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ANTI-BIOFILM STRATEGY: MEROPENEM MODULATES BIOFILM FORMATION IN *Acinetobacter lwoffii*

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ABSTRACT

Objectives: Numerous studies investigated multi-drug resistance and biofilm formation, but not determined the concentration of antibiotics which can be as anti-biofilm. *Acinetobacter lwoffii* is opportunistic pathogen that is caused increasing rate of hospital-acquired infections.

Methods: *A. lwoffii* isolates from urinary tract infection are MDR.

Results: *A. lwoffii* was resistance to all antibiotics that were tested. The planktonic cells of *A. lwoffii* 1 and *A. lwoffii* 7 show resistance (256 and 32) µg/ ml toward meropenem and levofloxacin respectively, as well as strong resistance (ND) for ceftazidime, amikacin and gentamicin. The biofilm cells MICs results were significantly $P < 0.005$ higher than the planktonic MICs. Pattern of biofilm ability for #1 and #7 with 2 µg/ ml meropenem and without showed great tendency to shape a biofilm, where *A. lwoffii* 1 (1.1) followed by *A. lwoffii* 7 (1.07).

Conclusion: An interesting observation was the formation of biofilm showed enormous decline at a concentration 32 µg /ml with the presence of meropenem. The intracellular secondary messenger c-di-GMP also significantly declined with meropenem 32 µg /ml, hence, this confirm the estimation that meropenem affect biofilm formation.

Key words: *A. lwoffii*, Biofilm, Meropenem, multi-drug resistance.

INTRODUCTION

In fact, due to increasingly resistant of nosocomial isolates to commonly used antibiotics the cure of *Acinetobacter spp.* infections is becoming more difficult. *A. lwoffii*, is aerobic gram-negative bacillus, non-fermentative that is seen as a normal flora of the oropharynx and skin in approximately 25% of the healthy individuals (Regalado *et al*., 2009; Ku *et al*., 2000). *A. lwoffii* plays an important role in opportunistic pathogen in patients with compromised immune systems, is spreading in clinical locations and causes varies types of infections, including bacteremia, acute gastroenterictis, urinary tract infections, pneumonia and meningitis (Regalado *et al*., 2009). *A. lwoffii* are clinically multi-drug-resistant are stated on many literatures, that have a menace to individual lives and general health. *A. lwoffii* defined as multidrug-resistant (MDR) isolates as it is shown resistance to four classes of drugs in antimicrobial pattern (Tega *et al*., 2007; Martí *et al*., 2011). Biofilm formation consider as one of the main mechanisms to increase an MDR isolates. *A. baumannii* clinical isolates have a robust ability to form biofilms (Burmølle *et al*., 2006). This feature is responsible for chronic infections. As drawback, biofilm formation becomes more common responding to a direct consequence of low-dose therapy (sub-inhibitory concentrations of antibiotics) (Kaplan, 2011). The biofilm formation been reported to connected with multidrug resistance, quorum sensing and efflux pumps (Martí *et al*., 2011; He *et al*., 2015). The formation of Biofilms occurs by the attachment of bacteria over the surfaces through their production of microbial products including proteins, nucleic acids and polysaccharides (Ryder *et al*., 2007). The regulation of biofilm formation, polysaccharide and pellicle formation is occurred by a second messenger, 3,5-cyclic diguanylic acid (c-di-GMP) level (Sakuragi and Kolter, 2007). This work considered these *A. lwoffii*, particularly the antibiotic-resistant isolates, in urinary tract infection after era of prolong treatment with antibiotic caused a high rise in resistance of antibiotic related to formation of biofilm. Therefore, this study was designed to investigate the effect of clinically important meropenem dosages on *A. lwoffii* biofilm.

MATERIALS AND METHODS

Isolation and identification: Eight of *A. lwoffii* isolated from 100 females in their mid-aged admitted with urinary tract infection, from four hospitals in Iraq. Samples collected period from 1st October 2014 to 1st December 2016. Samples of bacteria cultured with blood agar plates and incubated about 24h in order to gain single colonies, followed by sit in liquid medium for 24 h to get multiplied. Bacteria identification was done by API 2 0NE system and Vitek2 automatic bacterial identify-cation instrument.

Preparation of antibiotic stock solutions: Sigma-Ald-rich\ China was the provider of all antibiotics. Concentrations of stock solutions were 5.12 mg/ml with sterile distilled water, afterward it kept at -20°C ready for use.

Antibiotic susceptibility assays: According to the Clinical and Laboratory Standards Institute (CLSI) procedure (Clinical and Laboratory Standards Institute, 2009) the susceptibility of the biofilm cells

has been determined. In short, the solutions of antibiotic stock diluted to concentrations from 0.25 to 256 µg/ml. According to McFarland standard 0.5, the *A. lwoffii* planktonic and biofilm cells were adjusted. Based on CLSI guidelines, susceptibilities are clarified (Clinical and Laboratory Standards Institute, 2007).

Biofilm assay: The formation of Biofilm in 96-well micro-titer plates has been quantified and assayed as shown above (O'Toole and Kolter, 1998; Caiazza and O'Toole, 2004). M63 minimal medium were used for biofilm assays. Each isolate suspension was diluted (1: 50) into an aliquot of the Biofilm media. The wells were inoculated of the 96-well plate (100 µl/well) from the isolate mixture. The 96-well plate was incubated at 37°C for 24 hours. After then wells were shaken out to remove the unattached bacteria and then rinsed, subsequently, 125 µl of Crystal violate at 0.1% concentration was added to each well for 10-15 min. The excess stain was rinsed twice, and the plate was left to dry.

Biofilm induction by meropenem: Around 10^5CFU/ml in NB of *A. lwoffii* were inoculated. With (1/2, 1/32µg/ml the MIC) sub lethal concentrations of antibiotics. For 24 h at 37°C the plates were incubated. A positive control of the experiment was *A. baumannii* ATCC 19606 (He *et al*., 2015).

C-di-GMP measurement: The isolation of C-di-GMP done according to (Amikam *et al*., 1995). The growth of 1 L of LB medium was cultured for *A. lwoffii* in 18h at 250 rpm, then in order to inactivate degradation of c-di-GMP formaldehyde was added. Centrifuge were used at 8,000 g for 10 min at 4°C. As mentioned earlier Nucleotide extraction was prepared (Amikam *et al*., 1995).

RESULTS AND DISCUSSION

Sensitivity test of planktonic cells: It can be seen from table 1, the *A. lwoffii* antibiotics sensitivity according to the MICs of antibiotics against the planktonic cells. The *A. lwoffii* isolates display resistance patterns to different antibiotics classes. MICs of amikacin and gentamicin for all *A. lwoffii* isolates were >256 µg/ml. whereas, there is a fluctuation in the MIC between 128 and 256 µg/ml for ceftazidime and between 32 and 64 µg/ml for meropenem. In case of levofloxacin the fluctuation in the MICs was between 2 and 8 µg/ml. Our findings show similarity to (Martí *et al*., 2011) that stated only six out of ten *A. lwoffii* isolates, showed resistant to ceftazidime. Where three of the remaining *A. lwoffii* resist for meropenem MIC 1–2 µg/mL, and amikacin MIC 2-4 µg/mL. The last strain was susceptible to meropenem MIC 2 µg/mL, amikacin MIC 4 µg/mL. The resistance of Metallo-β-lactamase may relate to a mobile gene blaNDM-1 that have been spread worldwide in clinical bacteria and appeared in clinical *A. lwoffii* strains in china (Wang *et al*., 2012; Hu *et al*., 2012).

Table 1. MIC of antibiotics resistance in *A. lwoffii*.

Isolates no.	MIC µg/ ml				
	Ceft	Mero	Ami	Gent	Levo
A. lwoffii #1	256 (R)	32 (R)	> 256	> 256	8 (R)
A. lwoffii #2	128 (R)	32 (R)	> 256	> 256	4 (I)
A. lwoffii #3	128 (R)	32 (R)	> 256	> 256	4 (I)
A. lwoffii #4	256 (R)	64 (R)	> 256	> 256	8 (R)
A. lwoffii #5	256 (R)	64 (R)	> 256	> 256	8 (R)
A. lwoffii #6	256 (R)	32 (R)	> 256	> 256	8 (R)
A. lwoffii #7	256 (R)	64 (R)	> 256	> 256	4 (I)
A. lwoffii #8	256 (R)	64 (R)	> 256	> 256	2 (I)

The resistance breakpoint was ceftazidime as <8.16 and >32 µg/ml, Meropenem as <4.8 and >16 µg/ml, Amikacin as <16 and >32µg/ml, Gentamycin as <4 and >8µg/ml, Levofloxacin as <2.4 and >16 µg/ml, 1-Intermediate, R-Resistance

Biofilm and antibiotic susceptibility in *A. lwoffii:* The resistant *A. lwoffii* clinical isolates displayed biofilm rates as low as 0.82 and high at 1.2 which indicate a positive correlation among biofilm formation ability and meropenem resistance as showed in table 2. This result it seems disagreed with Perez (2014) study that concluded the meropenem resistance of *A. baumannii* clinical isolates was inversely related to biofilm production. The biofilm formation capacities of *A. lwoffii* were all adjusted to the control strain *A. baumannii* ATCC 19606.

Table 2. Resistant biofilm formation (RBF) in *A. lwoffii* clinical isolates

Clinical Isolates	RBF (A550±SD)*
A. lwoffii #1	1.2±0.283
A. lwoffii #2	0.95±0.041
A. lwoffii #3	0.82±0.043
A. lwoffii #4	0.91±0.014
A. lwoffii #5	0.97±0.024
A. lwoffii #6	0.99±0.008
A. lwoffii #7	1.04±0.045
A. lwoffii #8	1.05±0.041

*Standard deviation of the average of four experiments with four replicates per experiments. Data were analyzed by ANOVA with Turkeys post-test comparison. Significant different; $P < 0.005$ compared to the *A. baumannii* ATCC 19606

Table 3 shows the association of *A. lwoffii cell* and biofilm susceptibility of antibiotics. The biofilm cells of *A. lwoffii* #1 and *A. lwoffii* #7 show meropenem resistance and levofloxacin and intense resistance (ND) to ceftazidime, gentamicin and amikacin. The biofilm cells MICs were significantly higher than the planktonic MICs. This result is consistence with previous reports (Costerton *et al*.,

1999; Stewart, 2002) that demonstrated a direct association related to biofilm formation with a significant increment in the resistance of antibiotic. Changes in metabolic activity and membrane permeability might be attached to biofilm cells resistance.

Table 3. MIC against the biofilm of *A. lwoffii* clinical isolates

Antibiotics	MIC µg/ ml	
	A. lwoffii #1	***A. lwoffii #7***
Ceftazindime	ND	ND
Meropenem	256 (R)	256 (R)
Amikacin	ND	ND
Gentamicin	ND	ND
Levofloxacin	32 (R)	32 (R)

R: Resistant isolate; ND: Non determined.

Carbapenem induce ability of biofilm formation: The profiles of biofilm formation for 1 and 7 with 2 and 32 µg /ml meropenem and without it is showing in table 4. This study found that when the antibiotics is absent, the formation of biofilm tend to be high in *A. lwoffii* 1 (1.1) followed by *A. lwoffii* 7 (1.07). An interesting observation was at a concentration of 32 µg /ml of meropenem the formation of biofilm decreased significantly unlike with 2 µg /ml. Suggesting the low ability to form bio-films is not probably by coincidence, as similar findings have been reported by Nucleo *et al*. (2009) when same class of antibiotics (imipenem) been used with *A. baumannii*, indicated that Carbapenem induced ability of biofilm. Is good to know that other studies by Linares *et al*., (2006) and Boehm *et al*., (2009) exhibit an induction of biofilm formation using fluoroquinolone antibiotic in *E. coli* and *Pseudomonas aeruginosa*. Still, this phenomenon is studied in this work for first time by using *A. lwoffii*. The results of this work emphasis of how crucial the selection of meropenem antibiotic concentration for the treatment of *A. lwoffii* infections. This might be a rational justification for the eradication fail-ure of *A. lwoffii* from patients' treatment with low concentration despite a long time of antibiotic treatment.

Table 4. Effect of sub–MIC of meropenem on the biofilm value.

Isolates	Biofilm	Biofilm with sub–MIC of meropenem 32g/ml	Biofilm with sub–MIC of meropenem 2g/ml
A. lwoffii #1	1.3±0.08165	0.8±0.163299	1.1±0.08165
A. lwoffii #7	1.06±0.05099	0.6±0.244949	1.1±0.141421

*Standard deviation of the average of four experiments with four replicates per experiments. Data were analyzed by ANOVA with Turkeys post test comparison. Differences were considered a significant when $P \leq 0.005$.

The impact of meropenem on c-di-GMP level: Present study measured the cellular c-di-GMP concentrations of *A. lwoffii* 1 and A. *lwoffii* 7 as reported before by using high performance liquid chromatography (HPLC) Kulasakara *et al*., (2006). A concentration of 32 µg/ml Meropenem showed a significant decline in the intracellular secondary messenger c-di-GMP, hence, this confirm the estimation that meropenem affect biofilm forma-tion. The fig. 1 clearly illustrates that A. lwoffii 1 and 7 showed a decrease at (0.005 pmol/mg cells) when meropenem 32 µg/ml has been added.

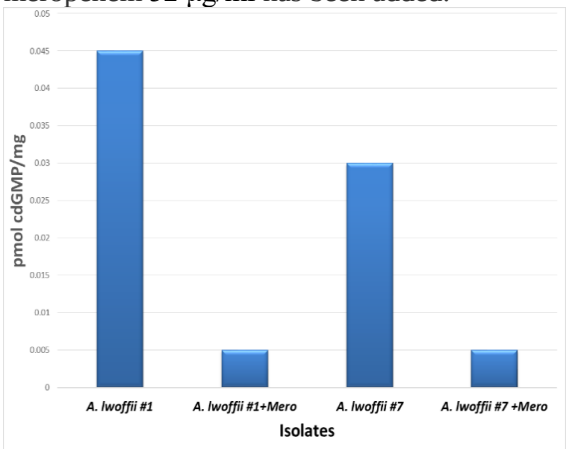

Figure – 1: Quantification of cellular c-di-GMP concentrations by HPLC from 30mg of cells. Differences were considered a significant when p< 0.001.

Conclusion

The aim of this study was investigated the effect of Carbapenem antibiotic in modulation of biofilm formation via c-di-GMP. The findings affirm that the meropenem modulates the biofilm formation and enhance the treatment of UTI with meropenem in high dose. As a result, using a low-dose of antibiotic therapy in *A. lwoffii* infections is discourage as it could be not effective as a treating method.

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Conflicts of Interest: No conflict of interest has been recorded.

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