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Optimal Antimicrobial Catheter Lock Solution, Using Different Combinations of Minocycline, EDTA, and 25-Percent Ethanol, Rapidly Eradicates Organisms Embedded in Biofilm▽

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Antimicrobial lock solutions may be needed to salvage indwelling catheters in patients requiring continuous intravenous therapy. We determined the activity of minocycline, EDTA, and 25% ethanol, alone or in combination, against methicillin-resistant *Staphylococcus aureus* and *Candida parapsilosis* catheter-related bloodstream infection strains in two established models of biofilm colonization. Biofilm-colonized catheter segments from a modified Robbins device and a silicone disk biofilm colonization model were exposed to these antimicrobial agents for 15 or 60 min, respectively. After exposure, segments were sonicated and cultured. To determine regrowth after incubation at 37°C, following the brief exposure to the antimicrobial agents, an equal number of segments were washed, reincubated for 24 h, and then sonicated and cultured. The triple combination of minocycline-EDTA (M-EDTA) in 25% ethanol was the only antimicrobial lock solution that completely eradicated *S. aureus* and *C. parapsilosis* in biofilm of all segments tested in the two models, and it completely prevented regrowth. In addition, M-EDTA in 25% ethanol was significantly more effective in rapidly eradicating the growth or regrowth of methicillin-resistant *S. aureus* and *C. parapsilosis* biofilm colonization in the two models than the other solutions—minocycline, EDTA, M-EDTA, 25% ethanol, and EDTA in ethanol. We conclude that M-EDTA in 25% ethanol is highly effective at rapidly eradicating *S. aureus* and *C. parapsilosis* embedded in biofilm adhering to catheter segments.

Central venous catheters (CVCs) providing long-term vascular access have become the lifeline for patients requiring dialysis, chemotherapy, or total parenteral nutrition (TPN). Infections, particularly catheter-related bloodstream infections (CRBSIs), are the most serious and frequent complications associated with indwelling CVCs (22, 23). Microbial organisms embedded in the intraluminal biofilm of indwelling CVCs eventually migrate, leading to CRBSI in long-term silicone CVCs (30). To salvage long-term CVCs, intraluminal antimicrobial lock therapy (ALT) has been proposed for the prevention and treatment of CRBSIs (2, 14, 18, 19, 25, 32, 35–37). In ALT, the lumen of the catheter is filled with 2 to 4 ml of antimicrobial solution at a concentration 100- to 1,000-fold higher than the MIC of the antibiotic or its usual target systemic concentration; the solution is then allowed to dwell (lock) for a period of time while the catheter is not in use to eradicate the bacteria and fungi embedded in the intraluminal biofilm of the catheter (36).

Although vancomycin and heparin have been frequently used as ALT in the salvage treatment of catheter-related staphylococcal bloodstream infections, several studies have reported the failure of response or salvage of CVCs with this combination (1, 13, 18, 19, 21). A combination of minocycline and EDTA (M-EDTA) has been shown to be synergistically active in eradicating microorganisms embedded in biofilm (29). It has also been shown to be effective in an animal model of catheter-related staphylococcal bacteremia, in the prevention of CRBSI, and in the salvage of CVCs in cases of CRBSI, in patients with cancer, and in those undergoing hemodialysis (3, 7, 31). However, like other antibiotic lock solutions, M-EDTA requires a dwell time of at least 4 h daily. Recently, ethanol lock techniques were suggested as a treatment for CRBSI in patients with cancer with long-term CVCs (8). Anecdotal reports have suggested that ethanol could be locked in for a dwell time of 1 h (22a).

The objective of the current study was to determine the role of minocycline, EDTA, and 25% ethanol, alone or in combination, in eradicating methicillin-resistant *Staphylococcus aureus* (MRSA) and *Candida parapsilosis* organisms embedded in biofilm on silicone segment surfaces within a short time period of up to 1 h.

MATERIALS AND METHODS

Modified Robbins device. We used a modified Robbins device (MRD) to determine the eradication of organisms embedded in the biofilm of catheter segments. The MRD has been previously described (10, 15); it is constructed from an acrylic block, 42 cm long with a lumen of 2 by 10 mm. It consists of 25 evenly spaced specimen plugs, each connected to a silicone catheter segment (Allegiance Healthcare Corp., McGaw Park, IL) whose anterior surface (0.3 cm^2) comes in contact with the flushed infusate.

The catheter segments were placed in the specimen plug of the MRD, and the entire apparatus was sterilized with ethylene oxide. A solution of 500 ml of 5% dextrose in water was connected to the MRD through an intravenous tubing administration set and infected with an inoculum of 10^8 CFU of MRSA to produce an infected infusate at the concentration of 2×10^5 CFU/ml. The MRSA isolates were obtained from patients with CRBSIs. In another series of experiments, 500 ml of a 5% dextrose in water bag was infected with *C. para-*

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TABLE 1. Activity of antimicrobial lock solutions against MRSA after 15 min of exposure in the MRD model

Drug combination tested	Eradication after 15 min in drug (mean CFU/ml ± SD)	*P* value vs control	Regrowth (24-h growth after 15 min in drug) (mean CFU/ml ± SD)	*P* value vs control
Minocycline (3 mg/ml)	324 ± 206	0.07	308 ± 165	0.04
EDTA (30 mg/ml)	480 ± 47	0.28	500 ± 0	0.99
Ethanol (25%)	138 ± 199	<0.01	500 ± 0	0.99
Minocycline (3 mg/ml), EDTA (30 mg/ml)	295 ± 188	0.02	171 ± 163	<0.01
Ethanol (25%), EDTA (30 mg/ml)	26 ± 65	<0.01	334 ± 249	0.08
Ethanol (25%), minocycline (3 mg/ml)	0	<0.01	0	<0.01
Ethanol (25%), EDTA (30 mg/ml), minocycline (3 mg/ml)	0	<0.01	0	<0.01
Control (MHB alone)	440 ± 117		500 ± 0	

psilosis; an inoculum of 10^5 CFU/ml produced an infected infusate at a concentration of 2×10^2 CFU/ml of *C. parapsilosis*. The whole system was incubated at 37°C, and the infected infusate was flushed through the MRD using a peristaltic pump that permitted the infusate to flow at the rate of 60 ml/h for 8 h. The MRD was left to incubate for another 10 h. Subsequently, the infected bag was removed, and a sterile bag of 250 ml of saline was infused through the MRD at 125 ml/h for 2 h to remove all free-floating organisms.

Catheter segments were then removed and placed in new tubes containing either Mueller-Hinton broth (Becton Dickinson & Co., Cockeysville, MD) as the control or 30 mg/ml of EDTA in broth (Abbott Laboratories, North Chicago, IL), 3 mg/ml of minocycline in broth (Wyeth Laboratories, Collegeville, PA), 3 mg/ml of M-EDTA in broth, 25% ethanol solution in broth, 3 mg/ml of minocycline in 25% ethanol solution in broth, 30 mg/ml of EDTA in 25% ethanol solution in broth, or M-EDTA in 25% ethanol solution in broth. The segments were soaked in their respective drug solutions for 15 min at 37°C. One set of catheter segments was removed from broth solution, scraped with a sterile stick, and placed along with the stick in a tube containing Trypticase soy broth, and both were sonicated for 5 min (scrape sonication method). A 100-μl aliquot was plated on Trypticase soy agar with 5% sheep blood and incubated at 37°C overnight. Plates were counted, and final counts were calculated taking the dilution factor into account.

A second set of catheter segments was removed from the drug solutions and reincubated in Mueller-Hinton broth at 37°C. After 24 h, catheter segments were scrape sonicated, and a 100-μl aliquot was plated on Trypticase soy agar with 5% sheep blood and incubated at 37°C overnight. Colonies were counted and recorded, taking the dilution factor into account. Numbers of CFU of 100 or greater were considered equivalent to 100 CFU. When the dilution factor was taken into consideration, the maximum number of CFU reported was 500.

Silicone disk biofilm colonization. The ability of ALT solutions to eradicate MRSA and *C. parapsilosis* organisms was determined by the silicone disk biofilm colonization model as previously described (20). Sterile silicone disks were placed in human plasma and incubated while shaking for 24 h at 37°C. The plasma was then replaced with 1 ml of bacterial inoculum and incubated while shaking for 24 h at 37°C. The bacterial inoculum was made by diluting the isolates to 5.5×10^5 cells/ml in Mueller-Hinton broth (MHB). Organisms were tested in 5 to 10 replicates. The inoculated broth was then removed, and silicone disks were washed with 0.9% saline while shaking for 30 min at 37°C. The silicone disks were then transferred to new tubes containing MHB or the drug solution to be tested. Drug solutions included 3 mg/ml of minocycline, 30 mg/ml of EDTA, 25% ethanol solution, 30 mg/ml of EDTA in 25% ethanol, 3 mg/ml of minocycline in 25% ethanol, 3 mg/ml of minocycline with 30 mg/ml of EDTA, and a triple combination of 3 mg/ml of minocycline and 30 mg/ml of EDTA in 25% ethanol. After 1 h of incubation at 37°C, the disks were placed in 5 ml of 0.9% saline and sonicated for 5 min. Finally, the tubes were vortexed for 30 s, a 100-μl aliquot of saline was plated on Trypticase soy agar with 5% sheep blood, and plates were incubated overnight at 37°C. Colonies were counted, and final counts were calculated, taking the dilution factor into account. To determine whether the drug solutions prevented the regrowth of organisms embedded in biofilm after a 60-min exposure of the disks to the drug, the disks were prepared in the same manner as the others. They were allowed to dwell in the drug solutions for 60 min, and the drug was then removed using a plastic transfer pipette. The pieces were washed with saline transferred to new sterile tubes containing Trypticase soy broth and then placed in the incubator at 37°C overnight. The disks were then sonicated for 5 min in the same Trypticase soy broth that had grown overnight. A 100-μl aliquot was then plated on Trypticase soy agar with 5% sheep blood and incubated overnight at 37°C. Colonies were counted, and final counts were calculated, taking the dilution factor into account. Numbers of CFU of 100 or greater were considered equivalent to 100 CFU. When the dilution factor was taken into consideration, the maximum number of CFU reported was 5,000.

Statistical analysis. The significance of differences between study groups was determined with Student's *t* test or the Wilcoxon rank-sum test for continuous variables. All *P* values were based on two-tailed tests of significance. A *P* value of ≤0.05 was considered significant. All computations were performed with the Statistical Package for the Social Sciences, SPSS software (version 11.00; SPSS, Inc., Chicago, IL).

RESULTS

Modified Robbins device. As shown in Table 1, EDTA alone failed to eradicate MRSA organisms embedded in biofilm after 15 min of exposure. Minocycline alone (3 mg/ml), with or without EDTA, resulted in some decrease in colonization. However, organisms continued to grow after 15 min of exposure and after 24 h reincubation in broth at 37°C, although the growth associated with M-EDTA was significantly less than that with the control ($P \leq 0.04$). A 25% ethanol solution

TABLE 2. Activity of antimicrobial lock solutions against *C. parapsilosis* after 15 min of exposure in the MRD model

Drug combination tested	Eradication after 15 min in drug (mean CFU/ml ± SD)	*P* value vs control	Regrowth (24-h growth after 15 min in drug) (mean CFU/ml ± SD)	*P* value vs control
Minocycline (3 mg/ml)	138 ± 122	0.01	500 ± 0	0.99
EDTA (30 mg/ml)	160 ± 86	0.01	500 ± 0	0.99
Ethanol (25%)	0	<0.01	142 ± 244	0.01
Minocycline (3 mg/ml), EDTA (30 mg/ml)	153 ± 177	0.01	500 ± 0	<0.01
Ethanol (25%), EDTA (30 mg/ml)	0	<0.01	0	<0.01
Ethanol (25%), minocycline (3 mg/ml)	0	<0.01	83 ± 204	<0.01
Ethanol (25%), EDTA (30 mg/ml), minocycline (3 mg/ml)	0	<0.01	0	<0.01
Control (MHB alone)	500 ± 0		500 ± 0	

TABLE 3. Activity of antimicrobial lock solutions against MRSA after 60 min of exposure in the silicone disk biofilm colonization model

Drug combination tested	Eradication after 60 min in drug (mean CFU/ml ± SD)	*P* value vs control	Regrowth (24-h growth after 60 min in drug) (mean CFU/ml ± SD)	*P* value vs control
Minocycline (3 mg/ml)	5,000 ± 0	NS[a]	5,000 ± 0	NS
EDTA (30 mg/ml)	5,000 ± 0	NS	5,000 ± 0	NS
Ethanol (25%)	2,900 ± 2,286	0.017	5,000 ± 0	NS
Minocycline (3 mg/ml), EDTA (30 mg/ml)	5,000 ± 0	NS	3,110 ± 2,016	0.013
Ethanol (25%), EDTA (30 mg/ml)	730 ± 1,199	<0.01	2,770 ± 248	0.012
Ethanol (25%), minocycline (3 mg/ml)	0	<0.01	85 ± 269	<0.01
Ethanol (25%), EDTA (30 mg/ml), minocycline (3 mg/ml)	0	<0.01	0	<0.01
Control (MHB alone)	5,000 ± 0		5,000 ± 0	

[a] NS, not significant.

suppressed growth initially to a mean concentration level of 138 CFU/ml ($P < 0.01$). However, upon reincubation in broth at 37°C for 24 h, there was remultiplication and growth of the staphylococcal organisms embedded in biofilm to 500 CFU/ml, which was comparable to the growth of control catheter segments. The EDTA in 25% ethanol solution resulted in a significant decrease in colonization immediately after 15 min exposure. However, regrowth occurred after reincubation in broth solution at 37°C for an additional 24 h. Minocycline in 25% ethanol, with or without EDTA, resulted in complete eradication of microorganisms embedded in biofilm after 15 min of exposure. In addition, reincubation of the catheter segments in broth for an additional 24 h at 37°C failed to allow regrowth of the organisms, indicating the complete eradication of the MRSA organisms embedded in biofilm.

Similarly, as shown in Table 2, EDTA alone, minocycline alone, and M-EDTA failed to completely eradicate *C. parapsilosis* organisms embedded in biofilm. The 25% ethanol solution, with or without minocycline, inhibited *C. parapsilosis* growth after 15 min of exposure. However, regrowth was noted after 24 h of incubation in broth. EDTA in 25% ethanol and M-EDTA in 25% ethanol completely eradicated *C. parapsilosis* in biofilm after 15 min of exposure, with no regrowth after reincubation in broth.

On the basis of the MRD model of in vitro colonization of MRSA and *C. parapsilosis*, the M-EDTA solution in 25% ethanol was the only formulation that resulted in the complete eradication of these two organisms after a rapid exposure of 15 min, with no growth thereafter.

Silicone disk biofilm colonization model. As shown in Table 3, exposure to minocycline alone, EDTA alone, or minocycline plus EDTA failed to suppress the bioprosthetic MRSA colonization of the silicone disks. EDTA in 25% ethanol resulted in partial suppression, but regrowth of the organisms occurred after 24 h of incubation, although to a lower extent than with the control. The control silicone disk segments were heavily colonized before and after 24 h of reincubation. Minocycline in 25% ethanol completely suppressed bacterial growth, with some regrowth after 24 h of incubation. However, the triple combination of M-EDTA in 25% ethanol eradicated the MRSA organisms completely, with a complete inhibition of regrowth after 24 h of incubation.

Table 4 shows a similar trend for *C. parapsilosis* on silicone disks. Furthermore, there was a complete or partial regrowth of the *C. parapsilosis* on silicone disks after exposure to these agents and reincubation for 24 h. Ethanol resulted in some suppression of *C. parapsilosis* on the silicone disks, but regrowth occurred after 24 h of reincubation. Similarly, EDTA in 25% ethanol resulted in some suppression of *C. parapsilosis* growth after 1 h of exposure, but further regrowth occurred after 24 h of incubation. The combination of M-EDTA and 25% ethanol completely eradicated the organisms on the silicone disks before and after reincubation.

DISCUSSION

Through two independent models of biofilm colonization, we showed that M-EDTA in 25% ethanol completely eradi-

TABLE 4. Activity of antimicrobial lock solutions against *C. parapsilosis* after 60 min of exposure in the silicone disk biofilm colonization model

Drug combination tested	Eradication after 60 min in drug (mean CFU/ml ± SD)	*P* value vs control	Regrowth (24-h growth after 60 min in drug) (mean CFU/ml ± SD)	*P* value vs control
Minocycline (3 mg/ml)	5,000 ± 0	NS[a]	5,000 ± 0	NS
EDTA (30 mg/ml)	5,000 ± 0	NS	5,000 ± 0	NS
Ethanol (25%)	1,933 ± 2,330	0.001	3,875 ± 1,955	0.068
Minocycline (3 mg/ml), EDTA (30 mg/ml)	4,080 ± 1,850	NS	5,000 ± 0	NS
Ethanol (25%), EDTA (30 mg/ml)	1,667 ± 2,440	<0.001	2,333 ± 2,582	0.006
Ethanol (25%), minocycline (3 mg/ml)	1,490 ± 1,715	0.068	5,000 ± 0	NS
Ethanol (25%), EDTA (30 mg/ml), minocycline (3 mg/ml)	0	<0.01	0	<0.01
Control (MHB alone)	5,000 ± 0		5,000 ± 0	

[a] NS, not significant.

cated MRSA and *C. parapsilosis* organisms grown on silicone catheter and disk surfaces and prevented their regrowth after 24 h of incubation in broth. Intraluminal colonization of the long-term CVC is a major source of bloodstream infections in catheters that remain in place for more than 30 days. Given this understanding of CRBSI and long-term CVC and the limited vascular access in chronically ill patients undergoing dialysis, chemotherapy, or TPN, catheter salvage through antimicrobial lock therapy has been widely practiced since 1988 (2, 4, 6, 9, 14, 24, 25, 32, 38).

Vancomycin in combination with heparin has been the most commonly used ALT combination (1, 2, 4, 6, 13, 18, 19, 21). However, glycopeptide antimicrobials, including vancomycin, have been previously reported to have limited activity against slime-producing microbial organisms embedded in biofilm on a catheter surface (10, 11). It is not known whether ALT with vancomycin and heparin results in effective salvage of long-term catheters (2, 4, 6, 9, 14, 24, 25, 32, 33, 38), and most of the clinical data are anecdotal. The only prospective randomized trial of ALT that included vancomycin and heparin for gram-positive infections found that catheter salvage occurred in 67% of the episodes in the ALT arm versus 43% in the placebo arm ($P = 0.1$) (33). Mermel et al. (23), on the other hand, reviewed 14 trials that included systemic therapy alone without ALT for 514 episodes of CRBSI and reported a salvage rate of 66.5%. Several studies that used a glycopeptide antibiotic (vancomycin or teicoplanin) as part of ALT for methicillin-resistant staphylococci in hemodialysis, AIDS, cancer, or TPN have reported successful salvage rates of only 31% to 64% (1, 13, 18, 19, 21).

Messing et al. (24, 25), on the other hand, reported a successful salvage rate of 91% to 93% when minocycline was used as one of the antistaphylococcal antibiotics in an ALT regimen used in patients receiving TPN. Recently, we found that minocycline was significantly more effective than vancomycin or vancomycin plus heparin in reducing the colonization of *S. aureus* or *Staphylococcus epidermidis* embedded in biofilm on catheter surfaces in an in vitro model (29). Therefore, in formulating antibiotic lock therapy, it might be essential to use a nonglycopeptide antibiotic that is active against staphylococcal organisms embedded in biofilm.

Heparin is an anticoagulant often used in combination with an antibiotic to flush the lumen of the CVC to prevent thrombotic occlusions. However, heparin has been shown to have no antimicrobial activity (5, 29) and, more recently, has been shown to stimulate *S. aureus* biofilm formation (37). On the other hand, EDTA, a known chelator, has been shown in vitro and clinically to have anticoagulant activity equivalent to that of heparin (3, 28). EDTA was also shown to have limited broad-spectrum activity against methicillin-resistant staphylococci, gram-negative bacilli, and even *Candida* (12, 16, 26, 29, 34). Recently, Percival et al. (26) found that tetrasodium EDTA at a concentration of 40 mg/ml applied for 21 to 25 h reduced the biofilm colonization of *S. epidermidis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Candida albicans* on CVC segments.

The combination of minocycline and EDTA has been shown to be synergistically active in vitro in eradicating microorganisms embedded in biofilm adhering to catheter surfaces, and the M-EDTA catheter lock solution was highly efficacious in preventing catheter-related bacteremia in an animal model and colonization or CRBSI in two clinical studies involving patients with pediatric cancer and those undergoing hemodialysis, respectively (3, 7, 29, 31).

On the basis of in vitro, animal, and clinical studies, for an ALT to be effective, irrespective of whether it contains EDTA, a novel antiseptic such as taurolidine, or a therapeutic antibiotic in combination with heparin, a dwell time of at least 4 h is often required (3, 7, 17, 27, 36). This might not be feasible among patients who continuously require fluids and therapeutic agents through the catheter. In the current MRD model, exposure to minocycline alone or M-EDTA reduced the biofilm colonization of MRSA and *C. parapsilosis* after 15 min of exposure but failed to prevent regrowth. Minocycline, EDTA, and M-EDTA failed to significantly reduce the heavy biofilm colonization associated with the silicone disks in the silicone disk biofilm colonization model involving MRSA and *C. parapsilosis*. Ethanol, alone or in combination with either EDTA or minocycline, significantly reduced the biofilm colonization in the two models but failed to completely and consistently eradicate regrowth of the MRSA and *C. parapsilosis*.

Several investigators have promoted the use of ethanol as an ALT alternative. Ethanol acts rapidly and has been used in the pediatric oncology patient population and patients requiring TPN. Crnich et al. (7a) showed that ethanol does not alter the mechanical properties of silicone and polyurethane catheters. Maki et al. (22a) used ethanol to successfully prevent a CRBSI relapse in a patient with recurrent CRBSI requiring TPN. However, in our study, although ethanol alone reduced biofilm colonization of MRSA and *C. parapsilosis* in the two in vitro models, it failed to prevent regrowth. This finding as it relates to MRSA is consistent with the results of a recent animal study whereby 50% ethanol catheter lock solution failed to prevent catheter-related colonization and infection due to methicillin-resistant staphylococci in a rabbit model (34a). However, a low concentration of ethanol (25%) expedited and complemented M-EDTA in completely and rapidly eradicating MRSA and *C. parapsilosis* organisms and preventing the regrowth of these organisms after a 15- to 60-min rapid exposure to this triple combination ALT. The quantitative decrease in bacterial burden, as seen with the use of the triple combination of M-EDTA in 25% ethanol, could correlate with a better efficacy; however, this correlation should be tested in an in vivo model.

Recently, Rijnders et al. (33), in a randomized, blinded, multicenter prospective trial, treated patients with parenteral antibiotic therapy and ALT consisting of vancomycin or ceftazidime versus a placebo. Although 85 patients with CRBSI were eligible, only 46 were included. The leading causes of exclusion were the requirement for a dwell time of at least 8 h per day and the limited spectrum of the ALT, which did not include fungi and mixed gram-positive and gram-negative organisms. The study failed to show any significant difference in outcome between the ALT and placebo arms ($P = 0.1$). The rapid activity of the M-EDTA in 25% ethanol—within 15 to 60 min—and its broad-spectrum activity would allow the rapid salvage of CVCs. Because of the limited data and the high failure rate, the recent Infectious Disease Society of America guidelines for the management of intravascular catheter-related bloodstream infections did not include ALT in the treatment of catheter-related candidemia (2, 19, 23). In addition, a recent study showed that ALT failure was significantly higher

in cases of *S. aureus* CRBSI versus *S. epidermidis* CRBSI (40% versus 75%; $P = 0.04$) (27). M-EDTA in 25% ethanol solution would provide a broad-spectrum alternative that would include the treatment of CRBSI caused by common and complicated organisms such as *S. aureus* and *Candida*. Our study could be limited by having a wide dispersion of some data, as indicated by some large standard deviations, and hence, its results should be verified through future studies. Furthermore, although we observed a slimy layer formed by the microorganisms when tested by the silicone disk model, and which is usually shown by electron microscopy to be due to biofilm formation, this layer may not have been a quite mature biofilm.

In conclusion, M-EDTA in 25% ethanol prevented the regrowth of MRSA or *C. parapsilosis* upon reincubation at 37°C for 24 h. It was also found to be significantly more effective than other tested solutions, namely 25% ethanol, M-EDTA, EDTA in 25% ethanol, and minocycline in 25% ethanol, in eradicating MRSA and *C. parapsilosis*. This triple combination may be highly useful as an antimicrobial catheter lock solution and its observed efficacy in this in vitro study should be tested further through future clinical studies.

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