Original Article

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Microbiology Section

Biofilm Formation and Colistin Susceptibility of Clinical Isolates of *Acinetobacter* Species in a Tertiary Care Hospital of Nepal

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ABSTRACT

Introduction: *Acinetobacter* species are a major cause of hospital acquired infections worldwide with remarkable level of resistance to various classes of antibiotics.

Aim: To evaluate the MIC of colistin against biofilm forming, Multi Drug Resistant (MDR) *Acinetobacter* species by E-test in a tertiary care hospital of Kathmandu.

Materials and Methods: Isolation and identification of *Acinetobacter* species was done by standard methods. Biofilms were developed using 96-well microtiter plates in Tryptic Soy Broth (TSB). Optical Density (OD) was measured at 570 nm after washing, fixation and staining. Antibiotic susceptibility test was performed by Kirby-Bauer disk diffusion method. Carbapenem resistance and Metallo B-Lactamase (MBL) production were tested by Modified Hodge Test (MHT) and Imipenem-EDTA combined disk method respectively. MIC was determined by E-test against colistin.

Results: Out of 573 bacterial isolates the number of *Acinetobacter* species was 73 (12.7%) and among them 72 (99%) were biofilm producers having significant relationship to multi drug resistance (p=0.01). All isolates were resistant to cephalosporins; 65 isolates (89%) were carbapenem resistant, 61 isolates (93.8%) gave positive MHT, 36 (56%) of total carbapenem resistant *Acinetobacter* isolates revealed positive for MBL, 72 (99%) of isolates were found sensitive to colistin by disc diffusion method whereas only 68 (93.1%) by MIC testing.

Conclusion: *Acinetobacter* clinical isolates have a strong ability to produce biofilm. Carbapenemases and MBL were also observed in this study. Only colistin and polymyxin B were effective against higher numbers of isolates, however, 5 (6.9%) of the isolates were found resistant as detected by MIC testing and indicated reduced susceptibility to colistin.

Keywords: Antibiotic susceptibility, Carbapenem resistance, Optical density

INTRODUCTION

Acinetobacter species are associated with consistently increasing rates of Healthcare Associated Infections (HAIs) in hospitalised patients [1-3]. Hospital acquired infections caused by *Acinetobacter* species are more common than community acquired infections. Common species associated with HAIs are *Acinetobacter baumannii*, *Acinetobacter pittii* and *Acinetobacter nosocomialis* [4].

Acinetobacter species are gram negative coccobacilli, with a great ability to acquire resistance to antibiotics. Thus, making it one of the more alarming pathogens today [5]. Risk factors for infections with MDR *Acinetobacter* species includes prolonged length of hospital stay, exposure to an Intensive Care Unit (ICU), receipt of mechanical ventilation, colonisation pressure, long term exposure to an antimicrobial agents, recent surgery, invasive procedures and underlying severity of illness [6,7].

Acinetobacter baumannii have shown a high degree of resistance to β-lactam antibiotics, fluoroquinolones, aminoglycosides and carbapenems. They have emerged as one of the most problematic pathogens to eradicate using available antibiotics [8]. Carbapenems are most commonly used antibiotics against MDR *Acinetobacter baumannii* infections. However, resistance to these agents is now a worldwide problem. Carbapenem resistance is due to a combination of different mechanisms. The most common is by carbapenemases mediated enzymatic hydrolysis [9-11].

Colistin and tigecycline are examples of last resort drugs used against carbapenem resistant *Acinetobacter* infections. However, resistance or reduced susceptibility to these agents has been reported recently in different countries, thus, making treatment of *Acinetobacter* infections extremely difficult [12].

The main objective of this study was to assess the in-vitro susceptibility of the biofilm producing MDR *Acinetobacter species* isolated from clinical samples to colistin.

MATERIALS AND METHODS

The descriptive type of study was carried from January 2016 to August 2016 in Norvic International Hospital; a tertiary referral Hospital in Kathmandu, Nepal. In the present study, already isolated bacteria from routine cultures were used. Any personal information from the patients was not included. No ethical clearance is applicable for this study. *Acinetobacter* species those were isolated from different samples such as endotracheal secretions, endotracheal tubes, blood, sputum, and pus during the study period. The isolation and identification of *Acinetobacter* species were done by standard microbiological procedures following the World Health Organisation (WHO) protocol [13]. After complete identification, isolates were preserved on TSB with 20% glycerol at -20ºC.

Biofilm production was determined in the laboratory using an overnight culture of *Acinetobacter* species in 200 µL TSB in 96-well microtiter plates and incubated at 37°C for 24 hours. Un-inoculated wells with TSB were used as negative controls. After incubation, wells were gently washed with normal saline to remove planktonic cells. Fixation of remaining biofilm was done by 2% sodium acetate and then stained with crystal violet (1% w/v) and quantified at 570 nm after solubilisation with ethanol (95%). Optical Density (OD) of each well and OD of the control (ODc) were measured by using 96 well ELISA readers (Multiskan EX, Thermo Electron Corporation, Waltham, MA). Results were interpreted following well established categories of biofilm formation as non-producer, weak-producer, moderate-producer and strong-producer [14].

Susceptibility testing of the clinical isolates of *Acinetobacter* species against several commercially available antibiotics discs (HiMedia Laboratories Pvt., Limited, India) was performed by Kirby-Bauer disk diffusion method on Mueller Hinton agar (MHA) following Clinical and Laboratory Standards Institute (CLSI) guidelines 2016 [15].

Phenotypic detection of carbapenemase activity was carried out following the Modified Hodge test (MHT) as described by Lee K et al., [16]. A MHA plate was inoculated with the indicator organism (*E. coli* ATCC 25922). An imipenem disk was placed at the centre of the plate and imipenem resistant *Acinetobacter* species isolates were streaked from the edge of the disk to the periphery of the plate. After 16 hours of incubation, an indentation forming clover leaf like appearance at the intersection of the test organism and indicator organism was interpreted as MHT positive. *Klebsiella pneumoniae* ATCC® BAA-1705™ was used as positive control and *Klebsiella pneumoniae* ATCC® BAA-1706™ was used as negative control.

Phenotypic detection of MBL was performed by combined disk method, Imipenem and Imipenem plus Ethylenediaminetetraacetic Acid (EDTA) as described by Yong D et al., [17]. For the analysis of MBL production, *Acinetobacter* species were incubated overnight at 37°C on a lawn culture of MHA plate with a disc containing only imipenem and another disc containing imipenem plus EDTA. The zone diameter was compared. The zone of clearance ≥7 mm produced by imipenem plus EDTA disc was considered as positive for MBL.

To determine the MIC of colistin against *Acinetobacter* species an E-test was performed following the manufacturer's instructions (HiMedia Laboratories Pvt., Limited, India) and another report [18] using strips impregnated with different concentration of colistin. The bacterial suspension, with a turbidity equivalent to 0.5 McFarland standards was prepared by suspending well-isolated colonies in 0.9% saline. The suspension was poured on a previously warmed, dry MHA (HiMedia Laboratories Pvt., Limited, India) plate. Excess liquid was aspirated using a sterile disposable pipette, after which the E-test strip (ranging from 0.06 to 1024 µg/mL of colistin was positioned and incubated for 16-18 hours at 37ºC. MIC values for colistin were interpreted according to manufacturer's instructions and CLSI guidelines 2016 [15].

STSTISTICAL ANALYSIS

Chi-square test was done to estimate the p-values applicable elsewhere; p-values ≤0.5 was considered as statistical significant.

RESULTS

Out of 573 bacterial isolates during the study period, 105 were Gram positive cocci and 468 were Gram negative bacilli, among Gram negative bacilli the number of *Acinetobacter* species were 73 (12.7%). The result of biofilm formation in the standard microtiter plate method showed that only one isolate did not produce a biofilm, a few isolates produced moderate and weak biofilms; however, the greatest number of isolates produced a biofilm strongly [Table/Fig-1]. There was a significant relationship between moderate to strong biofilm production and multi drug resistance (p=0.01).

Mean OD value	No. of isolates (%)	Biofilm formation
OD≤ODc	1 (1.4)	None
ODc<OD<2ODc	5 (6.8)	Weak
2ODc<OD<4ODc	2 (2.7)	Moderate
4ODc≤OD	65 (89)	Strong

[Table/Fig-1]: Classification of biofilm formation based on microtiter plate method. OD:Optical density

The antimicrobial susceptibility profiles of *Acinetobacter* species by modified disc diffusion method showed that all isolates were found resistant to the cephalosporin group [Table/Fig-2]. Highest susceptibility was observed against colistin and polymyxin B.

Antibiotics	No. of susceptible isolates (%)	No. of resistant isolates (%)
Colistin (10 µg)	72 (98.6)	1 (1.4)
Polymyxin B (300 units)	72 (98.6)	1 (1.4)
Minocycline (30 µg)	23 (31.5)	50 (68.5)
Tigecycline (15 µg)	20 (27.4)	53 (72.6)
Cefoperazone+sulbactam (75/30 µg)	15 (20.6)	58 (79.4)
Meropenem (10 µg)	12 (16.4)	61 (83.6)
Levofloxacin (5 µg)	12 (16.4)	61 (83.6)
Ciprofloxacin (5 µg)	11 (15.1)	62 (84.9)
Ofloxacin (5 µg)	10 (13.7)	63 (86.3)
Amikacin (30 µg)	10 (13.7)	63 (86.3)
Gentamycin (10 µg)	10 (13.7)	63 (86.3)
Cotrimoxazole (1.25/23.75 µg)	10 (13.7)	63 (86.3)
Piperacillin+tazobactam (100/10 µg)	9 (12.3)	64 (87.7)
Imipenem (10 µg)	8 (11)	65 (89)
Ceftazidime (30 µg)	0 (0)	73(100)
Ceftriaxone (30 µg)	0 (0)	73 (100)
Cefotaxime (30 µg)	0 (0)	73 (100)
Cefepime (30 µg)	0 (0)	73 (100)

[Table/Fig-2]: Antimicrobial susceptibility profiles of *Acinetobacter species*.

Among 65 carbapenem resistant isolates 61 (93.8%) gave positive MHT and were carbapenemase producers. There was a significant relationship between imipenem resistance and carbapenemase production (p=0.026) and between meropenem resistance and carbapenemase production (p=0.008). These carbapenemase producing isolates were also tested for MBL production, 56% of total carbapenem resistant *Acinetobacter* species isolates revealed a positive for MBL test. However, there was no statistically significant relationship between carbapenem and colistin susceptibility among *Acinetobacter* isolates (p>0.05). Since, the available antibiotics were tested by disc diffusion method which indicated several MDR (not presented on the table), Extensively Drug-Resistant (XDR) and Pan Drug-Resistant (PDR) as per the established classification system [19]. On other hand, there was a significant relationship between moderate to strong biofilm formation by MDR, XDR and PDR strains (p=0.01).

By disc diffusion method, 98.6% of the isolates were found susceptible to colistin, however, only 93.1% strains were found susceptible in the MIC results by the E-test. This result can be interpreted as evidence for a reduced level of susceptibility against colistin by *Acinetobacter* clinical isolates by this method [Table/Fig-3].

Antibiotic	Break point (µ/mL)	Resistant No. (%)	Sensitive No. (%)
Colistin	Sensitive ≤2 Resistant ≥4	5 (6.9)	68 (93.1)

[Table/Fig-3]: MIC result of colistin by the E-test method.

DISCUSSION

In this study the prevalence of *Acinetobacter* species among total bacterial isolates was 12.7% and among total gram negative bacilli was 15.5%. Similar prevalence was also reported by other studies from Nepal [20-22] and *Acinetobacter* was the third most commonly isolated gram negative bacilli after *E. coli* and *Klebsiella* species.

There was significant relationship between biofilm formation and antibiotic resistance (MDR) indicating serious threat of hospital acquired infections. The result is in accordance with another study carried in Iran [13]. To the best of our knowledge, this is the first study in Nepal that correlates the biofilm formation and MDR of *Acinetobacter* species with special reference to MIC against colistin by the E-test.

The antimicrobial susceptibility profile of *Acinetobacter* species from this study found that all isolates were resistant to third and fourth generation cephalosporins. Similar resistance (100%) to cephalosporins was reported by Joshi PR et al., from a tertiary hospital in Nepal [23]. This indicated that cephalosporins are no longer an effective treatment for *Acinetobacter* infections. This higher cephalosporin resistance among these organisms might be due to hyper production of ESBL [24]. The resistance to imipenem and meropenem was 89% and 83.6% respectively. Similar resistance to imipenem and to meropenem was also reported by other authors [25-27]. A study from Turkey in 2014 by Gundeslioglu OO et al., showed 91.3% carbapenem resistance among *Acinetobacter* isolates [28]. Rolain JM et al., showed 100% resistance to imipenem and meropenem [29]. However, lower carbapenem resistance (47.3%) of *Acinetobacter* species was shown by another study [22] from Nepal. Carbapenems group of antibiotics were very effective to treat *Acinetobacter* infections caused by MDR strains. However, the present result indicated that these drugs can be recommended if they are found effective by antibiotic susceptibility testing. Numerous mechanisms, including decreased permeability, efflux pump over expression and carbapenemase production, can be responsible for the resistance to carbapenems. Class B (IMP and VIM enzymes) and D (oxacillinases) β-lactamases are the most important group of enzymes able to hydrolyze carbapenems [30]. Resistance to carbapenems among these isolates left no options for treatment except colistin and polymyxin B, which have many adverse effects, including nephrotoxicity [29].

The emergence of *Acinetobacter* resistance against colistin has been reported by other studies [31,32]. However, this report indicates that colistin is still an option for the treatment of infections caused by *Acinetobacter* species.

All carbapenem resistant strains were tested for carbapenemase production and 93.8% of these gave a positive result by MHT. MBL is one of the most important enzymes responsible for carbapenem resistance in *Acinetobacter* species, among them, 60.7% of MHT positive isolates gave positive result for MBL production by combined disk method. A study conducted by Khanal S et al., observed that 54.5% of *Acinetobacter* species were MBL producers [33]. MBL producing bacteria are an increasingly public health problem worldwide with an increased mortality rate.

In the present study, an E-test for the determination of MIC of colistin against *Acinetobacter* isolates was performed, 6.9% isolates were resistant to colistin with an MIC value ≥4 µg/mL. More than 98% sensitivity to colistin was reported by another study [34]. The E-test is very simple and it greatly reduces the time for MIC testing, authors [35,36] who evaluated the sensitivity and specificity of E-test have recommended this technique to assess MIC in routine clinical setting. The conventional multiple dilution method is a time consuming and not conductive to routine application, whereas the E-test can be easily applied to obtain MIC values of any antibiotics in a routine clinical laboratory setting.

Present results show a high tendency of drug resistance in *Acinetobacter* species isolated in clinical samples. A continuous antibiotics stewardship program in health care settings is mandatory to control this alarming trend.

LIMITATION

Due to lacking molecular based platform and other newer approaches this study limited to characterise the *Acinetobacter* isolates only up to species level. The analysis of plasmids responsible for MDR in *Acinetobacter* clinical isolates for further studies is recommended.

CONCLUSION

High amount of biofilm and MBL production among MDR *Acinetobacter* isolates was observed in this study. Colistin and polymyxin B were effective against a greater number of isolates, however, 6.9% of the isolates were found resistant to colistin by MIC testing which indicated a reduced susceptibility to colistin by this method. Colistin and polymyxin B are potentially toxic compounds and have limited use in severely ill patients. Furthermore, there is urgent need for alternative treatment options against MDR Acinetobacter. Continuous antibiotics stewardships and proper infective control measures in the hospitals are extremely important to control the nosocomial infections caused by this pathogen.

Author Contributions

BRT conceived and designed the project. BRK and SW performed the experiments as guided by BRT. BRT reviewed the relevant literatures and drafted the manuscript. BRT prepared the final manuscript for submission. All authors read the final manuscript and provided their approval.

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