IJSCIA

International Journal of Scientific Advances
ISSN: 2708-7972
Volume: 5 | Issue: 6 | Nov – Dec 2024 Available Online: www.ijscia.com
DOI: 10.51542/ijscia.v5i6.57

The Potential of Alcohol and Triclosan, Alcohol and Chloroxylenol, and the Antibiotic Azithromycin Combinations as Anti-Biofilm Agents Against *Pseudomonas aeruginosa*

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ABSTRACT

Background: Pseudomonas aeruginosa is a gram-negative bacterium commonly found in the environment and known as an opportunistic pathogen capable of forming biofilms. Biofilm formation makes the bacteria more resistant to conventional treatments, necessitating effective anti-biofilm agents. This study aims to evaluate the potential of combining alcohol and triclosan, alcohol and chloroxylenol, and the antibiotic azithromycin as anti-biofilm agents against Pseudomonas aeruginosa. ***Method:*** The research was conducted using experimental methods in the laboratory, utilizing clinical isolates of Pseudomonas aeruginosa from Dr. Soetomo General Hospital. The bacteria were incubated in media containing these combinations of antibiofilm agents, and their effects were measured based on the optical density of the formed biofilm. ***Result:*** The combinations of alcohol and triclosan, alcohol and chloroxylenol, and azithromycin showed significant effects in inhibiting the formation of Pseudomonas aeruginosa biofilms. The combination of alcohol and triclosan had the strongest antibiofilm effect compared to the other combinations. ***Conclusion:*** The combinations of alcohol and triclosan and alcohol and chloroxylenol are effective as anti-biofilm agents against Pseudomonas aeruginosa, with the combination of alcohol and triclosan showing the highest potential. In contrast, azithromycin exhibited comparatively lower efficacy in inhibiting biofilm formation relative to the other combinations. Further research is needed to test the effectiveness and safety of these combinations in clinical settings.

Keywords: *pseudomonas aeruginosa*; biofilm; antimicrobial resistance; anti-biofilm; antibacterial; antibiotic.

INTRODUCTION

Pseudomonas aeruginosa belongs to a pathogenic bacterial group which is found to be abundant in human digestive and skin normal flora [1]. Pseudomonas infection has been a major health issue, especially in inpatient hospital settings. *P. aeruginosa* infection in particular accounts for 7.1-7.3% of nosocomial infection worldwide. Moreover, in the intensive care setting, *P. aeruginosa* caused 23% among ICU-acquired infections [2]. In the outpatient setting, people can also contract *P. aeruginosa* infection, which presents mostly as respiratory tract infection and musculoskeletal infection according to recent studies in 14 different Asia-Pacific countries [3].

One of the main challenges in the management of *P. aeruginosa* infection is the emergence of antimicrobial-resistant strains [4]. These infections present worse clinical conditions with worse clinical outcomes and prognosis. This emerging antimicrobial resistance is due to the environmental condition in the hospital with high use of a wide variety of antimicrobials [5]. Virulence factors include the ability of biofilm production in *P. aeruginosa*. Biofilm allows *P. aeruginosa* to adapt with hazardous environments such as pH instability, ultraviolet light exposure, hydrogen peroxide, and metal toxicity, and also host immune response, such as phagocytosis [6].

The definitive approach in the eradication of *P. aeruginosa* in the use of antimicrobials. Azithromycin in particular, is used in combination with other antimicrobial agents to effectively fight *P. aeruginosa,* especially with the presence of biofilm [7]. But with the emergence of antimicrobial resistance which has been mentioned earlier, earlier approaches should be considered, such as the prevention of infections in the first place. The most common approach in the prevention of infection is the use of antiseptic agents. The most common antiseptic agent is the use of 60-80% alcohol with the combination of several other antiseptic agents [8].

Active agents with the ability to eradicate microbes are triclosan and chloroxylenol. It is known that these agents work synergistically with alcohol as an antimicrobial agent [8]. Thus, this study aims to understand the antimicrobial potency of alcohol and triclosan combination, alcohol and chloroxylenol combination, and azithromycin to hinder the production of biofilm of *P. aeruginosa*.

METHODS

True experimental research was conducted in Laboratorium Medis Terpadu, Faculty of Medicine, Universitas Airlangga from April Until June 2024. *P. aeruginosa* strain was collected from the microbiology laboratorium, Faculty of Medicine Universitas Airlangga, with the strain coming from a clinical isolate Dr. Soetomo General Hospital.

The procedure involves inoculation of *P. aeruginosa* Mueller-Hinton agar overnight with a temperature of 37°C. Before continuing the procedure, the colony characteristic must follow these criteria: 1) Bluish green from the pyocyanin and pyoverdine pigment. 2) Wet and viscous due to the exopolysaccharide. 3) Grape or sweet corn scent. Natrium chloride with a concentration of 0.85% is added to the normal saline solution for the isolate concentration to be adjusted into 0.5 McFarland (~1,5×10^8 CFU/mL). For the cultivation of the biofilm, inoculation of *P. aeruginosa* was performed into 5 groups. Positive control groups contain microplates with 180µL Tryptic Soy Broth (TSB) and 20 µL of 0.5 McFarland isolate suspension, Negative control groups contain only microplates with 180µL TSB and three intervention groups: 1) Microplate with *P. aeruginosa* and Alcohol-Triclosan combination, 2) Microplate with Aeruginosa and Chloroxylenol, and 3) Microplate with *P. aeruginosa* and azithromycin.

Every intervention group will perform varied doses for 8 times each dose, resulting in a total of 24 times. Meanwhile, only 8 times were performed for the control groups. At the end of the procedure, the microplate will be sealed and incubated for 48 hours at 37°C.

Crystal violet assay method was conducted to find the biofilm, by expelling the suspension that doesn't adhere to the microplate and rinsing off the ones that adhere with phosphate buffer saline three times. Microplates were dried in air for 30 minutes, afterwards, methanol was poured into each microplate for 30 minutes. Dispose of the methanol, wait for ten minutes, and then 150 µL crystal violet 1% was added for 10 minutes before washing it with water. After the microplates dried, 150 µL ethanol 95% was added to fixate the biofilm. Biofilm will be measured using the Elisa reader on 630 nm for the absorbance in each microplate aiming to evaluate the optical density (OD). The OD from every group will be analyzed and statistically counted using GraphPad Prism 9.5.1 with Analysis of Variance (ANOVA).

Every result from the crystal violet OD will be reported in mean and standard deviation (SD). The first step of the analysis is finding the OD cutoff by using the equation of OD Cutoff= $\bar{x}$ODControl+3SDControl , then the OD cutoff is used to find the isolate of the OD with ODisolate= $\bar{x}$ODintervention+ODcutoff equation. The OD cutoff was used to determine the potential of each combination's ability to inhibit biofilm formation. If OD was below cutoff then the intervention has the potency to inhibit biofilm formation. Kruskal Wallis test followed by Dunn's post-hoc test was applied to find the difference of OD between groups. Statistically significant was determined if the p-value was < 0.05. All statistical analysis of this study was performed utilizing IBM SPSS for Windows.

RESULTS

Optical Density of Pseudomonas Aeruginosa from each group

The positive control group's OD was found to be 0.0943 ± 0.05736 and the negative control group with an OD of 0.0650 ± 0.00670. The purpose of these two control groups was to find the OD cutoff with the equation stated in the methods. The OD cutoff obtained was 0.0851. The Positive control and negative control are represented in Table 1.

TABLE 1: OD of Positive and negative control group.

Optical Density											
Group	Intervention								Mean	SD	OD Cut off
	1	2	3	4	5	6	7	8			
Positive Control	0.064	0.062	0.062	0.068	0.07	0.072	0.132	0.224	0.0943	0.05736	0.0851
Negative Control	0.059	0.059	0.059	0.063	0.075	0.075	0.064	0.066	0.0650	0 0.00670	

Results from the OD of each intervention group are depicted in Table 2. All the combination groups and their different doses showed a result that was below the determined cut-off. The lowest OD was found in Alcohol-Triclosan 100% combination with the results of 0.0473 ± 0.00568.

For the Alcohol-Chloroxylenol group, the 10-ppm dose showed the lowest OD compared to other doses with 0.0603 ± 0.02517. As for the Azithromycin group, the highest dose showed better performance on inhibiting biofilm formation with 0.0569 ± 0.00467.

TABLE 2: OD of Alcohol-Triclosan, Alcohol-Chloroxylenol, and Azithromycin.

Optical Density										
Group	Intervention								Mean	SD
	1	2	3	4	5	6	7	8		
Alcohol-Triclosan 100%	0.048	0.04	0.039	0.048	0.048	0.05	0.048	0.057	0.0473	0.00568
Alcohol-Triclosan 50%	0.05	0.052	0.049	0.049	0.049	0.122	0.059	0.052	0.0603	0.02517
Alcohol-Triclosan 33%	0.052	0.047	0.048	0.073	0.058	0.06	0.052	0.054	0.0555	0.00835
Chloroxylenol 10 ppm	0.061	0.048	0.045	0.053	0.049	0.051	0.046	0.057	0.0513	0.00552
Chloroxylenol 100 ppm	0.053	0.053	0.048	0.048	0.052	0.085	0.048	0.103	0.0613	0.02089
Chloroxylenol 1000 ppm	0.062	0.055	0.078	0.055	0.061	0.076	0.055	0.075	0.0646	0.01010
Azithromycin 64 µg/mL	0.057	0.054	0.048	0.059	0.06	0.061	0.054	0.062	0.0569	0.00467
Azithromycin 32 µg/mL	0.056	0.056	0.05	0.06	0.058	0.152	0.054	0.052	0.0673	0.03439
Azithromycin 16 µg/mL	0.06	0.056	0.073	0.075	0.059	0.058	0.059	0.058	0.0623	0.00736
Azithromycin1µg/mL	0.061	0.059	0.063	0.061	0.062	0.065	0.063	0.062	0.0620	0.00177

Statistical Analysis of the Intervention Group

The result of the analysis performed is displayed in Table 3. From the Kruskal-Wallis's test, it was found that $p<0.001$ which means that there is a statistically significant difference from at least three intervention groups. Therefore, the Dunn-Bonferroni test was performed to assess which of the intervention groups has a statistically significant difference in OD compared to the positive control group. Based on Dunn's post-hoc test, three groups showed a statistically significant result. Alcohol-Triclosan 100%, Alcohol-Triclosan 50%, and Alcohol-Chloroxylenol 10 ppm demonstrate $p < 0.05$. The test statistic value (Z) represents the magnitude of the intervention effect against biofilm formation, Alcohol-triclosan leads with a Z value of -4.840

DISCUSSION

Pseudomonas Aeruginosa Biofilm Production

P. aeruginosa is a gram-negative bacterium, with a high virulence as a pathogenic bacterium commonly associated with nosocomial infection [9]. This high virulence is partly caused by the ability of biofilm production observed in *P. aeruginosa* colony which gives the ability to suppress hazardous effects of environment and host immune response [6]. *P. aeruginosa* acquired the ability of quorum sensing (QS) which allows the colony to coordinate the transcription of specific genes which intensifies the adaptation traits of the colony [10].

Biofilm itself is a colony of bacteria that grows on the extracellular matrix covered with structural components of eDNA and biosurfactants which act as a protective barrier and ensure a stable environment for growth and colonization [11]. Biofilm can also occur in an external environment, particularly in hospital settings. This extremely adapted colony is able to withstand several antiseptic approaches while acquiring antimicrobial resistance to variations of agents in the hospital [12]. The use of newer or combination antiseptic agents to eradicate biofilm in *P. aeruginosa* is associated with superinfection in hospital settings, particularly in ICU [13]. In the pandemic era of COVID-19, one study found that *P. aeruginosa* superinfection happened in 46% of COVID-19 patients with a high antimicrobial resistance rate [14].

TABLE 3: Kruskas-Wallis and Post-Hoc Dunn-Bonferroni Test of the intervention group compared to the positive control.

Group	Kruskal-Wallis (p-value)	Post-Hoc Dunn-Bonferroni	
		Comparison with Positive Control (p-value)	Test statistic (Z)
Alcohol-Triclosan 100%	P < 0.001	**0.000***	**-4.840**
Alcohol-Triclosan 50%		**0.049***	**-3.375**
Alcohol-Triclosan 33%		0.094	-3.190
Alcohol-Chloroxylenol 10 ppm		**0.003***	**-4.058**
Alcohol-Chloroxylenol 100 ppm		0.126	-3.105
Alcohol-Chloroxylenol 1000 ppm		1.000	-1.218
Azithromycin 64 µg/mL		0.813	-2.503
Azithromycin 32 µg/mL		0.734	-2.539
Azithromycin 16 µg/mL		1.000	-1.582
Azithromycin 1 µg/mL		1.000	-0.917

Alcohol and Triclosan for Pseudomonas Aeruginosa

The high potency of antimicrobials was found in the alcohol-100% triclosan combination. One study has found that triclosan is an effective antiseptic agent to increase the vulnerability of *P. aeruginosa* biofilm [15]. It is known that triclosan is an adjuvant aminoglycoside which shows a good safety profile if used in humans to accelerate the eradication of biofilm. Triclosan has hydrophobic properties which could denature proteins and alter their biological role. Triclosan also shows the ability to alter the integrity of extracellular polymeric substances (EPS) in the biofilm with the effect of enhancing other antimicrobial penetration synergistically [16].

Even though effective antimicrobial effects were observed, another study shows that low-concentration of triclosan could increase the attachment ability of *P. aeruginosa* colony to their environment [17]. This is due to the difference in dose-response relationship in triclosan utilization. Low-concentration triclosan acts as a bacteriostatic agent through the inhibition of fatty acid synthesis [18]. In some conditions, even though the growth of the colony is altered, the attachment ability is increased and resistance could occur due to stress condition exposure [19]. The high concentration of triclosan on the other hand has a bactericidal effect which kills the bacteria through outer membrane lysis and inhibition of lipid synthesis [20].

The synergistic effect of triclosan and alcohol is due to the ability of alcohol to destroy the cell membrane of *P. aeruginosa.* In lower concentrations, such as in the 50% triclosan concentration, this synergistic ability could be disturbed due to limited penetration and uneven distribution of both agents [21].

As previously mentioned, the lower concentration combination could further increase the EPS production due to stress conditions and further increase the resistance of both agents [22]. It is also known that 70% alcohol concentration works best in almost all bacteria as an antimicrobial agent. Thus, a higher concentration combination of both agents altogether is needed to effectively eradicate the *P. aeruginosa* colony.

Alcohol and Chloroxylenol for Pseudomonas Aeruginosa

The usage of alcohol and 10 ppm chloroxylenol shows the best effectiveness in inhibiting biofilm production in *P. aeruginosa.* It is known from a recent study that chloroxylenol has a lethal effect on bacteria [23]. A high dose of chloroxylenol could inhibit biofilm production while a low concentration does not [24]. The mechanism of action of chloroxylenol is assumed to be similar to other phenol and halophenol which is to alter the integrity of bacterial cell membrane and cause leakage of cell plasma leading to death [18]. It is similar to the mechanism of action of triclosan which both alters cellular protein function and causes shutting down of vital cell function.

It is generally known that gram-negative bacteria cell membrane has different properties compared with gram-positive bacteria which causes resistance to several antibacterial agents. The complex cell wall composition acts as a barrier preventing the penetration of antibacterial agents [25]. Some studies show *P. aeruginosa* developing resistance to chloroxylenol [26]. One of the factors contributing to this condition is the wide usage of chloroxylenol as a common agent for hospital antiseptics.

Further research also shows that because of this increasing usage of chloroxylenol as a common antiseptic, chloroxylenol has been found abundant in aquatic environments [27]. Thus, even though chloroxylenol is an effective agent in *P. aeruginosa* eradication, several aspects should be considered, particularly antimicrobial resistance and environmental impact.

Azithromycin for Pseudomonas Aeruginosa

Azithromycin is a common antimicrobial agent used in the infection of *P. aeruginosa*. In one study, azithromycin could delay the production of biofilm but not inhibit it in any way [28]. This partial effect could support the development of resistance to azithromycin in *P. aeruginosa.* Recent study proves this theory showing that *P. aeruginosa* has high resistance to several macrolides, including azithromycin [29]. The tolerance of azithromycin *in P. aeruginosa* also results from the efflux pump gene mutation which could excrete azithromycin out of the cell, resulting in reduced antimicrobial activity [30].

In the event of *P. aeruginosa* infection, the colony could produce biofilm on the surface of the extracellular matrix. This process is the main contributing factor to the high prevalence of *P. aeruginosa* infection as a persistent nosocomial infection [31]. With this high resistance developed in azithromycin, several alternative treatment regimens have been proposed. One of these is the combination therapy of azithromycin with polymyxin B [32]. This study shows that this combination is effective even in the case of multi-drug resistance isolates of *P. aeruginosa.* Thus, to battle the antimicrobial resistance in *P. aeruginosa*, a combination drug should be utilized and hospital management of nosocomial infection should be further assessed, including in the protocol of antiseptic procedure.

CONCLUSION

The combination of Alcohol and Triclosan has proven to be the most effective for inhibiting *P. aeruginosa* biofilm formation compared to the Alcohol-Chloroxylenol combination and azithromycin. The study also revealed that the dose of the combination used is important to determine the inhibiting potency, 100% and 50% Triclosan, and only 10 ppm of chloroxylenol showed a statistically significant difference in OD in terms of inhibiting the biofilm formation. The Azithromycin group showed no significant inhibition of the biofilm formation which showed the high capability of *P. aeruginosa* for forming a biofilm that leads to resistance towards this antibiotic.

ACKNOWLEDGMENT

The authors would like to acknowledge and thank everyone involved for finishing this research until publication.

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