# Can metabolically generated $CO_2$ enhance *Candida albicans* biofilm formation within central venous catheters used in preterm infants?:An in vitro study

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## ABSTRACT

*Candida albicans is responsible for the majority of invasive fungal infections in preterm infants. Biofilm formation within indwelling central venous catheter (CVC) used for these preterm babies leads to CVC related infections and may lead to antifungal resistance. Metabolically generated $CO_2$ can act as a communicating molecule triggering the yeast to filamentous transition that is essential for the pathological effects caused by C. albicans. The aim of this study was to demonstrate that $CO_2$ generated by C. albicans contributes to biofilm-formation in central venous catheters utilised in preterm infants. An in vitro model with neonatal CVCs, using established $CO_2$ bio indicator strain ($CO_2$-BIS) to determine whether there is an accumulation of metabolic $CO_2$ within the catheters, which may be influencing C. albicans biofilm formation was developed. Biofilms formed within CVC lines showed an enhanced recovery of $CO_2$-BIS (p=0.06) when co-incubated with another $CO_2$ donor C. albicans strain CAI4. This indicated that the metabolically generated $CO_2$ from neighbouring CAI4 cells within the biofilm had generated sufficient $CO_2$ to complement the growth demand of $CO_2$-BIS. Therefore, these results highlight the importance of high concentrations of $CO_2$ in the circulatory system, which enhances fungal pathogenicity and may lead to invasive candidiasis.*

**KEYWORDS***: Candida albicans, biofilm, central venous catheters, metabolic $CO_2$*

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# 1. INTRODUCTION

Invasive fungal infection is an important cause of serious morbidity and mortality in the neonatal intensive care unit (Kaufman 2004, Clerihew *et al.* 2006) with *Candida albicans* being the most commonly isolated fungal pathogen in the preterm population (Kaufman 2010). Central Venous Catheters (CVCs) are frequently used in preterm infants for the provision of parenteral nutritional support whilst enteral feeds are established. As such, CVCs represent a focus for the development of microbial biofilms and provide a potential reservoir of antimicrobial resistant microbes (Chandra *et al.* 2001).

*C.albicans* is an opportunistic pathogen which has the ability to undergo reversible morphogenesis between yeast and hyphal forms, a characteristic essential for the pathobiology of the fungus (Chandra *et al.* 2001). In addition, *C. albicans* can exist as a biofilm, attached to a surface surrounded by an extracellular matrix (Donlan *et al.* 2002, Douglas 2003), e.g. on the surface of medical implants and catheter surfaces. Biofilm formation alters phenotype of the fungus and enhances its virulence (Douglas 2003) and resistance to both humoral and cellular host defences (Costerton *et al.* 1978, Donlan *et al.* 2002). Importantly, biofilms confer resistance to commonly used antifungal agents (Mukherjee *et al.* 2009), making such infections increasingly difficult to treat.

*C.albicans* morphogenesis is regulated by many host derived environmental cues including the availability of $CO_2$ (Hall *et al.* 2010). Two major enzymes are integral to the *C. albicans* $CO_2$ sensing system; the adenylyl cyclase Cyr1p and the carbonic anhydrase Nce103p (Klengel *et al.* 2005, Hall *et al.* 2010). Fungal adenylyl cyclase Cyr1p is involved in initial adhesion of fungal cells to surface and biomass formation and is a direct sensor of environmental $CO_2$ concentrations (Verstrepen *et al.* 2006). The carbonic anhydrase Nce103p of *C. albicans* catalyzes the hydration of $CO_2$ to bicarbonate, which is an essential metabolic intermediate required for growth in $CO_2$ levels of 0.03%, while elevated bicarbonate/$CO_2$ levels regulate the yeast to hyphal transition. Strains lacking carbonic anhydrase can only grow in environments enriched with $CO_2$, and are still responsive to hyphal inducing conditions (Odds 1988, Cutler 1991, Donlan *et al*. 2002, Hall *et al*. 2010).

The build-up of metabolically generated $CO_2$ in enclosed compartments such as CVCs may modulate biofilm formation of *C. albicans*. This, in turn, may influence the success of treatment of CVC related infections. Although there are several *in vitro* and *in vivo* animal model data regarding drug resistance of biofilms on medical devices (Deborah *et al.* 1998, Schinabeck *et al.* 2004, Mukherjee *et al.* 2009), relatively less is known about metabolically generated $CO_2$ production in fungal biofilms inside CVCs. Given this, it may be possible that manipulation of metabolic pathways involved in fungal specific $CO_2$ generation may be used as a target in drug development to help

prevent invasive fungal infection in the future.

The aim of this study was to demonstrate that $CO_2$ generated by *C. albicans* contributes to biofilm-formation in central venous catheters utilised in preterm infants. Thus, here we

present data from an *in vitro* model study which used CVCs commonly used in preterm infants in order to establish whether metabolically generated $CO_2$ production occurs inside them which may enhance the biofilm formation of *C. albicans*.

## 2. MATERIALS AND METHODS

### Strains and Growth Conditions

The *C.albicans* strains used in this study included CAI4 (*ura3:imm434/ura3:imm434*) and $CO_2$-BIS (*Δnce103; ura3::l imm434 ura3::l imm434 nce103::HisG/nce103::HisG (pSM2)*)( Hall *et al.* 2010). *C.albicans* strains were maintained as glycerol stocks at -80°C. When required for the study, strains were streaked onto YPD (1% yeast extract, 1% bactopeptone, 2% glucose, 2% agar) and grown at 37°C for 16 hours. Fresh plates were used for each experiment to ensure 100% viability.

When differentiation of the two strains ($CO_2$-BIS and CAI4) was required after co-incubation experiments, $CO_2$-BIS was selected by plating on YNB agar (0.67% yeast nitrogen base [without amino acids], 2% glucose, 2% agar), 5% $CO_2$, while CAI4 was selected by plating cells onto YPD agar and growing in atmospheric

concentrations of $CO_2$ (0.03%). All experiments were performed at 37°C unless stated otherwise.

### Colony co-incubation experiments

$CO_2$-BIS was incubated as a single biomass of YPD agar either as homogenous populations (2000 cells) or as heterogeneous populations (1000 $CO_2$-BIS\1000 CAI4 cells) for 48 hours. Cells were recovered from the plate and then $CO_2$-BIS fold recovery was estimated as below. First, the cells were washed from the plate and then the agar was homogenised to release invaded cells. Serial dilutions were plated onto YNB in 5% $CO_2$ (which selectively allows only the growth of $CO_2$-BIS) and YPD in 0.033% $CO_2$ (which selectively allows only the growth of CAI4). Colony forming units on plates were determined as relative cell number.

### Central venous catheter details

We studied three types of CVCs commonly used in preterm infants, namely Epicutaneo-Cava-Katheter (ECK), Premicath and Nutriline Twin Flow (NTF). The materials used for each catheter are as follows: ECK-silicone XRO (radio-opaque) with winged needle, Premicath-PUR (Polyurethane) XRO with splitting needle and NTF-PUR (Polyurethane) XRO with split cannula introducer. Further details of each catheter are given in Table 1. All CVCs studied were manufactured by Vygon® (Vygon UK Ltd, Bridge Road, Cirencester, Gloucestershire, GL7 1PT).

**Table 1: Central venous catheters used in the study**

| Central venous catheter type | Catheter length (cm) | $\varnothing$ (internal diameter) (mm) | Flow rate (ml/min) | Priming volume (ml) | Lumen gauge (French size) |
|---|---|---|---|---|---|
| Epicutaneo-cava-katheter (ECK) | 30 | 0.3 x 0.6 | 5.0 | 0.12 | 24G (2Fr) |
| Premicath | 20 | 0.17 x 0.35 | 0.50 | 0.15 | 28G (1 Fr) |
| Nutriline twin flow (NTF) | 30 | 2 x 0.20 x 0.60 | 2 x 1.45 | 2 x 0.2 | 24G (2Fr) |

### *In Vitro* Model

A simple *in vitro* model was made using a CVC to study the formation of biofilms on biomaterial surfaces by pathogenic fungi *C. albicans*. For each experiment, a different type of CVC was used as given above. The fungal strain was locked in the CVC for attachment for 24 hours and then replaced with horse serum (as an alternative to human serum) for further 48 hours. Then the removed CVC was examined under the microscope for biomass formation followed by estimation of the biomass by cell count.

### Biofilm formation within central venous catheters

*C.albicans* strains were grown overnight in YPD at 30°C, then re-suspended in fresh YPD and grown at 30°C to mid-exponential phase (O.D.$_{600nm}$ = 0.5). Cells were then harvested, washed twice in sterile phosphate-buffered saline in pH 7.4 and re-suspended at the desired cell density (given below) in YPD. For each experiment, one CVC was inoculated with only the $CO_2$-BIS strain and one CVC was inoculated with $CO_2$-BIS and CAI4 in a 1:1 ratio [final cell density was maintained at $1 \times 10^7$ cells/ml (Kuhn *et al.* 2002, Schinabeck et al. 2004)]. The inoculum was 'locked' in the catheter and allowed to dwell for 24 hours (Mukherjee et al. 2009) at 37°C in air, following which the inoculum was removed and replaced with horse serum for incubation for further 48 hours. This allowed the cells in the biomass to be bathed in serum simulating a CVC inside the venous system in humans. The tests were repeated six times.

### Estimation of Biofilm Formation

Catheters were examined under the microscope for biofilm formation. For a detachment of biomass from the surface, catheter segments were cut in half lengthwise using sterile technique (using sterile scalpel blades) and sonicated in 2 ml of PBS at 40,000 Hz (Ultra U50: Ultrawave Ltd, UK ) (Andes *et al.* 2004) for 6 min in 3 min sessions. After vortexing for 15s, serial dilutions were prepared and 50 µl aliquots were plated in YNB (Difco $^{TM}$, Becton, Dickton and Company Sparks, UK) and YPD agar incubated in 5.5% $CO_2$ in $CO_2$ incubator (Infors HT Minitron, Infors UK Ltd, surrey, UK) and air respectively, for 48 h

at 37°C. Cell numbers in the biofilm were assessed by manual counting of Colony Forming Units (CFU). The average of the repeated results was taken for analysis and the student's t-test was performed for statistical analysis.

## 3. RESULTS & DISCUSSION

### 3.1 RESULTS

**Exogenous $CO_2$ and fungal generated $CO_2$ rescue the growth of $CO_2$-BIS**

CAI4 is deficient in uridine biosynthesis and as a result, can only grow in uridine rich environments (YPD), whereas $CO_2$-BIS is proficient in uridine biosynthesis but defective in capturing bicarbonate. Therefore, we can utilise these auxotrophies to separate the two strains from mixed populations, as YPD plates grow in air (0.03% $CO_2$) will only support the growth of CAI4, while YNB grown in enriched atmospheres of $CO_2$ will only permit the growth of the $CO_2$-BIS strain. To confirm that we would be able to separate out mixed populations of CAI4 and $CO_2$-BIS into single populations, we tested the robustness of the two phenotypes. Strains were repeatedly plated onto selective environments and maintained their phenotype confirming that the phenotype was stable and could be used for separation of the two strains (Figure 1A). To confirm previous findings (Hall *et al.* 2010), that self-generated $CO_2$ concentrations in fungal biomasses were sufficient to promote the growth of the $CO_2$-BIS strain, CAI4 and $CO_2$-BIS were co-incubated or $CO_2$-BIS was incubated

alone as single biomasses on YPD agar plates, and the fold recovery of the $CO_2$-BIS strain was measured after 48 hours. We observed a 25 fold increase in $CO_2$-BIS biomass when co-incubated with CAI4 compared to homogenous growth, confirming that self-generated $CO_2$ within fungal biomasses was sufficient to promote the growth of the $CO_2$-BIS strain (Figures 1B & 1C).
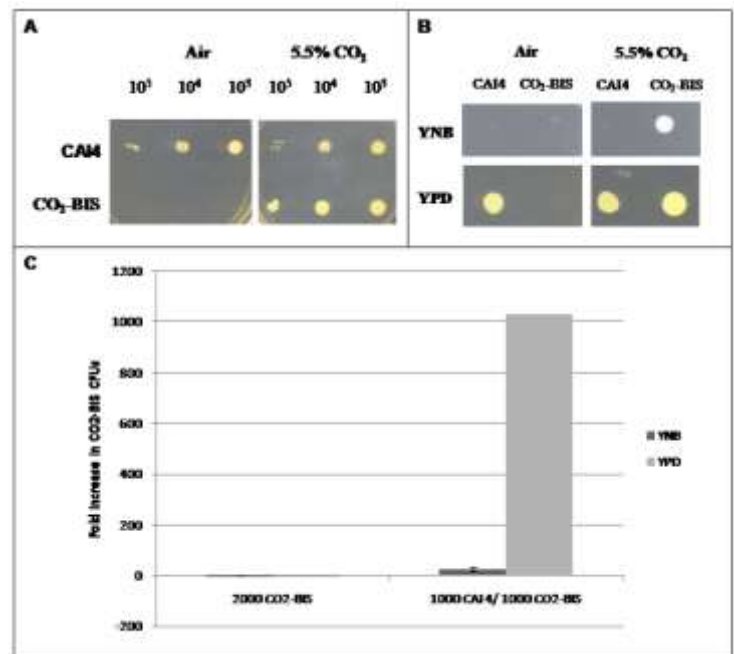


**Figure 1: Uridine dependant selective growth of CAI4 & CO₂-BIS in air and 5% CO₂**

(A) Cell dilutions (5 μl) of CAI4 and $CO_2$-BIS were spotted onto YPD plates and incubated in the presence of air or 5% $CO_2$ for 48 hours.
(B) Cell dilutions (5 μl) of CAI4 and $CO_2$-BIS were spotted onto basic media YNB lacking uridine and YPD plates

containing uridine and incubated for 48 hours.

(C) A total of 2000 cells $CO_2$-BIS alone and mixed with 1000 each cell of $CO_2$-BIS and CAI4 were incubated in air on YNB and YPD agar allowing to form colonies. Then they were washed off and plated on selective media to determine the numbers of each strain. The fold recovery of $CO_2$-BIS was calculated for analysis.

### Self-generated-fungal $CO_2$ accumulates in biofilms on CVC surfaces

Pathogenic fungal biomasses can develop on a variety of biomaterials, including contact lenses, urinary or venous catheters or prosthesis used *in vivo* (Donlan, 2001). To determine whether CVC biomaterial could support the formation of biomass on its surface we established an *in vitro* model using CVCs from different manufacturers, commonly used in neonatal care settings. Incubation of *C. albicans* inocula in these lines generated patchy biomasses on the interior surface of all CVC's studied (Figures 2A & 2B). Using the final cell recovery counts of each strain in the biomass, the initial attachment to biomaterial was calculated for both *C. albicans* strains comparing ECK (silicone) with Premicath (Polyurethane). This was done assuming that the conditions provided were similar in all CVCs except the plastic material. In this way, we found that the catheters made of silicone had more cells adhered to its surface than the polyurethane catheters (Table 2).
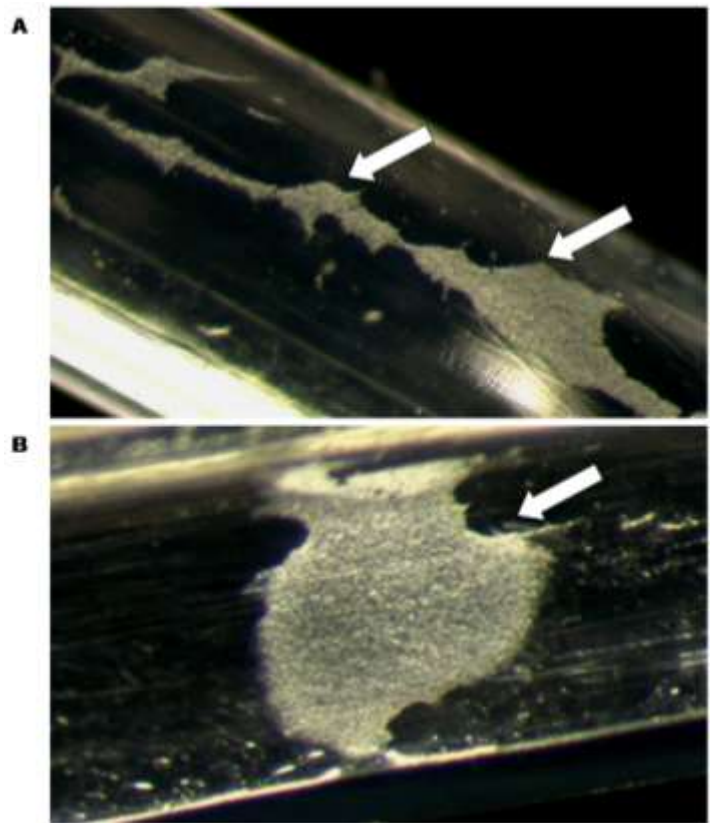
### Figure 2: Biomass formation inside central venous catheters

Images (A) and (B) show *C. albicans* biofilms (shown by the arrow) inside a central venous catheter. CVCs were inoculated with *Candida albicans* strains and allowed biomass formation. These were cut opened after 72 h (total duration) of incubation in the air at $37^0$C. The CVCs were observed through the LEICA MZ 75 stereomicroscope (x50) and photographs were taken using DC 180 digital imaging system (Leica Microsystems Ltd., Switzerland).

**Table 2: Adherence of fungal cells to biomaterial surface (cell count/1000 cells)**

| *Candida albicans* strain | Epicutaneo-cava-katheter (ECK) (cell count/1000 cells) | Premicath (cell count/1000 cells) |
|---|---|---|
| CAI4 in the mixture with CO$_2$-BIS | 21.07 | 7.42 |
| CO$_2$-BIS in the mixture with CAI4 | 67.98 | 12.89 |
| CO$_2$-BIS alone | 47.58 | 6.33 |

As the architecture of a fungal colony is very different to that of a biofilm, we tested whether metabolically generated CO$_2$ would accumulate with a biofilm on the surface of a CVC line. Co-incubation of CO$_2$-BIS with CAI4 formed patchy biofilms on the CVC line surface similar to that observed for CAI4 alone. Estimation of the CO$_2$-BIS cell number confirmed that there was a 1-2 enhancement ($p=0.06$) of the CO$_2$-BIS strain when co-incubated with CAI4 compared to when CO$_2$-BIS was incubated alone for all neonatal catheters examined (Figure 3). This indicates that the structure of the biofilm permits the accumulation of metabolically generated CO$_2$.
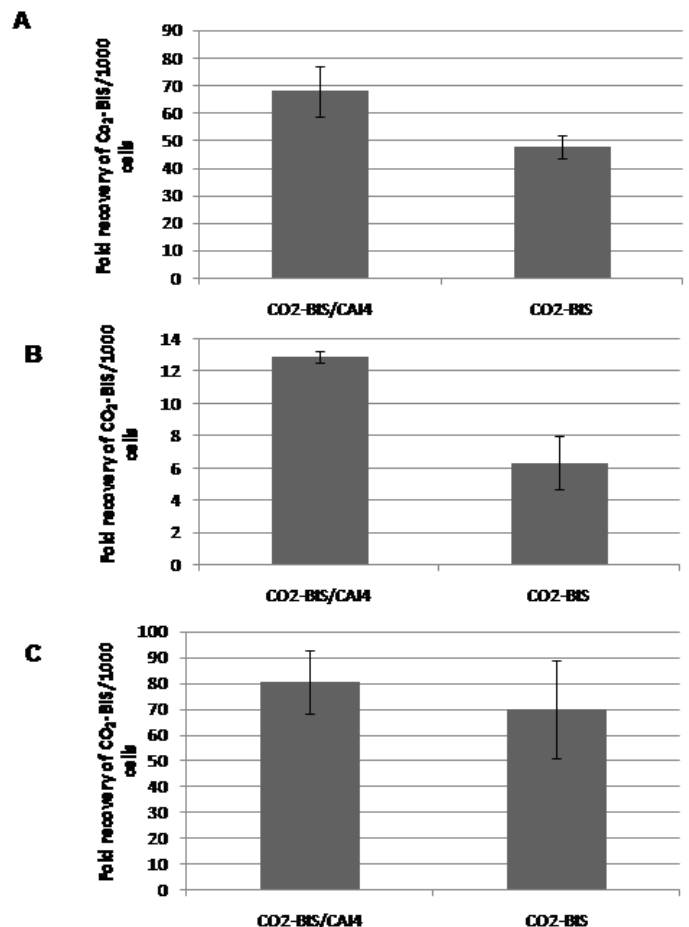


**Figure 3: Fold recovery of CO$_2$-BIS from neonatal CVC biofilms**

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(A)- Premicath Vygon® (polyurethane catheter) (B)-Nutriline twin flow Vygon® (polyurethane catheter), (C) - Epicutaneo-cava-katheter Vygon® (silicone catheter) One CVC line was inoculated with only the $CO_2$-BIS and one line with both $CO_2$-BIS and CAI4 in a 1:1 ratio (final cell density was maintained at $1 \times 10^7$ cells / ml). The inoculum was 'locked' in the

## 3.2 DISCUSSION

*C.albicans* is one of the major causes of fungal infections in preterm infants due to the use of intravenous lines (Saiman *et al.* 2000, Stoll *et al.* 2002, Feja *et al.* 2005). Despite the vast improvements in the generation of non-adherent surface materials, researchers (Lewis *et al.* 2002, Andes *et al.* 2004, Schinabeck *et al.* 2004, Nobile *et al.*, 2006, Mukherjee *et al.*, 2009) have shown that *C. albicans* is still capable of adhering to and forming biofilms on the luminal surface of CVCs used in the clinical setting for treatment of preterm infants and adults. Although there are several *in vitro* studies using different models for studying *C. albicans* biofilm formation, the majority have used pieces of biomaterial instead of a whole medical device (Hawser *et al.* 1994, Kuhn *et al.* 2002, Garcı´a-Sa´nchez *et al.* 2004, Chandra *et al.* 2008, Ramage *et al.* 2008, Uppuluri *et al.* 2009 ). *In vivo* animal studies of biomass formation within central venous lines have been performed, although this technique is not easily amenable to a longitudinal study (Andes *et al.* 2004, Schinabeck *et al.* 2004, Mukherjee *et al.* 2009) and hence we confined our study to an *in vitro* model using the full CVC line.

catheter for 24 h at 37$^{o}$C in air and then replaced with horse serum for further 48 hours incubation. Catheter segments were cut opened and sonicated for biomass detachment. Cell numbers of each strain in the biofilm were assessed by counting of Colony Forming Units (CFU). An average of repeated test results was used for analysis (p=0.06).

A number of parameters (surface material, medium, incubating conditions) affect the structure and morphology of biofilms. Biofilm formation varies with surface material. For example polyvinyl chloride resists biomass formation more than latex or silicone elastome (Hawser *et al*. 1994). We observed that *C. albicans* biofilm formation was more prevalent on silicone surfaces than polyurethane. This observation is consistent with reports from Hawser and Douglas (Hawser *et al*. 1994), but Schinabeck *et al* (Andes *et al*. 2004, Schinabeck *et al.* 2004, Mukherjee *et al.* 2009) did not observe differences in adherence between the two biomaterials. Therefore, other environmental conditions may influence the adhesion to the biomaterial.

Hall *et al* and other researchers (Odds 1988, Cutler 1991, Donlan *et al.* 2002, Hall *et al.* 2010) have demonstrated the virulence enhancing nature of metabolically generated gaseous $CO_2$ in *C. albicans*. They conclude that such $CO_2$ sensing could represent a mechanism for enhancing virulence when the host's immune system is impaired. Build-up of metabolically generated $CO_2$ in an enclosed compartment such as CVC lines may also have a role in stabilizing the *C.*

*albicans* biofilm. Here, we have shown that metabolic $CO_2$ does accumulate with biofilms on medical devices. Given that biofilms confer resistance to commonly used antifungal agents (Odds 1988, Cutler 1991, Donlan *et al.* 2002, Hall *et al.* 2010) and thus adversely impact upon the efficacy of antifungal therapy, further studies are warranted to establish the role of gaseous $CO_2$ in this process. For example, it would be interesting to see whether exogenous $CO_2$ enhances the biomass of the biofilm. Also of interest and would be the impact mixed biofilms in our system as bacteria are predicted to generate greater concentrations of $CO_2$ than fungal species, which may be of considerable importance in the hospital setting where biofilms are likely to formed from a mixture of species.

## 4. CONCLUSIONS

*C. albicans* is capable of forming biofilms inside CVCs irrespective of the biomaterial used. The architecture of the biofilm allows sufficient metabolically generated $CO_2$ to accumulate to concentrations where it can act as a signalling molecule and may therefore enhance the virulence and biofilm formation of *C. albicans*.

We identified a few areas for improvements in future studies. The time allowed for the adherence of cells to the catheter could be reduced, dry weight instead of colony counting could be used for biofilm quantification, horse serum which is not a well-defined medium and might influence the growth of the catheter surface-attached cells could be replaced

with a suitable alternative. The fold recovery calculated in our study could be the result of two independent events, adherence and proliferation, which should have been evaluated independently. It will also be important to test the materials that form the CVCs and then, test the CVC line implanted in animal models to reproduce what is happening in a patient.

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