Evaluation of Biofilm Production in *Acinetobacter baumannii* with Reference to Imipenem Resistance

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***Abstract*- Background**

Acinetobacter baumannii has emerged as an important and problematic nosocomial pathogen. It is responsile for most of infections associated with medical devices, e.g. vascular catheters, cerebrospinal fluid shunts or Foley catheters. It causes several types of infections including pneumonia, meningitis, septicemia, and urinary tract infections. Carbapenems are the drug of choice for treatment but now resistance to these group of drugs is spreading. Mechanism of resistance may be due to production of carbapenemase enzyme or due to biofilm production.

Aims: The study aims to detect and examine the correlation between biofilm production and imipenem resistance among the clinical isolates of *Acinetobacter baumannii* and also to detect inhibitory effects of EDTA on biofilm production.

Materials and Methods:

A prospective cross-sectional study was conducted to detect imipenem resistance and biofilm production from various clinical samples. A total of 130 clinical isolates of *A. baumannii* from various clinical specimens like pus, tracheal aspirate, sputum, wound swab, urine, blood, endotracheal tip, etc. were screened for imipenem resistance by Kirby Bauer disk diffusion method and further confirmed by agar dilution method according to CLSI guidlines 2015. Bioflim production was done by microtitter plate method and bioflim inhibition was further done by microtittre plate method using ethylenediaminetetraacetic acid (EDTA).

Results and Conclusion

In our study imipenem resistance was 47.69% by agar dilution method and biofilm production was seen among 88 isolates out of 130 isolates. Most of imipenem resistant strains were biofilm producers. There was strong association between biofilm production and imipenem resistance (p value: 0.000005 at $p<0.05$).55-75% reduction of biofilm was seen by using EDTA.

***Index Terms*-** bioflim, imipenem, EDTA

I. Introduction

Acinetobacter are ubiquitous, Gram-negative bacteria found in wide range of sources such as soil, water, food products and medical environments (Bergogne-Berenzin&Towner, 1996). *Acinetobacter baumannii* is sophisticated nosocomial weapon to jeopardize health care settings of 21st century.[1] It occupies second position after *Pseudomonas aeruginosa* for being most common nosocomial, aerobic, non-fermentative, Gram negative bacilli pathogen.[2] Infection caused by *A. baumannii* is hard to treat since it can acquire resistance to multiple antimicrobial agents. [3] Someof the challenges in the prevention and treatment of theinfections caused by this opportunistic pathogen are its remarkable widespread resistance to different antibiotics and its ability to persist in nosocomial environments and medical devices. [1]

Carbapenems are often used as a last resort for the treatment of infections due to multiresistant *Acinetobacter baumannii* isolates. However, acinetobacters may develop resistance to carbapenems through various combined mechanisms, including AmpC stable derepression, decreased permeability, altered penicillin-binding proteins (PBPs) and, rarely, efflux pump overexpression.[4]

Costerton et al. define biofilm as “a structured community of bacterial cells enclosed in a self-produced polymeric matrix adherent to an inert or living surface.”[5] Organisms producing biofilms are highly resistant to antimicrobial drugs than non-biofilm producing organisms. In some extreme cases, the concentrations of antimicrobials required to achieve bactericidal activity against adherent organisms can be three- to four-fold higher than for those bacteria which do not produce biofilm, depending on the species and drug combination.[6]

Mechanisms responsible for antimicrobial resistance in organisms producing biofilms may be delayed penetration of the antimicrobial agents through the biofilm matrix, altered growth rate of biofilm organisms, and other physiological changes due to the biofilm mode of growth.[7]

In addition, due to the emergence of multidrug-resistant (MDR) and pan-resistant strains of this species; there is rapid growth of interest in *A. baumannii*. [8-10].

Therefore, this very study was done to determine the frequency of biofilm formation by different methods and correlate biofilm formation with development of multiple antibiotic resistances among clinical isolates of *A. baumannii.*Furthermore the study also investigated about the inhibitory effects of chelating agent like EDTA on biofilm production.

II. Materials and Methods

The present study was conducted in department of Microbiology, SRM Medical Hospital College and Research Center, Potheri, Kattankulathur for one year and 2 months duration period from Dec 2013 to April 2015. A total of 130 isolates of *A. baumannii* from various clinical specimens like pus, tracheal aspirate, sputum, wound swab, urine, blood, endotracheal tip, etc. were included in this study. During this period all isolates of genus Acinetobacter satisfying the criteria being oxidative in oxidative-fermentative test, production of acid

from lactose 1% and 10%, growth at 31^0C, 41^0C and 44^0C included in the study.

Test for Imipenem Resistance:

Kirby Bauer Disk diffusion method was used for testing the isolates for imipenem (10 µg) resistance along with other antibiotics. Interpretation of antimicrobial susceptibility testing by disc diffusion test was done as per Clinical Laboratory and Standard Institute (CLSI) Guidelines 2014.

Determination of MIC:

MIC determination was done only for imipenem by agar dilution method. Muller Hinton agar with following concentration of the antibiotics was prepared 1, 2, 4, 8, 16, 32, and 64µ/ml. The strains were incubated at 37^0C for 4 hours in peptone water. The turbidity was adjusted to 0.5 McFarland standard and the surface of each plate inoculated with 1×10^4/spot. Incubate at 37^0C aerobically for 24hrs. Plates are examined for growth and compared with positive growth control plate without the antibiotic agent.

Interpretation: (as per CLSI guidelines)

According to CLSI standard the expected breakpoints are follows:

- Imipenem:
  - ≤4µg/l: Sensitive.
  - = 8µg/l: Intermediate.
  - ≥16µg/l: Resistance.

BIOFILM PRODUCTION (using microtiter plate method):

2-3 similar colonies of *Acinetobacter baumannii* was inoculated into 5 ml tripticase soy broth and was incubated overnight at 37^0C.230 µl of tripticase soya broth was dispensed in flat bottomed microtiter plate wells.20 µl of tripticase soy broth (TSB) culture of test isolate was added in the wells containing 230 µl of TSB and incubated for 24 hours at 37^0C.Then the plate was decanted and washed thrice with Phosphate Buffer Saline (PBS) pH 7.2. 230 µl methanol was dispensed into the wells and incubated for 20 minutes. The wells were then decanted and stained with safranin for 20 minutes. The wells were again decanted and washed with PBS. The stained cells adherent to the wells was resolubilized with 33% Glacial acetic acid. Blank, Positive control, Negative control was also included followed by the isolates which were tested in triplicate for each. The optical density (O.D) of each well was quantified at 490 nm using automated BioRad laboratory ELISA reader, India.

Interpretation

The classifications were:

- No biofilm formation: OD≤ODc;
- Weak biofilm formation: 2×ODc≥OD>ODc;
- Moderate biofilm formation: 4×ODc≥OD>2×ODc;
- Strong biofilm formation: OD>4×ODc, according to Stepanovic*et al*. (2004).

Inhibition of biofilm formation by EDTA

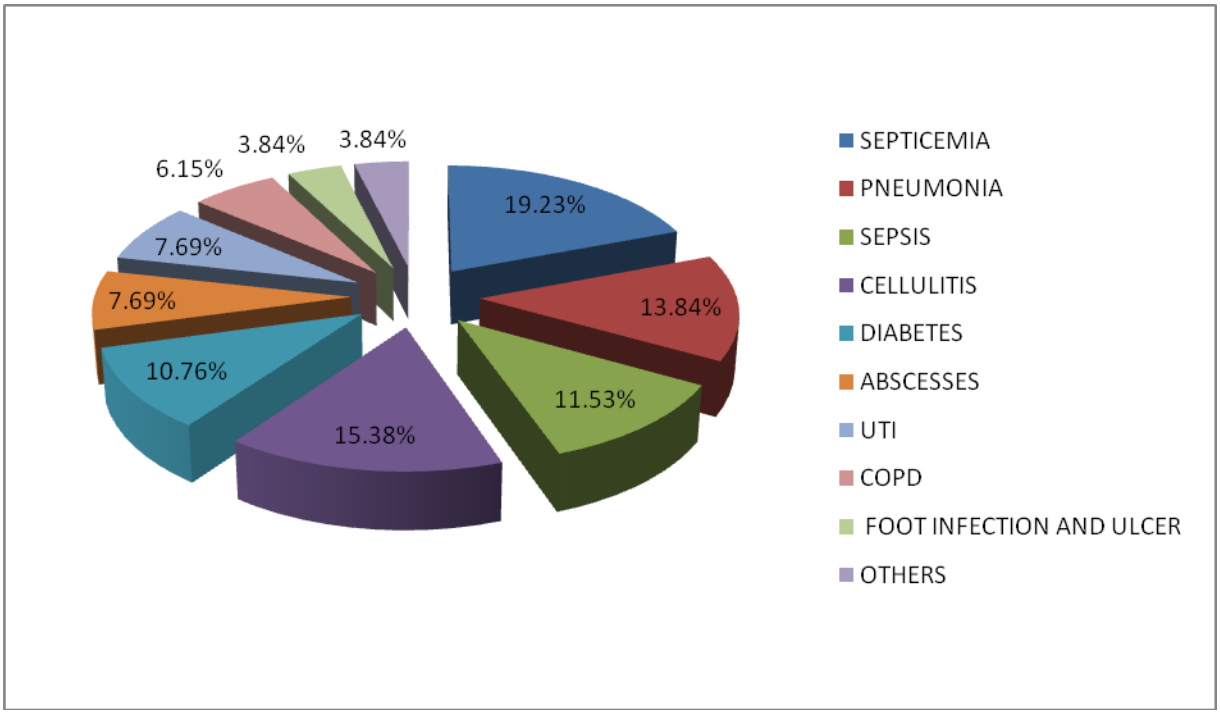
The inhibitory effect of EDTA on bioflim formation was evaluated using *A. baumannii* 19606 which formed the highest amount of bioflim. A 200 µl aliquot of *A. baumannii* suspension was inoculated into each well of a flat bottomed 96-well polystrene plate and was incubated overnight at 37^0C in both with and without EDTA 125 mg/L, after which the amount of bioflim produced was measured as described above.

III. Results

DISTRIBUTION OF *ACINETOBACTER BAUMANNII* IN VARIOUS CLINICAL SPECIMENS:

Clinical Specimen	Number of Isolates
Pus	41 (31.53%)
Tracheal aspirate	37 (28.46%)
Sputum	23 (17.69%)
Wound Swab	10 (7.69%)
Urine	10 (7.69%)
Blood	7 (5.38%)
ET tip	1 (0.76%)
Broncheal wash	1 (0.76%)

CLINICAL PROFILE OF PATIENTS :



Determination of Imipenem resistance by Disk Diffusion method:

Out of 130 isolates of *A. baumannii* 38.46% (50) were imipenem sensitive, 3.07% (4) were intermediate and 58.46% (76) were resistant.

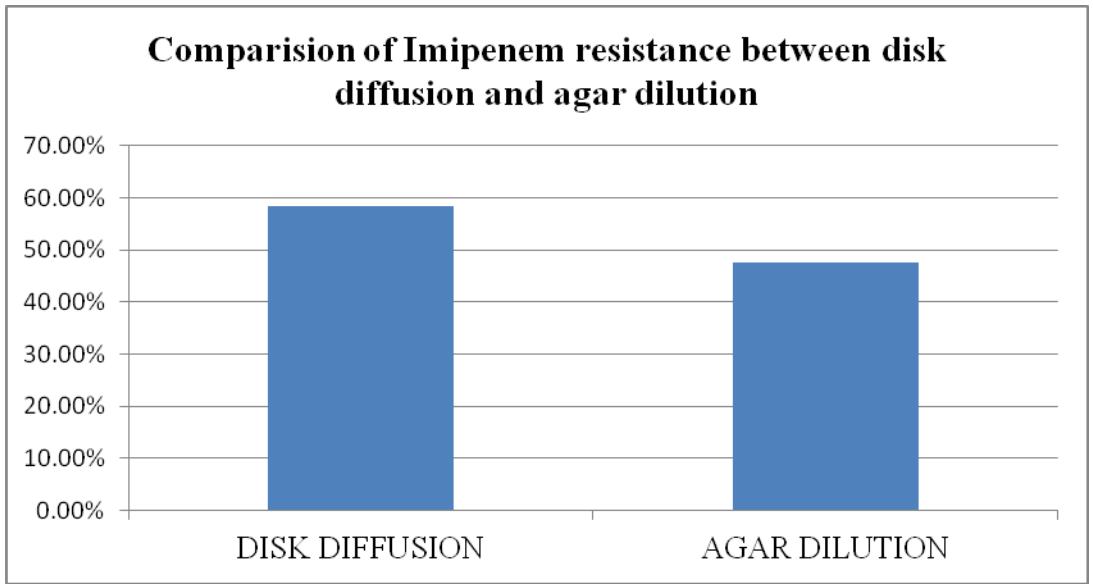
DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION BY AGAR DILUTION METHOD:

DRUG	No. of isolates Sensitive	No. of isolates Intermediate	No. of isolates Resistance
IMIPENEM	5	9	62 (47.69%)

COMPARISON BETWEEN KIRBY BAUER DISC DIFFUSION METHOD AND MINIMUM INHIBITORY CONCENTRATION BY AGAR DILUTION METHOD:

The resistance pattern of the isolates varies in Kirby Bauer disk diffusion and Minimum inhibitory concentration (MIC) by agar dilution method as follows:

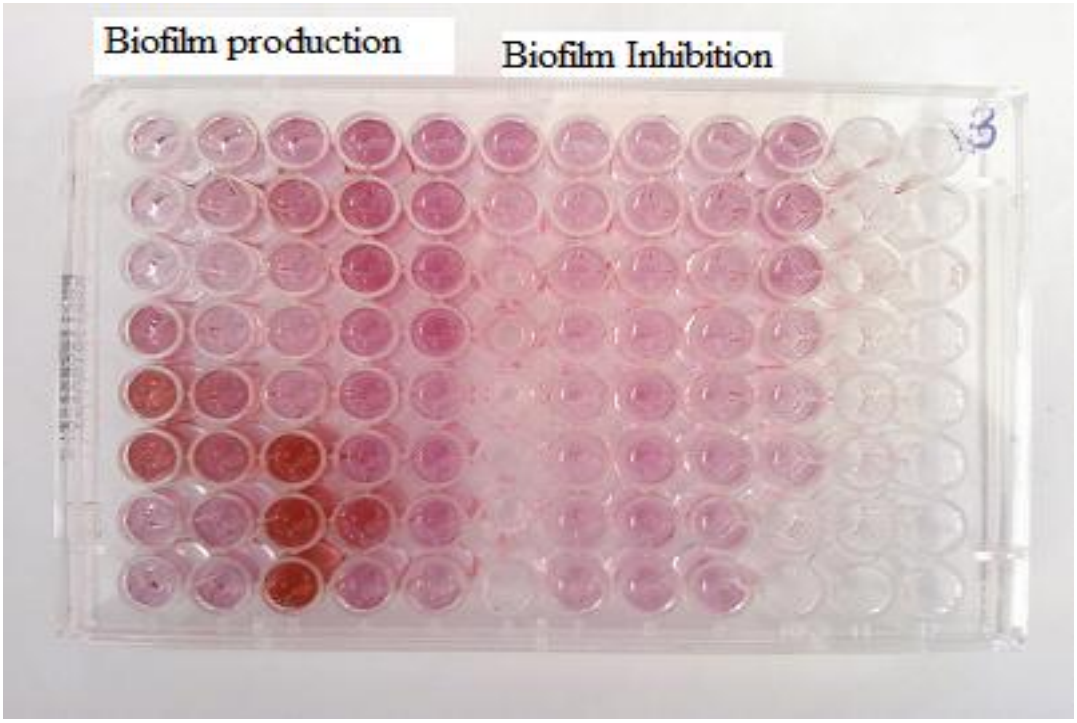
DRUG	DISK DIFFUSION	AGAR DILUTION
IMIPENEM	76 (58.46%)	62 (47.69%)



BIOFILM PRODUCTION AND IMIPENEM RESISTANCE IN ACINETOBACTER BAUMANNII:

Biofilm production was tested for all 130 isolates of *A. baumannii*. Out of 130 isolates; 23 isolates were strong, 50 isolates were moderate and 27 isolates were weak and 30 isolates were non biofilm producers. Out of 62 imipenem resistant isolates by agar dilution, 20 were strong, 32 were moderate, 4 were weak biofilm producers and 6 were non biofilm producer . Out of 13 imipenem intermediate strains, 5 were moderate and 8 were weak biofilm producers. There was significant co-relation between biofilm production and imipenem resistance (p value: 0.000005) at $p<0.05$.

Biofilm production	Imipenem Sensitive (n= 55)	Imipenem resistance (n=62)
Strong	2	20
Moderate	12	32
Weak	15	4
NON-Biofilm	26	6



INHIBITION OF BIOFILM FORMATION BY EDTA:

A total of 45 isotates comprising 15 each of strong, moderate and weak biofilm producers were taken for study. Biofilm production was markedly reduced (55-70%) in presence of EDTA.

All 15 strong biofilm producers became moderate biofilm producers, of 15 moderate biofilm producers; 13 became weak and 2 were moderate and all 15 weak biofilm producer became negative after addition of EDTA with significant reduction in biofilm production ranring from 55-75%.

IV. DISCUSSION

130 isolates of *Acinetobacter baumannii* was isolated from various clinical samples in the present study and 47.69% samples were from respiratory (Tracheal aspirate 28.46%, Sputum17.69%, ET tip 0.76%, Bronchial wash 0.76%), followed by pus (28.46%) and wound (7.69%) , urine (7.69%) and blood (5.38%) samples.

In study done by Anil Chaudhary et al. maximum 59 (57.8%) isolates were obtained from respiratory secretions followed by blood 16 (15.7%) and pus 10 (9.8%).

In study of SM Amudhan, U Sekar, Arunagiri K, B Sekar, *A. baumannii* were isolated from clinical specimens such as blood (n = 25), respiratory secretions (n = 62), pus and wound swab (n = 18), cerebrospinal fluid (n = 4), body fluids (n = 3) and urine (n = 4).

Similar results were seen in study conducted by Abhisek Routray et al. maximum isolates were from pus samples (43.07%), tracheal aspirate (23.07%), sputum (15.38%) and urine (7.69%).47.69% imipenem resistance was found in our study by agar dilution in our study.

While in study done by Abhisek Routray, P. Lavanya et al. resistance to imipenem was 21.71% by agar dilution.

Among the 113 isolates, 80 (70.8%) isolates were resistant to imipenem with MICs ranging from 64 to 512 mg/ml in the study done by Drissi.

Resistance to imipenem by agar dilution was 46% in the study done by Anu Madanan Sunu Kumari et al. which is similar to our study. Nahid H Ahmed et al stated 100% resistance to imipenem in his study. The MIC values for imipenem were over 16 mg/ml for 97.4% of the isolates, Ahmed et al.

Out of 76 Imipenem Resistance isolates 15 (19.73%) were MBL producer and 66 (50.76%) in our study. However MBL production and carbapenemase production was lower 13.3% and 26.6% respectively in study done by Anu Madanan Sunu Kumari et al.

Biofilm production was tested for all 130 isolates collected in this study, of which 25 were strong, 52 were moderate and 27 were weak and 26 were non biofilm producers. Out of 62 imipenem resistant isolates by agar dilution, 23 were strong, 35 were moderate, 4 were weak biofilm producers. Out of 13 imipenem intermediate strains, 5 were moderate and 8 were weak biofilm producers in our study.

Anu Madanan Sunu Kumari et al. found that out of 65 isolates tested; 7 were strong biofilm producers,18 were moderate biofilm producers, 20 were weak biofilm producers and 20 were non- biofilm producers. Among 30 strains which were resistant to imipenem by agar dilution method 7 were strong, 13 were moderate and 10 were weak biofilm producers.

Fifty-six (63%) isolates formed biofilm in study done by J. Rodrı´guez-Ban et al. In the current study, 90% of strains were biofilm former (56.7% strong, 23.3% moderate, 10% weak biofilm former) in study of Nermin H. Ibrahimet al.

Overall the present study demonstrated high level of carapenemase resistance (47.69%) among the strains of *Acinetobacter baumannii.* Biofilm producers showed increased level of resistance towards imipenem than the non-biofilm producers. Moreover, use of chelating agents like EDTA can be used for flushing solution of devices. Biofilm production might be associated with high rate of colonization and persistence related to high rate of device related infection.

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