



Assessment of Biofilm Production in Carbapenem Resistant Acinetobacter Species Isolated from Different Clinical Specimens

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Abstract

Introduction: Microbial biofilms are important virulence factors contributing not only to the severity of the disease process but also to the antibiotic resistance. Acinetobacter species, particularly Acinetobacter baumannii is an important pathogen causing nosocomial outbreaks of infection in healthcare settings. Herein, we evaluated the biofilm forming ability of Carbapenem resistant Acinetobacter isolated from various clinical specimens.

Methods: A total of 109 phenotypically identified Acinetobacter isolates from different clinical specimens were studied, in which, Carbapenem resistance was determined as per the CLSI guidelines. Those isolates were then quantitatively assessed for their biofilm forming ability using the Microtitre Plate Assay.

Results: Of the 109 Acinetobacter isolates studied, 64.2% were Carbapenem resistant. Among them, 77.1% were biofilm formers. There was significant association seen between Carbapenem resistance and biofilm formation ($p = 0.024$). Odds ratio calculated was 2.6.

Conclusion: Our study showed that among the Carbapenem resistant strains of Acinetobacter, a significant number were biofilm producers. Further genetic analysis may provide a better understanding of the virulence, multidrug resistance and survival of the bacteria in the hospital environment.

Keywords: Acinetobacter, Carbapenem resistant, Modified Hodge Test, Combined Disc Test, Microtitre Plate Assay, Biofilm.

Introduction

Acinetobacter is a heterogeneous group of Gram negative bacilli and has recently become the focus of the clinicians worldwide due to its pathogenic potential¹. They are commonly isolated from the hospital environment and from colonised or infected individuals². Their environmental

resilience and the wide range of resistance determinants make them successful nosocomial pathogens³. Not only do they cause severe infections but also show resistance to major antibiotic classes⁴.

Most of the isolates are resistant to broad-spectrum Cephalosporins, other β -lactam agents,

Aminoglycosides, Quinolones and the Carbapenems⁵. Carbapenem resistance is associated with high mortality, primarily due to delays in administration of effective treatment and the limited availability of effective treatment options, especially in the resource poor settings in the developing nations. Also, the Carbapenem resistant strains are adapted to spread rapidly in healthcare settings as well as in the community⁶.

Acinetobacter frequently causes infections associated with medical devices like the endotracheal tube, central venous catheters, Foley’s catheter, etc. Biofilm formation is a well-known virulence factor in such infections. Also, their environmental survival may be facilitated by biofilm formation on abiotic surface⁷. Two properties are often associated with biofilm producing bacteria, namely, the increased synthesis of exopolysaccharide (EPS) and the development of antibiotic resistance. It can be assumed that increased production of EPS in Acinetobacter might be creating a protective environment leading to poor antibiotic penetration which in turn leads to the development of resistance. Also, there appears to be some differences in the cellular physiology of cells within the biofilm that may also result in increased resistance to the drug⁸.

In this study we attempt to evaluate the association of biofilm production and Carbapenem resistance in Acinetobacter species isolated from various clinical specimens.

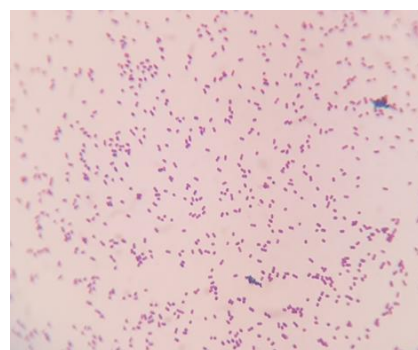
Materials and Methods

Bacterial Strains

109 strains of Acinetobacter were isolated from different samples like sputum, endotracheal aspirate, wound swab, blood, urine etc. Isolates were identified as Acinetobacter based on Gram stain, colony morphology on the MacConkey’s agar, growth at 42°C, catalase, oxidase, nitrate, citrate, arginine dihydrolase, malonate utilisation and Hugh Leifson’s oxidation – fermentation tests.



Picture 1 – Acinetobacter Colonies on the Mac Conkey’s Agar



Picture 2 – Gram Stain Showing Gram Negative Coccobacilli

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by Kirby Bauer disk diffusion method using routine drugs including Imipenem and Meropenem as per CLSI guidelines⁹.

Isolates resistant to Imipenem and Meropenem or any one of them was further tested with Meropenem E (Epsilometric) strips. Results were interpreted by the zone of inhibition in the form of ellipse.

Table 1: MIC Interpretation for Imipenem and Meropenem

Antibiotic	Sensitive (mcg/ml)	Intermediate (mcg/ml)	Resistance (mcg/ml)
Imipenem	<= 4	8	>= 16
Meropenem	<= 4	8	>= 16

The resistant isolates were further screened for Carbapenemase and Metallo beta lactamase (MBL) production by modified Hodge test (MHT) and Meropenem – EDTA Combined Disc Test, respectively¹⁰.

Modified Hodge Test

Two to three identical colonies of *Escherichia coli* (ATCC 25922) were inoculated into saline and incubated at 37°C for 4-6 hours to obtain optical density matching that of 0.5 McFarland turbidity standards. This suspension of the test organism was then diluted 1:10, by adding 0.5ml of 0.5 McFarland to 4.5 ml of the test suspension. A lawn culture of 1:10 diluted *E. coli* ATCC 25922 was done on the Mueller Hinton Agar (MHA) plates with a sterile cotton swab. The plate was allowed to stand for a period of 5 minutes at room temperature. A 10 mcg Meropenem disc was placed at the centre and the test organism was streaked in a straight line from the edge of the disc to the edge of the plate. The plate was incubated at 35°C \pm 2°C in ambient air for 16-22 hours. The presence of distorted zone of inhibition or clover leaf type of indentation at the intersection of the test organism and *E. coli* ATCC 25922, within the zone of Meropenem susceptibility disc was interpreted as positive result¹¹.

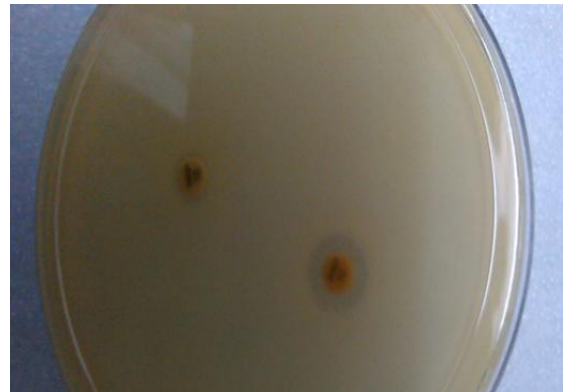


Picture 3 – Modified Hodge Test

Meropenem-EDTA Combined Disc Test (CDT)

0.5 M EDTA solution was prepared by dissolving 18.61 gram of disodium EDTA.2H₂O in 100 ml of distilled water and its pH was adjusted to 8. The mixture was then sterilized by autoclaving. 10 μ l EDTA solution was poured on 10mcg Meropenem disc. Two to three identical colonies of test organism were inoculated in the nutrient broth and incubated at 37°C for 4-6 hours. The turbidity was adjusted to 0.5 McFarland. Lawn culture of this suspension of test organism was done on Muller Hinton Agar (MHA) plate with a

sterile cotton swab. One 10 μ g meropenem disc was placed on MHA plate. EDTA impregnated meropenem disc was also placed on the same MHA plate at the distance of 20-25 mm from centre to centre. The plate was incubated at 37°C for 16-18 hours. An increase in zone size of \geq 7mm around the Meropenem – EDTA disc compared to Meropenem without EDTA was recorded as an MBL producing strain¹².



Picture 4 – Meropenem – EDTA Combined Disc Test

Detection of biofilm production (Microtiter plate assay)

Each isolate was grown overnight in trypticase soy broth (TSB) with 0.25% glucose at 37°C. The overnight growth was diluted in a ratio of 1:40 in TSB-0.25 % glucose. Two hundred microlitre of cell suspension was inoculated in sterile 96 well polystyrene microtitre plates. After 24 hours of incubation, the wells were gently washed three times with 200 microlitre of phosphate buffered saline (PBS), dried in an inverted position and stained with 1% crystal violet for 15 min. The wells were rinsed again in 200 microlitre of ethanol-acetone (80:20 v/v) to solubilise crystal violet. The optical density at 620 nm (OD 620) was determined using microplate reader. Each assay was performed in triplicate and the average optical density was considered.

The following values were assigned for biofilm determination:

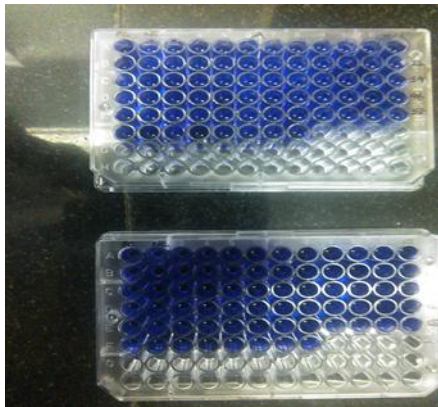
Non-biofilm producer: OD₆₂₀ < 0.248(OD_c)

Weak biofilm producer: 0.248(OD_c) \leq OD 620 < 0.496(2OD_c)

Medium biofilm producer: $0.496(2ODc) \leq OD620 < 0.744(3ODc)$

Strong biofilm producer: $0.744(3ODc) \leq OD620$

The value 0.248 was chosen as the baseline because it was three standard deviations above the mean OD of a clean microtitre plate stained by the above method¹³.



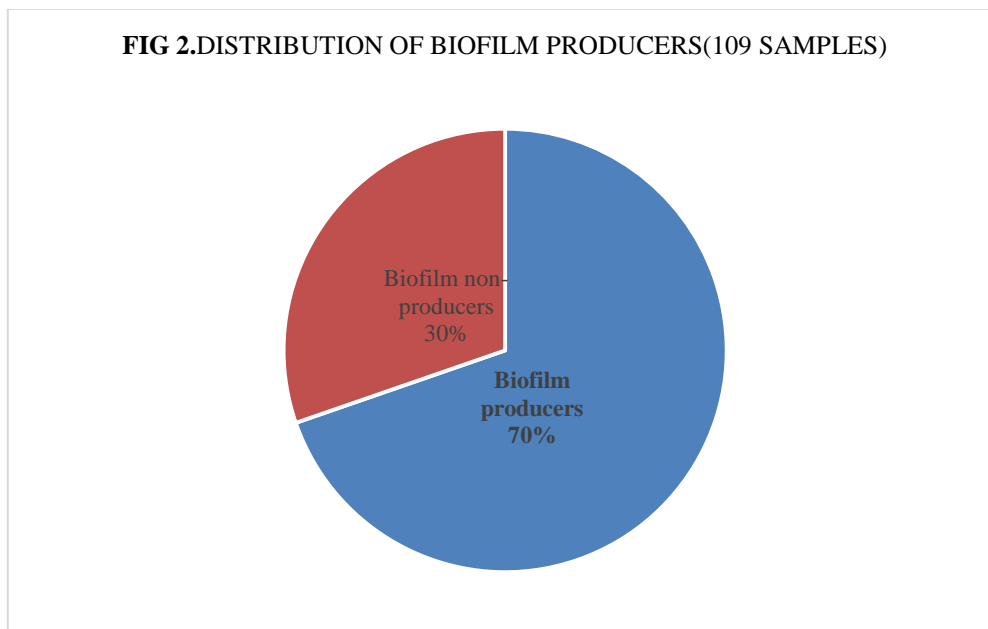
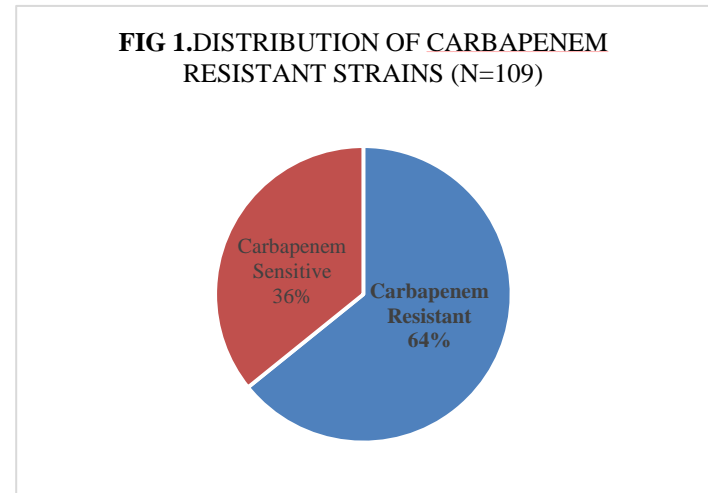
Picture 5 – Microtitre Plate Assay For Biofilm Detection

Statistical Analysis

Using SPSS 16 the association between biofilm formation and Carbapenem resistance was analysed with the help of Chi Square Test.

Results

Of the 109 Acinetobacter isolates studied, 93.8% were Acinetobacter calcoaceticus baumannii complex. 64.2% were Carbapenem resistant and 69.7% of the 109 isolates were biofilm producers.



Among the Carbapenem resistant strains, 77.1% were biofilm formers.

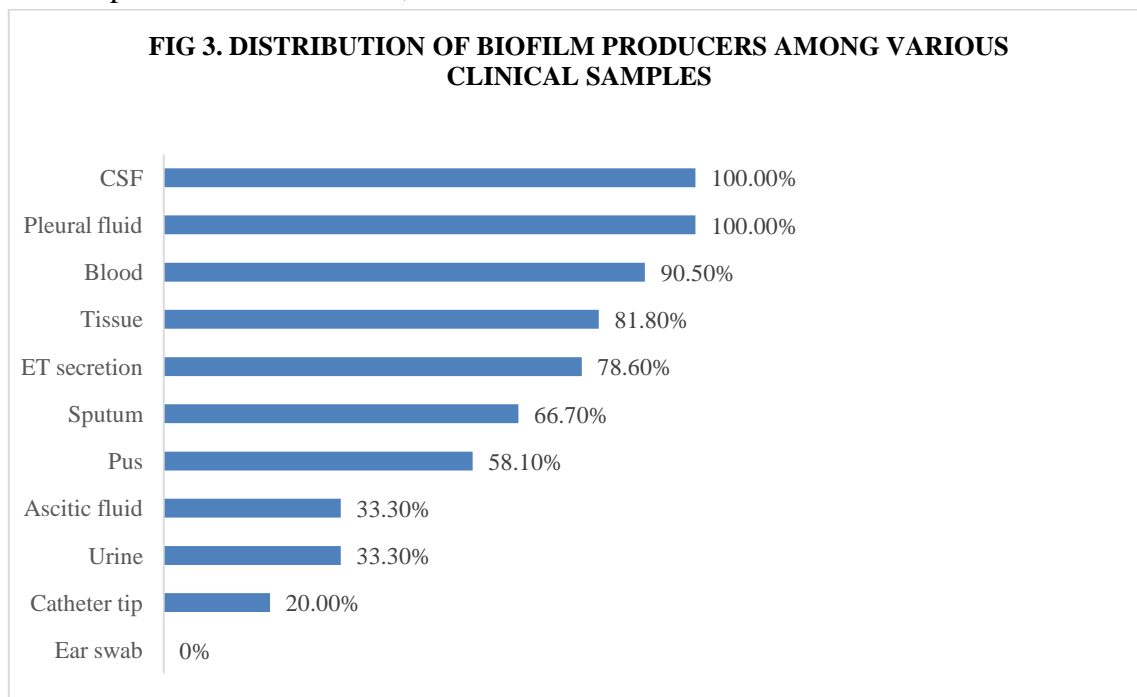
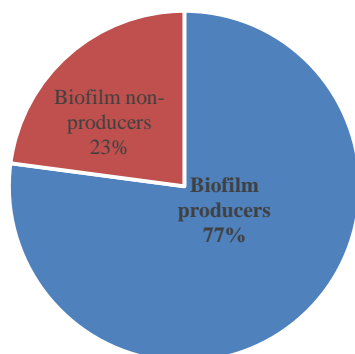


FIG 4. DISTRIBUTION OF BIOFILM PRODUCERS AMONG THE CARBAPENEM RESISTANT STRAINS



There was a significant association seen between Carbapenem resistance and biofilm formation ($p = 0.024$). Odds ratio calculated was 2.6

Discussion

In our study, the species most frequently isolated was *Acinetobacter calcoaceticus baumannii* complex (93.8%) following which was *Acinetobacter lwoffii*. Predominance of *A. baumannii* (90.6%) was reported by Raina et al¹⁴. Singla et al¹⁵ have reported an isolation rate of 74.6% for *A. baumannii* in clinical samples. 64.2%

of the isolates in our study were carbapenem resistant. Carbapenem resistance is emerging as a huge threat not only in ICUs but also in the wards. In a similar study by Shareek et al¹⁶, 75% of the *Acinetobacter* strains were resistant to Carbapenems.

Carbapenems are generally the last resort in the treatment of life threatening infections caused by multidrug resistant *Acinetobacter* isolates. Emergence of Carbapenem hydrolysing β -lactamases of Ambler class B (MBLs) and class D (Oxacillinases/CHDLs) have been proven to be the most important mechanism of carbapenem resistance and thus have caused difficulty in the treatment. Simple and accurate tests are needed to detect MBL producers. Meropenem-EDTA combined disc test and Modified Hodge test have been used in this study for MBL detection. Though CLSI does not advocate the use of MHT for detection of Carbapenemase production in non-fermenting gram negative bacilli, several authors have found MHT with Imipenem, EDTA and ZnSO₄ as a useful screening test for Carbapenemase production^{17, 18}.

Screening for carbapenem resistance and detecting carbapenemase and MBL producers among *Acinetobacter* isolates in resource limited setting

helps to avoid unnecessary use of broad spectrum antibiotics and thereby prevent treatment failures and development of resistance. The global spread of multidrug resistant *Acinetobacter* spp. is a major challenge in the clinical setting. Drugs such as Colistin, Polymyxin B, Tigecycline and Doripenem, are being tried for treating such infections¹⁹.

Biofilm production was assessed in all the 109 isolates using the microtitre plate method, which is considered the gold standard, 70% of the isolates were biofilm producers. In a similar study by Bala et al²⁰, 62.5% were biofilm producers among the clinical *Acinetobacter* isolates screened. Similar occurrence of 63% and 62% biofilm formers have also been reported by Rodriguez et al²¹, and Rao et al⁸, respectively.

There was a significant association seen between Carbapenem resistance and biofilm formation ($p = 0.024$). This was in concordance with studies conducted by Abdi et al²² and Rao et al⁸. Biofilms on surfaces result in decreased penetrability of antibiotics and makes managing infections a clinical challenge. In a similar study, Rao et al⁸ and Rong et al²³, reported a significant association between multidrug resistance and biofilm. The study by Rao et al⁸ showed that the presence of blaPER-1 was more critical for cell adhesion than the formation of bacterial biofilms on abiotic surfaces.

Further research should concentrate on the genetic and molecular mechanisms associated with the formation of biofilm. Understanding biofilm formation and the genetic basis for control of this process is required to develop new strategies for dealing with infections caused by these opportunistic and often multi-drug resistant nosocomial pathogens²⁴. Novel treatment strategies such as phage therapy, quorum-sensing inhibition, and induced biofilm-dispersion have been worked upon²⁵.

Acknowledgement

Principal and Dean, RMC, Bangalore; Professor and Head of Department of Microbiology, RMC, Bangalore

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