Comparison of Different Culture Methods on Bacterial Recovery in Hemodialysis Fluids

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To examine the culture method that could provide the highest bacterial recovery, 143 reverse osmosis water samples used in hemodialysis were collected for comparison of the media (Tryptic Soy Agar, TSA vs Reasoner's 2A Agar, R2A), the temperature (20 C vs 37 C), the duration of incubation (48-hour vs 7-day), and the culture technique (membrane filtration vs spread plate methods). The European Best Practice Guideline method, R2A at 20 C for 7-day incubation provided higher bacterial recovery than the Association for the Advancement of Medical Instrumentation (AAMI) method, TSA at 37 C for 48-hour incubation. The membrane filtration method gave better yield than the spread plate method. As such, the European Best Practice Guideline method in combination with the membrane filtration technique would be the culture method of choice for hemodialysis fluids.

Keywords : *The european best practice guideline, The association for the advancement of medical instrumentation, Tryptic soy agar, Reasoner's 2A agar*

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The purity of the hemodialysis fluids is crucial for hemodialysis patients who are inevitably exposed to a large volume of water during hemodialysis. Bacteria-contaminated hemodialysis fluids could induce bacteremia as well as endotoxin-mediated pyrogenic reaction[1,2], and might also eventually cause a chronic inflammatory state resulting in dialysis-related amyloidosis, hypoalbuminemia, and atheroscle-rosis[3-6]. For standard hemodialysis, the Association for the Advancement of Medical Instrumentation (AAMI) has recommended that the viable colony count of bacteria should be less than 200 CFU/ml for the water used to prepare dialysate and be less than 2,000 CFU/ml for the dialysate[7]. Recently, the European Best Practice Guideline has suggested using the "ultrapure" quality of water, containing a viable colony count of bacteria below 0.1 CFU/ml, in both standard and new hemodialysis techniques including high flux dialysis and hemodiafiltration[8,9].

The AAMI has recommended that the hemodialysis fluids should be cultured on either Tryptic Soy Agar (TSA) or standard method agar (SMA) and be incubated at 37°C for 48 hours[7]. The European Best Practice Guideline, however, has suggested using the Reasoner's 2A Agar (R2A), a nutrient-poor media, incubated at 20-22°C for 7 days[8]. Most but not all recent studies have demonstrated that the AAMI-recommended culture method might not be optimal for the recovery of bacteria[10-12]. At present, there are still several unestablished issues regarding the recommended value of bacterial recovery, the type of media, the temperature as well as the duration of incubation, and the culture-related technical procedure[1,13]. By using the criteria of the AAMI and the European Best Practice Guideline, the present study was carried out to compare the bacterial recovery between two different types of agar (TSA vs R2A); temperature (20°C vs 37°C); duration of incubation (48 hours vs 7 days), and culture-related technical procedure (spread plate

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vs membrane filtration method). Then, the bacterial recovery from the culture method recommended by AAMI, TSA at 37 for 48-hour incubation[7] was compared with that recommended by the European Best Practice Guideline, R2A at 20°C for 7-day incubation[8].

Material and Method

The study was conducted for a period of 3 years (2000-2003) at the hemodialysis unit of the Division of Nephrology, Department of Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok Thailand. The water samples prepared by reverse osmosis (RO) system were collected fort-nightly by an aseptic method for an amount of 150 ml in sterile pyrogen-free plastic bottles, temporarity stored at 4°C within 30 minutes, and incubated within 5 hours[12].

Medium preparation

The Tryptic Soy Agar (BIOTEC, code 3/186) was prepared by suspending 37 gram of the powder in 1 liter of purified water. After the powder was allowed to soak for 10 min, the solution was swirled to mix. The Reasoner's 2A Agar (DIFCO, code 218263) was prepared by suspending 18.2 gram of the powder in 1 liter of purified water. The solution was well mixed, heated with frequent agitation, and boiled for 1 minute to completely dissolve the powder. The final pH of the solution was adjusted until it achieved the value of 7.2 ± 0.2.

Both solutions were then taken to autoclave at 121°C for 15 minutes. After waiting until the temperature decreased to 47°C, both solutions were well mixed and then poured into 140 x 20 mm Petri dishes (50 ml/plate).

Cultivation

One hundred and forty three RO water samples were obtained for comparison of the media (TSA vs R2A), the temperature (20°C vs 37°C), and the duration of incubation (48-hour vs 7-day). After shaking the samples, 0.1 ml of the undiluted samples were pipetted and inoculated in a quarter plicate on R2A at 20°C and 37°C and on TSA at 20°C and 37°C. By spread plate method, the samples were spread thoroughly over the surface of the agar plates by a spreader and incubated for 7 days. The colony numbers were observed every day and recorded at the time after 48 hours and 7 days of incubation[10].

Two parameters were used for the comparison. The first was the percentage of samples that had the viable colony count of bacteria exceeding the AAMI value, 200 CFU/ml for the water used to prepare dialysate. The percentage of samples that was positive for culture was constituted as the second parameter and was defined by the colony growth more than zero CFU/ml. This number, thus, exceeded the ultrapure cut-off value according to the European Best Practice Guidelines for hemodialysis.

In the next step, to compare the quantitative microbiology methods between membrane filtration and spread plate methods, one hundred and seventy five RO water sample were collected and used for culture. The samples were processed in duplicate on R2A by the spread plate method described above and by the membrane filter method. In brief, for the membrane filter method, 100 ml of undiluted sample was filtered in Plastic Sterifil Funnel (Millipore, USA, MPXX 1104710) using 0.45 micron sterile MCE Membrane Filter (Millipore, USA, MPHAWG047S1*5). Then, the MCE Membrane Filters were placed on R2A. The medium was incubated at 20°C for 7 days that could provide the highest yield of bacterial recover according to the results of the first part of the study. The numbers of colony were observed daily and counted on membrane filter by stereoscopic microscope (magnification power 10x15) after 7 days of incubation.

Statistical analysis

The descriptive values of both parameters in all figures were expressed as percentage. Comparisons between different culture methods were performed by McNemar test or Wilcoxon signed ranks test where appropriate. All statistical analyses were performed by SPSS version 11.0, and statistically significant level was defined when the p-value was less than 0.05.

Results

Microorganism Data

Of the one hundred and forty three RO water samples, Table 1 details various genuses of bacteria discovered from the positive-culture hemodialysis fluids. The most common bacteria recovered was *Pseudomonas spp*.

Comparison of the temperature and the time of incubation for each media

The percentage of CFU values exceeding the AAMI value and the percentage of positive culture exceeding the ultrapure value according to the European Best Practice Guidelines for hemodialysis between two incubation temperatures, 20°C vs 37°C, and between two incubation times, 48-hour vs 7-day were depicted in figure 1 for TSA and figure 2 for R2A.

Table 1. The various species of bacteria discovered from the positive-culture hemodialysis fluids

Species of bacteria	Percent
Pseudomonas spp.	40
Moraxella spp.	23
Acinetobacter spp.	16
Staphylococcus spp.	16
Alcaligenase spp.	14
Gram negative rod	7
Corynebacterium spp.	3
Micrococcus spp.	3
Bacillus spp.	1
Chromobacterium spp.	1
Gram positive rod	1
Rhodococcus spp.	1
Streptococcus spp.	1

For TSA, at 48-hour incubation, the values of both parameters were higher in 37°C than 20°C temperature (Fig. 1, 16.1 vs 9.1, p = 0.013 for percentage of CFU exceeding AAMI value and 25.9 vs 12.6%, p < 0.01 for the percentage of CFU exceeding ultrapure value). No significant differences were observed at 7-day incubation. At 20°C incubation, the values of both indicators were higher in 7-day than 48-hour duration (Fig. 1, 16.1 vs 9.1, p = 0.002 and 21.7 vs 12.6, p < 0.01, respectively). At 37°C incubation, however, no significant disparity was noted (16.1 vs 16.1%, NS and 27.3 vs 25.9%, NS, respectively).

Regarding R2A, at both 48-hour and 7-day periods, there were no significant differences in both parameters between the two incubation temperatures (Fig. 2). Nevertheless, the values of these two parameters on R2A after 48 hours of incubation were significantly lower than after 7 days of incubation at both incubation temperatures (11.2 vs 22.4% at 20°C and 16.8 vs 25.9% at 37°C for the percentages of the CFU values exceeding the AAMI value; 15.4 vs 36.4% at 20°C and 21.7 vs 35.0% at 37°C for the percentages of CFU values exceeding the ultrapure value, p < 0.01 for all comparisons).

Comparison of the media at various culture conditions

The percentages of the CFU values exceeding the AAMI value detected on TSA and R2A in the water samples are shown in Fig. 3, while the percentages of CFU values exceeding ultrapure value are demonstrated in Fig. 4.

The percentages of the CFU values exceeding the AAMI value on R2A were higher than TSA at both temperatures (22.4 vs 16.1% at 20°C, p = 0.02 and 25.9 vs 16.1% at 37°C, p = 0.01) after 7 days of incubation. Also, the percentages of CFU values exceeding the ultrapure value on R2A were greater than on TSA with statistically significant differences at both 20°C and 37°C (36.4 vs 21.7%, p < 0.01 and 35.0 vs 27.3, p = 0.013, respectively). On the other hand, no statistical significance was attained with percentages of both

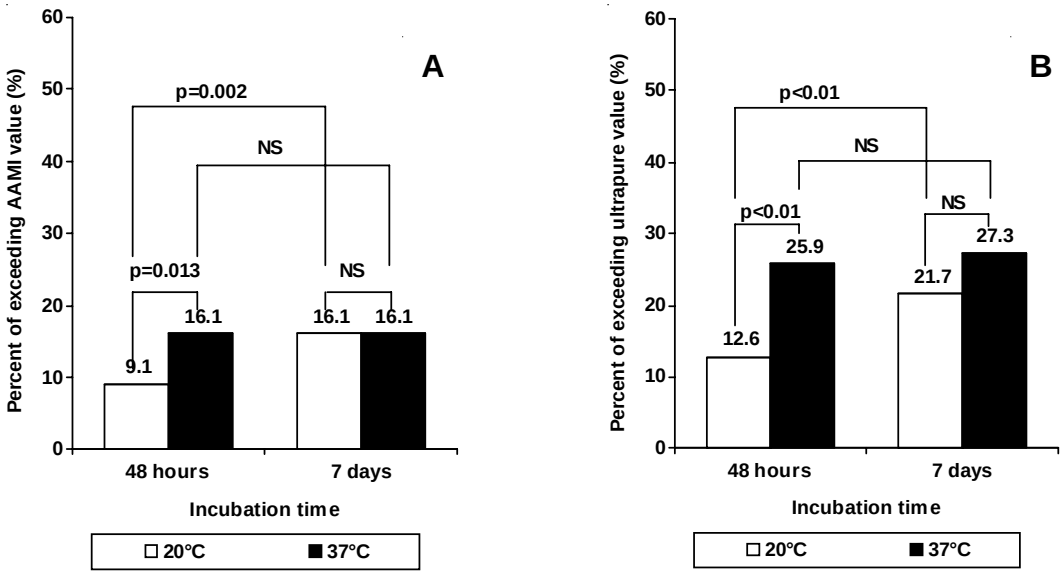

Fig. 1 The comparison between the two incubation temperatures (20vs37 C) and two incubation times (48 hours vs 7 days) using TSA
A. Comparison of percent of exceeding AAMI value, B. Comparison of percent of exceeding ultrapure value

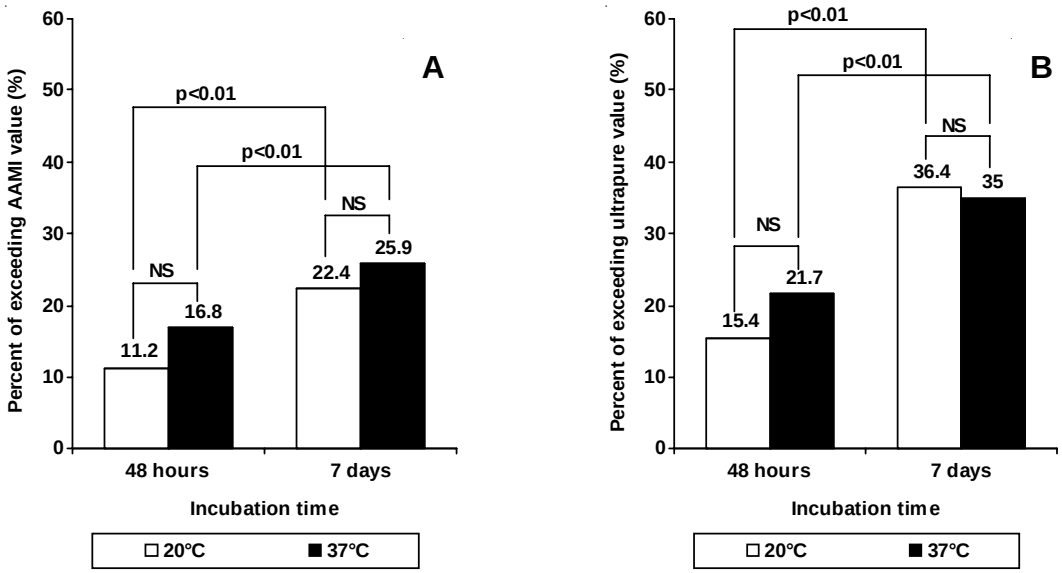

Fig. 2 The comparison between the two incubation temperatures (20vs37 C) and two incubation times (48 hours vs 7 days) using R2A
A. Comparison of percent of exceeding AAMI value, B. Comparison of percent of exceeding ultrapure value

CFU values when compared between incubation at 20°C and 37°C for 48 hours (Fig. 3, 4).

Comparison between the culture method recommended by AAMI and European Best Practice Guideline

When one compared between the AAMI standard method, culture with TSA at 37°C for 48 hours, and the European Best Practice Guideline method, culture with R2A at 20°C for 7 days, the results in the present study demonstrated that R2A could provide a higher percentage of CFU value exceeding the AAMI cut off value than TSA (22.4 vs 16.1%, $p = 0.049$) (Fig. 3). Also, if the ultrapure cut off value was used to determine the quality of water, culture with R2A at 20°C for 7 days also clearly provided a higher yield than TSA (36.4 vs 25.9%, $p = 0.014$) (Fig. 4).

Comparison of the quantitative microbiology methods between membrane filtration and spread plate methods

From the above studies, by the spread plate method, culture with R2A at 20°C for 7 days could provide the highest yield of bacterial recovery. In the next step, to compare the quantitative microbiology methods between membrane filtration and spread plate methods, one hundred and seventy five RO water samples were collected and used for culture. The samples were processed in duplicate on R2A by the spread plate method described above and by the membrane filter method and then incubated at 20°C for 7 days.

The percentages of CFU values exceeding the AAMI value and ultrapure value cultured on R2A by membrane filtration and spread plate methods at 20°C for 7 days are illustrated in Fig. 5. The percentages of positive culture and percentages of CFU values exceeding the ultrapure value on R2A by the membrane filtration method were significantly higher than the spread plate method (91.4 vs 60.0%, $p < 0.01$ and 87.4 vs 60.0%, $p < 0.01$). In contrast, the percentages of the CFU values exceeding the AAMI value on R2A by the membrane filtration method were significantly lower than by the spread plate method (4.0 vs 26.3%, $p < 0.01$). Of note, the counting area on the media of the membrane filtration method was smaller than the spread plate method.

Discussion

Contamination of hemodialysis fluids with bacteria, especially gram-negative organisms like Pseudomonas, is associated with bacteremia, dialysis-related fevers, and several chronic inflammatory-related disorders. In agreement with previous studies [11], the most common bacteria recovered in the present work also was *Pseudomonas species*.

The routine quality assurance culture method approved by AAMI is TSA at 37°C for 48-hour incubation[7]. Most following studies, however, have

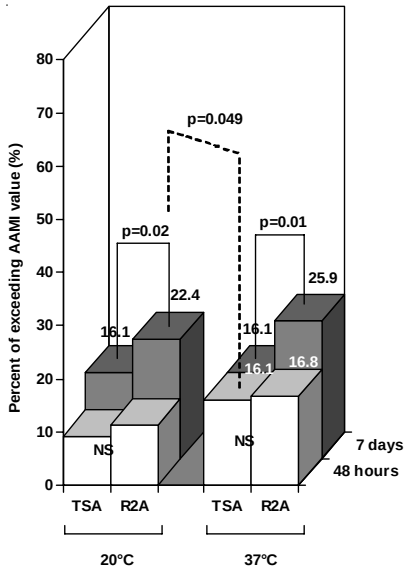

Fig. 3 The comparisons of percent of exceeding AAMI value on TSA and R2A in the RO water samples incubated at 20°C and 37°C for 48 hours and 7 days.
NS = not-significant

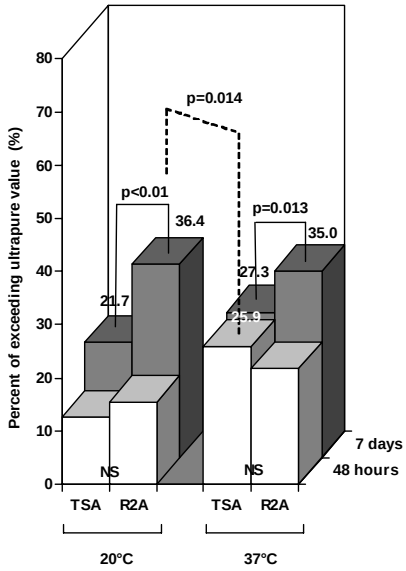

Fig. 4 The comparisons of percent of exceeding ultrapure value on TSA and R2A in the RO water samples incubated at 20°C and 37°C for 48 hours and 7 days.
NS = not-significant

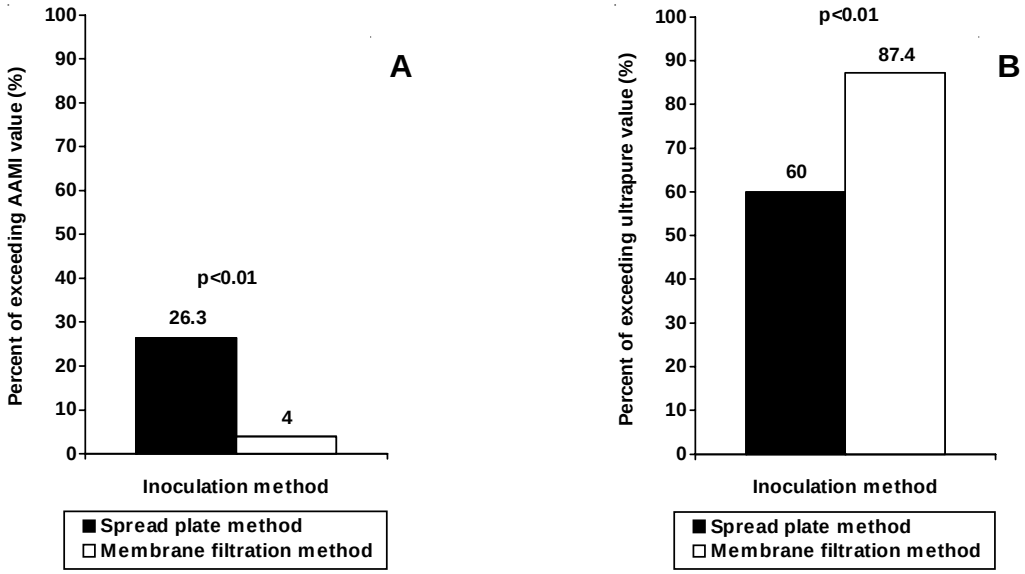

Fig. 5 The comparisons of positive culture between membrane filtration method and spread plate method on R2A incubated at 20°C for 7 days.
A. Comparison of percent of exceeding AAMI value, B. Comparison of percent of exceeding ultrapure value

demonstrated that the AAMI-recommended culture method may not be adequate for the recovery of bacteria[10-13]. The nutrient rich media including TSA tended to underestimate the bacterial contamination when compared with the low nutrient agar including R2A. It has been hypothesized that the bacterial floras of water have become adapting to a carbon and nutrient-poor environment. Besides the type of media, lower temperature and extended incubation tended to produce higher colony counts[10-12]. One recent report, however, could not show the differences in bacterial recovery with different incubation times as well as temperatures[14], the small sample size and the statistical analysis method were the main flaws in such study[11].

Recently, thus, the culture method recommended by the European Best Practice Guideline is R2A at 20-22°C for 7-day incubation[8].

From the present study, if the ultrapure water, the viable colony count of bacteria lower than 0.1 CFU/ml, is the goal, culture with using R2A for 7 days at 20°C, recommended by the European Best Practice Guideline, or at 37°C is the best method (Fig. 4). Both incubation temperatures in the present study, 20 °C and 37 °C, did not offer the difference in sensitivity when cultured on R2A. This was not in agreement with a previous study[10] and may be caused by differences in geographic conditions which could influence the habitual growth of microorganisms. Of note, culture with the European Best Practice Guideline[9] could provide much higher statistical significance than culture with using TSA for 48 hours at 37 °C, recommended by AAMI[8] (Fig. 4). In the present study, the results from the TSA, which showed that 37°C provided more sensitivity than 20°C differed from the previous study which demonstrated the superiority of the temperature at 20°C[10]. Such discrepancy might be caused by the difference in geographic environment.

Also, if the AAMI cut off value, the viable colony count of bacteria lower than 200 CFU/ml for water and 2,000 CFU/ml for dialysate, is the aim, culture with using R2A for 7 days at either 20 or 37°C is still the most preferable method (Fig. 3). In this regard, culture with the European Best Practice Guideline[8] showed marginally higher statistical significance than the AAMI guideline[7] ($p = 0.0498$, Fig. 3).

When ultrapure water is required, the membrane filtration method gave higher bacterial recovery than the spread plate method (Fig. 5). This may be explained by two reasons: 1) the more sensitive criteria, the less CFU values, is used to diagnose the quality of ultrapure water 2) the more volume of water is used in the membrane filtration method. However, when the AAMI value was the reference, the membrane filtration method provided lower yield than the spread plate method. It should be noted that the counting area on the medium of the membrane filtration method was smaller while the number of colonies was much higher than the spread plate method. Thus, some colonies growing at the same location could not be separately identified.

In conclusion, the European Best Practice Guideline method, R2A with 20°C and 7-day incubation, in combination with the use of membrane filtration method provides the highest yield of bacterial recovery and would be the culture method of choice for hemodialysis fluids.

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เปรียบเทียบการเพาะเชื้อแบคทีเรียจากน้ำที่ได้จากการฟอกเลือด

นภาวรรณ ปุณกบุตร, ผ่องพรรณ นันทาภิสุทธิ์, ไตรรักษ์ พิสิษฐ์กุล, ขจร ตีรณธนากุล, เกรียง ตั้งสง่า, สมชาย เอี่ยมอ่อง

เพื่อหาวิธีการเพาะเชื้อแบคทีเรียที่ดีที่สุด ได้เก็บตัวอย่างน้ำรีเวอร์สออสโมซิสที่ใช้ในการฟอกเลือด 143 ตัวอย่าง เพื่อเปรียบเทียบระหว่างชนิดสารอาหาร (ทริปทิค ซอยอาการ์ กับ รีซันเนอร์ ทูเอ อาการ์) อุณหภูมิ (20 กับ 37 องศาเซลเซียส) ระยะเวลาการเพาะเชื้อ (48 ชั่วโมง กับ 7 วัน) และ เทคนิคการเพาะเชื้อ (วิธีเมมเบรนฟิลเตรชั่น กับ วิธีสเปรดเพลท) วิธีการเพาะเชื้อของทวีปยุโรป ซึ่งใช้รีซันเนอร์ ทูเอ อาการ์ 20 องศาเซลเซียส เป็นเวลา 7 วัน ให้ผลการเพาะเชื้อดีกว่า วิธีการเพาะเชื้อของประเทศสหรัฐอเมริกา ซึ่งใช้ทริปทิค ซอยอาการ์ 37 องศาเซลเซียส เป็นเวลา 48 ชั่งโมง นอกจากนี้ยังพบว่าเทคนิคการเพาะเชื้อวิธีเมมเบรนฟิลเตรชั่น ให้ผลการเพาะเชื้อดีกว่าวิธีสเปรดเพลท ดังนั้น วิธีการเพาะเชื้อของทวีปยุโรปร่วมกับเทคนิคการเพาะเชื้อวิธีเมมเบรนฟิลเตรชั่นเป็นวิธีการเพาะเชื้อแบคทีเรียที่ดีและเหมาะสมที่สุดสำหรับน้ำยาที่ใช้ในการฟอกเลือด