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EVALUATION THE EFFICACY OF DILUTED CHLOROXYLENOL AGAINST BIOFILM PRODUCING PSEUDOMONAS AERUGINOSA IN CLINICAL ISOLATES

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Abstract

The extensive use of disinfectant and their causing in dissemination at the hospitals can contribute to alterations in bacteria leading to the expansion of highly resistant microorganisms to antibacterial agents. The mechanisms of resistance in bacteria are similar for both antibacterial agents and disinfectant. The main objective of this study was to assess the activity of the various dilution of the chloroxylenol as an ordinarily used disinfectant against P. aeruginosa at hospitals and their association to biofilm production. This study was carried out on 91 P. aeruginosa obtained from different clinical specimens at hospitals in Erbil city. All clinical isolates of P. aeruginosa were screened for biofilm formation in different concentration of disinfectants. The activity of chloroxylenol on P. aeruginosa to the biofilm was found to be concentration reliant. The isolates showed to be a non-biofilm producer to dilution factor of 1:10 and 1:20, while in the range between 1:40 to1:160 the ability was higher to biofilm formation. The maximum inhibition rate of chloroxylenol was documented 43% of isolates for 1/2 MIC, while the lowest inhibition 17% was established for 1/32 MIC. It might be probable that P. aeruginosa modifies to resistant which leads to their survival even at high concentrations of disinfectant. Therefore, it is observable that resistance to disinfectant especially in the hospital settings could be due to multi-resistance bacteria then it can be easily conveyed to patients who admitted to hospital.

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EVALUATION THE EFFICACY OF DILUTED CHLOROXYLENOL AGAINST BIOFILM PRODUCING *PSEUDOMONAS AERUGINOSA* IN CLINICAL ISOLATES

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ABSTRACT

The extensive use of disinfectant and their causing in dissemination at the hospitals can contribute to alterations in bacteria leading to the expansion of highly resistant microorganisms to antibacterial agents. The mechanisms of resistance in bacteria are similar for both antibacterial agents and disinfectant. The main objective of this study was to assess the activity of the various dilution of the chloroxylenol as an ordinarily used disinfectant against P. aeruginosa at hospitals and their association to biofilm production. This study was carried out on 91 P. aeruginosa obtained from different clinical specimens at hospitals in Erbil city. All clinical isolates of P. aeruginosa were screened for biofilm formation in different concentration of disinfectants. The activity of chloroxylenol on P. aeruginosa to the biofilm was found to be concentration reliant. The isolates showed to be a non-biofilm producer to dilution factor of 1:10 and 1:20, while in the range between 1:40 to1:160 the ability was higher to biofilm formation. The maximum inhibition rate of chloroxylenol was documented 43% of isolates for 1/2 MIC, while the lowest inhibition 17% was established for 1/32 MIC. It might be probable that P. aeruginosa modifies to resistant which leads to their survival even at high concentrations of disinfectant. Therefore, it is observable that resistance to disinfectant especially in the hospital settings could be due to multi-resistance bacteria then it can be easily conveyed to patients who admitted to hospital.

Key Words: ***Biofilm formation; Chloroxylenol; Disinfectant; Drug resistance; P.aeruginosa.***

1. INTRODUCTION:

Pseudomonas aeruginosa is a well-known opportunistic pathogen in hospitalized entities and considered as one of the most significant nosocomial pathogen (Pramodhini *et al.*, 2016),

because of its ubiquitous has been frequently associated with outbreaks in hospital settings among hospitalized patients mainly people with cystic fibrosis and burns (Alkolaibea *et al.*, 2015). Consequently, treatment options are narrowed down to only a few antibiotics, due to its virulence factor, intrinsic and acquired resistance genes against antibiotics, which successively limit the selection of the current antimicrobial agents (Zavascki *et al.*, 2005; Okesola & Olola, 2011). However, multidrug resistance to the novel antibiotics is increasing worldwide (Aryanezhad *et al.*, 2016). Bacteria can colonize the surfaces and grow as biofilm embedded in a polysaccharide matrix leading to the suggestion that biofilm formation plays a key role to emerge and re-emerge infections (Schellenberg *et al.*, 2003). The effective eradication of these highly resistant pathogens with antimicrobial agents has been problematical by the development of multi-resistant pathogens (Corehtash *et al.*, 2015). Some antimicrobial agents of various preparations have been developed and introduced with the purpose of breaking the sequence of infections in and hospital setting (El-Mahmood & Doughari, 2009). A wide variety of disinfectants and antiseptics are now available commercially to combat bacterial existence in healthcare settings (Okesola & Olola, 2011). Considerable progress has been made in the antiseptics and disinfectants mode of action as antimicrobial agents and Dettol included (Higgins *et al.*, 2001; Ogbulie *et al.*, 2008). As a result of widespread use of biocides, a significant proportion of the pathogens have not only developed resistance microorganism, but they also survive in the solutions of these antiseptics and disinfectants and the consequent cross-resistance to antibiotics (McDonnell & Russell, 1999). There is convincing evidence that *Pseudomonas aeruginosa* has an existence strategy by forming biofilm communities, not only on abiotic surfaces (e.g., glass and plastics) but also on biotic surfaces such as epithelial cells, leading to the suggestion that biofilm formation plays a key role to emerge and re-emerge infections (Ogbulie *et al.*, 2008; Gowrishankar *et al.*, 2012). The antimicrobial action of disinfectants and antiseptics have been Influenced by their construction effects, level of organic component, interaction, temperature, concentration rate and experimental methods (Davin-Regli & Pagès, 2012). The objective of this study was to evaluate the activity of the different dilution of the chloroxylenol as a frequently used disinfectant against clinical isolates of *P. aeruginosa* at hospitals of Erbil city and their correlation to biofilm formation.

2. MATERIALS AND METHODS

2.1. Specimen collection and bacterial isolates

A total of 91, non-repetitive isolates of *Pseudomonas aeruginosa* from all clinical samples were included in this study. Identification was done by conventional biochemical test using standard methods and confirmed by Vitek II system (Rani *et al.*, 2015).

2.2. Selection of disinfectants

The disinfectant used in this study included chloroxylenol (Dettol) (Batch 3096, Beckith benckiser Pharmaceuticals ltd, South Africa) was selected based on wide suitability and commonly of use in the most hospital setting (Okesola & Olola, 2011; Okore *et al.*, 2014).

2.3. Preparation of diluted disinfectants samples

Fifty milliliters (50ml) of the diluted chloroxylenol was prepared inside a sterile container and used for microbiological analysis. Standard antibiotic-micro broth 96-flat well plates were used. Two-fold serial dilutions ranged from 1:5 to 1:160 for the chloroxylenol were prepared. Control plates were prepared as two sets of free chloroxylenol plate that all wells dispensed with 200µl of nutrient broth without chloroxylenol (Alkolaibea *et al.*, 2015).

2.4. Detection of biofilms production of P. aeruginosa

The activity of chloroxylenol on *P. aeruginosa* biofilms was determined using comparable cultures as described by (Tote *et al.*, 2010). The microtiter plates were incubated for 72 h at 37 °C. After removal of the neutralizer and subsequent rinsing with PBS pH 7.2, then air dried, treated adherent populations were fixed using 200µl of 0.1% crystal violet solution (Sigma-Aldrich, Bornem, Belgium) per well. After 30 min of incubation, followed by a wash under running tap water, crystal violet was removed and plates exposed to air-dry. Next, 200µl of ethanol 70% per well was added to resolubilize the biofilm-bound crystal violet. Following short incubation, the optical density (OD) was measured at 480 nm. ELISA micro-plate reader ELX800 (Biotek / USA) was used to assess *P. aeruginosa* biofilm formation (Sánchez *et al.*, 2013). The biofilm value was assessed using the following **Table 1** while the inhibition percentage of biofilm was calculated by the formula.

Percentage of biofilm inhibition = (Control–Test) / Control x 100

Table 1: Interpretation of biofilm formation

Mean OD value	Adherence	Biofilm Formation
<0.120	**Non**	**Non/weak**
0.120-0.240	**Moderate**	**Moderate**
> 0.240	**Strong**	**Strong**

2.5. Statistical analysis of data

The mean ±SD of biofilm inhibition was measured and the paired sample t-test was applied for comparison the means.

3. RESULTS:

3.1. Antibacterial potency

Minimal inhibitory concentration (MIC) was used as a comparative measure of the effectiveness of chloroxylenol against a total of 91 isolates of *P. aeruginosa*. The MIC for this disinfectant in culture media estimated to (1:5) as dilution factor.

3.2. Anti-biofilm effects

In the current study, the disinfectant activity on *P.aeruginosa* to the biofilm was found to be concentration dependent. The biofilm pattern of *P.aeruginosa* to the active constituent of chloroxylenol at different sub-MICs ranged from 1:10, 1:20, 1:40, 1:80 and 1:160, which also measured as (1/2MIC, 1/4MIC, 1/8MIC, 1/16MIC, and 1/32MIC) comparing with the control for biofilm inhibition. In our study, at the practice dilution of 1:10 and 1:20 *P.aeruginosa* demonstrated adequate results with the majority of the isolates were non-weak biofilm producer while in the range between 1:40 to1:160, the ability was higher for production of moderate to strong. The percentage of biofilm formation in both degrees, strong and moderate to the different dilution factors of chloroxylenol is shown in **Table 2 and Figure 1**

Table 2: Different dilution factors of chloroxylenol and biofilm formation

Biofilm degree	Diluted chloroxylenol					
	1:10	**1:20**	**1:40**	**1:80**	**1:160**	**Control**
Non/Weak	54	33	33	26	17	20
Moderate	12	26	20	29	43	33
Strong	25	32	38	36	31	38
Total	**91**	**91**	**91**	**91**	**91**	**91**

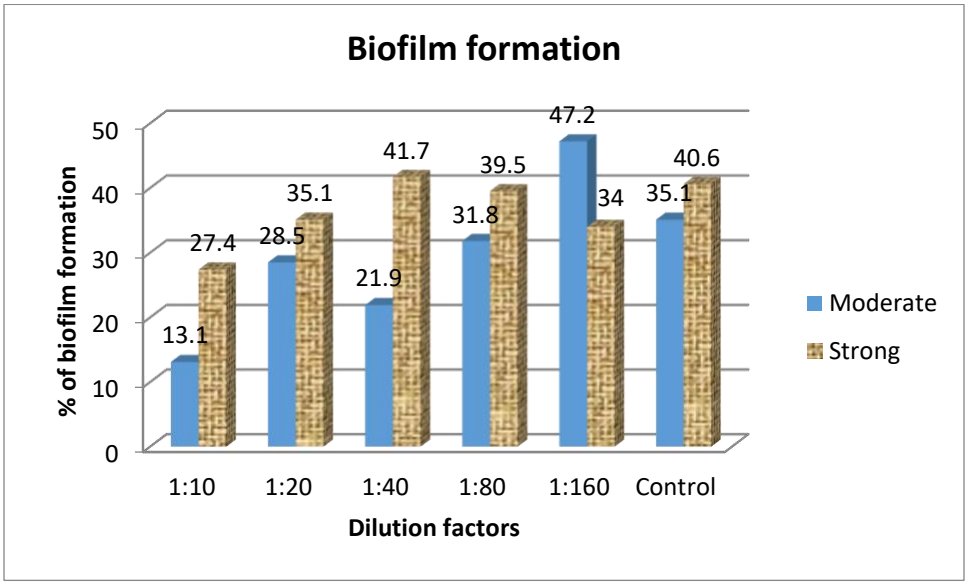

Figure 1: *The percentage of biofilm formation of 91 isolates in different dilution factors*

Anti-biofilm activity of chloroxylenol at sub-MIC values showed biofilm inhibition at different dilutions ranged from 1/32 MIC to 1/2 MIC. The results exhibited significantly difference

(P<0.001) against 91 isolates of *P. aeruginosa* at 1/2MIC, while a nonsignificant inhibition exhibited at the rest of sub-MICs **Table 3.**

Table 3: Mean of biofilm inhibition at different Sub-MICs of chloroxylenol

Mean biofilm inhibition OD_{480}		
Sub-MIC	**Mean±SD**	***P-value***
1/2MIC	0.1829±0.1777	0.001
1/4 MIC	0.2561±0.2189	0.121
1/8 MIC	0.2609±0.2250	0.151
1/16 MIC	0.2527±0.2294	0.108
1/32 MIC	0.2643±0.2392	0.189
Control	**0.3199±0.32337**	

On the other hand, the percentage of biofilm inhibition by five sub-MIC levels of chloroxylenol against 91isolates of *P. aeruginosa* was evaluated as shown in Figure 2. The highest Inhibition rate of chloroxylenol was recorded 43% of isolates for 1/2MIC, while the lowest inhibition 17% was demonstrated for 1/32MIC.

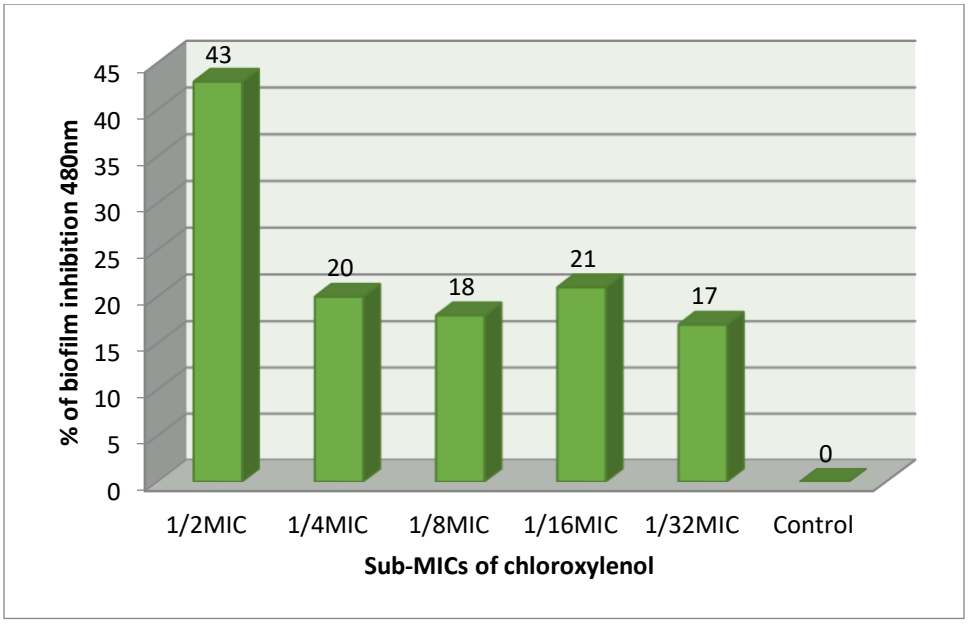

Figure 2: *Percentage of biofilm inhibition to the sub-MICs of chloroxylenol.*

4. DISCUSSION:

Pseudomonas aeruginosa is one of the most common contaminant found on the skin of hospitalized patients, laboratory surfaces, toilets and pools in the hospital environment (Pramodhini *et al.*, 2016). It is also known to be one of the microorganisms associated in nosocomial outbreaks which involve a broad spectrum of infections including respiratory and urinary tracts as well as a wound from burn infections and sepsis (Gowrishankar *et al.*, 2012; Vahdani *et al.*, 2012). Furthermore, it's an enormously adaptable microorganism that can

promptly develop resistance to different sorts of antimicrobial agents and can easily adapt environment, physical and chemical condition and grows in hospital environments certain disinfectants are designated to share the comparable mechanism of action with some antimicrobial agents and this can cause resistance to sterilizers used in cleaning hospitals environments (Ogbulie *et al.*, 2008; Norouzi *et al.*, 2010). Therefore, based on this fact, it is noticeable that resistance to disinfectants especially in the hospital setting could be multi-resistance consequently it can be transmitted rapidly among hospitalized patients. This study has further established that the biofilm producing abilities in the commonly used disinfectants (chloroxylenol) at most hospitals in Erbil city, against clinical isolates of *P. aeruginosa*, are concentration-dependent, distinguish between matrix formation and viable microbial burden, as very few study demonstrated the association between biofilm formation and disinfectants. The antibacterial activity of chloroxylenol has been described by numerous researchers including (Ayres *et al.*, 1998; Higgins *et al.*, 2001; Olorode & Okpokwasli, 2012). The mechanism of action of disinfectant or antiseptic on the bacteria remains the same regardless of the kind and is used through the diffusion into the cell and action at the target sites (Smith *et al.*, 2009; Masri *et al.*, 2013). The susceptibility of pathogenic bacteria, therefore, can be a very important feature in estimating the crucial consequence of the treatment with the proposed disinfectant in the hospital settings. Some of these disinfectants also work by making of destructive chemicals in contradiction of bacteria to attack cell membrane, nucleic acid and other essential cell constituents (Pramodhini *et al.*, 2016). The effectiveness of disinfectants in controlling hospital-acquired infections are often given by the fact that some of the antiseptics used in the clinic and hospital settings have been stated to be dirtied with organisms during the preparation processes (Higgins *et al.*, 2001; Guenther *et al.*, 2015). It was proved experimentally that specific concentration of disinfectant has bacteriostatic activity and can inhibit the growth of bacteria, it was clearly evidenced that Gram-negative bacteria were killed at high concentration of disinfectant especially *P. aeruginosa* (Riaz *et al.*, 2009). Some other reports have also proposed a reduced susceptibility to some disinfectants and antibiotic resistance have been associated with mobile genetic elements (Olowe *et al.*, 2004; Okesola & Olola, 2011). Furthermore, the use of sub-optimal dilution might yield the development of virulent and resistant microorganism (El-Mahmood & Doughari, 2009). Due to the ability to survive under unfavorable environmental conditions and it is high resistance to the antimicrobial (Alkolaibea *et al.*, 2015), who reported the resistance pathogenic bacteria has arisen due to inadequate cleaning, improper product use and unsuccessful infection control practices, which can be underestimated. Therefore we expecting that resistance to widely used antiseptics and disinfectants have crucial role in the adaptation of microorganisms to a variety of environmental, physical and chemical conditions.

5. CONCLUSION:

The widespread use and misuse of disinfectant resulting dissemination of resistance in the hospital setting, chloroxylenol can either eradicate or inhibit the bacteria. It might be possible that *P. aeruginosa* alters to resistant which leads to their existence even at extraordinary concentrations of disinfectant. Therefore, it is obvious that resistance to antiseptics particularly in

the hospitals could be multi-resistance subsequently then it can be transferred rapidly among patients who admitted to hospital.

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