Journal of Hospital Infection (2003) **53**: 64–71
doi:10.1053/jhin.2002.1320

Biofilm removal from silicone tubing: an assessment of the efficacy of dialysis machine decontamination procedures using an in vitro model

K. Marion-Ferey*, M. Pasmore†, P. Stoodley†, S. Wilson†, G. P. Husson* and J. W. Costerton†

**College of Pharmacy, Paris, France; and †Center for Biofilm Engineering, Bozeman, Motana, USA*

Summary: The aim of this study was to assess the efficacy of 21 decontamination procedures, for the removal of a multispecies biofilm. Experiments were performed on five-day-old biofilms grown inside silicone tubing, using a reactor system that mimics a dialysis machine. The treatments were tested on 5 cm tubing samples. Effects of treatment were measured using direct microscopy following staining. Bacterial viability and endotoxin removal were determined using conventional microbiological methods following biofilm detachment by scraping. The 21 procedures were classified into four groups based on the amount of biofilm removed. The most effective treatment was an acid pre-treatment, followed by use of a concentrated bleach solution. Acid pre-treatment removes calcium and magnesium carbonate crystals that are always found in dialysis biofilms. Treatments performed at high temperature did not increase the efficacy of biofilm removal. Most treatments caused at least a 10^5-fold reduction in bacterial viability with a few resulting in complete kill. Autoclaved and bleach-treated samples gave the best results for viability reduction, with both treatments providing an equally effective and complete kill. In addition, autoclaving led to a significant decrease in endotoxin level (removal of 99.99%).

Keywords: Biofilm; haemodialysis; silicone tubing; endotoxins.

Introduction

The clinical procedure of haemodialysis involves bringing a patient's blood into contact with a semi-permeable membrane (dialyser) and mineral salt solution containing essential trace elements called dialysate, prepared with non-sterile, purified water. The presence of bacteria, which may release endotoxins, demands vigilance in a dialysis centre water purification system and dialysate delivery systems. Bacteria are not expected to cross the dialysis membrane because of their size, but even moderate levels of endotoxin, have been found to do so, stimulating the production of cytokines. Many pyrogenic reactions have been associated with the practice of dialyser re-use.[1,2] Indeed, even when re-use is not practised, pyrogenic reactions associated with endotoxin penetration across intact dialysis membranes may still occur.[3,4] Moreover, repetitive induction of cytokines may contribute to some of the long-term complications amongst dialysis patients including chronic cardiovascular and articular disorders and carpal tunnel syndrome.[3]

Received 25 September 2001; revised manuscript accepted 8 August 2002; published online 11 November 2002.
Author for correspondence: Karine Marion-Ferey, CH Lyon-Sud, Service Pharmacie, Secteur Ste Eugénie 165 Chemin du Grand Revoyet, 69 495 PIERRE-BENITE cedex, France. Tel.: 33-4-78-86-12-15; Fax: 33-4-78-86-66-66; E-mail: karine.marion@laposte.net

0195–6701/03/010064 + 08 $35.00/0

This chronic stimulation of the inflammatory system is known as bio-incompatibility.[5] The finding that endotoxin in haemodialysis fluid increases bio-incompatibily has resulted in a demand for the improvement of microbiological quality of such fluids. Standards have been developed by the Association for the Advancement of Medical Instrumentation (AAMI) setting the upper level of bacteria and endotoxin to 200 cfu/mL and 2 endotoxin units (EU)/mL respectively in haemodialysis fluid.[6] The European Pharmacopoeia defines the microbiological quality of dialysis fluids as no more than 100 cfu/mL and 0.25 EU/mL for the water and 0.5 cfu/mL for the dialysate.[7] With improved detection limits, more recent publications call for decreasing these thresholds to obtain an ultrapure dialysate containing less than 0.1 cfu/mL and 0.03 EU/mL.[4] Such levels are very difficult to reach because of the regular re-contamination of dialysate.[8,9] Some studies have shown evidence of biofilm development in fluid pathways of haemodialysis systems, with the most significant formation occurring inside the silicone tubing of dialysis machines.[10–12] Despite regular decontamination, the rough inner surface of silicone tubing, the 37°C temperature and the presence of bicarbonate in the dialysate, provide ideal conditions for promoting biofilm growth.

Once a biofilm is formed, the penetration barrier provided by the polymeric matrix, along with increased microbial resistance, reduces the efficacy of disinfectants.[13,14] Moreover, the biofilm is difficult to remove because of the presence of exopolysaccharides, which help anchor the bacteria to the surface. In haemodialysis systems, there is the added difficulty, resulting from calcium and magnesium carbonate crystals which promote bacterial adherence.

Pharmaceutical firms producing dialysis machines each recommend a different procedure for decontamination. Although most disinfectant agents used inside haemodialysis machines have proven efficacy in killing planktonic bacteria, few have been tested for biofilm activity.[10,15–17] Indeed, after an extensive search of the literature, only a few studies examining bacterial attachment to a dialysis system were found.[10–12,15] This project aims to fill this gap by examining the efficacy of 21 decontamination procedures for the removal of biofilm from silicone tubing in dialysis machines. The study was performed with the help of an experimental model leading to the development of a multispecies biofilm. Changes in thickness, coverage, bacterial viability and endotoxin levels were assessed for each of the treatments tested.

Materials and methods

Biofilm reactor system

A continuously stirred tank reactor was set up to mimic a dialysis machine (Figure 1). The system was supplied with dialysate made from non-sterile, reverse osmosis water mixed with dialysis acid and bicarbonate concentrate solutions. The bacterial and endotoxin contamination of the reverse osmosis water was 187 cfu/mL and 1.8 EU/mL respectively. These levels were in accordance with the AAMI standards but were higher than the upper limits of the European standards.[6,7] The concentrate solutions were prepared with different salts according to the composition of the Bieffe Medital concentrates bags (Clearflex®, Bieffe Medital, Sabinanigo, Spain). The bicarbonate concentrate was sterilized by filtration (0.2 μm autoclavable capsule filter, No.12122, Pall-Gelman Sciences, Ann Arbor, MI, USA). To accelerate the biofilm growth, a 1/50 diluted Luria Bertani (LB) broth, a common bacteria growth medium (BD Bioscience, Sparks, MD, USA) was added. The LB broth and the dialysate effluent flow rates were respectively 0.5 and 5 mL/min, giving a resulting mean residence time in the entire reactor system of 60 min. The medium circulated through

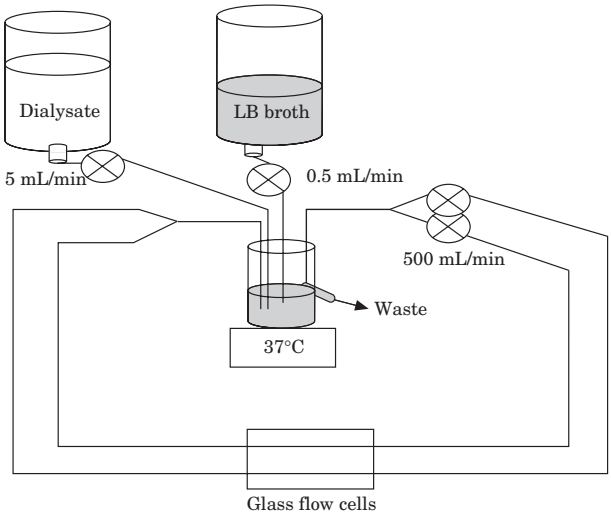

Figure 1 Biofilm reactor system. The medium made of non sterile dialysate and LB broth circulates through two 1.5 m recycle loops that are heated to 37°C with a hot plate. Glass flow cells are incorporated into the loops.

two 1.5 m recycle loops made of silicone tubing (Masterflex® 7015, 5 mm ID, Cole Parmer, USA) at a flow rate of 500 mL/min (Reynold's Number = 2000, Reynold number is a unitless value for a flow rate that allows for comparison between different tubing and flow systems) provided by a dual-head peristaltic pump (Masterflex® L/S, Cole Parmer, USA). The loops and system were sterilized by autoclaving at 121°C and 2 atms 15 min prior to inoculation. The dialysate was the only component that was not sterilized and was the only source of inoculate. The resulting biofilm is therefore very close to those that develop inside dialysis silicone tubing.[10]

The whole system was maintained at a temperature of 37°C by use of a hot plate and a regularly monitored thermometer. Square glass flow cells (3× 3 mm, S-103 Camlab, Cambridge, UK) were incorporated into the recycle loops to allow the dailymonitoring of biofilm growth. The flow cells were attached to a polycarbonate holder, which was mounted on the stage of an Olympus fluorescence microscope (model: BH2) with a high resolution digital camera attachment (COHU 4612-5000 CCD, Cohu Inc. Electronics Division, San Diego, CA, USA).

The system was allowed to run for five days. The LB broth peristaltic pump was then turned off and the recycle loop was rinsed with dialysate alone at a flow rate of 100 mL/min for 24 h to remove the excess of LB broth. Then, the tubing was treated as described below.

Sampling

The tubing covered with the biofilm was cut into 5 cm long segments. Each segment was randomly selected to undergo one of the 21 different treatment procedures. Each treatment was performed in duplicate with one untreated segment used as a control.

Sample treatment

The 21 decontamination procedures studied are summarized in Table I, the eight most commonly used being marked with an asterisk. It is notable that treatments were performed at different temperatures and that two mineral acid solutions (nitric and hydrochloric) were tested in addition to citric acid (most commonly used to decontaminate dialysis machines). A high-concentration bleach solution containing 3% of active chlorine was also tested. Nitric and hydrochloric acids were diluted to reach the pH of a 3% citric acid solution (pH = 2.2). Acid

Table I *List of the decontamination procedures tested*

Procedure	Pre-treatment	Temperature (°C)	Time (min)	Treatment	Temperature (°C)	Time (min)
1	Citric acid 3%	20	5	Autoclave	121	30
2	Nitric acid 0.15%	20	5	Autoclave	121	30
3	Hydrochloric acid 0.15%	20	5	Autoclave	121	30
4	Actril® 3%	20	40	Autoclave	121	30
5	Citric acid 3%	20	5	Actril® 3%	20	40
6	Nitric acid 0.15%	20	5	Actril® 3%	20	40
7	Hydrochloric acid 0.15%	20	5	Actril® 3%	20	40
*8	Citric acid 3%	20	5	Bleach 0.3%	20	40
*9	—			Bleach 0.3%	20	40
*10	—			Actril® 3%	20	40
*11	Citric acid 3%	90	40	Bleach 0.3%	20	40
*12	Citric acid 3%	37	5	Autoclave	121	30
13	Citric acid 3%	37	5	—		
*14	Citric acid 3%	90	40	—		
15	Hydrochloric acid 0.15%	37	5	—		
16	Hydrochloric acid 0.15%	90	40	—		
*17	—			Glycolic acid 0.6%	84	30
18	Glycolic acid 0.6%	84	30	Autoclave	121	30
*19	—			Water	90	40
20	Citric acid 3%	20	5	Bleach 3%	20	40
21	—			Bleach 3%	20	40

* The eight most usual procedures.

solutions and powders were purchased from Merck. Actril® was obtained from Minnteck Renal System, Minneapolis, MN, USA, and the bleach solution was a 5.25% sodium hypochlorite solution (Clorox®, Oakland, CA, USA).

The duplicate 5 cm tubing samples that underwent the same treatments were connected together and attached to a peristaltic pump allowing the different chemicals, except bleach, to run through the samples at a flow rate of 500 mL/min from 5–40 min, according to the contact times shown in Table I. Bleach treatments were performed without any flow, the tubing samples being clamped at both ends, bleach injected with a syringe and the samples allowed to remain static for the duration of treatment.

Temperature was controlled with a water-bath and after each treatment, samples were rinsed with reverse osmosis water[1,4] for 10 min at room temperature at a flow rate of 500 mL/min.

Samples analysis

Direct observations

Segments, 1 cm in length, were cut from the treated 5 cm tubing sample and stained using live–dead Baclight bacterial viability kits (L7012, Molecular Probes, OR, USA) according to the methodology described by Decker.[18]

After staining, the tubing samples were cut lengthwise, pulled open, and attached to a glass slide with tape which was placed at the tubing edges. The exposed inner surface was imaged with an epifluorescence microscope (λ exc: 465/490 nm; λ em: 515/555 nm) and an attached CCD camera (model ST-133, Princeton Instruments Inc., Trenton, NJ, USA). The surface coverage was then determined using the image analysis software Scion Image. The samples and untreated controls were compared to determine the efficacy of treatments.

The samples were additionally analysed with a confocal scanning laser microscope (Model TCS-NT, confocal Leica Microscope, Bannockburn, USA) providing three-dimensional images and the measurement of biofilm thickness.

Biofilm quantification

The biofilm was quantified by cfu counting and endotoxin level measurement following biofilm detachment by scraping. The methodologies are described elsewhere.[19]

Results

Biofilm growth

Adherent cells were observed within a few hours, isolated micro-colonies appearing within 48 h. After five days, the micro-colonies had merged to form an extensive biofilm of environmental bacteria on the surface. Pictures of the biofilm that formed on the glass flow cells [Figure 2(a)] and the silicone tubing are shown below [Figure 2(b)].

After five days the biofilm was 7 μm (±2 μm) thick after drying (this was approximately equivalent to a 15 μm thin hydrated biofilm), it contained an average of 1.7×10^9 ($\pm0.6\times10^9$) cfu/cm^2 of culturable bacteria and covered an average of 96% (±4%) of the total inner surface of the tubing sample. The standard deviations calculated from the 21 different control samples showed that the biofilm development was quite homogenous along the whole surface of the 1.5 m silicone tubing. The three dominant

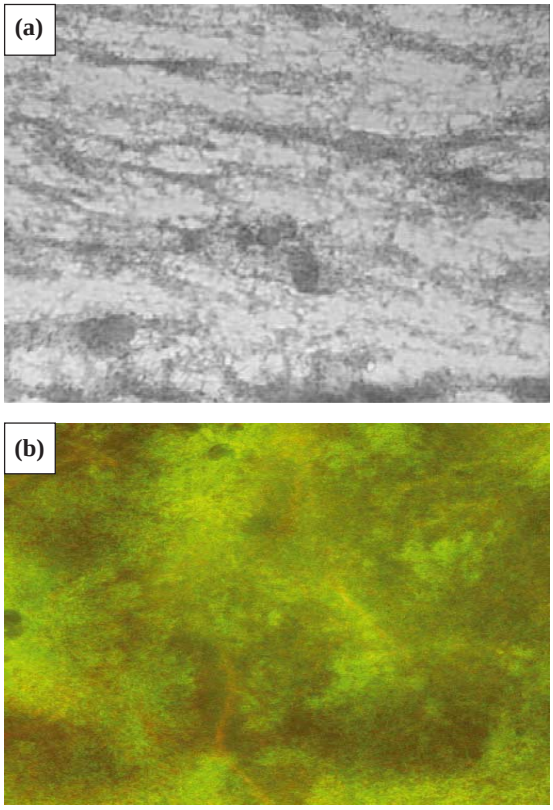

Figure 2 Biofilm development in the reactor system. Picture (a): Biofilm after a 5-days-growing time on glass flow cells (optical microscope ×200). Picture (b): Biofilm after a 5-days-growing time on silicone tubing (live–dead staining, confocal scanning laser microscope ×200).

organisms isolated from the biofilm were all Gram-negative bacteria, identified as *Pseudomonas putida*, *Pseudomonas fluorescens* and *Flavimonas orizihabitans*. These bacterial strains are commonly found in haemodialysis waters and dialysates.[10]

Biofilm removal

Table II summarizes the biofilm thickness and coverage reduction from the 21 treated samples, compared with controls. For procedures 20 and 21, extreme values are given because of the heterogeneity of residual biofilm following treatment.

These results led to a division of treatments into four groups: (1) very good efficacy (75–100%), (2) good efficacy (50–75%), (3) intermediate efficacy (25–50%), (4) poor efficacy (0–25%). In cases of discrepancy between coverage and thickness, coverage is considered to be the determining factor for classification. Representative pictures obtained with the confocal scanning laser microscope following the treatments are shown below [Figure 3(a–d)].

Figure 4 gives the correlation between thickness and coverage for the treated samples, with the exception of samples 20 and 21. The resulting correlation coefficient ($R^2 = 0.84$) shows that these two parameters are well correlated, giving strength to our determination of efficacy.

Table II *Compared with controls, following treatment, biofilm removal*

Procedure	Thickness reduction (%)	Coverage reduction (%)	Group
1	54	50	2
2	60	59	2
3	50	63	2
4	24	39	3
5	54	68	2
6	54	61	2
7	50	53	2
8	60	65	2
9	50	58	2
10	19	15	4
11	62	61	2
12	67	62	2
13	20	14	4
14	0	7	4
15	0	15	4
16	0	17	4
17	0	12	4
18	0	39	3
19	0	7	4
20	67–100*	98	1
21	33–100*	76	1

Averages percentage reduction for thickness and coverage. Classification into four groups of efficacy. Group 1: very high efficacy (75–100%); group 2: high efficacy (50–75%); group 3: moderate efficacy (25–50%); group 4: low efficacy (0–25%). The * indicates extreme values.

Bacterial viability

Plate count results are shown in Table III. Most samples still contained living cells after treatment, the extent of kill compared with controls being >99.999%, corresponding to a 5 log reduction. Most autoclaved and bleach-treated samples led to a 100% kill. Conversely, treatments using acid or water alone had a low efficacy (kill ranged from 99.900% to 99.990%).

Endotoxin levels

Control samples contained an average of 6028 EU/cm^2. The endotoxin levels after treatment (EU/cm^2) and the percentage of endotoxin removal related to controls are shown in Table III. Most treatments resulted in a significant decrease in endotoxin level (>90%), with autoclaved samples having the greatest decrease (>99.99%). A single descaling treatment with citric or hydrochloric acid left a high level of endotoxin on the tubing surface, even at high temperature (removal ranged from 22.60–94.57%). Moreover, bleach-treated samples retained a significant level of endotoxin, despite the effective removal of biofilm.

Discussion

We studied the efficacy of various dialysis machine decontamination procedures in removing biofilm from silicone tubing. The results provide useful data to guide professionals in making a choice in the many different procedures recommended by manufacturers.

A continuously stirred tank reactor was used to accelerate biofilm growth. This model was chosen due to ease of performance and the ability to simultaneously test numerous procedures. The results indicate that a low level of nutrients was sufficient to develop a mature biofilm within a few days. The flow rate, temperature, reactor materials and solutions were all selected to mimic the conditions in dialysis units, whilst, LB broth was used to accelerate biofilm formation. Because of nutrient supplementation, the resulting biofilms may be thicker than expected in actual dialysis units and represents the worst case scenario for the procedures tested.

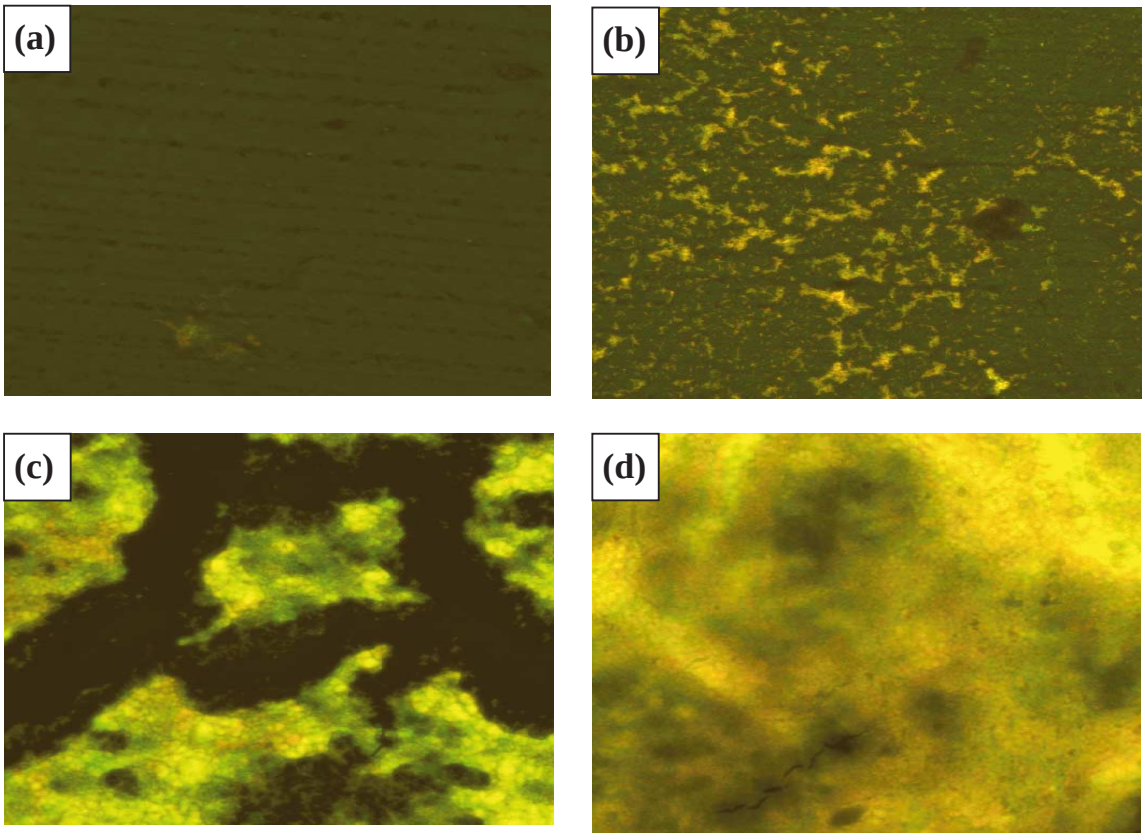

Figure 3 Efficacy of the 21 cleaning procedures on biofilm removal. Classification into four groups. Picture (a): group 1, very high efficacy (samples 20, 21); Picture (b): group 2, high efficacy (samples 1, 2, 3, 5, 6, 7, 8, 9, 11, 12). Picture (c): group 3, moderate efficacy (samples 4, 18); Picture (d): group 4, low efficacy (samples 10, 13, 14, 15, 16, 17, 19).

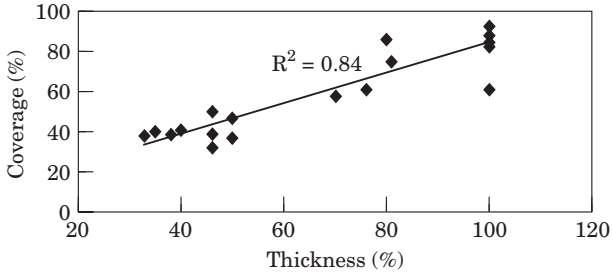

Figure 4: Correlation between the remaining coverage and thickness after treatments. Data from 19 out of the 21 different procedures tested.

The 21 treatments chosen included those recommended by the major dialysis machine manufacturers. The most common of these procedures currently used are: citric acid and autoclave (used inside Miroclav® machines), Actril®, hot glycolic acid, hot water and bleach. Temperature and treatment times were chosen in accordance with the recommendations of the pharmaceutical manufacturers. Additional chemicals such as nitric and hydrochloric acids were tested to replace citric acid, which is an organic acid and a nutrient source for bacteria. A high-concentration bleach solution was tested in an attempt to remove the biofilm completely.

In this study, only a few treatments led to a complete kill of bacteria within the biofilm, in agreement with other studies.[14,16,20–22] Most treatments caused a 10^5-fold bacterial reduction corresponding to the French AFNOR standard for bactericidal activity assessment.[23]

The efficacy of a decontamination procedure used in haemodialysis machines however, must be determined by both the level of biofilm and endotoxin removal, not just by the efficacy of bacterial kill as endotoxins are not necessarily destroyed by conditions that kill bacteria.[24] These endotoxins can remain in the tubing and be released at a later date, crossing the dialysis membrane.[4,5] Among all the procedures tested, autoclaving appeared to be the best way to both kill bacteria and reach a low level of

Table III *Endotoxin level and number of culturable bacteria after treatment*

Cleaning procedure	Culturable bacteria (cfu/cm^2)	Kill (%)	Endotoxin level (EU/cm^2)	Endotoxin removal (%)
1	6.8×10^2	>99.999	0.16	99.99
2	2.2×10^2	>99.999	1.15	99.98
3	<1	>99.999	5.20	99.91
4	<1	>99.999	0.80	99.98
5	2.1×10^3	>99.999	70.20	98.83
6	5.8×10^4	>99.999	224.00	96.28
7	9.5×10^2	>99.999	162.00	97.30
8	<1	>99.999	22.30	99.63
9	22	>99.999	354.00	94.13
10	8.6×10^3	>99.999	470.00	92.20
11	2.2×10^4	>99.999	74.40	98.76
12	<1	>99.999	5.20	99.91
13	1.5×10^6	99.928	3617.00	40.00
14	3.6×10^5	99.982	2618.00	56.57
15	2.0×10^6	99.904	4663.00	22.60
16	3.2×10^4	>99.999	327.00	94.57
17	1.5×10^4	>99.999	80.00	98.67
18	<1	>99.999	0.10	99.99
19	9.1×10^4	99.995	1400.00	76.70
20	18	>99.999	26.60	99.56
21	30	>99.999	266.00	95.58
Controls ($N=21$)	1.7×10^9 ($\pm0.6\times10^9$)		6028.00 ($\pm$1265.00)	

Averages of percentages kills and endotoxin removal compared with controls.

endotoxin. Combinations of an acid decontamination pre-treatment followed by a disinfection treatment being the most effective method. This is probably due to the acid pre-treatment removing calcium and magnesium carbonate crystals formed from the dialysate, which attach to the silicone and promote bacterial adherence. The use of an acid solution provides a descaling step prior to disinfection.[10] Citric acid was first chosen by suppliers because of its chelating properties, but this study shows that the efficacy of the two mineral acids is similar. Therefore, 3% citric acid might usefully be replaced with 0.15% hydrochloric acid, which is not a bacterial nutrient.

Other treatments also showed promise for dialysis machine cleaning. A chemical oxidizer such as Actril® (peroxyacetic acid, acetic acid and hydrogen peroxide) used alone without pre-treatment has a limited efficacy in biofilm removal although, its efficacy significantly increased when a descaling step was added prior to treatment. In addition, the efficacy of Actril® was also improved when used as a pre-treatment followed by autoclaving.

This study shows that high-temperature treatments (80–90°C) are not effective for biofilm removal. Increasing the temperature is believed to lead to a 'baking' of the biofilm, which becomes highly adherent to the surface of the tubing. This environment also promotes subsequent biofilm growth by providing nutrients to the bacteria, increasing adherence to the surface, and also limiting removal of endotoxins. Such high-temperature cleaning procedures should always be combined with a disinfection treatment with bleach or autoclave.

In this study, bleach was the most efficient disinfectant tested for biofilm removal. The higher the concentration, the more efficient the treatment with efficacy further improved by citric acid pre-treatment. We noted that a chlorine level 10 times higher than usual (0.1–0.3%) results in total removal of the biofilm. Such a level cannot be used regularly however because of possible damage to the machine including the pump heads, silicone tubing and measurement instruments such as pH meter, temperature probes and flow-rate meters. This treatment should therefore be reserved for occasional use. A 10-fold increase in concentration of most of the other chemicals tested (citric, nitric, hydrochloric, glycolic acids, and Actril®) did not significantly improve their efficacy in biofilm removal (data not shown). The results obtained with bleach are in agreement with other studies that have also shown the high efficacy of chlorine.[17,25] Even though most suppliers are trying to develop new decontamination methods, bleach remains the most efficient product.

Further experiments are required to improve the efficacy and safety of the treatments. Interactions between dialysis machine components and chemicals should be studied to identify unwanted reactions, including modification of the CT values (CT = concentration × contact time).

We note that the use of ozone for the disinfection of dialysis water treatment systems and dialysis membrane prior to re-use suggests that this treatment could be included in a cleaning procedure for haemodialysis machines.[26] However, its efficacy on biofilm and endotoxin removal should be assessed first.

Additional research needs to test long-term performing and clinical applications of these results. First, the model for biofim growth should be adjusted to include regular cleaning sessions and an extended run should be performed to assess the efficacy of most procedures for preventive maintenance against biofilm formation. The most efficient procedures from such an extended study should then be confirmed against biofilms from a clinical dialysis unit.

Acknowledgements

We are grateful to Martine Leguyader and her team from the microbiological department of the CRECEP for the bacterial strains identifications. We thank Mary Fitzgerald, Jill Petik and John Neuman for their technical assistance.

References

1. Rudnick JR, Arduino MJ, Bland LA *et al*. An outbreak of pyrogenic reactions in chronic hemodialysis patients associated with hemodialyzer reuse. *Artif Organs* 1995; **19**: 289–294.
2. Matos JP, Andre MB, Rembold SM *et al*. Effects of dialyzer reuse on the permeability of low-flux membranes. *Am J Kidney Dis* 2000; **35**: 839–844.
3. Capelli G, Inguaggiato P, Perrone S *et al*. Retention of limulus amoebocyte lysate reactive bacterial products by polysulfone dialyzers is affected by the type of disinfectant. *ASAIO J* 1998; **44**: M587–M591.
4. Ledebo I, Nystrand R. Defining the microbiological quality of dialysis fluids. *Artif Organs* 1999; **23**: 37–43.
5. Berland Y, Brunet P, Ragon A *et al*. Dialysis fluids and water: their roles in biocompatibility. *Nephrol Dial Transplant* 1995; **10**(Suppl. 10): 45–47.
6. Anonymous. Association for the Advancement of Medical Instrumentation, AAMI/ANSI RD 62: 2001. Standards for hemodialysis water quality.
7. European Pharmacopoeia, European Council Ed, Strasbourg, France, 4th Edn, 2002. Monograph 01-167 Eau pour hémodialyse; Solutions concentrées pour hémodialyse.
8. Costerton JW, Lewandowski Z, Caldwell DE *et al*. Microbial biofilms. *Ann Rev Microbiol* 1995; **49**: 711–45.
9. Stickler D. Biofilms. *Curr Opin Microbiol* 1999; **2**: 270–275.
10. Man NK, Degremont A, Darbord JC *et al*. Evidence of bacterial biofilm in tubing from hydraulic pathway of hemodialysis system. *Artif Organs* 1998; **22**(7): 596–600.
11. Phillips G, HudsonS, Stewart WK. Persistence of microflora in biofilm within fluid pathways of contemporary hemodialysis monitors (Gambro AK-10). *J Hosp Infect* 1994; **27**: 117–125.
12. Cappelli G, Ballestri M, Perrone S *et al*. Biofilms invade nephrology. *Blood Purif* 2000; **18**: 224–230.
13. Costerton JW, Stewart PS, Greenberg EP *et al*. Bacterial biofilms: a common cause of persistent infections. *Science*, 1999; **284**: 1318–1322.
14. Stewart PS, Grab L, Diemer JA. Analysis of biocide transport limitation in an artificial biofilm system. *J Appl Microbiol* 1998; **85**: 495–500.
15. Vincent FC, Tibi AR, Darbord JC. A bacterial biofilm in an hemodialysis system. Assessment of disinfection and crossing of endotoxin. *ASAIO Trans* 1989; **35**: 310–313.
16. Norwood DE, Gilmour A. The growth and resistance to sodium hypochlorite of *Listeria monocytogenes* in a steady-state multispecies biofilm. *J Appl Microbiol* 2000; **88**: 512–520.
17. Merritt K, Hitchins VM, Brown SA. Safety and cleaning of medical materials and devices. *J Biomed Mater Res* 2000; **53**: 131–136.
18. Decker DM. The ability of direct fluorescence-based, two colors assays to detect the different physiological states of oral streptococci. *Lett Appl Microbiol* 2001, **33**: 188–192.
19. Marion-Ferey K, Enkiri F, Husson GP, Vilagines R. Methods for biofilm analysis on silicone tubing of dialysis machines. *Artificial Organs* (In press).
20. Lechevallier MW, Cawthon CD, Lee RG. Factors promoting survival of bacteria in chlorinated water supplies. *Appl Envir Microbiol* 1988; **54**: 649–65.
21. De Beer D, Srinivasan R, Stewart PS. Direct measurement of chlorine penetration into biofilms during disinfection. *Appl Environ Microbiol* 1994; **60**: 4339–4344.
22. Ntsama-Essomba C, Bouttier S, Ramaldes M *et al*. Resistance of *Escherichia coli* growing as biofilms to disinfectants. *Vet Res* 1997; **28**: 353–363.
23. Anonymous. Recueil des normes Françaises. Antiseptiques et désinfectants. Paris: AFNOR Ed, 1995.
24. Dawson ME, Novitzki JT, Gould MJ. Microbes, endotoxins and water. *Pharmaceut Eng* 1988; **8**: 8–10.
25. Karpay RI, Plamondon TJ, Mills SE *et al*. Combining periodic and continuous hypochlorite treatment to control biofilms in dental unit water systems. *J Am Dent Assoc* 1999; **130**: 957–965.
26. Gal G, Kiss E, Foldes J *et al*. Disinfection of regenerated dialyzers with ozone. *Int J Artif Organs* 1992; **15**: 461–464.