Destructive Effect of Calcium Hypochlorite against *Pseudomonas aeruginosa* Biofilm

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Abstract

Background: *Pseudomonas aeruginosa* is the most common bacteria contaminating the hemodialysis water and has high capability to form a biofilm. The presence of biofilm is hazardous because it becomes a constant source of bacterial and toxin release toward the hemodialysis patient's blood. Calcium hypochlorite (Ca(OCl)2) is an easily obtained disinfectant. This study was aimed to detect the destructive effect of Ca(OCl)2 against *P. aeruginosa* biofilm and the optimal disinfectant concentration required to achieve significant effect.

Methods: This experimental study was conducted in six replicates from September to October 2015 in Microbiology Laboratory of Faculty of Medicine Universitas Padjadjaran Bandung. A modified tissue culture plate method was performed to grow *P. aeruginosa* biofilms which were subsequently treated with Ca(OCl)2 in various chlorine concentrations, namely 20, 30, 40, and 500 parts per million (ppm). The data was analyzed using Welch Analysis of Variance (ANOVA) and Games-Howell post-hoc tests and presented in tables.

Results: Data were obtained from 36 flat-bottomed polystyrene wells. There was a statistically significant mean difference between groups [$F(4, 11.92)= 91.198$, $p<0.001$)]. All of the tested chlorine concentrations caused significant decreases in biofilm optical densities ($p = 0.027$ for 20 ppm and $p< 0.001$ for 30, 40, and 500 ppm).

Conclusions: Ca(OCl)2 with chlorine concentrations of 20, 30, 40, and 500 ppm have significant destructive effect against *P. aeruginosa* biofilm. The mean differences among treated groups were not significant. The most optimum concentration is 30 ppm.

Keywords: Biofilm, calcium hypochlorite, *Pseudomonas aeruginosa*

Introduction

In the year of 2012, there were 15,980 patients who required hemodialysis in order to replace the kidney's function in eliminating the circulating toxins.[1] During a hemodialysis session, the patient's blood is in contact with 80-160 liters of dialysate through the dialysis membrane.[2] Therefore, adequate cleaning and disinfecting of the dialysate are required in order to protect the patients from blood borne virus and pathogenic bacteria.[3] Despite the vigorous attempt in purification and disinfection, the bacteria have evidently adapted to low nutrient niches such as the hemodialysis system and produce biofilm in order to survive.[4] The presence of biofilm is hazardous because it becomes a constant source of bacterial release toward the patient's blood and may induce chronic inflammatory reaction in hemodialysis patients.[5,6]

Several studies have concluded that *Pseudomonas aeruginosa* is the most common bacteria contaminating the hemodialysis water samples and has high tendency to form biofilm.[5,7] In fact, there was a *P. aeruginosa* bacteremia outbreak in hemodialysis facility in Israel[8] in 2013. These facts supports that a careful surveillance of *P. aeruginosa* biofilm in hemodialysis system is required.

A recent study in Bandung[9] revealed that practice of improper disinfecting of hemodialysis unit is still happening in Indonesia. Improper disinfecting effort not only causes chronic inflammation to the

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patients,[5,6] but also increases *P. aeruginosa* resistance against antibiotics or even increases the biofilm production.[4,10] Therefore, it is clear that a study needs to be performed in order to help determining the adequate disinfecting process to eliminate *P. aeruginosa* biofilms. Calcium hypochlorite ($Ca(OCl)_2$) is a common disinfectant which is cheap and easily obtained. It is relatively steady and has greater available chlorine than sodium hypochlorite (NaOCl). This study was aimed to detect the destructive effect of $Ca(OCl)_2$ against *P. aeruginosa* biofilm and its maximum concentration to achieve significant effect.

Methods

This experimental study with post-test only control group design was carried out from September–October 2015 in Microbiology Laboratory of Faculty of Medicine Universitas Padjadjaran Bandung. It had been approved by the Health Research Ethics Committee of Faculty of Medicine Universitas Padjadjaran Bandung.

Biofilm detection method using tissue culture plate as described by Christensen was used in this study with slight modification, namely addition of glucose[11] and prolonged incubation period.[12] In addition, the content of the well was increased from 200 µl to 250 µl. The sample size was calculated according formula of Federer. For five groups (four different chlorine concentrations and negative control), the minimal number of replication required is five.

Bacterial preparation was conducted by adding a loopful of *P. aeruginosa* American Type Culture Collection (ATCC) 27853 in lyophilized form to tryptone soy broth (OXOID) mixed with 1% glucose (Merck) which was incubated for 18 hours. The resulting broth was then stroken to sheep blood agar to maintain the viability of the bacterial isolate.[4,11]

The biofilm formation using modified tissue culture plate method[11,12] was performed by first adding a loopful of isolates from sheep blood agar which were inoculated to the tryptone soy broth with 1% glucose and incubated for 18 hours at 37°C. Then, the broth was diluted with fresh tryptone soy broth 1:100 and poured into the polystyrene 96-well flat-bottomed tissue culture plate with lid (Iwaki). Eight wells for positive controls were filled with 250 µl fresh medium only without bacteria, whereas the remaining eight wells for negative controls and thirty-two wells for treatment were filled with 250 µl aliquots of the diluted broth. Next, the edges of the tissue culture plate were covered and sealed using a parafilm to avoid evaporation, and then incubated at 37 °C for 48 hours. The contents of the wells were emptied by pipetting without touching the base. Each well then washed with 250 µl of phosphate buffer saline for four times to remove the free floating bacteria.[11]

The disinfecting process was done by treating the wells with $Ca(OCl)_2$ (Bratachem) in different chlorine concentrations. Previous experiment showed that single-species biofilms could be inactivated by 30 parts per million (ppm) of chlorine,[13] and very high concentration of chlorine (>500 ppm) could cause corrosiveness to metals.[3] Thus, the chlorine concentrations of 20, 30, 40, and 500 ppm were chosen. The chlorine concentrations were obtained by freshly diluting the 60% $Ca(OCl)_2$ powder with deionized water according to the World Health Organization (WHO)14 fact sheet on environmental sanitation number 2.19. In order to create a 2% (20,000 ppm) $Ca(OCl)_2$ solution, 3.33 grams of 60% $Ca(OCl)_2$ powder were added to 100 ml of deionized water. The resulting supernatant were diluted in 1:1000, 1:660, 1:500, and 1:4 to yield the desired chlorine concentrations. Each concentration was tested on eight individual wells, resulting in 32 treated wells. The disinfecting process was performed by filling the designated wells with 250 µl of $Ca(OCl)_2$ and left for 30 minutes. The excess disinfectant was removed by gently tilting and tapping the plate, and washed using tap water. The fixation was achieved by adding sodium acetate 2% to each well for 15 minutes and air-dried.

The reading of remaining biofilm optical density on the plate was conducted using microplate photometer (Thermoscientific). Initial staining process was performed by pipetting crystal violet 0.1% to each well and left for 15 minutes. The remaining stain was removed by washing with tap water. Once the plate was dried, isopropyl hydrochloric acid was added to each well. The biofilm optical density was read using microplate photometer at 550 nm wavelength.[12]

The data were recorded and subsequently analyzed using statistical analysis software. The initial data distribution was not normal, so it was normalized using log10 function. As the Levene's test statistic showed non-homogeneity in variances (p-value = 0.002), Welch Analysis of Variance (ANOVA) was conducted instead of One-Way ANOVA in order to detect significant mean difference between

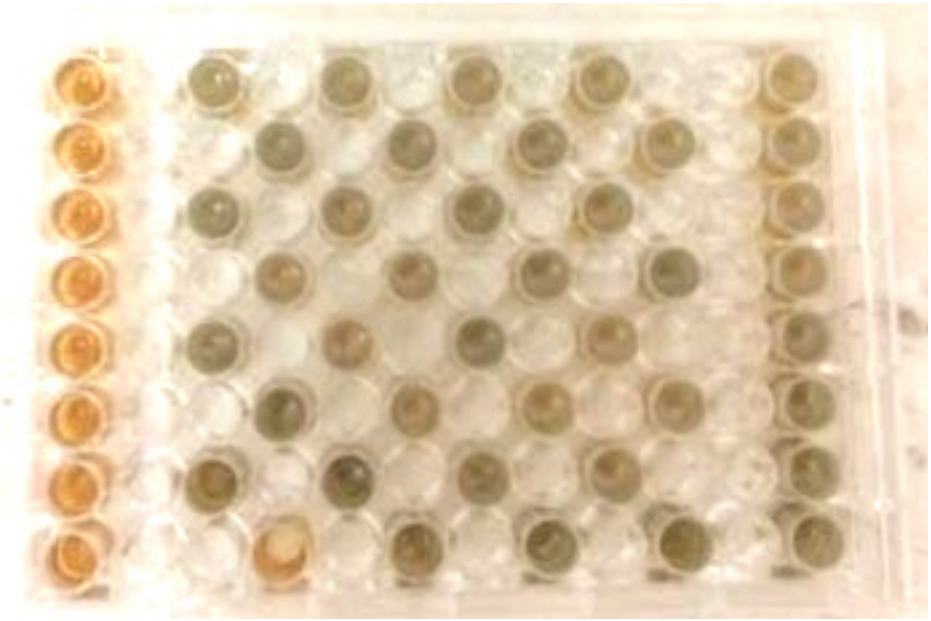

Figure 1 Tissue culture plate after 48 hours of incubation, showing biofilms growth (green) and positive control (yellow). One of the well at the bottom showed complete evaporation

groups. Games-Howell test was carried out as the post-hoc test. The p-values of < 0.05 were considered significant. The results of the analysis were presented in tables.

Results

There were one well for negative control, one well for positive control, and 4 treated wells for each chlorine concentration (20, 30, 40, and 500 ppm). The experiment was originally run in eight replicates with 48 wells in total, but the last two sets were omitted due to presence of content evaporation which could possibly interfered with the analysis, resulting in 36 wells (six replicates) in total (Figure 1).

The non-treated biofilm in negative control wells had very high optical density compared to the treated wells and the positive control wells (Table 1). In general, the mean optical density was decreased in response to the increase of chlorine concentration. However, an exception occurred at the wells treated with chlorine 40 ppm which showed higher optical density compared to those of chlorine 30 ppm.

Table 1 Optical Density of Pseudomonas aeruginosa Biofilm

	Positive Control	Chlorine 20 ppm	Chlorine 30 ppm	Chlorine 40 ppm	Chlorine 500 ppm	Negative Control
OD_{550nm}	0.107	1.121	0.336	0.267	0.302	2.703
	0.117	0.359	0.457	0.571	0.366	2.762
	0.107	2.233	0.383	0.476	0.488	2.66
	0.121	0.284	0.372	0.364	0.288	2.847
	0.121	0.454	0.534	0.531	0.223	3.353
	0.113	0.410	0.544	0.569	0.785	2.076
Mean OD_{550nm}	0.114	0.810	0.438	0.463	0.409	2.734
Standard Deviation	0.006	0.760	0.088	0.123	0.205	0.409

Note: *OD_{550nm}: *P. aeruginosa* biofilm optical density read in microplate photometerat 550nm

Table 2 Multiple Comparison using Games-Howell Post-Hoc Test

Comparison	Mean Difference	Standard Error	P-value
Negative Control – Chlorine 20 ppm	0.653*	0.144	0.027
Negative Control – Chlorine 30 ppm	0.799*	0.045	< 0.001
Negative Control – Chlorine 40 ppm	0.782*	0.060	< 0.001
Negative Control – Chlorine 500 ppm	0.860*	0.084	< 0.001
Chlorine 20 ppm – Chlorine 30 ppm	0.146	0.145	0.846
Chlorine 20 ppm – Chlorine 40 ppm	0.129	0.151	0.904
Chlorine 20 ppm – Chlorine 500 ppm	0.207	0.162	0.710
Chlorine 30 ppm – Chlorine 40 ppm	-0.165	0.064	0.999
Chlorine 30 ppm – Chlorine 500 ppm	0.061	0.087	0.948
Chlorine 40 ppm – Chlorine 500 ppm	0.078	0.096	0.920

Note: *Significant mean difference

performed prior to analysis. Welch ANOVA result showed a statistically significant mean difference between groups [F(4, 11.92) = 91.198, p < 0.001)].

Games-Howell post-hoc showed significant difference between non-treated group (negative control) and treated groups ((Ca(OCl)2 with chlorine concentrations of 20, 30, 40, and 500 ppm). However, the mean differences among treated groups were not significant (Table 2).

Discussion

Biofilm needs to be removed because its presence in the hemodialysis system is hazardous to the hemodialysis patients.[5,6] Several disinfectant containing chlorine and monochloramine have been proved to be effective in inactivating bacterial biofilm.[3]

The $Ca(OCl)_2$ is a chlorine-based disinfectant which is very affordable and widely available for purchase. Its use includes industrial sterilization, water purification, and bleaching. When it is freshly diluted in water, the following reaction will occur: $Ca(OCl)_2$ + 2 H2O --> 2 HOCl + $Ca(OH)_2$.[15] The resulting undissociated hypochlorous acid (HOCl) is the active disinfecting component which has bactericidal properties.[3,16]

According to study by Tote et al.[15] both hydrogen peroxide and hypochlorite solution were active on both the biofilm matrix and the viable mass of *P. aeruginosa*. Although their study used NaOCl instead of $Ca(OCl)_2$, the results might be comparable because the active agent of both biocides is hypochlorous acid. A 1% hypochlorite solution can markedly reduce *P. aeruginosa* biofilm matrix, reaching a 66% reduction at 1 minute and 91% reduction after 15 minutes of treatment.[17]

The HOCl and its dissociated form, hypochlorite ion (OCl-), exert damaging consequences to the *P. aeruginosa's* cell by several mechanisms. First, HOCl can cause deleterious effect on DNA as it readily reacts with highly nucleophilic sites.[18] Second, HOCl severely repressed several genes involved inprimary metabolic processes, (glucose transport, oxidative phosphorylation, and electron transport) resulting in minimal energy production.[19] Third, HOCl induces active transport of several organic sulfur compounds, possibly in order to manage the sulfur starvation issue (as HOCl reacts strongly with sulfhydryl groups in many substrates) and to find alternative carbon source needed for energy production.[18,19] Finally, HOCl stress generate deleterious oxidative species such as superoxide anions (O2-) and hydroxyl radicals (`OH) which can damage cellular components.[18,19]

In regard to the mean optical density in each treatment groups of this study, it is possible that the destruction of biofilm is concentration-dependant. The higher the chlorine concentration used, the lower the optical density would become. However, an exception occurred in the treatment group with 40 ppm chlorine. Instead of a lower optical density, it was actually showing higher optical density (mean 0.463, SD0.123) compared to those treated with 30 ppm chlorine (mean 0.438, SD 0.088). This result was possibly caused by the limitation of this study.

According to the statistical analysis, there were significant mean difference between

groups [F(4, 11.92) = 91.198, p < 0.001)]. Post-hoc result showed that in comparison to the non-treated group (negative control), all of the tested chlorine concentrations caused significant decreases in mean biofilm optical densities (p = 0.027 for 20 ppm and p < 0.001 for 30, 40, and 500 ppm).

The chlorine concentration of 20 ppm yielded significant mean difference, but still the p-value was not as significant as those of 30, 40, and 500 ppm. This result is consistent with the findings of Behnke et al.[18] which stated that single-species biofilms were readily inactivated with 30 ppm of chlorine with contact time of 30 minutes.

However, according to a study by Borges et al.[19] *P. aeruginosa* were resistant to hypochlorite solution at a concentration of 500 ppm for 10 minutes contact time. This disinfection routine was used monthly for disinfecting the water distribution system in its local dialysis unit. With such a high chlorine concentration, the possible explanation for the result was that 10 minutes of contact time were not enough to cause sufficient damage against *P. aeruginosa*.[19] In addition, the usage of hypochlorite in high concentrations (> 500 ppm) should be avoided as it can cause corrosiveness to metals.[3]

At present, there is no specific intervention which is intended to specifically manage the presence of *P. aeruginosa* and its biofilm in hemodialysis system. However, according to Agar et al.[20] any water used for hemodialysis should meet the International Organization for Standardization (ISO) guidelines 13959:2014 to ensure the patient's protection. The current standard for maximum allowable bacteria levels and endotoxin levels are at 100 colony forming unit (CFU)/ml and 0.25 endotoxin unit (EU)/ml, respectively. In addition, Agar et al.[20] also stated that biofilm prevention should be performed by heat-disinfecting the water distribution system in regular basis to limit bacterial proliferation. Hard-to-reach areas within the equipment need to be manually cleaned. In some cases, harsh chemicals (e.g., peracetic acid) is required. Finally, it is advised to annually replace the inflow hoses to prevent biofilm formation.

The limitation of this study includes an inability to measure the exact chlorine concentration in each $Ca(OCl)_2$ solution. The assumed chlorine concentration in this study heavily relied on the manual dilution process which may be subjected to human error.

In conclusion, $Ca(OCl)_2$ with chlorine concentrations of 20, 30, 40, and 500 ppm have significant destructive effect against P. aeruginosa biofilm. The most optimum chlorine concentration for disinfecting *P. aeruginosa* biofilm is 30 ppm as the lowest concentration with high significance result. Further study using chlorine-based disinfectant should use specific method such as iodometric titration in order to determine the actual chlorine concentration in the disinfecting solution. It should include more variables such as by incorporating more chlorine concentrations with various contact time, adding water shear stress, and testing it to other single-species bacterial biofilm and multi-species bacterial biofilms whose combination includes *P. aeruginosa* and other bacteria which frequently contaminate the hemodialysis system. If possible, the bacteria should be obtained from an actual hemodialysis water sample.

References

1. Perkumpulan Nefrologi Indonesia. 5th report of Indonesian renal registry. Jakarta: Perkumpulan Nefrologi Indonesia; 2012.
2. Kwan BCH, Chow KM, Ma TKW, Cheng PMS, Leung CB, Li PKT, et al. Effect of using ultrapure dialysate for hemodialysis on the level of circulating bacterial fragment in renal failure patients. Nephron Clin Pract. 2013;123(1):246–53.
3. Centers for Disease Control and Prevention. Guideline for disinfection and sterilization in healthcare facilities. Atlanta: Centers for Disease Control and Prevention; 2008.
4. Suman E, Varghese B, Joseph N, Nisha K, Kotian MS. The bacterial biofilms in dialysis water systems and the effect of the sub inhibitory concentrations of chlorine on them. J Clin Diagn Res. 2013;7(5):849–52.
5. Montanari LB, Sartori FG, Cardoso MJ, Varo SD, Pires RH, Leite CQ, et al. Microbiological contamination of a hemodialysis center water distribution system. Rev Inst Med Trop Sao Paulo. 2009;51(1):37–43.
6. Oumokhtar B, Lalami AEO, Mahmoud M, Berrada S, Arrayhani M, Houssaini TS. Prevent infection linked to the dialysis water in a hemodialysis center in Fez city (Morocco). Pan Afr Med J. 2013;16(1):122–6.
7. Arvanitidou M, Vayona A, Spanakis N, Tsakris A. Occurrence and antimicrobial resistance of Gram-negative bacteria isolated in haemodialysis water and dialysate of renal units: results of a Greek multicentre study. J Appl Microbiol.

2003;95(1):180–5.
8. Ciobotaro P, Fialko A, Nadir E, Oved M, Bardenstein R, Gershkoviz P, et al. P205: an outbreak of polyclonal pseudomonas aeruginosa bacteremia in hemodialysis patients. Antimicrob Resist Infect Control. 2013;2(Suppl 1):205.
9. Al-Jailawi MH, Ameen RS, Al-Jeboori MR. Effect of Disinfectants on Antibiotics Susceptibility of Pseudomonas aeruginosa. J Appl Biotechnol. 2013;1(1):54–63.
10. Hassan A, Usman J, Kaleem F, Omair M, Khalid A, Iqbal M. Evaluation of different detection methods of biofilm formation in the clinical isolates. Braz J Infect Dis. 2011;15(4):305–11.
11. World Health Organization. Fact sheet 2.19: calcium hypochlorite. [cited 2015 December 1]. Available from: http://www.who.int/water_sanitation_health/hygiene/emergencies/fs2_19.pdf.
12. Mathur T, Singhal S, Khan S, Upadhyay DJ, Fatma T, Rattan A. Detection of biofilm formation among the clinical isolates of Staphylococci: an evaluation of three different screening methods. Indian J Med Microbiol. 2006;24(1):25–9.
13. Dutta A, Saunders WP. Comparative evaluation of calcium hypochlorite and sodium hypochlorite on soft-tissue dissolution. J Endod. 2012;38(10):1395–8.
14. Wang L, Bassiri M, Najafi R, Najafi K, Yang J, Khosrovi B, et al. Stabilized hypochlorous acid: a component of the inorganic armamentarium of innate immunity. J Burns Wounds. 2007;6(1):65-79.
15. Tote´ K, Horemans T, Berghe DV, Maes L, Cos P. Inhibitory effect of biocides on the viable masses and matrices of Staphylococcus aureus and Pseudomonas aeruginosa biofilms. Appl Environ Microbiol. 2010;76(10):3135–42.
16. Fukuzaki S. Mechanisms of actions of sodium hypochlorite in cleaning and disinfection processes. Biocontrol Sci. 2006;11(4):147–57.
17. Small DA, Chang W, Toghrol F, Bentley WE. Toxicogenomic analysis of sodium hypochlorite antimicrobial mechanisms in Pseudomonas aeruginosa. Appl Microbiol Biotechnol. 2007;74(1):176–85.
18. Behnke S, Parker AE, Woodall D, Camper AK. Comparing the chlorine disinfection of detached biofilm clusters with those of sessile biofilms and planktonic cells in single and dual-species cultures. Appl Environ Microbiol. 2011;77(20):7176–84.
19. Borges CR, Lascowski KM, Filho NR, Pelayo JS. Microbiological quality of water and dialysate in a haemodialysis unit in Ponta Grossa-PR, Brazil. J Appl Microbiol. 2007;103(5):1791–7.
20. Agar JW, Perkins A, Heaf JG. Home hemodialysis: infrastructure, water, and machines in the home. Hemodial Int. 2015;19(Suppl 1):S93–111.