An Optimized Lock Solution Containing Micafungin, Ethanol and Doxycycline Inhibits Candida albicans and Mixed C. albicans – Staphyloccoccus aureus Biofilms

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Abstract

Candida albicans is a major cause of catheter-related bloodstream infections and is associated with high morbidity and mortality. Due to the propensity of C. albicans to form drug-resistant biofilms, the current standard of care includes catheter removal; however, reinsertion may be technically challenging or risky. Prolonged exposure of an antifungal lock solution within the catheter in conjunction with systemic therapy has been experimentally attempted for catheter salvage. Previously, we demonstrated excellent in vitro activity of micafungin, ethanol, and high-dose doxycycline as single agents for prevention and treatment of C. albicans biofilms. Thus, we sought to investigate optimal combinations of micafungin, ethanol, and/or doxycycline as a lock solution. We performed two- and three-drug checkerboard assays to determine the in vitro activity of pairwise or three agents in combination for prevention or treatment of C. albicans biofilms. Optimal lock solutions were tested for activity against C. albicans clinical isolates, reference strains and polymicrobial C. albicans-S. aureus biofilms. A solution containing 20% (v/v) ethanol, 0.01565 μg/mL micafungin, and 800 μg/mL doxycycline demonstrated a reduction of 98% metabolic activity and no fungal regrowth when used to prevent fungal biofilm formation; however there was no advantage over 20% ethanol alone. This solution was also successful in inhibiting the regrowth of C. albicans from mature polymicrobial biofilms, although it was not fully bactericidal. Solutions containing 5% ethanol with low concentrations of micafungin and doxycycline demonstrated synergistic activity when used to prevent monomicrobial C. albicans biofilm formation. A combined solution of micafungin, ethanol and doxycycline is highly effective for the prevention of C. albicans biofilm formation but did not demonstrate an advantage over 20% ethanol alone in these studies.
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**Introduction**

*Candida albicans* is a major source of nosocomial bloodstream infections in the United States [1] and treatment can be complicated by the presence of indwelling medical devices, particularly central venous catheters [1–4]. Persistent infection of medical devices with *Candida* spp. can occur despite treatment, leading to recurrent infections [2]. Treatment of catheter-related infection may be challenging due to the formation of biofilms, which are dense communities of hyphal and yeast cells characterized by increased resistance to host immune responses and antifungal therapy [5–7]. *Candida* biofilms are up to 1000-fold more resistant to standard azole treatment [8] and up to 20 times more resistant to echinocandin treatment compared to susceptibilities when in planktonic form [9–11]. Increased resistance within biofilms is a multifactorial process; in early stages of biofilm formation, the expression of drug efflux pumps encoded by *CDR1*, *CDR2*, and *MDR1* is increased [12] and during later stages, the extracellular matrix may prevent antifungal drugs from reaching underlying cells [13]. The extracellular matrix has three essential polysaccharide components including α-mannan, β-1,3 glucan and β-1,6 glucan [14]. Resistance in *C. albicans* may also be due to mutations in drug targets or alterations in membrane sterol composition, metabolic salvage pathways [15–16] and a reduction in metabolic activity inherent to the inner biofilm cells [17]. Due to the limited presence of nutrients and oxygen in this environment, antimicrobials that depend on cell viability are rendered ineffective. Thus, biofilm-related infections in medical devices, including those caused by *C. albicans* are often difficult to treat.

In the event of a suspected catheter-related bloodstream infection due to *Candida* species, current Infectious Diseases Society of America guidelines recommend catheter removal in most cases [18]. However, central venous catheter removal and re-insertion can be associated with a number of serious and not infrequent complications, such as bleeding, arterial puncture, hematoma, and pneumothorax [19]. An investigational alternative to catheter removal is antifungal lock therapy (AfLT). AfLT, like antibacterial lock therapy, attempts to sterilize an infected catheter or medical device through prolonged exposure to a solution containing high concentrations of antifungals in conjunction with systemic antifungal therapy. Locally instilled lock solutions may contain up to 1000 times the concentration of an antimicrobial drug than that given systemically [4], thereby increasing the potential for microbial eradication.

A number of drug combinations have been tested *in vitro* as potential antimicrobial lock solutions, using the checkerboard technique. The checkerboard assay is designed to test the activity of multiple drugs in combination and has been a successful approach to determining *in vitro* combination efficacy, including evaluation of echinocandin-resistant *Candida* strains [20–21]. The checkerboard technique has uncovered several combinations that demonstrate synergistic activity, which is defined as an increase in the antimicrobial activity produced by a combination of drugs which is greater than the sum activity of each single drug alone [21]. Some drug combinations previously tested and which demonstrated synergistic activity include fluconazole combined with finasteride [22], doxycycline [23], terpenes [24], and others. However, a number of antifungal agents that may be used in a lock solution have shortcomings. For instance, resistance to fluconazole has been widely documented in biofilms and certain non- *albicans* *Candida* species [25–27] and higher concentrations of ethanol may compromise the integrity of polyurethane catheters (see discussion) [28].

Micafungin (MICA) is a potent fungicidal echinocandin with activity against *Candida* spp., *Aspergillus* and some other pathogenic molds [29]. Echinocandins are a class of antifungal drug that target cell wall synthesis by inhibiting 1,3-β glucan synthase [29]. Ethanol (EtOH) is an inexpensive sterilizing agent [30] with the advantage of reduced potential for resistance through its non-specific activity of protein denaturation [31]. Similarly, doxycycline (DOX) is
a widely used broad-spectrum antibiotic [32] and has demonstrated in vitro antifungal activity in combination with fluconazole [23]. Tetracycline exerts antifungal activity though ribosomal inhibition in mitochondria, which are similar in structure to the bacterial 30S ribosomal subunit. Concentrations required for antifungal activity are much higher than used for gene induction [33]. As a component of an antifungal lock solution, DOX could potentially broaden the utility of the solution to include antibacterial activity, particularly against medically important bacteria such as *Staphylococcus aureus*. DOX has demonstrated both enhanced and antagonistic activity with the antifungal amphotericin B [34–35], suggesting that DOX may be useful in combination with other antifungals, although an antagonistic interaction is also theoretically possible.

*S. aureus* is a leading contributor to nosocomial bloodstream infections [1] with strong biofilm forming capabilities. One important review estimated that 27% of nosocomial *Candida* bloodstream infections were polymicrobial, and *S. aureus* was the third most common species co-isolated with *C. albicans* [36]. This review also reported a 36–57% incidence of candidemia-bacteremia co-infection, when the isolation of bacteria occurred 48 hours before or after *Candida* spp. isolation. *Staphylococcus* species represented 20% of these dual infections. An in vivo animal study demonstrated a synergistic increase in virulence when *S. aureus* and *C. albicans* were present together [37]. Specifically, a combination of 1/4 the lethal dose (50%) of *C. albicans* and 1/8 the lethal dose (50%) of *S. aureus* was enough to cause 100% mortality in a murine model. Additionally, *C. albicans* may confer vancomycin resistance to *S. aureus* via excretion of extracellular matrix [38]. The presence of a polymicrobial biofilm may complicate treatment by augmenting resistance to monomicrobial therapies; thus a lock solution with both antifungal and antibacterial activity for prevention and treatment may be advantageous.

In this study, we sought to determine the optimal combination of MICA, DOX and/or EtOH for use as an antifungal lock solution for the prevention and treatment of *C. albicans* biofilms in vitro. We further tested optimized solutions against *C. albicans*--*S. aureus* mixed biofilms. MICA, EtOH, and DOX were chosen because they have been shown to have strong antifungal activity against *C. albicans* and *S. aureus* [35, 39–42] and are clinically widely available. Our objective was to determine whether the potent antifungal activity of MICA combined with the activity of DOX would allow us to minimize the concentration of EtOH, thereby reducing the potential risk to the integrity of polyurethane catheters [30]. To establish an optimized antifungal lock solution, we performed two drug (2D) and three drug (3D) checkerboard assays as well as fungal colony regrowth assays. Optimized antifungal solutions were then tested for activity against additional *C. albicans* laboratory reference strains, two clinically-derived echinocandin resistant isolates as well as forming and mature *C. albicans*--*S. aureus* mixed biofilms. We demonstrate that the combination of 20% EtOH, 0.01565 μg/mL MICA and 800 μg/mL DOX does not improve the efficacy of the lock solution compared to ethanol alone at preventing the regrowth of forming and mature *C. albicans* monomicrobial biofilms in a silicone disk model at microbiologically relevant concentrations. Synergistic activity was only detected in combination with much lower concentrations of ethanol.

**Materials and Methods**

**Microbial strains and growth conditions**

*C. albicans* reference strains ATCC10231, ATCC14053 (fluconazole resistant) and ATCC 24433 were purchased from ATCC (Manassas, VA). Clinically derived echinocandin resistant isolates 42379 and 53264 [43] were also studied in addition to the standard laboratory strain SC5314 [44]. All strains were grown and maintained at 30°C in yeast peptone dextrose (YPD, 1% yeast extract, 2% peptone, and 2% glucose, Becton, Dickinson and Company, Sparks, MD).
Overnight cultures used in biofilm treatment, prevention and regrowth studies were re-suspended to a final density of 1.0×10^6 cells/mL in RPMI-1640 media (Media Tech, Inc, Corning, NY). Concentrated (4X) RPMI media (20.8g RPMI-1640 with L-glutamine and 69g MOPS in 500mL H2O) was buffered to pH 7.0 with NaOH and stored at 4°C in a light-impermeable container. MICA was kindly provided by Astellas Pharma (Northbrook, IL); EtOH (100%) and DOX were purchased from Sigma-Aldrich (St. Louis, MO).

*S. aureus* strain M2 was used for all polymicrobial biofilm studies [45]. *S. aureus* was maintained as a glycerol stock at -80°C, streaked onto trypticase soy agar (TSA), and incubated at 37°C prior to use. A single colony was inoculated into 5 mL of trypticase soy broth (TSB) and incubated overnight at 37°C with shaking at 200 rpm. The following day, *S. aureus* was sub-cultured at a 1:100 dilution in fresh TSB and incubated at 37°C with shaking for three hours until the cells reached the early log phase of growth. Following growth, microbes were washed in phosphate buffered saline (PBS), counted on a hemocytometer, and adjusted to 1×10^7 CFU/mL in 0.6X TSB containing 0.2% glucose (TSB-g). TSB-g was previously determined to be an optimal medium for supporting biofilm growth of both *S. aureus* and *C. albicans* [46–47].

**Antifungal activity assays**

**Preparation of drug solutions.** All drug stock solutions were prepared in sterile ultrapure water using 1:2 serial dilutions, in accordance with Clinical Laboratory and Standards Institute guidelines [48]. Each drug was prepared as a concentrated solution and then diluted in the wells of a 96-well plate by the addition of media, cells and other drug(s) to complete each antifungal lock solution. For use in 2D checkerboards, ten stock solutions (1:2 dilutions) of MICA were prepared at concentrations four times that of the final concentration. The highest concentration of MICA tested was 0.0313 μg/mL. DOX and EtOH stock solutions were prepared at twice the final concentration, which ranged from 1600 μg/mL to 3.125 μg/mL, and 40% to 0.625% (v/v), respectively. Each well was brought to a final volume of 200 μL. When DOX was used in combination with EtOH, working stocks of EtOH were prepared as described above, while DOX was prepared at four times its final concentration. Final DOX concentrations tested ranged from 800 μg/mL to 1.625 μg/mL.

All drug stocks used in 3D checkerboards were made at four times the final concentration tested and a single concentration of EtOH (5, 10, or 20%) was applied to every well in a single plate. The highest concentration of MICA tested was 0.0313 μg/mL with a total of twelve different concentrations utilized. DOX was used at eight different concentrations beginning with 800 μg/mL.

**2D checkerboard assays.** An overnight culture of SC5314 was washed twice with PBS and re-suspended to a density of 4.0×10^6 cells/mL in 4X RPMI. Media containing cells were added to a 96-well, flat bottomed, polystyrene micro dilution plate (Costar 3595, Corning Inc. Corning, NY) and diluted four-fold in the plate with single-drug stock solutions to create unique combination AfLT solutions. The plate was then incubated for 24 hours at 37°C. Each concentration of MICA was added to a column (1–10) while either DOX or EtOH were added to the plate in rows (1–7), producing a different combination of drug concentrations in each well. One column in the plate was reserved for positive (cells with media) and negative (media-only) control wells. Another column was reserved as a DOX or EtOH only control (single drug activity against *C. albicans* cells). One row was reserved as a MICA or DOX only control. Controls were diluted to the final concentration with water.

The antifungal activity of each solution against mature biofilms was then examined [49]. First, 4×10^6 cells/mL in 4X RPMI were diluted to a final concentration of 1×10^6 cells/mL in RPMI with sterile water, and allowed to incubate at 37°C for 24 hours. Mature biofilms were
then washed three times with PBS before media and single-drug solutions were applied as described above. The plate was then incubated for an additional 24 hours before metabolic activity was assayed. Each 2D checkerboard (prevention and treatment) was performed independently three times.

**3D checkerboard assays.** The arrangement of the 3D checkerboard assay was similar to that of the 2D. A separate control plate was necessary for calculating the activity of the three drugs in combination. The control plate included positive (cells with RPMI), negative (RPMI only), and single drug control wells. Every drug concentration tested in the checkerboard had three technical replicates performed to establish the activity of each drug concentration alone. Incubation conditions, times, and washes were consistent between 2D and 3D checkerboards. Each assay was repeated three times independently.

**XTT reduction assay.** The antifungal activity of MICA+DOX, MICA+EtOH and DOX+EtOH in the prevention and treatment of biofilm formation was assessed using the colorimetric XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] reduction assay as previously described [49]. Briefly, following incubation with an antifungal lock combination solution, each plate was washed three times with 200 μL PBS. Menadione was added to XTT solution and 100 μL of the resulting mixture was added to each well. The plate was then wrapped in aluminum foil and incubated for an hour and a half to two hours at 37°C. Absorbance was read in a plate reader at 490nm (BioTek model ELx808, Winooski, VT).

**Antifungal activity of combination solutions against C. albicans isolates.** Strains SC5314, ATCC10231, ATCC14053, ATCC24433, 42379, and 53264 [50–51] were resuspended to a concentration of 4×10⁶ cells/mL in 4X RPMI. Two optimized solutions were tested: ("OS 1") 20% EtOH, 0.01565 μg/mL MICA, and 800 μg/mL DOX, and ("OS 2") 10% EtOH, 0.0313 μg/mL MICA, and 800 μg/mL DOX. Each strain was added to wells 1–12 of a designated row in two 96-well plates. One row was reserved for a negative control. Then, 50 μL of four-fold concentrated MICA, DOX and EtOH were added to the first three columns of each plate to generate OS 1 and OS 2. The remainder of each plate was used for single drug and positive/negative controls. Each strain was challenged with each drug alone, including 0.0313 μg/mL MICA, 0.01565 μg/mL MICA, 20% EtOH, 10% EtOH, and 800 μg/mL DOX. Metabolic activity was calculated using the XTT reduction assay as described. Each prevention and treatment assay contained three technical replicates and was repeated three times independently.

**C. albicans metabolic activity in a silicone disk model.** Several solutions were tested for activity as a prevention or treatment of SC5314 biofilms on silicone disks. These solutions included 20% EtOH with 800 μg/mL DOX and either 0.0313 or 0.01565 μg/mL MICA, solutions with 10% EtOH, 800 μg/mL DOX and either 0.0313 or 0.01565 μg/mL MICA and a solution of 10% EtOH with 200 μg/mL DOX and 0.0313 μg/mL MICA. Finally, a solution of 10% EtOH with the lowest MICA and DOX concentrations tested in the 3D checkerboards was also used. Drug solutions were made using 1:2 dilutions as described for the 2D checkerboard assay. Medical-grade silicone (Invotec, Jacksonville, FL) was washed with mild detergent then rinsed with deionized water, cut into disks that fit the well diameter, autoclaved, then aseptically inserted into the bottom of each well. The plate was then UV sterilized for 15–20 minutes before the addition of cells, media and drug solutions.

**C. albicans colony regrowth assay.** Promising solutions which decreased metabolic activity by ≥ 98% in the 3D checkerboard assay were chosen in addition to positive and negative controls to study C. albicans reference strain SC5314 regrowth from silicone disks [40]. These solutions were: 20% EtOH with 800 μg/mL DOX and either 0.0313 or 0.01565 μg/mL MICA, 10% EtOH with 800 μg/mL DOX and 0.0313 or 0.01565 μg/mL MICA, 20% EtOH alone, 10% EtOH alone, and 10% EtOH with 0.0313 μg/mL MICA and 200 μg/mL DOX. Each solution was made as in the 3D checkerboard assay with the addition of a sterilized, medical-grade
silicone disk inserted into each well. After 24-hour incubation with a drug combination, disks were washed three times with PBS and then added to 5 mL of Sabouraud’s dextrose broth. The disk was then sonicated for three minutes and incubated at 30°C for 24 hours with shaking at 250 rpm. Absorbance was measured at 600 nm and then 100 μL of each culture was plated to Sabouraud’s dextrose agar. Plates were incubated at 30°C for an additional 24 hours. Highly turbid cultures, including the positive controls and mature biofilms treated with 10% EtOH alone and in every combination, were plated as 1:400 dilutions to obtain an accurate CFU count. The number of observed CFU was then multiplied by the dilution factor to obtain the actual CFU. The colony regrowth assays on silicone disks were performed twice independently.

**Treatment of mature polymicrobial biofilm with an optimized combination solution.** Mono- and polymicrobial biofilms were grown as previously described with minor modifications [40, 42]. Briefly, wells of a 96-well plate were fitted with sterilized silicone discs. Wells harboring monomicrobial biofilms were inoculated with 100 μL of 1×10^6 cells/mL of either C. albicans or S. aureus in TSB-g. TO this, 100 μL of sterile TSB-g was added for a total volume of 200 μL per well. For wells harboring polymicrobial biofilms, 100 μL of C. albicans and S. aureus adjusted cell suspensions in TSB-g were added per well. Control disks were inoculated with 200 μL of sterile TSB-g. Plates were incubated at 37°C for 24 hours to allow for mature biofilm development.

**XTT reduction assay of mature polymicrobial biofilms.** Following mature monomicrobial and polymicrobial biofilm development, silicone disks were washed three times with 200 μL sterile PBS to remove non-adherent cells. One group of disks was inoculated with TSB-g, while the other was challenged with TSB-g containing 20% EtOH, 0.01565 μg/mL MICA, and 800 μg/mL DOX (OS 1). Plates were returned to a 37°C incubator for 24 hours. The following day, disks were washed three times with 200 μL sterile PBS and incubated with 200 μL of XTT working reagent for two hours at 37°C protected from light. Absorbance was read in a plate reader at 490 nm (VersaMax, Molecular Devices, Sunnyvale, CA).

**Polymicrobial colony regrowth assay.** Regrowth assays were performed as described previously [40,42]. Biofilms were grown on silicone disks as described above. Disks were challenged with either TSB-g (control) or OS 1 and incubated for 24 hours at 37°C. After washing three times with 200 μL PBS, disks were transferred to sterile 50-ml conical tubes containing 5 ml of YPD supplemented with 2 μg/mL vancomycin (VAN, for disks harboring C. albicans biofilms) or TSB containing 2.5 μg/mL amphotericin B (AMB, for disks harboring S. aureus biofilms). In order to obtain both C. albicans and S. aureus counts individually, polymicrobial biofilms were grown in parallel, so that one biofilm disk was regrown in YPD containing VAN and the other was regrown in TSB containing AMB. This step was necessary to inhibit the growth of either organism in the nonselective medium because overgrowth of the non-targeted microbe may have confounded the results. Samples were sonicated, and the tubes were returned to 30°C (C. albicans) or 37°C (S. aureus) and incubated with shaking at 200 rpm for 20 hours. After incubation, the CFUs were enumerated on selective agar media as described above; the lower and upper limits of detection were approximately 10^2 CFU/mL and 10^8 CFU/mL, respectively.

**Calculations of fractional inhibitory concentrations**

**2D checkerboards.** Fractional inhibitory concentrations (FICs) were calculated for each combination of drugs: MICA+EtOH, MICA+DOX, DOX+EtOH. The FIC value was calculated by dividing the MIC90 or MIC50 (the minimum inhibitory concentration of each drug which produces ≤ 10 or ≤ 50% metabolic activity, respectively) of one drug when used in combination by the MIC90 or MIC50 of the same drug when used alone, FIC of drug A = [Drug A_combination]/ [Drug A_alone] [52]. Both MIC90 and MIC50 were calculated for each two-drug combination. The
summation of each FIC value, $FICI = FIC_{A} + FIC_{B}$, determined whether the combination of drugs produced a synergistic, indifferent or antagonistic effect. The combination was considered synergistic when the $FICI$ was $\leq 0.5$, indifferent when the $FICI$ was $> 0.5$ to $\leq 4$, and antagonistic when the $FICI$ was $> 4$ [21]. When the highest concentration of drug tested did not produce a 90% or 50% decrease in metabolic activity, the highest concentration of drug tested was used in place of the MIC90 or MIC50 to calculate FICI according to published methods [21, 52].

3D checkerboards. FICI calculations for three drugs in combination were calculated using the equation above with the addition of a third FIC term ($FICI = FIC_{A} + FIC_{B} + FIC_{C}$) and the expansion of the definition of synergy up to $FICI \leq 0.75$ [53]. A FICI value in the $> 0.75$ to $\leq 4$ range was considered indifferent and a FICI of $> 4$ was considered antagonistic.

Statistical analyses

The metabolic activities of forming and mature biofilms treated with optimized solution candidates were compared to the controls using two-way multiple comparisons analysis of variance (ANOVA) followed by a Tukey comparison post-test. One-way ANOVA was used to analyze monomicrobial colony regrowth from silicone disks. A Kruskal-Wallis one-way ANOVA and Dunn’s multiple comparisons post-test was used to analyze polymicrobial colony regrowth. Differences between groups were considered significant at a $P$ value of $< 0.05$. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA).

Results

2D checkerboard assays

An indifferent effect was found in all two-drug combinations for both prevention and treatment of $C. albicans$ monomicrobial biofilms when MIC90 (Table 1) and MIC50 (Table 2) criteria were utilized. Generally, all FICI values were approximately equal to one, except the combination of 5% (v/v) EtOH and 1.5626 μg/mL DOX, which had a FICI value approaching, but not meeting, the criteria for synergy (Table 1).

3D checkerboard assays. The combination of 2.5% EtOH, 0.0000153 μg/mL MICA and 12.5 μg/mL DOX demonstrated synergistic activity when used to prevent $C. albicans$ biofilm formation (Table 3). An indifferent effect was observed for all other three-drug combinations tested (Tables 3 and 4) for prevention and treatment of biofilm formation regardless of which MIC criterion was used (i.e. MIC50 or MIC90).

*C. albicans* metabolic activity measured by XTT reduction in a silicone disk model.

Solutions with 20% EtOH, 800 μg/mL DOX and either 0.0313 or 0.01565 μg/mL MICA (MIC90 and MIC50 of MICA, respectively) as well as solutions with 10% EtOH, 800 μg/mL DOX and either 0.0313 or 0.01565 μg/mL MICA all reduced metabolic activity to less than five percent when used to prevent biofilm formation (Fig 1), except the combination of 10% EtOH with 0.01565 μg/mL MICA and 800 μg/mL DOX, which produced more than ten percent metabolic activity. When the combination solutions were tested against mature biofilms, they demonstrated a greater reduction in metabolic activity compared to MICA and DOX alone, but an equal reduction in metabolic activity compared to 20% EtOH alone (Fig 2).

*C. albicans* colony regrowth in a silicone disk model. The four combination solutions tested, as well as 20% and 10% EtOH alone were equally successful at preventing all $C. albicans$ regrowth (Fig 3). Solutions containing 20% EtOH, either alone or in combination, prevented the regrowth of cells from mature biofilms (Fig 4). However, solutions containing 10% EtOH alone or in combination did not consistently eliminate fungal regrowth from mature biofilms.
Metabolic activity of optimized lock solutions against multiple \textit{C. albicans} strains. Two solutions selected from the preceding 3D checkerboard assays were further tested against all six strains (SC5314, ATCC10231, ATCC14053, ATCC24433, 42379, and 53264): 20\% EtOH with 0.01565 \( \mu \)g/mL MICA and 800 \( \mu \)g/mL DOX (designated as “OS 1”), and 10\% EtOH with 0.0313 \( \mu \)g/mL and 800 \( \mu \)g/mL DOX (designated as “OS 2”), (Prevention, Fig 5A; Treatment, Fig 5B). Across all strains, 20\% EtOH alone was the most effective in preventing and treating mature biofilms, producing \(< 1\% \) metabolic activity for prevention and \(< 5\% \) metabolic activity for treatment. Similarly, 10\% EtOH alone was also highly effective, producing \(< 2\% \) metabolic activity.

### Table 1. Antifungal activity of two-drug combinations against forming and mature \textit{C. albicans} biofilms, sMIC90.

| Biofilm   | Agent\(^a\) | sMIC90\(^b\) of Each Drug | FICI\(^c\) | Outcome   |
|-----------|-------------|----------------------------|------------|-----------|
|           | Alone       | Combination                |            |           |
| Prevention| MICA        | 0.01565 \( \mu \)g/mL     | 0.01565 \( \mu \)g/mL  | 1.031     | Indifference |
|           | ETOH (v/v)  | 10\%                       | 0.3125\%    |           |
|           | MICA        | 0.01565 \( \mu \)g/mL     | 0.01565 \( \mu \)g/mL  | 1.004     | Indifference |
|           | DOX         | 400 \( \mu \)g/mL         | 12.5 \( \mu \)g/mL    |           |
|           | ETOH (v/v)  | 10\%                       | 5\%          | 0.502     | Indifference |
|           | DOX         | 800 \( \mu \)g/mL         | 1.5625 \( \mu \)g/mL |           |
| Treatment | MICA        | 0.0313 \( \mu \)g/mL      | 0.0313 \( \mu \)g/mL  | 1.031     | Indifference |
|           | ETOH (v/v)  | 20\%                       | 0.3125\%    |           |
|           | MICA        | 0.0313 \( \mu \)g/mL      | 0.0313 \( \mu \)g/mL  | 1.016     | Indifference |
|           | DOX         | 800 \( \mu \)g/mL         | 12.5 \( \mu \)g/mL    |           |
|           | ETOH (v/v)  | 20\%                       | 20\%         | 1.002     | Indifference |
|           | DOX         | 800 \( \mu \)g/mL         | mL           |           |

\( a \). micafungin (MICA); ethanol (ETOH); doxycycline (DOX)

\( b \). Sessile minimum inhibitory concentration (sMIC90) is the concentration of each drug alone or in combination which inhibited 90\% of metabolic activity.

\( c \). FICI \(< 0.5 = \) synergy, 4 \( > \) FICI \( > 0.5 = \) indifference, FICI \( > 4 = \) antagonism

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### Table 2. Antifungal activity of two-drug combinations against forming and mature \textit{C. albicans} biofilms, sMIC50.

| Biofilm   | Agent\(^a\) | sMIC50\(^b\) of Each Drug | FICI\(^c\) | Outcome   |
|-----------|-------------|----------------------------|------------|-----------|
|           | Alone       | Combination                |            |           |
| Prevention| MICA        | 0.01565 \( \mu \)g/mL     | 0.01565 \( \mu \)g/mL  | 1.031     | Indifference |
|           | ETOH (v/v)  | 10\%                       | 0.3125\%    |           |
|           | MICA        | 0.01565 \( \mu \)g/mL     | 6.11\times10^{-5} \( \mu \)g/mL  | 1.004     | Indifference |
|           | DOX         | 400 \( \mu \)g/mL         | 400 \( \mu \)g/mL    |           |
|           | ETOH (v/v)  | 5\%                        | 0.625\%      | 0.513     | Indifference |
|           | DOX         | 800 \( \mu \)g/mL         | mL           |           |
| Treatment | MICA        | 0.0313 \( \mu \)g/mL      | 2.45\times10^{-4} \( \mu \)g/mL  | 1.008     | Indifference |
|           | ETOH (v/v)  | 10\%                       | 10\%         |           |
|           | MICA        | 0.0313 \( \mu \)g/mL      | 0.0313 \( \mu \)g/mL  | 1.016     | Indifference |
|           | DOX         | 800 \( \mu \)g/mL         | 12.5 \( \mu \)g/mL    |           |
|           | ETOH (v/v)  | 7\%                        | 7\%          | 1.002     | Indifference |
|           | DOX         | 800 \( \mu \)g/mL         | mL           |           |

\( a \). micafungin (MICA); ethanol (ETOH); doxycycline (DOX)

\( b \). Sessile minimum inhibitory concentration (sMIC50) is the concentration of each drug alone or in combination which inhibited 50\% of metabolic activity.

\( c \). FICI \(< 0.5 = \) synergy, 4 \( > \) FICI \( > 0.5 = \) indifference, FICI \( > 4 = \) antagonism

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metabolic activity for prevention of biofilm formation and < 50% metabolic activity for treatment of mature biofilms. Using a Tukey comparison test, we observed a statistically significant decrease in metabolic activity between the no-drug control and both optimized solutions, across all strains for both prevention and treatment. No statistically significant difference in metabolic activity was found between 20% and 10% EtOH treatment groups when used alone or in combination (OS 1 vs. OS 2) to prevent biofilm formation. When used to treat a mature biofilm, a statistically significant difference was observed between OS 1 compared to OS 2, 20% EtOH compared to 10% EtOH, OS 1 compared to 20% EtOH, and OS 2 compared to 20% EtOH for strains 24433 and 53264. SC5314 was similar in that there was a statistically significant difference between the 20% and 10% EtOH alone, OS 1 and 20% EtOH, and OS 2 and 10% EtOH solutions. The optimized lock solutions were not more effective than 20% ethanol alone. This may be due to the increased activity of 20% vs 10% EtOH, and it is worth noting that there was no statistically significant difference between these solutions in the other three strains tested.

**Metabolic activity of mature polymicrobial biofilms.** Incubation of mature polymicrobial (C. albicans + S. aureus) biofilms with OS 1 reduced the metabolic activity to less than 10% observed in control biofilms incubated with sterile media (Fig 6). There were no significant differences between metabolic activities of monomicrobial versus polymicrobial biofilms. Thus, OS 1 is highly effective at inhibiting proliferation of mature S. aureus and C. albicans mixed biofilms.

### Table 3. Antifungal activity of three-drug combinations against forming and mature C. albicans biofilms, sMIC50.

| Biofilm  | Agenta | ssMIC50b of Each Drug | FICIc | Outcome        |
|----------|---------|-----------------------|-------|----------------|
|          |         | Alone                 | Combination |                  |
| Prevention | MICA    | 0.0313 μg/mL          | 1.53×10^{-5} μg/mL | 0.516 | Synergy        |
|          | EtOH    | 5%                    | 2.5%          |                 |
|          | DOX     | 800 μg/mL             | 12.5 μg/mL    |                 |
| Treatment | MICA    | 0.0313 μg/mL          | 1.53×10^{-5} μg/mL | 1.008 | Indifference  |
|          | EtOH    | 20%                   | 10%           |                 |
|          | DOX     | 800 μg/mL             | g/mL          |                 |

a. micafungin (MICA); ethanol (EtOH); doxycycline (DOX)
b. Sessile minimum inhibitory concentration (ssMIC50) is the concentration of drug alone or in combination which inhibited 50% of metabolic activity.
c. FICI ≤ 0.75 = synergy, 4 ≥ FICI > 0.75 = indifference, FICI > 4 = antagonism

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### Table 4. Antifungal activity of three-drug combinations against forming and mature C. albicans biofilms, sMIC90.

| Biofilm  | Agenta | ssMIC90b of Each Drug | FICIc | Outcome        |
|----------|---------|-----------------------|-------|----------------|
|          |         | Alone                 | Combination |                  |
| Prevention | MICA    | 0.0313 μg/mL          | 6.11×10^{-5} μg/mL | 0.752 | Indifference  |
|          | EtOH    | 10%                   | 2.5%          |                 |
|          | DOX     | 800 μg/mL             | 400 μg/mL     |                 |
| Treatment | MICA    | 0.0313 μg/mL          | 1.5×10^{-5} μg/mL | 1.008 | Indifference  |
|          | EtOH    | 20%                   | 20%           |                 |
|          | DOX     | 800 μg/mL             | g/mL          |                 |

a. micafungin (MICA); ethanol (EtOH); doxycycline (DOX)
b. Sessile minimum inhibitory concentration (ssMIC90) is the concentration of drug alone or in combination which inhibited 90% of metabolic activity.
c. FICI ≤ 0.75 = synergy, 4 ≥ FICI > 0.75 = indifference, FICI > 4 = antagonism

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Polymicrobial regrowth in a silicone disk model. Optimized solution 1 (OS1) exhibited fungicidal activity by preventing regrowth of *C. albicans* in both monomicrobial and polymicrobial biofilms (Fig 7, CA, open circles) as compared to drug-free controls (Fig 7, CA, closed circles). Interestingly, despite significant reduction of metabolic activity observed in the XTT assay, regrowth of *S. aureus* was not completely inhibited in either monomicrobial or polymicrobial biofilms (Fig 7, SA, open circles). Staphylococcal regrowth potential was greater in polymicrobial biofilms (five of the eight disks) compared to monomicrobial biofilms (three of the eight disks); however, this difference was not statistically significant.

Discussion

In this study, we demonstrate a combination solution that is highly effective against forming and mature *C. albicans* biofilms comprised of 20% EtOH, 0.01565 μg/mL MICA and 800 μg/mL DOX (OS 1), but was not more effective than 20% EtOH alone. This solution reduced the metabolic activity of forming biofilms to ≤ 2%, and prevented fungal regrowth from both forming and mature biofilms. These results suggest that OS 1 has potential for antifungal lock therapy and has a low ethanol concentration, therefore reducing the concern of structural...
However, 20% EtOH alone produced the greatest reduction in clinical isolate and reference strain metabolic activity, both when used for prevention of biofilm formation and for treatment of mature biofilms. Additionally, OS 1 was effective in eliminating *C. albicans* from *C. albicans-* *S. aureus* polymicrobial biofilms and reduced *S. aureus* metabolic activity to <10%, although the solution did not demonstrate complete bactericidal activity. This is unsurprising given that the activity of DOX is dependent on cell viability and cells within the biofilm are metabolically inert.

Each component of the lock solution was carefully selected. Preliminary experiments in our laboratory demonstrated a ≥ 50% reduction in metabolic activity of sessile SC5314 when 0.0313 μg/mL MICA is used (see S1 File, MICA only experiments), which is consistent with prior studies in the literature. Studies which measured the susceptibility of invasive clinical isolates in planktonic form have reported MIC$_{90}$s of ≤ 0.03 μg/mL [54–55] and MIC$_{50}$s of ≤ 0.015 for MICA [55–56]. A number of other studies have reported similar MICs [29, 57–59]. AflT employs supra-therapeutic concentrations of antifungals [4] and a concentration of 5 μg/mL has demonstrated success in an *in vitro* lock model [39]. However, the potent fungicidal activity of MICA, particularly when used in combination with EtOH, did not warrant a greater dose. Concentrations of EtOH and DOX were chosen for 3D checkerboard assays based on established single-drug activity against *C. albicans* [35, 40] and the 2D checkerboard assay.
assays. Furthermore, the activity of two and three drugs in combinations was investigated for synergistic activity by decreasing concentrations of each component. Synergistic activity was found with low dose MICA in combination with low doses of EtOH and DOX (Table 3), although such a combination would be ineffective as a lock solution.

The synergistic activity we observed with low concentrations of MICA, DOX and EtOH may be due to the combined activity of EtOH and DOX only, which together produced a FICI close to synergy (0.502, Table 1). With the addition of a third drug the FICI definition of synergy increases to include values ≤ 0.75. Given the extremely low concentration of MICA in the solution that produced synergy, we may be observing only the indifferent activity of EtOH and DOX combined. However, in the 2D checkerboards, a four-fold decreased concentration of EtOH is required to produce an MIC50 in combination compared to the MIC50 of EtOH alone (FIC\text{EtOH} = 0.0125). When all three drugs are used in combination, there is a six-fold decrease in the amount of DOX required for 50% metabolic activity inhibition when used in a three-drug combination compared to the MIC50 of DOX alone (FIC\text{DOX} = 0.0156). The concentration of the second drug (either EtOH or DOX) in the 2D and 3D checkerboards is halved when

Fig 5. Prevention and treatment of clinical isolate and reference strain biofilm formation with optimized solutions. Fig 5a. Optimized solutions (OS 1, 20% ethanol, 0.015625 μg/mL micafungin, 800 μg/mL doxycycline, and OS 2, 10% ethanol with 0.0313 μg/mL micafungin and 800 μg/mL doxycycline) were highly effective in preventing the formation of echinocandin-resistant clinical isolate and reference strain biofilms. Optimized solutions were incubated with C. albicans isolates for 24 hours at 37°C to prevent biofilm development and untreated positive controls were compared to drug treated groups with the same strain. Metabolic activity was measured with XTT reduction. * indicates p value of < 0.05. **0.0313 μg/mL was not significant compared to the control for strain 53264 only. Fig 5b. Optimized solutions (OS 1, 20% ethanol, 0.015625 μg/mL micafungin, 800 μg/mL doxycycline, and OS 2, 10% ethanol with 0.0313 μg/mL micafungin and 800 μg/mL doxycycline) significantly reduce the metabolic activity of echinocandin-resistant clinical isolates and reference strains. Optimized solutions were incubated with mature C. albicans biofilms for 24 hours at 37°C. Metabolic activity was measured with XTT reduction and untreated positive controls were compared to drug treated groups with the same strain. * indicates p value of < 0.05.
Fig 6. Treatment of mature *C. albicans* and *S. aureus* polymicrobial biofilms on silicone disks with OS 1. Monomicrobial (CA, SA) and polymicrobial (CA+SA) biofilms of *C. albicans* and *S. aureus* were challenged with drug free (DF) or optimized solution (OS 1, 20% ethanol, 800 μg/mL doxycycline, and 0.015625 μg/mL micafungin) prepared in sterile growth medium for 24 hours. Metabolic activity was assessed using the XTT assay and reported as % inhibition of the drug-free control. Error bars represent SDs. Data is cumulative of 4 independent experiments performed in triplicate. ***, P ≤ 0.001, n.s., not significant using a one-way ANOVA and Tukey’s multiple comparisons post-test.

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Fig 7. Polymicrobial regrowth after treatment with OS 1. Regrowth of mono- and polymicrobial biofilms on silicone disks after treatment with optimized lock solution. Monomicrobial (CA, SA) and polymicrobial (CA + SA) biofilms of *C. albicans* and *S. aureus* were challenged with drug free (DF, closed circles) or optimized solution (OS 1, 20% ethanol, 800 μg/mL doxycycline, and 0.015625 μg/mL micafungin, open circles) prepared in sterile growth medium for 24 hours. Immediately following lock solution treatment, disks were briefly sonicated and cultured in selective liquid medium for 24 hours. Serial dilutions were plated onto selective medium to quantify microbial regrowth. Horizontal line in dataset represents the median. Data is cumulative of four independent experiments performed in triplicate. *, P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001 using a Kruskal-Wallis one-way ANOVA and Dunn’s multiple comparisons post-test.

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used in combination vs. alone. The decrease between FIC terms in two and three drug combinations suggests the activity is synergistic.

We chose a three-drug approach to AFLT to maximize the broad-spectrum activity of the solution to include yeasts and bacteria, to reduce the theoretical concern for development of resistance, and to minimize the ethanol concentration used. Even in the event that a catheter infection is resistant to one component of a lock solution, in principle, the other drugs should be effective, although this has yet to be tested. AFLT is not without risks and the selection of antimicrobial components should be carefully made. Concerns surrounding the use of a lock solution include the compatibility and stability of the selected agents as well as the effect on catheter integrity [60].

Limited investigation has been performed on the effect of high concentrations of ethanol to central venous catheters, including polyurethane and silicone. One study reported that 70% EtOH had no significant effect on the clinical performance of either catheter type after up to ten weeks of exposure [61]. In another study, silicone catheters exposed to 95% EtOH for 15 days showed no lumen degradation as assessed by scanning electron microscopy [62]. A recent review identified reports describing the elution of 1,4-butanediol from polyurethane catheters and minor structural alterations to carbothane catheters after prolonged exposure to concentrations of ethanol greater than 60% or 30%, respectively [28]. A statistically significant decrease in force at break and elongation at break were observed for carbothane catheters exposed to 30% EtOH for nine weeks. However, the change in force and elongation at break was not significant enough to prevent clinical utility [63]. While not explicitly tested in catheter integrity studies, the concentration of ethanol required for *C. albicans* sterilization in this study are well below the concentrations implicated in catheter damage, suggesting that the EtOH component of our proposed solution is less likely to affect catheter integrity.

These data demonstrate a highly effective optimized solution (20% EtOH, 0.01565 μg/mL MICA, 800 μg/mL DOX, OS 1), which prevents the formation of *C. albicans* biofilms, but was not more effective than 20% EtOH alone. Our study is limited by the static nature of biofilm prevention and treatment assays and the lack of physiological conditions in this *in vitro* model. The activity of EtOH, MICA and DOX in combination against polymicrobial biofilms is the subject of ongoing investigation. Further investigation of the clinical utility of other combination solutions is warranted.

**Supporting Information**

**S1 File.** Biofilm metabolic activity data for 2D and 3D checkerboard experiments, used to generate values given in Tables 1–4.

(XLSX)

**S2 File.** Biofilm metabolic activity and CFU data, summarized graphically in Figs 1–7.

(XLSX)

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**Author Contributions**

Conceived and designed the experiments: LL BMP SAL. Performed the experiments: LL BMP. Analyzed the data: LL BMP. Contributed reagents/materials/analysis tools: MCN SAL. Wrote the paper: LL BMP CW MCN SAL.
References

1. Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin Infect Dis Off Publ Infect Dis Soc Am. 2004 Aug 1; 39(3):309–17. PMID: 15084500

2. Kojic EM, Darouiche RO. Candida infections of medical devices. Clin Microbiol Rev. 2004 Apr; 17(2):255–67. PMID: 15084500

3. Ramage G, Saville SP, Thomas DP, López-Ribot JL. Candida biofilms: an update. Eukaryot Cell. 2005 Apr; 4(4):633–8. PMID: 15821123

4. Walraven CJ, Lee SA. Antifungal lock therapy. Antimicrob Agents Chemother. 2013 Jan; 57(1):1–8. doi: 10.1128/AAC.01351-12 PMID: 23070153

5. Chandra J, McCormick TS, Imamura Y, Mukherjee PK, Ghannoum MA. Interaction of Candida albicans with Adherent Human Peripheral Blood Mononuclear Cells Increases C. albicans Biofilm Formation and Results in Differential Expression of Pro- and Anti-Inflammatory Cytokines. Infect Immun. 2007 May 1; 75(5):2612–20. PMID: 17339351

6. Katragkou A, Kruhlak MJ, Simitisopoulos M, Chatzimoschou A, Taparkou A, Cotten CJ, et al. Interactions between Human Phagocytes and Candida albicans Biofilms Alone and in Combination with Antifungal Agents. J Infect Dis. 2010 Jun 15; 201(2):1941–9. doi: 10.1086/652783 PMID: 20415537

7. Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA. Biofilm formation by the fungal pathogen Candida albicans: development, architecture, and drug resistance. J Bacteriol. 2001 Sep; 183(18):5385–94. PMID: 11514524

8. Lamfon H. Susceptibility of Candida albicans biofilms grown in a constant depth film fermentor to chlorhexidine, fluconazole and miconazole: a longitudinal study. J Antimicrob Chemother. 2004 Jan 16; 53(2):383–5. PMID: 14729749

9. Taft HT, Mitchell KF, Edward JA, Andes DR. Mechanisms of Candida biofilm drug resistance. Future Microbiol. 2013 Oct; 8(10):1325–37. doi: 10.2217/fmb.13.101 PMID: 24059922

10. Nett JE, Crawford K, Marchillo K, Andes DR. Role of Fks1p and Matrix Glucan in Candida albicans Biofilm Resistance to an Echinocandin, Pyrimidine, and Polyene. Antimicrob Agents Chemother. 2010 Aug 1; 54(8):3505–8. doi: 10.1128/AAC.00227-10 PMID: 20516280

11. Tobudic S, Kratzer C, Lassnigg A, Graninger W, Presterl E. In vitro activity of antifungal combinations against Candida albicans biofilms. J Antimicrob Chemother. 2010 Feb 1; 65(2):271–4. doi: 10.1093/jac/dkp429 PMID: 19996142

12. Ramage G, Bachmann S, Patterson TF, Wickes BL, López-Ribot JL. Investigation of multidrug efflux pumps in relation to fluconazole resistance in Candida albicans biofilms. J Antimicrob Chemother. 2002 Jun; 49(6):973–80. PMID: 12039889

13. Nett JE, Sanchez H, Cain MT, Andes DR. Genetic basis of Candida biofilm resistance due to drug sequestering matrix glucan. J Infect Dis. 2010 Jul 20; 202:171–175. doi: 10.1086/651200 PMID: 20497051

14. Mitchell KF, Zarnowski R, Sanchez H, Edward JA, Reinicke EL, Nett JE, et al. Community participation in biofilm matrix assembly and function. Proc Natl Acad Sci U S A. 2015 Mar 13;

15. Mukherjee PK, Chandra J, Kuhn DM, Ghannoum MA. Mechanism of fluconazole resistance in Candida albicans biofilms: phase-specific role of efflux pumps and membrane sterols. Infect Immun. 2003 Aug; 71(8):4333–40. PMID: 12874310

16. White TC, Marr KA, Bowden RA. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clin Microbiol Rev. 1998 Apr; 11(2):383–402. PMID: 9564569

17. Davies D. Understanding biofilm resistance to antibacterial agents. Nat Rev Drug Discov. 2003 Feb; 2(2):114–22. PMID: 12563302

18. Pappas PG, Kauffman CA, Andes D, Benjamin DK Jr., Calandra TF, Edwards JE Jr., et al. Clinical Practice Guidelines for the Management of Candidiasis: 2009 Update by the Infectious Diseases Society of America. Clin Infect Dis. 2009 Mar; 48(5):503–35. doi: 10.1086/596757 PMID: 19191635

19. McGee DC, Gould MK. Preventing complications of central venous catheterization. N Engl J Med. 2003 Mar 20; 348(12):1123–33. PMID: 12646670

20. Liu S, Hou Y, Chen X, Gao Y, Li H, Sun S. Combination of fluconazole with non-antifungal agents: a promising approach to cope with resistant Candida albicans infections and insight into new antifungal agent discovery. Int J Antimicrob Agents. 2014 May; 43(5):395–402. doi: 10.1016/j.ijantimicag.2013.12.009 PMID: 24503221

21. Odds FC. Synergy, antagonism, and what the checkerboard puts between them. J Antimicrob Chemother. 2003 Jun 12; 52(1):1–1. PMID: 12805255
22. Chavez-Dozal AA, Lown L, Jahng M, Walraven CJ, Lee SA. In vitro analysis of finasteride activity against *Candida albicans* urinary biofilm formation and filamentation. Antimicrob Agents Chemother. 2014 Oct; 58(10):5855–62. doi: 10.1128/AAC.03137-14 PMID: 25049253

23. Fiori A, Van Dijck P. Potent Synergistic Effect of Doxycycline with Fluconazole against *Candida albicans* Is Mediated by Interference with Iron Homeostasis. Antimicrob Agents Chemother. 2012 Jul 1; 56(7):3785–96. doi: 10.1128/AAC.00617-11 PMID: 22564841

24. Pemmaraju SC, Pruthi PA, Prasad R, Pruthi V. *Candida albicans* biofilm inhibition by synergistic action of terpenes and fluconazole. Indian J Exp Biol. 2013 Nov; 51(11):1032–7. PMID: 24416942

25. Mukherjee PK, Chandra J. *Candida* biofilm resistance. Drug Resist Updat Rev Comment Antimicrob Anticancer Chemother. 2004 Oct; 7(4–5):301–9.

26. Akins RA. An update on antifungal targets and mechanisms of resistance in *Candida albicans*. Med Mycol. 2005 Jun; 43(4):285–318. PMID: 16110776

27. Pfaller MA, Diekema DJ, Messer SA, Hollis RJ, Jones RN. In Vitro Activities of Caspofungin Compared with Those of Fluconazole and Itraconazole against 3,959 Clinical Isolates of *Candida* spp., Including 157 Fluconazole-Resistant Isolates. Antimicrob Agents Chemother. 2003 Mar 1; 47(3):1068–71. PMID: 12604543

28. Metcalf SC, Chambers ST, Pithie AD. Use of ethanol locks to prevent recurrent central line sepsis. J Hosp Infect. 2004 Jul; 59(4):401–5. PMID:17446287

29. Chopra I, Roberts M. Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. Microbiol Mol Biol Rev. 2001 Jun 1; 65(2):232–60. PMID:11381101

30. Oliver BG, Silver PM, Marie C, Hoot SJ, Leyde SE, White TC. Tetracycline alters drug susceptibility in *Candida albicans* biofilms. Antimicrob Agents Chemother. 2008 Apr 1; 52(4):1256–62. doi: 10.1128/AAC.03137-14 PMID: 25049253

31. Harriott MM, Noverr MC. Antifungal Agents against *Candida albicans* Biofilms. Antimicrob Agents Chemother. 2012 Apr; 56(4):1333–40. doi: 10.1128/AAC.05774-11 PMID: 22615286

32. Peters BM, Ward RM, Rane HS, Lee SA, Noverr MC. Efficacy of ethanol against *Candida albicans* and *Staphylococcus aureus* polymicrobial biofilms. Antimicrob Agents Chemother. 2013 Jan; 57(1):74–82. doi: 10.1128/AAC.01599-12 PMID: 23070170
43. Wiederhold NP, Grabinski JL, Garcia-Effron G, Perlin DS, Lee SA. Pyrosequencing To Detect Mutations in FKS1 That Confer Reduced Echinocandin Susceptibility in Candida albicans. Antimicrob Agents Chemother. 2008 Nov 1; 52(11):4145–8. doi: 10.1128/AAC.00959-08 PMID: 18794385

44. Fonzi WA, Irwin MY. Isogenic strain construction and gene mapping in Candida albicans. Genetics. 1993 Jul; 134(3):717–28. PMID: 8349105

45. Brady RA, Leid JG, Camper AK, Costerton JW, Shirliff ME. Identification of Staphylococcus aureus Proteins Recognized by the Antibody-Mediated Immune Response to a Biofilm Infection. Infect Immun. 2006 Jun 1; 74(6):3415–26. PMID: 16714572

46. Adam B, Baillie GS, Douglas Lj. Mixed species biofilms of Candida albicans and Staphylococcus epidermidis. J Med Microbiol. 2002 Apr; 51(4):344–9. PMID: 11926741

47. Shanks RMQ. Catheter lock solutions influence staphylococcal biofilm formation on abiotic surfaces. Nephrol Dial Transplant. 2008 Aug 1; 21(8):2247–55. PMID: 16627606

48. Fothergill AW. Antifungal Susceptibility Testing: Clinical Laboratory and Standards Institute (CLSI) Methods. In: Hall GS, editor. Interactions of Yeasts, Moulds, and Antifungal Agents [Internet]. Totowa, NJ: Humana Press; 2012 [cited 2015 Mar 30]. p. 65–74. Available from: http://link.springer.com/10.1007/978-1-59745-134-5_2

49. Pierce CG, Uppuluri P, Tristan AR, Wormley FL, Mowat E, Ramage G, et al. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. Nat Protoc. 2008 Aug; 3(9):1449–500. doi: 10.1038/nprot.2008.141 PMID: 18772877

50. Gillum AM, Tsay EY, Kirsch DR. Isolation of the gene for orotidine-5-phosphate decarboxylase by complementation of Saccharomyces cerevisiae ura3 and E. coli pyrF mutations. Mol Gen Genet. 1984; 198:179–18251. PMID: 6394964

51. Wiederhold NP, Grabinski JL, Garcia-Effron G, Perlin DS, Lee SA. Pyrosequencing to detect mutations in FKS1 that confer reduced echinocandin susceptibility in Candida albicans. Antimicrob Agents Chemother. 2008 Sep; 52(11):4145–1448. doi: 10.1128/AAC.00959-08 PMID: 18794385

52. Berenbaum MC. A Method for Testing for Synergy with Any Number of Agents. J Infect Dis. 1978 Feb 1; 137(2):122–30. PMID: 627734

53. Rey-Jurado E, Tudó G, de la Bellacasa JP, Espasa M, González-Martín J. In vitro effect of three-drug combinations of antituberculous agents against multidrug-resistant Mycobacterium tuberculosis isolates. Int J Antimicrob Agents. 2013 Mar; 41(3):278–80. doi: 10.1016/j.ijantimicag.2012.11.011 PMID: 23312604

54. Ostrosky-Zeichner L, Rex JH, Pappas PG, Hamill RJ, Larsen RA, Horowitz HW, et al. Antifungal Susceptibility Survey of 2,000 Bloodstream Isolates in the United States. Antimicrob Agents Chemother. 2003 Oct 1; 47(10):3149–54. PMID: 14506023

55. Tawara S, Ikeda F, Maki K, Morishita Y, Otomo K, Teratani N, et al. In vitro activities of a new lipopeptide antifungal agent, FK463, against various fungal pathogens. J Antibiot (Tokyo). 2000 Oct; 53(10):1175–83. PMID: 10980180

56. Messer SA, Diekema DJ, Boyken L, Tendolkar S, Hollis RJ, Pfaller MA. Activities of Micafungin against 315 invasive Clinical isolates of Fluconazole-Resistant Candida spp. J Clin Microbiol. 2006 Feb 1; 44(2):324–6. PMID: 16455878

57. Uchida K, Nishiyama Y, Yokota N, Yamaguchi H. In vitro antifungal activity of a novel lipopeptide antifungal agent, FK463, against a variety of clinically important fungi. Antimicrob Agents Chemother. 2000 Sep; 44(1):57–62. PMID: 10602723

58. Uchida K, Nishiyama Y, Yokota N, Yamaguchi H. In vitro antifungal activity of a novel lipopeptide antifungal agent, FK463, against various fungal pathogens. J Antibiot (Tokyo). 2000 Oct; 53(10):1175–83. PMID: 10980180

59. Messer SA, Diekema DJ, Boyken L, Tendolkar S, Hollis RJ, Pfaller MA. Activities of Micafungin against 315 invasive Clinical isolates of Fluconazole-Resistant Candida spp. J Clin Microbiol. 2006 Feb 1; 44(2):324–6. PMID: 16455878

60. Bell AL, Jayaraman R, Vercaigne LM. Effect of ethanol/trisodium citrate lock on the mechanical properties of carbothane hemodialysis catheters. Clin Nephrol. 2006 May; 65(5):342–8. PMID: 16724655