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Original Article

Microbial inactivation properties of a new antimicrobial/antithrombotic catheter lock solution (citrate/methylene blue/parabens)

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Abstract

Background. Microbial infections are the most serious complications associated with indwelling central venous catheters. A catheter lock solution that is both antibacterial and antithrombotic is needed. The goal of this study was to determine whether a new catheter lock solution containing citrate, methylene blue and parabens has antimicrobial properties against planktonic bacteria and against sessile bacteria within a biofilm. These effects were compared to the antimicrobial properties of heparin at 2500 units/ml.
Methods. The tested solution (C/MB/P comprising 7% sodium citrate, 0.05% methylene blue and 0.165% parabens) and individual components were challenged against gram-positive and gram-negative organisms and fungi. Control solutions were heparin with preservatives. Studies included evaluation of eradication of planktonic bacteria and sessile organisms in a biofilm grown on polymeric and glass coupons. Biofilm samples were inspected by scanning electron microscopy, atomic force microscopy and vital stains.
Results. The C/MB/P solution, contrary to heparin, kills most tested planktonic microorganisms within 1 h of incubation. All tested organisms have an MIC of 25% or less of the original concentration of a new catheter lock. Bacteria strains did not develop resistance over more than 40 passages of culture suspensions. The C/MB/P solution is able to kill nearly all sessile bacteria in biofilm growth on plastic or glass discs in 1 h. Microscopic methods demonstrated extensive physical elimination of biofilm deposits from treated coupons. In contrast, heparin had a minimal effect on planktonic or biofilm organisms.
Conclusions. The new multicomponent lock solution has strong antimicrobial properties against both planktonic and sessile microorganisms. By comparison, heparin with preservative has weak antibacterial properties against planktonic and biofilm bacteria. The tested catheter lock may have usefulness in preventing bacterial colonization of haemodialysis catheters and diminishing the incidence of catheter-related bacteraemia.

Keywords: biofilm; catheter infection; CRBSI; haemodialysis; lock solution

Introduction

Catheter-related blood stream infection (CRBSI), especially in haemodialysis (HD) patients, is a problem of epidemic proportion and serious morbidity and mortality [1,2]. Every year, more than 320 000 patients in the United States undergo HD treatment and >25% (80 000) use dialysis catheters for access [3]. The incidence of CRBSI ranges from 1.6 to 6.1 episodes per 1000 catheter-days [4–7]. Assuming three CRBSI events per 1000 catheter-days for HD patients, there are about 100 000 CRBSI episodes per year among HD patients. With a mortality rate of 5–10% for each CRBSI event [5], there are 5000–10 000 HD patient deaths per year as a result of CRBSI. About one-third of CRBSI episodes require in-patient hospitalization. A number of strategies have been tried to reduce the incidence of CRBSI, including the use of strict hygienic measures, maximal barrier precaution, catheter dressing, catheter coatings, nasal decolonization and prophylactic antibiotic ointment at the exit site [8,9]. In order to prevent intraluminal colonization and the development of a biofilm, installation of an antimicrobial catheter lock solution (CLS) has been recently proposed [10]. Eight randomized clinical trials have compared the frequency of catheter-related bacteraemia in patients receiving a prophylactic antimicrobial CLS versus patients receiving standard heparin locks. Six studies used an antibiotic lock (gentamicin, cefazolin with gentamicin, minocycline, minocycline/EDTA or cefotaxime) [6,7,11–14], one study used taurolidine, [15] and one used 30% citrate [5]. These trials have shown more than 3-fold reduction in the occurrence of CRBSI, together with decreases

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in morbidity and mortality versus use of heparin as CLS [16–18].

Despite promising results with antibacterial CLS, there are a number of potential problems with prophylactic use of antibiotics. There are growing numbers of patients with microbial isolates resistant to antibiotics such as methicillin, gentamicin and vancomycin [19,20]. The spillage of up to 15% of the catheter volume into the patient's blood [21] can result in the development of antibiotic-resistant strains of *Staphylococcus* or *Pseudomonas* [22], systemic toxicity and aminoglycoside ototoxicity. Other disadvantages include the incompatibility of antibiotics such as gentamicin and cephalosporins with heparin [6,7]. Heparin as a CLS causes a bleeding risk due to systemic anticoagulation and, in some patients, heparin-induced thrombocytopenia [5,23,24].

CRBSI results both from intraluminal contamination with transfer of planktonic bacteria to the blood and release of bacteria within biofilms. Microorganisms embedded in a biofilm encounter a unique microenvironment with higher cell density, growth rates and gene transcription versus planktonic bacteria. Sessile bacteria in biofilms have an inherent resistance to antibiotics. As many as 60% of bacterial infections treated by physicians are related to biofilm formation and are not easily eradicated by conventional antibiotic therapy [25].

There is a great need for an antibacterial CLS based on antiseptics rather than antibiotics [26–28]. Antiseptics kill bacteria through physical effects rather than specific biochemical pathways and may not induce microbial resistance. In this publication, we describe *in vitro* tests of antibacterial properties of the new CLS against planktonic and sessile bacteria, and microscopic studies of physical effects of the lock solution on a biofilm. We compared these results to antibacterial properties of heparin with parabens as a preservative.

Methods

Composition of the citrate/MB/parabens lock solution (C/MB/P), and heparin solution

A new CLS (C/MB/P) comprises 7% (w/v) sodium citrate buffer (0.24 M of pH 6.2), 0.05% methylene blue (MB), 0.15% methyl paraben (MP) and 0.015% propyl paraben (PP). A solution of heparin 5000 units/ml with 1.5 mg/ml MP and 0.15 mg/ml of PP (Abraxis Pharmaceutical Products, Schaumburg, IL) was diluted 1:1 with sterile water just before experiments, yielding a concentration of 2500 units/ml (a medium dose concentration).

Planktonic bacteria tests

Antibacterial properties of C/MB/P, individual components of a new lock solution and heparin were tested against gram-positive and gram-negative strains of bacteria and fungi obtained from ATCC and from hospital isolates. Single colonies from fresh Trypticase Soy Agar (TSA) plates with sheep blood were used for preparation of overnight inocula. Twenty millilitre C/MB/P solutions containing 5% bovine serum albumin (BSA) were mixed with 1 ml of Luria-Bertani (LB) broth to augment bacterial growth as might occur with blood entry to the dialysis catheter tip. Finally, 10 μl of each microbial inoculum ($\geq 10^6$ CFU/ml for bacteria or $\geq 10^4$ for fungi) was added separately to each solution, mixed and 10 μl withdrawn immediately for preparation of serial 10× dilutions that were spread onto TSA plates in order to enumerate colony forming units per ml (CFU/ml) in time 0. Undiluted solutions were also plated and the remaining volumes filtrated through a 0.45 μm filter that, after washing with sterile saline, was placed on TSA and incubated overnight. The limit of detection was $\leq 10^1$ CFU/ml. A saline control prepared as above was also included for each experiment.

Erlenmeyer flasks with test solutions were placed on a shaker incubator (for bacteria 200 RPM, 37°C; for fungi 50 RPM and 25°C), and samples were taken at time intervals of 0.5, 1.0, 2.0 h and after 1, 2, 3 days for plating as described above. For comparison, a heparin solution was tested in a similar manner. Each experiment was repeated three times. We also ran identical experiments with each individual compound that was part of a new lock solution. They included citrate only, citrate/MB and citrate/mixture of parabens.

Minimum inhibitory concentration (MIC) study

The MIC of a C/MB/P solution against a panel of gram-positive and gram-negative pathogens was determined by microtitre plate dilution assay as described by the National Committee for Clinical Laboratory Standards [29]. Overnight inocula were diluted to match the 0.5 McFarland standards (1 to 2 × 10^8) and then further diluted 1:10 with Mueller Hinton (MH) broth. The microtitre tray contained 100 μl of 2-fold serial dilutions of the full-strength test solution distributed in six rows (i.e. concentrations of 50% down to 1.56% of the initial concentration) and 5 μl broth suspensions of the test microorganisms in the wells. Positive controls contained saline instead of a test solution; negative controls contained sterile broth instead of microbial suspension. MIC end points were determined by visual inspection after overnight incubation at 37°C for 24 h (bacteria) and 28°C for 24/48 h (yeast). Where colour intensity interfered with interpretation, cultures on MH agar were employed as confirmatory tests. Final results were expressed as the mean of three repetitions and presented as the percent of the original test solution concentration. The MIC was the lowest concentration of the test solution that completely inhibited growth of the microorganism in the well.

Bacterial resistance study

Resistance development studies were designed on the basis of MIC results. Gram-negative (*Escherichia coli* ATCC 25922) and gram-positive (*Staphylococcus aureus* ATCC 29213—MRSA) organisms were tested in capped sterile tubes. A single colony of each tested bacteria from fresh TSA plates containing sheep blood was used to prepare overnight inoculum in LB broth. The suspension of cells was next diluted with broth to a concentration of

$\sim$1–2 × 10^6 CFU/ml. Concentrations of the test solution close to the previously established MIC were prepared in LB broth with 0.2% glucose as an additional carbon source. One milliliter of each concentration was dispensed into sterile capped tubes, and 10 μl of the adjusted cell suspension was added to all tubes. After 24 h of incubation, 10 μl of cell suspension was passed to a new tube with the same dilution of the test solution to check continued survival in that concentration. Also, an additional 10 μl of bacteria from the strongest concentration showing survival was passed to a tube containing an even higher concentration; this was to determine if continued exposure to the lock solution made them able to adapt and survive. The control was passed to a new fresh broth with glucose. In concentrations too dark to assess visually (due to the concentration of MB), subcultures were employed each day to confirm viability of cells in tested solutions. The experiment was carried out in duplicate for 40 days to assess any change over time that might indicate increasing resistance that would allow continued survival in a higher concentration of the test solution than at Day 1.

Biofilm studies with various organisms

The biofilm in preliminary studies was developed in Erlenmeyer flasks on circular carbothane or glass coupons (d = 1.27 cm). Sterile coupons were incubated with microbes in 10 ml LB broth containing 0.2% glucose at 37°C and 160 RPM (25°C and 50 RPM for *Candida albicans*). The biofilm formation was tested for a few hours and up to 7 days. If incubation was longer than 1 day, coupons were transferred into fresh medium at 24-h intervals. On the basis of attained results, all further experiments were performed on biofilms developed within 24 h of incubation. At the end of incubation, coupons were gently rinsed in sterile saline to remove planktonic bacteria. Coupons were divided into two groups: control that served as a baseline for biofilm growth and tested discs that were additionally exposed to a new CLS for 1 h. The biofilm was removed from coupons by short 15 s vortexing followed by a 2 min sonicating procedure and again vortexing. Next, serial dilutions were applied to TSA plates for bacterial enumeration. Other coupons with a biofilm before and after 1-h treatment with C/MB/P were examined by various optical methods: scanning electron microscopy (SEM), atomic force microscopy (AFM) and fluorescence microscopy (FM) follow standard method protocols i.e. fixation with glutaraldehyde and critical drying for SEM, and staining with LIVE/DEAD® BacLight™ bacterial viability dye for FM. The LIVE/DEAD stain makes use of two different stains: Cyto 9 and propidium iodide. Cyto 9 is taken up by cells having charged membranes due to the presence of proton motive force (results in cells stained green). Propidium iodide is a DNA stain that only enters cells having a compromised/breached membrane (results in cells stained in red).

Biofilm studies staphylococcus in flow cell

The effectiveness of C/MB/P and heparin against a clinical isolate of *S. aureus* was also investigated in a flow cell bioreactor for a period of 16 days. In long-lasting experiments,

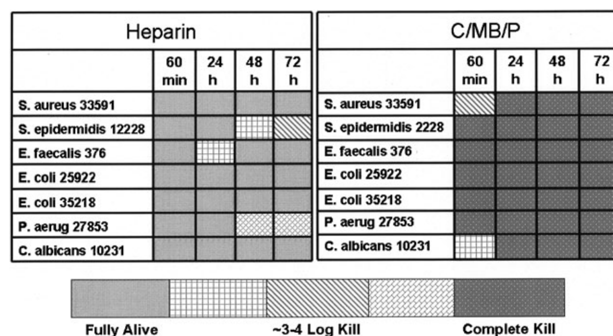

Fig. 1. Comparison of antimicrobial effectiveness of heparin and C/MB/P after incubation with microorganisms' broth suspension for 60 min, 24, 48 and 72 h.

hydraulic shear is a crucial factor in the development of a biofilm and a stable shear rate is best achieved in a bioreactor. The techniques were designed to replicate exposure of catheters to blood-side contamination during dialysis and locks with CLS between treatments. Sterile 1 × 2 cm carbothane strips presoaked with human serum were aseptically loaded into flow cells. The strips were challenged for 3 h with $\sim10^3$ CFU/ml bacterial suspensions before the appropriate lock solutions were introduced. The locks were left in place for 24-, 48- or 72-h periods simulating a catheter lock during three times weekly HD, and 3 h bacterial challenges in-between simulating dialysis with contaminated blood. So-called dirty samples were subjected to 48 h of bacterial challenge with *S. aureus* before they were inserted to the bioreactor cells for testing. After a predetermined number of cycles of plasma and lock solution, each strip was manually scraped on the outer surface in a consistent and repeatable manner and then sonicated (1 min) and vortexed (30 s) in a neutralizing solution. The resultant bacterial solutions were diluted with sterile saline and plated in triplicate on TSA for colony enumeration.

Statistical analysis

For planktonic bacteria, the log colony count at 24 h was compared for various combinations of the components of the lock solution. The standard deviation was determined by standard formulas. P-values were calculated using Student's t-test (two-tailed, paired) with Excel software.

Results

The results of the comparison study of antimicrobial effectiveness between heparin used commonly to lock the lumen of catheters after HD sessions and our solution are shown in Figure 1. Heparin is mostly ineffective in the inhibition of growth of tested microorganisms. A modest reduction in the range of 3–4 logs of living cells was achieved only with *Staphyloccus epidermidis* and *Pseudomonas aeruginosa* after prolonged 48–72 h of incubation. In contrast, C/MB/P revealed potent antimicrobial properties against all checked microorganisms. Most noticeably, 1-h contact of microbes with this solution is sufficient to eliminate the majority of planktonic cells in test solutions. Fungi like *C. albicans* or

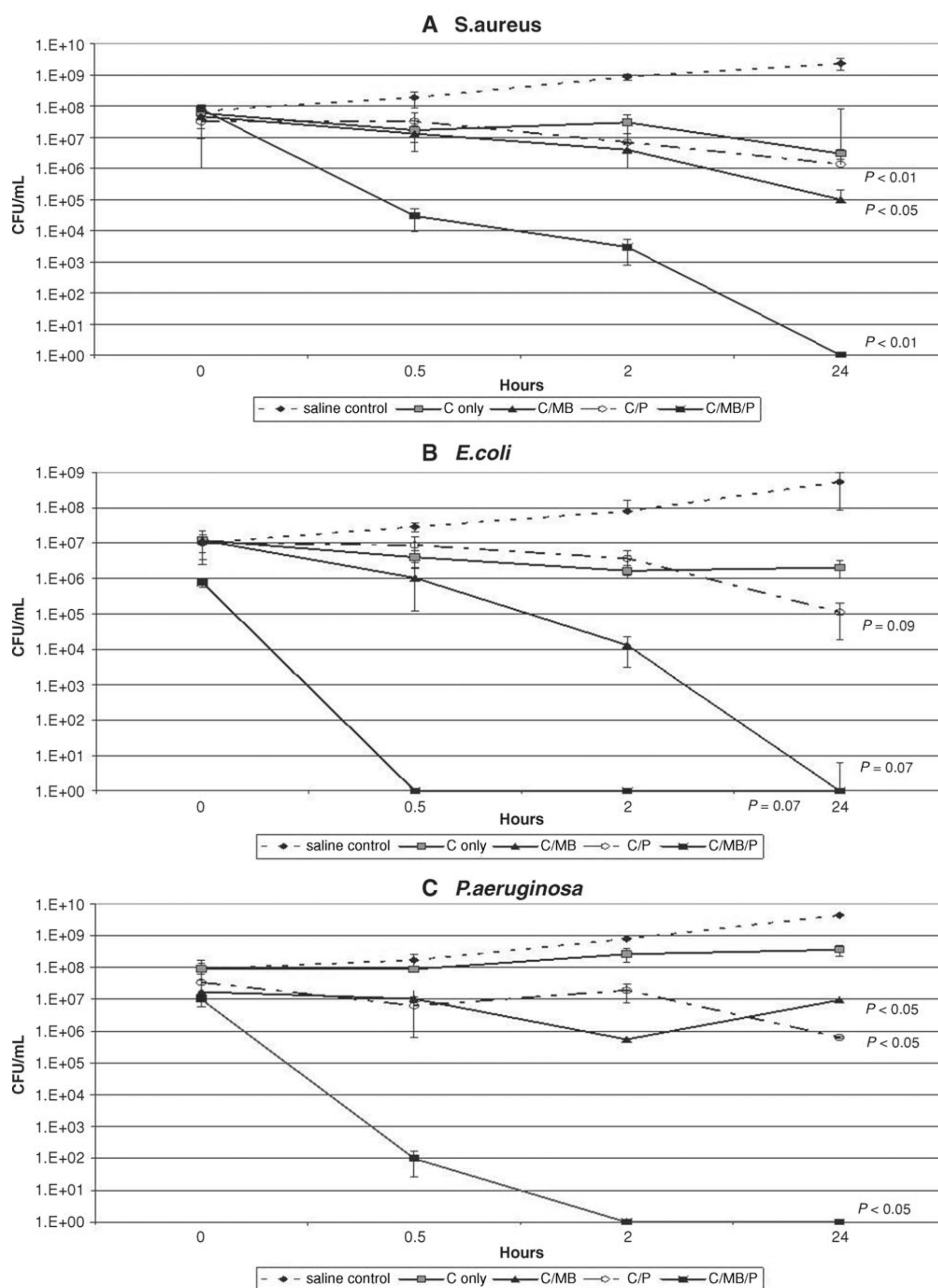

Fig. 2. Antimicrobial effectiveness C/MB/P and its components in the presence of media and albumin versus *Staphylococcus aureus* (**A**), *Escherichia coli* (**B**) and *Pseudomonas aeruginosa* (**C**).

Aspergillus niger survived a little bit longer, but there was no subcultured fungal activity after 24-h' exposure of cells to the solution.

The C/MB/P solution is composed of several compounds. Each of them, if used separately in the concentration employed in the final product, discloses a weak antimicrobial effect. This effect depends upon the strain of microorganisms and additional composites in the mixture. For example, if a small volume of broth were added to the reaction mixture, the life of cells might be prolonged. Serum proteins like albumin also extend the life span of microbes. Figure 2 represents data on the antibacterial effects of various components of the C/MB/P solution challenged alone and together, against three selected bacteria strains. In the case of *S. aureus* (Figure 2A), a small trend of inhibition of cell growth in the presence of each compound individually is visible over the period of 24 h. However, the C/MB/P acts much faster, lowering the concentration of bacteria by a few logs in 30 min. Furthermore, after 24 h viable bacteria were not detected ($P < 0.01$, C/MB/P versus sodium citrate alone). The combination of citrate and MB progressively reduced the *E. coli* count (Figure 2B) to an undetectable level after 24 h, but the C/MB/P acts more quickly. The exposure of *E. coli* for only 30 min to C/MB/P

	% of original concentration of C/MB/P						
	50%	25%	12.50%	6.25%	3.12%	1.56%	Controls**
S.aureus 33591	-	-	-	-	-	-	-/+
S.aureus 29213	-	-	-	+	+	+	-/+
MRSA*	-	-	-	-	+	+	-/+
S.epidermidis*	-	-	-	-	-	-	-/+
E.coli 35218	-	+	+	+	+	+	-/+
E.coli 25922	-	+	+	+	+	+	-/+
P.aeruginosa 27853	-	-	+	+	+	+	-/+
E.faecalis*	-	-	-	+	+	+	-/+
P.mirabilis*	-	+	+	+	+	+	-/+
C.albicans 10231	-	-	-	-	+	+	-/+
C.albicans*	-	-	-	-	-	+	-/+

*Hospital isolates
**Neg/Pos controls

Fig. 3. MIC of C/MB/P versus several pathogens (ATCC and hospital isolates) expressed as % of the original concentration.

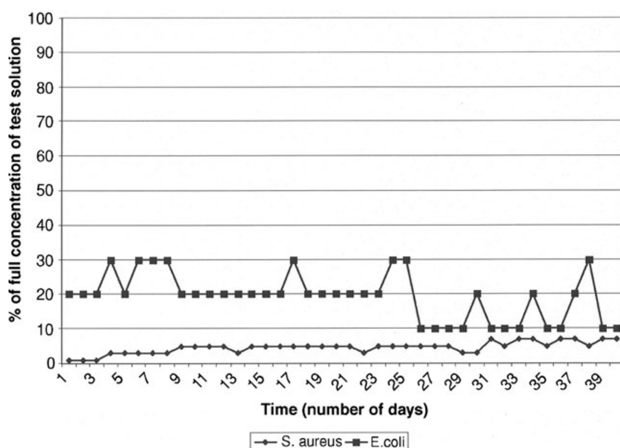

Fig. 4. Resistance study. Selected bacteria were grown in three different concentrations of C/MB/P (MIC, below and above MIC) for 40 days. Points on graph represent the highest concentration of the C/MB/P in which cells constantly survived.

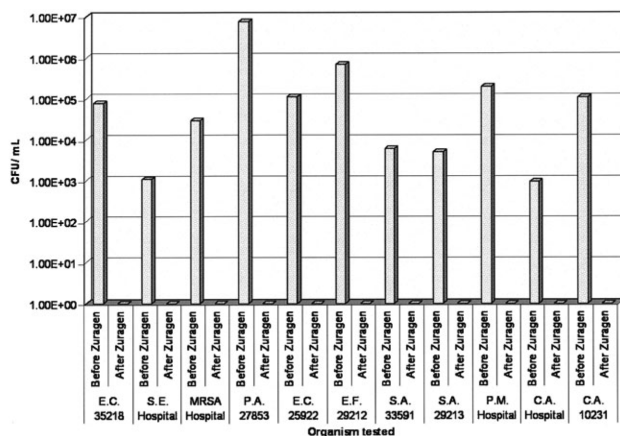

Fig. 5. Formation and elimination of a biofilm on carbothane coupons for different strains of bacteria and fungus. Duplicate coupons with a biofilm were treated for 1 h with C/MB/P. Similar results were obtained if carbothane coupons were presoaked in human plasma overnight before the experiment.

resulted in total elimination of living cells, as judged by the ability to form colonies on an agar plate ($P = 0.07$, C/MB/P versus sodium citrate alone). The *P. aeruginosa* strain (Figure 2C) was completely killed after 1–2-h exposure to C/MB/P ($P < 0.05$ for C/MB/P versus sodium citrate alone). The *P. aeruginosa* strain seems to be resistant to citrate at pH 6.2 or citrate/MB mixture. Overall, these results strongly suggest synergistic rather than additive effects among components in tested product.

As can be seen from Figure 3, all tested organisms have an MIC of 25% or less of the original concentration. Tested bacteria did not develop resistance above this earlier determined MIC over a long period of time. Figure 4 represents the first 40 days of the resistance development studies with examples of gram-positive and gram-negative strains. Both experiments indicate the high level of effectiveness of this new lock solution in eliminating microorganisms and thereby preventing catheter colonization with planktonic microorganisms.

The C/MB/P also demonstrated antibacterial activity against sessile microorganisms in a biofilm. One-hour treatment of biofilm growth on carbothane or glass coupons with the new CLS diminished viable cells practically to zero, as shown in Figure 5. This was also true with antibiotic-resistant strains from hospital isolates. To evaluate whether human plasma proteins may enhance the biofilm resistance to a new CLS, sterile coupons were immersed in 10 ml of human plasma and incubated overnight (37°C and 160 RPM). After that they were rinsed with saline, dried and treated as described in the Methods section. The pretreatment of the coupons in human plasma did not reduce potency of the C/MB/P solution (data not shown).

The microscopic studies demonstrated that C/MB/P not only kills bacteria within a biofilm but also removes the biofilm and bacteria from the surface of coupons to a high degree, as shown in Figure 6. As can be seen, the *P. aeruginosa* biofilm was almost totally removed after treatment with the new CLS and only a few bacteria cells were found to have survived (Figure 6A and B). Those that remained on the coupon surface were seen in highly degraded form using AFM (Figure 6C and D) and were probably dead. The high killing efficiency of C/MB/P was confirmed by FM with LIVE/DEAD® BacLight™ bacterial viability dye. As can been seen in Figure 6E, the *S. aureus* biofilm before treatment stained mainly green because of unchanged membrane structure of the cells. However, after exposure to C/MB/P (Figure 6F), the biomass on the coupon was reduced drastically and remaining cells were stained red indicating cellular death.

The results from the continuous flow cell bioreactor show that the new CLS reduces biofilm formation by more than four logs over 16 days in spite of repeated contamination, compared to control and heparin samples. The results of this study also demonstrate reduction of established biofilm (48 h) on the surface of carbothane coupons (Figure 7).

Discussion

Heparin is used as a CLS to prevent or reduce thrombosis of the catheter. It has been proposed that heparin, having

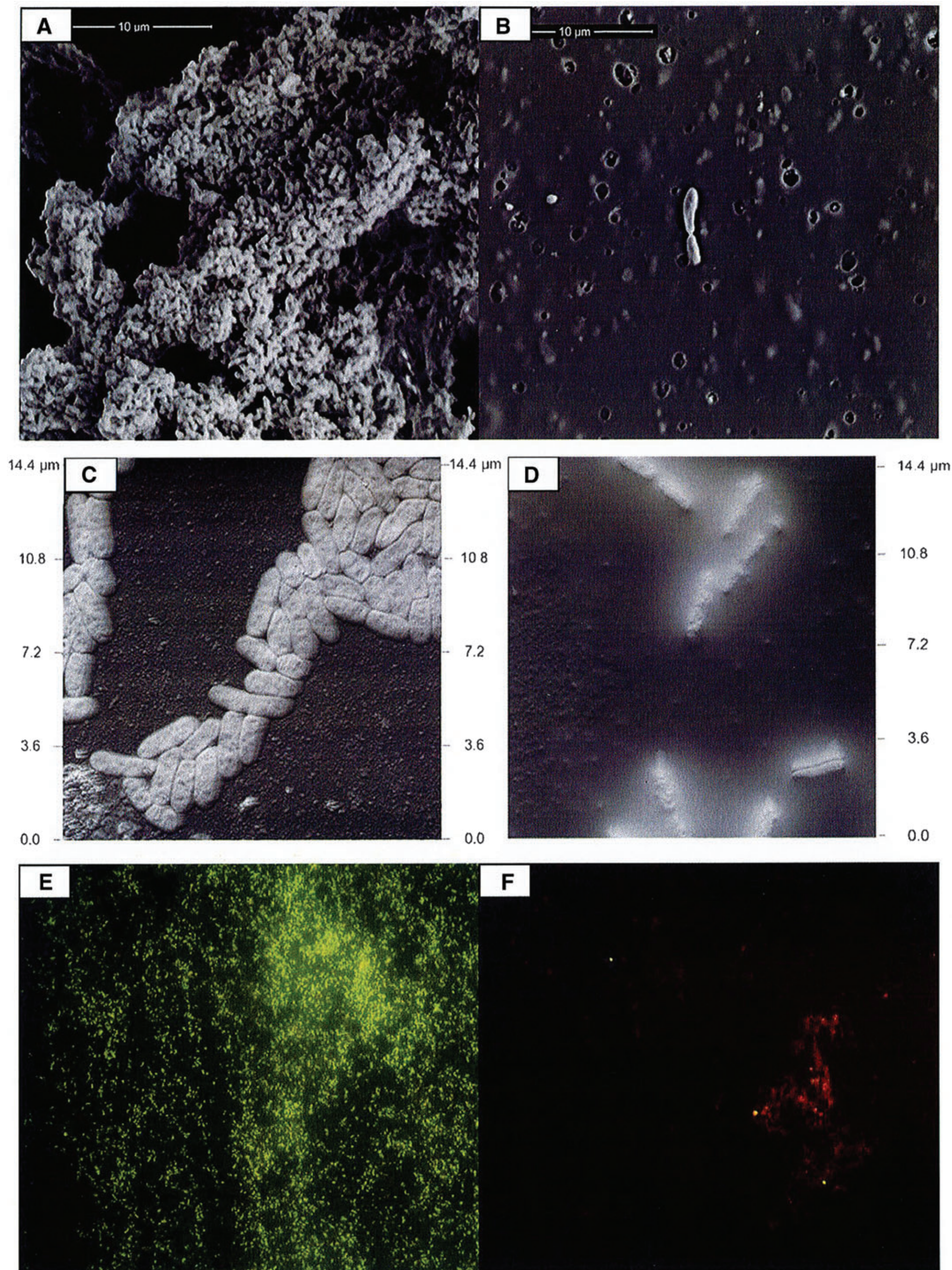

Fig. 6. Microscopic studies. Scanning electron microscopy (SEM) of the *P*. *aeruginosa* biofilm on carbothane coupons before (**A**) and after (**B**) treatment with C/MB/P. Atomic force microscopy (AFM) of the *P. aeruginosa* biofilm developed on glass discs before (**C**) and after (**D**) treatment with C/MB/P. Fluorescence microscopy (FM) of the *S. aureus* biofilm staining with LIVE/DEAD® BacLight™ bacterial viability dye. Dense biofilm with mainly live cells staining green (**E**) was drastically reduced after 1 exposure to C/MB/P. Remaining cells are stained red (**F**) indicating the antibiofilm efficacy of C/MB/P.

antibacterial properties, may avoid bacterial infection with some strains [30,31]. Most available heparin solutions contain preservatives such as benzyl alcohol (15 mg/ml) or a mixture of MP (0.15%) and PP (0.015%). In our comparison studies, we challenged microorganisms against the heparin solution containing 2500 units/ml and 0.75 mg/ml MP and 0.075 mg/ml PP. The possible binding of divalent cations by heparin [30], and the presence of antiseptics may have some impact on microbial inhibition of heparin solutions. However, in recent publications heparin is noted to promote biofilm formation, especially for *Staphylococcus* [32]. Some have proposed that heparin contributes to

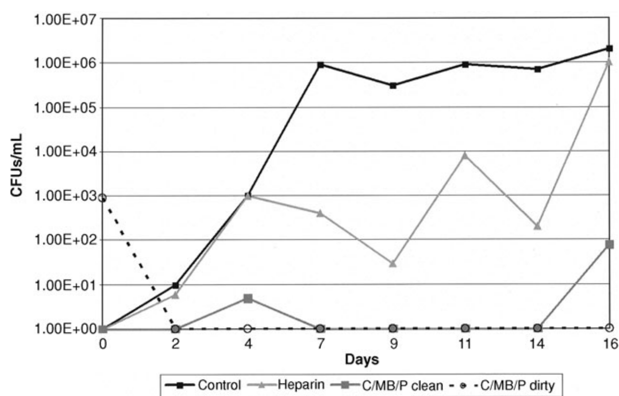

Fig. 7. Comparison of C/MB/P and heparin effectiveness against the *S. aureus* biofilm developed in a flow cell reactor. C/MB/P 'clean' refers to the experiment when originally clean coupons were daily challenged for 3 h with *S. aureus*. C/MB/P 'dirty' refers to the experiment when coupons first were challenged in a bioreactor with *S. aureus* for 48 h and then exposed continuously to test CLS.

the risk of CRBSI whenever it is used as a lock solution between dialysis treatments [33]. Our studies did not show any antibacterial effectiveness of heparin with parabens (Figure 1), which agrees with many recently published data [34–36]. A weak antimicrobial efficacy of heparin with parabens was observed only for a few organisms and after a long time of incubation (48–72 h).

Because of the development of bacterial resistance, antibiotic CLSs are considered to be inappropriate for some bacterial strains (e.g. *S. aureus*) that have an elevated rate of therapeutic failure [37,38]. Sensitization of the patients to aminoglycosides may also take place, and continuous exposure to these antibiotics in CLS is not advisable.

There is a renewed interest in citrate as an alternative to heparin in a CLS, because of its antithrombotic and antibacterial properties, reduced costs relative to heparin and lack of heparin complications. Citrate as CLS has been used in a wide range of concentrations from 4 to 46.7% [5,39–41]. In 2000, we demonstrated antimicrobial activity of sodium citrate, especially in higher concentrations (10–47%) [40]. Clinical trials have confirmed a decrease in CRBSI using 30% sodium citrate contrasted to heparin [35], but there are potential risks when used in such range of strength [42]. Report of a fatal cardiac arrest following the direct injection of 10 ml of a high concentration of trisodium citrate (46.7%) led to the restriction of use of a commercially marketed product, Tricitrosol, by the FDA [43]. Therefore, it is unlikely that higher concentrations of citrate, such as 30–47%, will be allowed by FDA because of persistent fears of myocardial toxicity from hypocalaemia if an administration error is made.

Studies of dilute sodium citrate formulation as CLS (4% or less) have demonstrated efficacy as an anticoagulant with generally improved safety and minimal to no risk of bleeding [44–47]. Sodium citrate at 4–5% concentration has been shown to maintain patency of HD catheters at least as effectively as heparin and is completely safe [48–50]. There have been several comparisons of the efficacy, safety and cost of citrate versus heparin as CLS [50,51]. Conversely, differences were not detected with respect to number of infections when comparing citrate 4% with heparin. An *in vitro* study demonstrated that sodium citrate at a concentration $>0.5\%$ might inhibit biofilm formation and cell growth of *S. aureus* and *S. epidermidis* and by so doing reduce the risk of biofilm-associated complications in indwelling catheters [52]. On the other hand, it was determined that even 4% citrate did not disrupt pre-existing biofilms.

C/MB/P is a unique combination of several compounds with a carefully balanced concentration to assure safety. Seven percent citrate (trisodium citrate/citric acid pH 6.2) has excellent anticoagulation properties, capability to prevent growth of many microorganisms as compared to a saline control (Figure 2), and has a density of 1.040 very close to the blood of patients with ESRD and mild anaemia. This density diminishes the hydraulic forces that can replace the CLS with blood. MB, one of a large group of phenothiazine dyes, has inherent microbial toxicity through reactive singlet oxygen [53–55] and redox potential of the dye [56]. It is a cationic dye and may interact with the membrane or cell wall of bacteria [57]. MB is often given intravenously at a dosage of 1–7 mg/kg of body weight. The new CLS contains only 2.5 mg of MB in the total volume of catheter lock. In our experiments, the combination of 7% citrate with 0.05% MB resulted in partial (*S. aureus*) to total (*E. coli*) elimination of bacteria strains, but this required up to 24-h period of incubation. However, as is also shown in Figure 2(C), the *P. aeruginosa* strain seems to be not affected by citrate or citrate/MB mixture. Similar behaviour of *P. aeruginosa* in a lower concentration of citrate was observed earlier [35] and may have been due to utilization of the citrate ion as a nutrient by these bacteria in the range of neutral pH. Parabens are well-known antibacterial agents, and this is the reason that they are used as preservatives in medicine, cosmetic and food industries. The biocidal activity increases with the length of the hydrocarbon alkyl chain, but solubility drastically decreases. Thus, the parabens are often used in combination, mainly methyl and propyl parabens [58,59]. They are used widely for intravenous fluids contained in multiple use vials, as well as topical applications in concentrations generally $<1\%$ (w/w) [58,59]. They are considered to be safe [60] although at a high concentration in skin treatments there is uncertainty about estrogenic potential. Parabens concentrations in tested formulation are much lower than the concentration used in skin preparations. Alone in the citrate solution, they preserve growth of microbes slightly better than citrate by itself, but elimination of living cells is limited. On the other hand, when all components are mixed together in the final product, microbial inactivation properties are surprisingly high. They reveal a synergistic effect as a mixture by eradicating in a short time all tested strains of bacteria and fungi. Microbial reductions above 99% were obtained in the first few minutes of incubation. One to two hours contact of the new CLS with tested planktonic bacteria strains irreversibly eliminates all living bacteria. The time interval for killing fungi was somewhat longer but still did not exceed 24 h. All organisms have an MIC of 25% or less of the original concentration of C/MB/P, and antimicrobial properties are not diminished in the presence of media and 5% albumin. This leads to the conclusion that this lock solution should be

effective in killing bacteria even in the presence of blood at the tip of the catheter. There was no change in the MIC over a long period of time as shown by the microbial resistance experiment. Other studies (not shown) have demonstrated safety and lack of toxicity of the new lock solution.

The C/MB/P solution also appears to prevent formation of a biofilm or to eradicate sessile bacteria from the surface of polymers used in catheter manufacturing. When polymeric or glass coupons were exposed to media inoculated with bacteria, biofilm formation was confirmed within hours for all tested microorganisms. After biofilms were grown for a 24-h period, coupons with a biofilm were immersed in C/MB/P. One-hour treatment of biofilm growth on carbothane or glass coupons with C/MB/P diminished viable cells to nearly undetectable levels. This happened also with antibiotic-resistant strains from hospital isolates. Pretreating the coupons with human plasma before creation of the biofilms did not reduce the potency of C/MB/P. Microscopic methods disclosed that the C/MB/P not only kills microorganisms on carbothane or glass discs but also physically removes a biofilm from the surface to a high degree. After treatment, few bacterial cells were found to have survived. Those that remained on the coupon surface were mainly dead as confirmed by FM and appeared highly degraded on AFM. These results were supported by flow cell bioreactor experiments with a clinical isolate of *S. aureus*. C/MB/P significantly reduced biofilm formation by four logs even if challenged daily by bacteria for 3 h. It also has a significant impact in eradication of living organisms from already established biofilms, in the flow-cell environment.

In conclusion, an antimicrobial study of a new multicomponent lock solution and comparison with heparin confirms superior properties of C/MB/P in elimination of both planktonic and sessile microorganisms. The study also confirms physical elimination of a biofilm and lack of development of resistance. Elimination of bacteria from a catheter lumen, prevention of biofilm formation and eradication of bacteria in the existing biofilm are all important if a CLS is to diminish the incidence of CRBSI. A clinical trial (AZEPTIC) with 415 enrolled patients has just been completed, to compare effects of C/MB/P versus heparin on CRBSI incidence and patency of CVC for dialysis.

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