
>70	6	2	4	
Total	**690**	**460**	**145(21)**	**105(22.8)**

A total of 258 bacterial isolates were conformed from 250 growth positive cases. Of 258 total isolates, Gram-negative bacteria were predominant constituting 204 (79%). Overall prevalence of ACB complex among total growth positive isolates were found to be 45 (17.5%).

Table 2: Frequency of bacterial isolates

Bacterial spp	Frequency	Percent
Escherichia coli	72	28
ABC complex	45	17.5
Klebsiella spp.	40	15.5
Pseudomonas aeruginosa	15	5.8
Proteus mirabilis	15	5.8
Proteus vulgaris	8	3
Citrobacter freundii	6	2.3
Enterobacter spp.	3	1.2
Staphylococcus aureus	40	15.5
Coagulase Negative Staphyloccoci (CoNS)	8	3
Enterococcus spp.	6	2.3
Total	**258**	**100**

Out of 45 isolates maximum number of ACB complex were isolated from sputum 16 (35.6%), followed by urine 11 (24.4%), pus 8 (17.8%), wound swab 6 (13.3%) and others 4 (8.9%) (2 from catheter tip, 1 from blood and 1 from pleural fluid) (Figure 1).

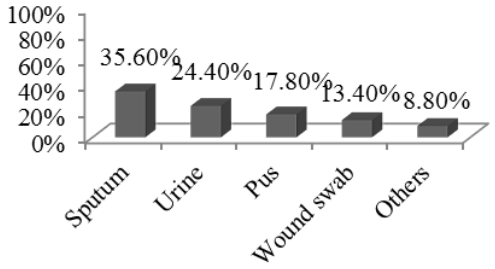

Figure 2: Distribution of ACB complex among different clinical specimens

Out of total 45 ACB complex isolates, 24 (53.3%) were obtained from ICU patients, 29 (40.3%) from general ward patients, and 4 (5.6%) from OPD patients (Figure 2).

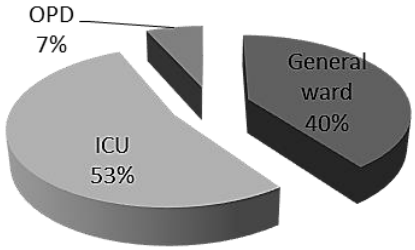

Figure 3: Distribution of ACB complex at different hospital sites

Out of 12 antibiotics tested, ACB complex was found to be more resistant to ceftazidime 36 (80%) and piperacillin 36 (80%) followed by ciprofloxacin (68.8%) and cefotaxime (66.6%) amikacin (57.8%) and tigecycline (24.5%). Colistin was found to be the most effective antibiotic with the susceptibility of 97.7% and followed by polymixin B most effective (100%) (Table 3).

Table 3: Antibiotic resistance pattern of ACB complex

Antibiotic tested	Resistant (%)	Susceptible (%)
Amikacin	26(57.8)	19(42.8)
Ampicillin/Sulbactum	26(57.8)	19(42.8)
Cefotaxime	30(66.6)	15(33.4)
Ceftazidime	36 (80)	9(20.0)
Ciprofloxacin	31 (68.8)	14(31.2)
Colistin	1 (2.3)	44(97.7)
Imipenem	21 (46.7)	24(53.3)
Meropenem	21 (46.7)	24(53.3)
Piperacillin	36 (80)	9(20.0)
Piperacillin/Tazobactam	31 (68.8)	14(31.2)
Polymixin B	0	45(100)
Tigecycline	11 (24.5)	34(75.5)

Out of 45 isolates of ACB complex, 32 (71.2%) were found to be MDR (Figure 3)

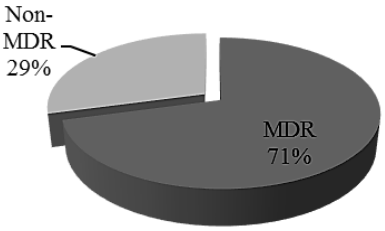

Figure 4: Prevalence of MDR ACB complex

Out of 45 isolates of ACB complex, both methods detected 2 isolates as strong biofilm producers and 18 isolates as biofilm non-producers. Out of 11 moderate biofilm producers detected by tube culture method, 8 isolates were detected as moderate and 3 isolates as weak biofilm producers by microtitre plate culture method and among 14 weak biofilm producers detected by tube culture method, 10 isolates were detected as weak biofilm producers and 4 isolates as biofilm non-producers by microtitre plate culture method. Biofilm production detected by both the methods 60% (n=27) were considered as positive (Table 4).

Table 4: Biofilm detection by microtitre plate culture method and tube culture method

		Microtitre plate culture method (MPC)				
		Strong	**Moderate**	**weak**	**Negative**	**Total**
Tube culture method (TCM)	Strong	2	0	0	0	**2 (4.5%)**
	Moderate	0	8	3	0	**11(24.5 %)**
	Weak	0	0	10	4	**14 (31%)**
	Negative	0	0	0	18	**18 (40%)**
	Total	**2 (4.5%)**	**8 (17.8%)**	**13 (28.9%)**	**22 (48.9%)**	**45 (100%)**

Interpretation of biofilm production for microtitre plate culture method

Based on cut-off optical density (ODc) the interpretation was done according to the criteria of Stepanovic et al.

Non-producer: $OD \leq 0.2867$

Weak: $0.2867 < OD \leq 0.5733$

Moderate $0.5733 < OD \leq 1.144$

Strong: $1.144 < OD$

Discussion

The prevalence of ACB complex in this study was found to be 17.5% among total growth positive isolates. This prevalence rate is relatively higher than that reported in study conducted at BPKIHS and KMC of Nepal, where the prevalence of *Acinetobacter* spp. was 19.4% and 13.4% respectively (Bhandari 2012; Ghimire et al 2002).

A study by Shrestha and Khanal (2010) showed the isolation of *Acinetobacter* were highest from blood sample, followed by urine, device, pus, wound swab, sputum, high vaginal swab, cerebrospinal fluid, CAPD fluid and throat swab. In another study by Ghimire et al (2002) at BPKIHS, maximum isolates were from pus, followed by ET tube. The variable prevalence of *Acinetobacter* spp in different studies might be due to difference in geography, arrangement of specimens in the study groups, condition, use of antibiotic and sanitary conditions, etc.

In present study, maximum numbers of isolates were from ICU (53.3%) patients. This finding was supported by other studies (Van et al 2014; Gaynes and Edwards 2005).

In this study, among the 27 biofilm positive isolates, majority of isolates were isolated from sputum (40.7%) followed by urine (25.9%) specimens, as similar to other study (Babapour et al 2016).

Biofilms increases desiccation tolerance and may confer antibiotic resistance. Biofilm formation is suspected of being one of the key pathogenic features of *A. baumannii*, particularly with device-related infections. This study has used microtitre plate culture method and tube culture method for the detection of biofilm producing strains. Rodrıguez-Banòo et al (2008) reported 63% biofilm positive isolates, which was comparable with this study conducted by this similar methodology. Similar results have been reported from Nepal, Dheepa et al (2011) (60%), and India (62%) (Rao et al 2008). However, Abdi-Ali et al (2014) has reported 22% biofilm positive isolates. The difference may be due to the difference in location and study methodology. What is common in all these studies is the observed intrinsic ability of this bacterium to form biofilms (Abdi-Ali et al 2014).

Also in this study, biofilm formers showed greater resistance to ceftazidime 25(92.6), piperacillin 25(92.6) and piperacillin/tazobactum 24(88.9) in compared to amikaci 21(77.8), ampicillin/sulbactum 20(74.1) and imipenem 18(66.7). Nehara et al (2013) from India, has reported 100% resistance to amoxicillin, ceftazidime and ceftriaxone in biofilm forming *Acinetobacter* species. In this study, isolates were highly sensetive to colistin and polymyxin B as similar to study reported by Evan et al 2008.

Conclusion

The results showed that most clinical isolates of *Acenetobacter* spp. have the ability to produce biofilms. This could potentially increase colonization of antibiotic-resistant bacteria in hospital environments. Increased hospital acquired infections can result in increased morbidity and mortality. Thus, a continuous monitoring of antibiotic susceptibility in *Acinetobacter* isolated from different clinical sources is necessary.

Acknowledgement

I gratefully acknowledge Mr Sunil lekhak Head of Department of Pathology, ANIAS Hospital, Kathmandu and Associated professor Mr. Binod Lekhak from Tribhuvan University for their valuable support during this research.

References

Babapour E, Haddadi A and Mirnejad R (2016). Biofilm formation in clinical isolates of nosocomial Acinetobacter baumanii and its relationship with multidrug resistance. Asian Pacific Journal of Tropical Biomedicine 6: 528-533.

Blossom DB and Srinivasan A (2008). Drug-Resistant *Acinetobacter baumannii-calcoaceticus* Complex. Infect Dis Clin Pract **16**: 1–3.

Chang HC, Dijkshoorn L, Vaneechoutte M, Tanq CT and Chanq TC (2005). Species-level identification of isolates of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex by sequence analysis of the 16S-23S rRNA gene spacer region. J. Clin. Microbiol **43**: 1632–1639.

Clinical and Laboratory Standards Institute (2014). Performance standards for

antimicrobial susceptibility testing; twenthy-fourth informational supplement. Wayne, PA: M100-S24.

Dijkshoorn L, Van Harsselaar B, Tjernberg I and Vaneechoutte (1998). Evaluation of amplified ribosomal DNA restriction analysis for identification of Acinetobacter genomic species. Syst Appl Microbiol **21**: 33-39

Evan ME, Feola DJ and Rapp RP (2008). Polymyxin B and Colistin: Old antibiotic for emerging multidrug resistant gram negative bacteria. Ann Pharmacother 3: 960-967.

Gaddy JA, Tomaras AP and Actis LA (2009). The *Acinetobacter baumannii* 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. Infect Immun **77**: 3150–3160.

Gaynes Rand Edwards JR (2005). Overview of nosocomial infections caused by gram-negative bacilli. Clin Infect Dis **41**: 848-854.

Ghimire G, Magar JKG, Bhattacharya S and Mahapatra TM (2002). Acinetobacter spp: A major isolates of nosocomial infections clinical significance and antimicrobial susceptibility. J Clin Antimicrobio **1**: 20-3.

Howard A, Michael O, Feeney A and Ray D (2012). *Acinetobacter baumanii*: An emerging opportunistic pathogen. **3**: 243-250.

Lee HW, Koh YM, Kim J, Lee JC, Lee YC and Seol SY (2008). Capacity of multidrug-resistant clinical isolates of *Acinetobacter baumannii* to form biofilm and adhere to epithelial cell surfaces. Clin Microbiol Infect **14**: 49–54.

Lewis K (2001). Riddle of biofilm resistance. Antimicrob Agents Chemother **45**: 999–1007

McQueary CN and Actis LA (2011). *Acinetobacter baumannii* biofilms: variations among strains and correlations with other cell properties. J Microbiol **49**: 243-250.

Nehara A, Anwar S and Ruhul M (2013). Association of Biofilm Formation with Antimicrobial Resistance Among the *Acinetobacter* species in a Tertiary Care Hospital in Bangladesh. J Medicine **14**: 28-32.

Peleg AY, Seifert H and Paterson DL (2008). *Acinetobacter baumannii*: emergence of a successful pathogen. Clin Microbiol Rev **21**:538–582.

Rao RS, Karthika RU, Singh SP, Shashikala P and Kanugo R (2008). Correlation between biofilm production and multiple drug resistsnce in imipenem resistsnt clinical isolates of *Acinetobacter baumanii*. J Med Microbiol **26**: 333-337.

Rungruanghiranya S, Somboonwit C and Kanchanapoom T (2005). *Acinetobacter* infection in the intensive care unit. J Infect Dis Antimicrob Agents **22**: 77–92.

Sanchez CJ, Mende K and Beckius ML (2013). Biofilm formation by clinical isolates and the implications in chronic infections. BMC Infectious Diseases 13: 47.

Shin JH, Lee HW, Kim SM and Kim J (2009). Proteomic analysis of *Acinetobacter baumannii* in biofilm and planktonic growth mode. J Microbiol **47**: 728–735.

Stepanovic S, Vukovic D, Dakic I and Savic B (2000). A modified microtiter plate test for quantification *Staphylococcal* biofilm formation. J Microbiol Methods **40**: 175-179.

Towner A (2006). The genus *Acinetobacter* The Prokaryotes **6**: 746–758.