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In vitro kill-time test of disinfectants against *Pseudomonas aeruginosa* recovered from water associated with hemodialysis applications

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Abstract

Bacterial contamination causes various problems in patients with kidney disease undergoing hemodialysis. *Pseudomonas aeruginosa* is found as a major contaminant in dialysis apparatus, therefore, effective disinfection to prevent the buildup of this bacteria in the system is needed. The aim of this study was to evaluate the effective concentration and required kill time of disinfectants commonly used for disinfection of dialysis apparatus against *Pseudomonas aeruginosa*. Antibacterial activities of five disinfectants, sodium hypochlorite, hydrogen peroxide, formalin, Perxania 2505 (5% peracetic acid) and Benzalkonium chloride were evaluated. They were tested against *P. aeruginosa* isolated from contaminated dialysis apparatus. The evaluation procedures included minimal bactericidal concentration (MBC) assay and kinetics of killing assessment. It was shown that Perxania possesses the lowest MBC required for killing *P. aeruginosa* (0.2 ppm) while the MBCs of sodium hypochlorite, hydrogen peroxide, formalin and Benzalkonium chloride are 200, 2, 370 and 1000 ppm, respectively. Kinetics of killing of each disinfectant was performed at MBC values of each disinfectant to kill 10^6 CFU/ml inoculum of tested bacteria. The results showed that Perxania killed 3 logs (99.9%) of *P. aeruginosa* in 58 minutes. In conclusion, the in vitro kinetics of kill studies demonstrate that Perxania 2025 (PAA) rapidly kills *P. aeruginosa* recovered from water associated with hemodialysis system. The speed of kill observed for PAA is faster than that for other test disinfectants under the same test conditions.

***Keywords*:** *Pseudomonas aeruginosa, hemodialysis, kill-time*

บทคัดย่อ

การปนเปื้อนของแบคทีเรียในน้ำที่ใช้ในเครื่องฟอกไตเป็นปัจจัยสำคัญที่ทำให้เกิดการติดเชื้อในผู้ป่วยที่รับการฟอกไต แบคทีเรียที่พบปนเปื้อนได้บ่อยในเครื่องฟอกไตและมักก่อให้เกิดอันตรายร้ายแรงกับผู้ป่วย คือ *Pseudomonas aeruginosa* ดังนั้นจึงจำเป็นที่ต้องมีการป้องกันการสะสมของเชื้อนี้ในระบบเครื่องฟอกไต วัตถุประสงค์ของงานวิจัยนี้เป็นการศึกษาความเข้มข้นและเวลาที่เหมาะสมในการฆ่าเชื้อ *Pseudomonas aeruginosa* ของน้ำยาฆ่าเชื้อที่นิยมใช้กำจัดเชื้อปนเปื้อนในเครื่องฟอกไต น้ำยาฆ่าเชื้อ 5 ชนิด คือ sodium hypochlorite, hydrogen peroxide, formalin, Perxania 2505 (5% peracetic acid) และ Benzalkonium chloride ได้ถูกนำมาศึกษาหาความเข้มข้นต่ำสุดที่สามารถฆ่าเชื้อ (minimal bactericidal concentration, MBC) และ เวลาที่ใช้ในการทำให้เชื้อ *P. aeruginosa* ลดลงร้อยละ 99.9 ผลการวิจัยพบว่า Perxania แสดงค่า MBC ต่ำสุดคือ 0.2 ppm ขณะที่ sodium hypochlorite, hydrogen peroxide, formalin และ Benzalkonium chloride แสดงค่า MBC เท่ากับ 200, 2, 370 และ 1000 ppm ตามลำดับ และจากการทดสอบ Kinetic of killing ของน้ำยาทั้ง 5 ชนิดที่ความเข้มข้นเท่ากับค่า MBC เพื่อฆ่าเชื้อ *P. aeruginosa* ด้วยจำนวนเชื้อเริ่มต้น 10^6 CFU/ml พบว่า Perxania สามารถกำจัดเชื้อ *P. aeruginosa* ได้ร้อยละ 99.9 ภายในเวลา 58 นาที ขณะที่น้ำยาชนิดอื่นๆที่ใช้ในการทดสอบต้องใช้เวลามากกว่านี้ในการทำให้เชื้อนี้ลดลงในจำนวนที่เท่ากัน โดยสรุปแล้วการศึกษานี้เป็นการแสดงให้เห็นถึงความเข้มข้นของน้ำยาฆ่าเชื้อที่เหมาะสมในการกำจัดเชื้อ *Pseudomonas aeruginosa* ที่ปนเปื้อนในเครื่องฟอกไต และ Perxania ฆ่าเชื้อ ได้ในเวลาที่สั้นที่สุด

***คำสำคัญ*:** *Pseudomonas aeruginosa, hemodialysis, kill-time*

1. Introduction

Water used in hemodialysis applications should be marked as a critical product since multi-resistant bacteria contaminated in hemodialysis water system can be one of the major sources of nosocomial infection in hemodialysis patients. Proper infectious control practices must be employed to improve hospital hygiene and reduce the infection risk in patients with chronic renal failure. Generally, these patients are more susceptible to nosocomial infection than others due to the dialytic treatment to which they are

submitted. During hemodialysis patients are exposed to large volumes of dialysis fluid with the dialyzer membrane being the only barrier preventing transfer of harmful contaminants from the dialysis fluid to the patient. Therefore, comprehensive control of water quality in hemodialysis services is extremely important in order to guarantee a better quality of life of the patients submitted to this treatment.

Water associated with hemodialysis applications includes dialysis water and dialysis fluid. The dialysis water means water that has been treated to meet the specified limits for chemical and microbial contaminants described by the Association of Renal Technologies and is suitable for use in hemodialysis applications. Hemodialysis applications include the preparation of dialysis fluid, reprocessing of dialyzers, preparation of concentrates, and preparation of substitution fluid for online convective therapies. The dialysis fluid refers to the fluid made from dialysis water and concentrates that is delivered to the dialyzer by the dialysis fluid delivery system. Dialysate and dialysis solution are synonyms that may be used in place of dialysis fluid (Mactier et al., 2009).

The genus *Pseudomonas* have been found to contaminate a number of aqueous solutions including reverse osmosis water used for dialysis applications (Morrison and Wenzel, 1984; Gilardi, 1991). Among members of the genus, *Pseudomonas aeruginosa* is recognized as one of the most persistent nosocomial pathogens. It is considered the most significant human pathogen in the genus and develops resistance to many disinfectants and antibiotics (Ayliffe and Collins, 1992; Lubello et al., 2002; Brooks et al., 2007). Use of effective disinfectants play an important role in preventing hospital acquired infections caused by these bacteria. There has been an interest in improving the sterilization and disinfection procedures to reduce the infection risk for patients undergoing renal hemodialysis. Many techniques have been used for evaluation of the effectiveness of disinfecting agents. These include the disk diffusion method, minimal inhibitory concentration test (MIC), kill-curve technique, and other methods. In this study the time-kill methodology was used to compare the rate of kill of several chemical disinfectants, commonly used for disinfection of dialysis machine and water system, using an in vitro *Pseudomonas aeruginosa* culture as a model.

2. Materials and methods

2.1 Bacterial isolates

Pseudomonas aeruginosa strains used in this study were originally isolated from water associated with dialysis machines sent from several hospitals' dialysis units to Watec Laboratories, Watec Company, Thailand. Cetrimide Agar plates were used for bacterial isolation. *P. aeruginosa* strain ATCC 27853 was used as a positive control strain for the expression of all biological traits tested.

2.2 Phenotypic identification of *Pseudomonas aeruginosa*

Morphological analysis, Gram staining, catalase and oxidase activities, biochemical tests such as indole, methyl red, Voges Proskauer, motility, oxidation-fermentation tests, starch hydrolysis, arginine, tween 80 and malonate utilization tests were completed according to standard methods described by Harrigon and McCance (1976) and MacFaddin (2000). Cultures were also streaked onto Difco *Pseudomonas* agar F to detect fluorescein production and Difco *Pseudomonas* agar P to detect pyocyanin production.

2.3 Genotypic identification of *Pseudomonas aeruginosa*

2.3.1 DNA extraction

Genomic DNA was prepared from 30 samples of *P. aeruginosa* obtained phenotypically. The extraction was performed according to Hassan et al. (2012) with slight modification. Ten ml overnight cultures were prepared from a single colony. Cells were harvested in a centrifuge for 5 min at 5000 rpm and suspended in 200 µl TE buffer (pH 8). Then 30 mg/ml lysozyme to the cell suspensions was added and incubated for 2 hours at 37 ºC. After the incubation, 370 µl of TE containing Proteinase K (1 mg/ml) were added and incubated for 1 h, then 30 µl of 10% SDS were added. The samples were then incubated for 1 h at 37 °C. After that phenol-chloroform extraction was performed using one equal volume of phenol/ chloroform/ isoamyl alcohol (24/24/1) for 30 minutes. The samples were then centrifuged for 5 min at 5000 rpm. The aqueous phase was transferred into a clean microcentrifuge tube and

the genomic DNA was precipitated by the addition of cold isopropanol (one equal volume). Finally, the pellet was redissolved in TE buffer.

2.3.2 Primer selection

16S rDNA-based primer sets were selected based on previously published studies (Spillker et. al. (2004). A primer pair, PA-GS-F (5′GACGGGTGAGTAATGCCTA3′) and PA-GS-R (5′CACTGGTGTTCCTTCCTATA3′), was designed for *Pseudomonas* genus specific having a product size of 618 bp. PA-SS-F (5′GGGGGATCTTCGG ACCTCA3′) and PA-SS-R (5′TCCTTAGAGTG CCCACCCG3′) were designed to amplify only *P. aeruginosa* with a product size of 956 bp.

2.3.3 PCR conditions

Amplification of targeted DNA was carried out in 25µl reaction volumes, each containing 2 mM $MgCl_2$, 50 mM Trizma (pH 8.3), 250 µM (each) deoxynucleoside triphosphates, 0.4 µM (each) primer, 1 U of *Taq* polymerase, and 2µl DNA sample, and adjusted to 25 µl by the addition of sterile distilled water. Amplification was carried out as follow: initial denaturization at 95 °C for 2 min, 25 cycles were completed, each consisting of 20 s at 94 °C, 20 s at 58 °C, and 40 s at 72°C, and a final extension at 72 °C for 1 min. The amplified product was subjected to 1.2% agarose gel electrophoresis.

2.4 Time-kill study

2.4.1 Disinfectants

The disinfecting agents used in this study were 10% sodium hyperchlorite, 35% hydrogen peroxide, 37% formalin, Perxania 2505 (5% peracetic acid), and Benzalkonium chloride. The first three chemical biocides were purchased from Aldrich Chemical Company, U.S.A. The latter two commercially available disinfectants were from Watec Company, Thailand. All studied disinfectants are currently and widely used for the disinfection of hemodialysis system. They were tested for their effectiveness against *Pseudomonas aeruginosa* using time-kill technique. The test disinfectant concentration for time-kill studies was based on the minimal bactericidal concentration (MBC) determined according to the Clinical and Laboratory Standards Institute (CLSI).

2.4.2 Preparation of test organisms

Five isolates that were identified *as P. aeruginosa* by both phenotypic and genotypic methods were selected for disinfectant time-kill test. *P. aeruginosa* strain ATCC 27853 was also tested as a reference. A 0.1 ml aliquot of overnight culture was transferred to a fresh Tryptic Soy Broth (TSB) (referred to as "test culture"). At the same time, 0.1 ml of the overnight culture was transferred to 10 ml of TSB in a 20 ml cuvette (referred to as "cuvette culture"). Both test and cuvette cultures were incubated at 37 °C. The cuvette culture was used to monitor the growth of the test culture. This was done by determining the absorbance at 525 nm in a spectrophotometer every 60 minutes. A sterile cuvette containing 10 ml of sterile TSB was served as the blank for the spectrophotometric readings. The test culture is ready to initiate the actual test when it has reached a cell density of 10^7 to 10^8 CFU/ml (OD_{525} 0.40-0.45).

2.4.3 Kinetics of kill testing

One ml of the test culture was added to 9 ml of fresh TSB. Kinetics of kill testing was conducted by exposing 10^6 to 10^7 cells of bacteria to the test agent at MBC for various periods of time. Care was taken to insure that each strain was in "log phase" growth in TSB at 37°C at the time of the exposure to the test agent. At the end of the exposure time (i.e. 30, 60, 90, 120, 150, and 180 minutes), the "treated" cells were serially diluted and then collected on a membrane filter and washed with five 100 ml aliquots of 0.9% sterile saline solution using Millipore filter funnel with 0.45 µm membrane filter (Millipore Corp., Bedford, MA, USA.). The liquid was filtered through the membrane by the aid of vacuum pump. The membrane, containing cells, was then rinsed with 0.9% sterile saline solution to remove all entrapped test agent. After the washing steps, the membrane filter containing the "treated" cells was place on to Total Plate Count Agar and incubated at 37 °C for 24 hours to determine the surviving colony forming unit (CFU) per ml.

3. Results

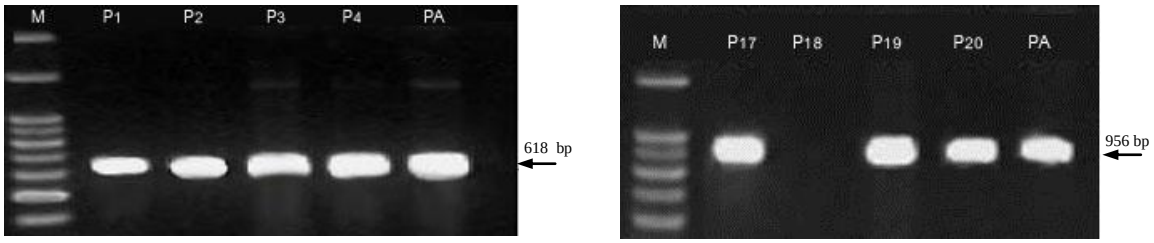
3.1 Identification of *Pseudomonas aeruginosa*

Isolates that were Gram negative bacilli showing fluorescein and pyocyanin pigments and

positive results for catalase, oxidase, motility, starch hydrolysis, arginine, tween 80, and malonate utilization tests but negative results for indole and glucose fermentation tests (Table 1) were identified as *P. aeruginosa*. All 30 isolates, P1-P30, that were randomly selected for PCR assay yielded 618 bp products after DNA amplification using *Pseudomonas* genus-specific primers. Two isolates, P18 and P24 showed no PCR product after DNA amplification using *Pseudomonas aeruginosa* species-specific primers indicating that they are of other species. *P. aeruginosa* ATCC 27853 that was included as a control showed positive results on both genus and species-specific primers (Figure 1).

Table 1 Charcteristics of *Pseudomonas aeruginosa*

Test	Result
Gram stain & morphology	Gram negative bacilli
Fluorescein pigment	+
Pyocyanin pigment	+
Oxidase	+
Catalase	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Utilization of Arginine	+
Tween 80	+
Malonate	+
Glucose O/F	+/-
Lactose fermentation	-
Motility	+
Indole	-
Urease	-
MR	-
VP	-
Citrate	+



(A) (B)

Figure 1 (A) PCR analysis of *Pseudomonas* species using *Pseudomonas* genus-specific primers PA GS-F and PA GS-R. Lanes M, reference marker; PA, *Pseudomonas aeruginosa*; P1-P4, Isolates P1-P4. All other isolates also showed positive results (data not shown). (B) PCR analysis of *Pseudomonas aeruginosa* using *P. aeruginosa*-specific primers PA SS-F and PA SS-R. Lanes M, reference marker; PA, *Pseudomonas aeruginosa*; P17-P20, isolates P17-P20. P17, P19, P20, and *P. aeruginosa* showed PCR product of 956 bp. P18 and P24 did not show PCR product. All other isolates showed positive results (data not shown).

3.2 Minimal bactericidal concentration (MBC)

The MBC profiles of the test disinfectants against *Pseudomonas aeruginosa* are shown in Table 2.

Table 2 Minimal bactericidal concentration (MBC) of test disinfectants against *Pseudomonas aeruginosa*

Disinfectants	MBC (ppm)
Benzalkonium chloride	1000
Formalin	370
Hydrogen peroxide	2
Perxania	0.2
Sodium hypochlorite	200

3.3. Kinetics of kill testing

Kinetics of kill studies showed that peracetic acid was superior to others in the rate at which it killed *pseudomonas aeruginosa* (Table 3 and Figure 2).

In summary, for *Pseudomonas aeruginosa*, peracetic acid (0.2 ppm) killed 3 logs (99.9%) in 58 minutes whereas sodium hypochloride (200 ppm) and hydrogen peroxide (2 ppm), formalin (370 ppm) and Benzalkonium chloride (1000 ppm) took longer time to achieve the same kill.

Table 3 Kill rate of disinfectants against *Pseudomonas aeruginosa* ATCC 27853 and 5 isolates of *Pseudomonas aeruginosa* recovered from hemodialysis system

Test disinfectant	Disinfectant concentration (ppm)	Time (minutes) to kill 99.9% of exponentially growing cells (mean±SD)
Benzalkonium chloride	1000	116±3
Formalin	370	112±4
Hydrogen peroxide	2	86±2
Perxania (Peracetic acid; PAA)	0.2	58±3
Sodium hypochlorite	200	83±2

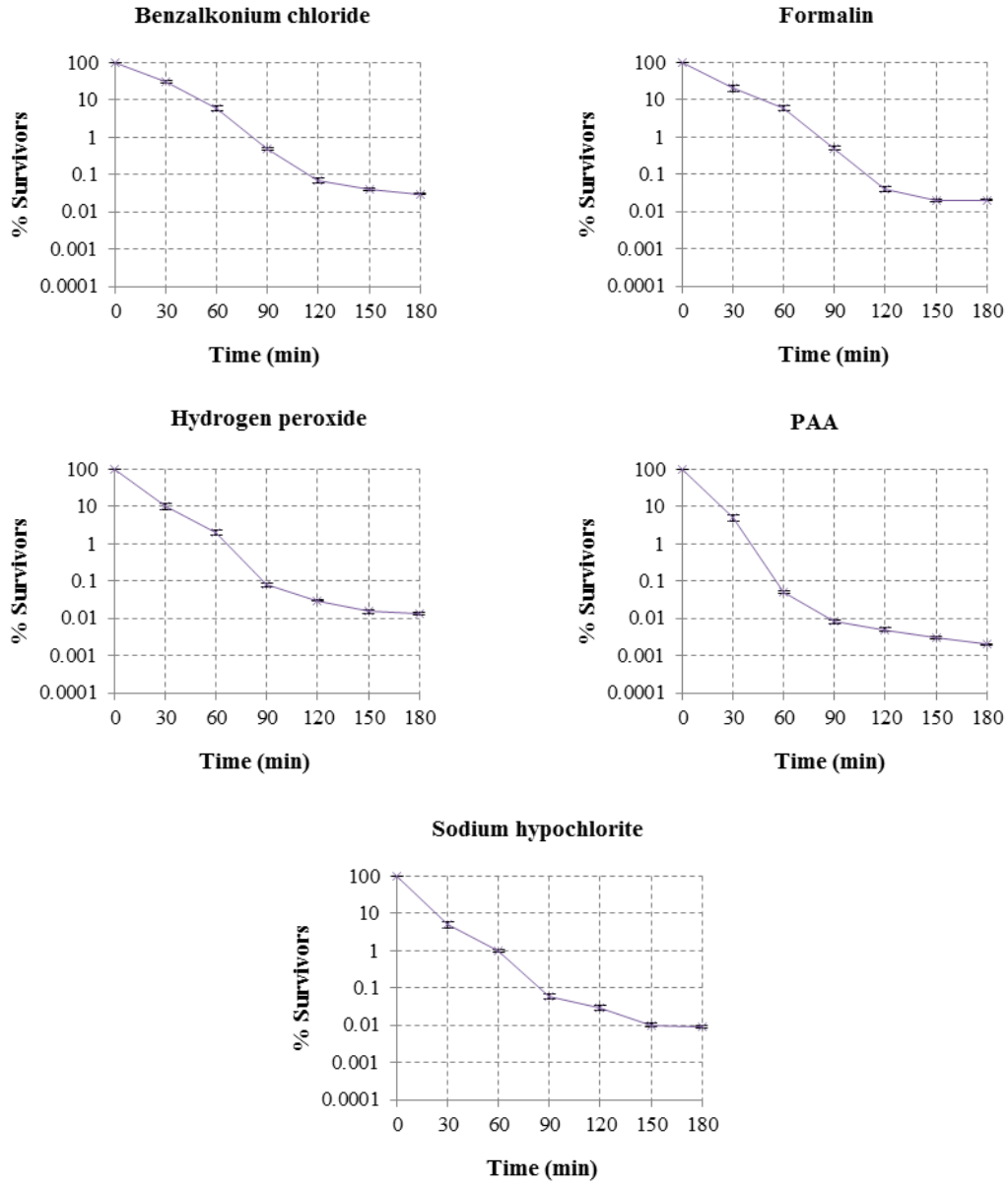


Figure 2 Kill rate of disinfectants against *Pseudomonas aeruginosa.* Values shown are average kill time of *Pseudomonas aeruginosa* ATCC 27853 and 5 isolates of *Pseudomonas aeruginosa* recovered from hemodialysis system.

4. Conclusions

These in vitro kinetics of kill studies demonstrate that Perxania 2025 (PAA) rapidly kills *Pseudomonas aeruginosa* recovered from water associated with hemodialysis system. The speed of kill observed for PAA is faster than that for other test disinfectants under the same test conditions.

5. Discussion

The disinfection of dialysis system must be carried out with adequate kill time for problem organisms. The levels of contamination in dialysate are even greater due to the fact that the recirculating system allows carbon- and nitrogen-containing waste products dialyzed from the patient to accumulate, be used as nutrients by microorganisms, and subsequently support for a few log increases of contaminants during a dialysis treatment. Since renal dialysis machine can serve as vehicles to infectious agents, care must be taken to assure the competence of disinfection procedures to minimize the risk of infection. Chemical biocides, reverse osmosis and/or deionization are the most widely used methods to achieve the water quality. However, water and dialysate do not have to be completely free of microorganisms because the dialyzer membrane acts as a barrier to acceptable number of infectious agents. The Association for the Advancement of Medical Instrumentation (AAMI) recommends the maximum acceptable level of bacteria to be 200 colony forming units (CFU) per milliliter of water while the recommendation by the European Pharmacopoeia is 100 CFU/mL (Ahmad, 2005).

Mode of disinfection by PAA is oxidation of the outer cell membrane of vegetative bacterial cells, endospores, yeast, and mold spores (Block, 1991). The mechanism of oxidation is the transfer of electrons, therefore the stronger the oxidizer, the faster electrons are transferred to the microorganism and the faster the microorganism is inactivated or killed. PAA also inactivates enzymes that are responsible for discoloration and degradation, such as peroxidase in the browning of potatoes (Greenspan and Margulies, 1950). PAA provides not only its rapid action in killing bacteria but also the benefit of leaving no harmful decomposition products and leaves no residue. Its effectiveness is not interfered by the presence of organic matter. It is also used in many automated systems to chemically sterilize medical, surgical, and dental instruments. In addition, PAA is used in food processing and handling as a sanitizer for food contact surfaces and as a disinfectant for fruits, vegetables, meat, and eggs (Evans, 2000). PAA can also be used to disinfect recirculated flume water (Lokkesmoe and Olson, 1993). Other uses of PAA include removing deposits, suppressing odor, and stripping biofilms from food contact surfaces (Block, 1991; Mosteller and Bishop, 1993; Marriot, 1999; Fatemi and Frank, 1999).

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