Destructive Effect of Calcium Hypochlorite against *Pseudomonas aeruginosa* Biofilm

Ilma Arifani,1 Gita Widya Pradini,2 Insi Farisa Desy Arya,3 Adi Imam Cahyadi2
1Faculty of Medicine Universitas Padjadjaran, 2Department of Microbiology and Parasitology Faculty of Medicine Universitas Padjadjaran, 3Department of Public Health Faculty of Medicine Universitas Padjadjaran

**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. In a hemodialysis session, the patient's blood is in contact with 80-160 liters of dialysate through the dialysis membrane. Therefore, adequate cleaning and disinfecting of the dialysate are required in order to protect the patients from blood borne virus and pathogenic bacteria. 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. 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.

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

A recent study in Bandung revealed that practice of improper disinfecting of hemodialysis unit is still happening in Indonesia. Improper disinfecting effort not only causes chronic inflammation to the patients but also may lead to the outbreak of *P. aeruginosa* bacteremia. Therefore, the appropriate disinfecting method should be performed in order to reduce the risk of bacteremia in hemodialysis patients.
patients,\textsuperscript{5,6} but also increases \textit{P. aeruginosa} resistance against antibiotics or even increases the biofilm production.\textsuperscript{4,10} Therefore, it is clear that a study needs to be performed in order to help determining the adequate disinfecting process to eliminate \textit{P. aeruginosa} biofilms. Calcium hypochlorite (Ca(OCl)\textsubscript{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)\textsubscript{2}, against \textit{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\textsuperscript{11} and prolonged incubation period.\textsuperscript{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 \textit{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.\textsuperscript{4,11}

The biofilm formation using modified tissue culture plate method\textsuperscript{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 of phosphate buffer saline for four times to remove the free floating bacteria.\textsuperscript{11} The disinfecting process was done by treating the wells with Ca(OCl)\textsubscript{2} (Bratachem) in different chlorine concentrations. Previous experiment showed that single-species biofilms could be inactivated by 30 parts per million (ppm) of chlorine,\textsuperscript{13} and very high concentration of chlorine (>500 ppm) could cause corrosiveness to metals.\textsuperscript{2} 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)\textsubscript{2} powder with deionized water according to the World Health Organization (WHO)\textsuperscript{14} fact sheet on environmental sanitation number 2.19. In order to create a 2% (20,000 ppm) Ca(OCl)\textsubscript{2} solution, 3.33 grams of 60% Ca(OCl)\textsubscript{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)\textsubscript{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.\textsuperscript{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
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**

<table>
<thead>
<tr>
<th>Positive Control</th>
<th>Chlorine 20 ppm</th>
<th>Chlorine 30 ppm</th>
<th>Chlorine 40 ppm</th>
<th>Chlorine 500 ppm</th>
<th>Negative Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD$_{550\text{nm}}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.107</td>
<td>1.121</td>
<td>0.336</td>
<td>0.267</td>
<td>0.302</td>
<td>2.703</td>
</tr>
<tr>
<td>0.117</td>
<td>0.359</td>
<td>0.457</td>
<td>0.571</td>
<td>0.366</td>
<td>2.762</td>
</tr>
<tr>
<td>0.107</td>
<td>2.233</td>
<td>0.383</td>
<td>0.476</td>
<td>0.488</td>
<td>2.66</td>
</tr>
<tr>
<td>0.121</td>
<td>0.284</td>
<td>0.372</td>
<td>0.364</td>
<td>0.288</td>
<td>2.847</td>
</tr>
<tr>
<td>0.121</td>
<td>0.454</td>
<td>0.534</td>
<td>0.531</td>
<td>0.223</td>
<td>3.353</td>
</tr>
<tr>
<td>0.113</td>
<td>0.410</td>
<td>0.544</td>
<td>0.569</td>
<td>0.785</td>
<td>2.076</td>
</tr>
<tr>
<td>Mean OD$_{550\text{nm}}$</td>
<td>0.114</td>
<td>0.810</td>
<td>0.438</td>
<td>0.463</td>
<td>0.409</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.006</td>
<td>0.760</td>
<td>0.088</td>
<td>0.123</td>
<td>0.205</td>
</tr>
</tbody>
</table>

Note: *OD$_{550\text{nm}}$*: *P. aeruginosa* biofilm optical density read in microplate photometer at 550nm
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)\textsubscript{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. Several disinfectant containing chlorine and monochloramine have been proved to be effective in inactivating bacterial biofilm. The Ca(OCl)\textsubscript{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: \(\text{Ca(OCl)}_{2} + 2 \text{H}_{2}\text{O} \rightarrow 2 \text{HOCl} + \text{Ca(OH)}_{2}\). The resulting undissociated hypochlorous acid (HOCl) is the active disinfecting component which has bactericidal properties.

According to study by Tote et al., 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)\textsubscript{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.

The HOCl and its dissociated form, hypochlorite ion (OCl\textsuperscript{-}), exert damaging consequences to the *P. aeruginosa* cell by several mechanisms. First, HOCl can cause deleterious effect on DNA as it readily reacts with highly nucleophilic sites. Second, HOCl severely repressed several genes involved in primary metabolic processes, (glucose transport, oxidative phosphorylation, and electron transport) resulting in minimal energy production. 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. Finally, HOCl stress generate deleterious oxidative species such as superoxide anions (O\textsubscript{2}-) and hydroxyl radicals (\'OH) which can damage cellular components.

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, SD 0.123) 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.\(^2\)

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


