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Biofilm Alterations on the Stepwise Acquisition of Fluconazole-resistant *Candida Albicans* Isolates

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Abstract

Objectives: By assessing and comparing the phenotypic changes on the stepwise acquisition of fluconazole resistant *Candida albicans* isolates, we could find and describe the relationship between drug resistance and biofilm formation ability in a series of clonal strains.

Methods: We performed antifungal susceptibility of five drugs (fluconazole, itraconazole, voriconazole, caspofungin and amphotericin B) to further verify the antifungal activity of the six isolates *in vitro*. Then we combined hyphal formation assay, cell surface hydrophobicity test positively related to adherence ability, and biofilm assays *in vitro* to observe and compare the phenotypic characteristics of our six clonal strains.

Results: Biofilm capability is enhanced for four drug- intermediate strains, whereas the initial susceptible strain and the final resistant strain are both poor in adherence, hyphal growth and biofilm formation.

Conclusions: It was suggested that the biofilm formation ability were not absolutely related to the degree of fluconazole resistance.

Keywords: *Candida albicans*, stepwise acquired azole resistance *in vivo*, adherence, hyphal formation, biofilm

Introduction

Microbial biofilms are considered to be the most common growth state for many microbial species, and 80% of human microbial infections are related to biofilms.[1-2] *Candida albicans* is the most commonly-diagnosed fungal pathogen of the human microbiota and can cause pervasive infections ranging from superficial mucosal and dermal infections to life threatening systemic infections.[3-4] The ability to grow biofilms complicates the eradication of *C. albicans* infections, particularly regarding its tendency to colonize the surfaces of medical devices and its persistence in different host tissues.[1-2] Cells in *C. albicans* biofilm are also often resistant to a variety of conventional antifungal agents, whose minimal inhibitory concentrations (MICs) can then be up to 1000 times higher than those found for planktonic cells.[5] Current studies related to *C. albicans* biofilms mainly focus on describing differences in the biofilm formation process for *Candida* species, or on comparing the biofilm-forming ability of *C. albicans* strains in different niches in the host, or in exploring interactions between *C. albicans* and bacteria species in the context of dual-species biofilms.[2,6-7] However, the relationship between biofilm formation ability and the degree of

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drug resistance *in vivo* in sequential clonal *C. albicans* isolates has never been investigated. Then how did the biofilm change during the evolution of acquired drug resistance *in vivo*? Here, we select a series of six *C. albicans* considered as having acquired azole resistance *in vivo*. These isolates were taken from an HIV-infected patient receiving progressively higher doses of fluconazole for recurrent oropharyngeal candidiasis over two years.[8] The biofilm-related parameters we employed in this study include adherence represented by cell surface hydrophobicity here, hyphal growth and biofilm mass.

Materials and methods

Ethical statement

No ethical approval was required as the research in this article related to micro-organisms.

Strains and growth conditions

Candida albicans strains (Ca1, Ca2, Ca5, Ca8, Ca14 and Ca17) used in this study were kindly provided by professor T. C. White (Seattle Biomedical Research Institute. Seattle). A colony was obtained from SDA medium (1% peptone, 2% dextrose, 1.5% agar, PH 7.0) after a 72 hours culture at 30 °C and transferred to YPD medium (1% yeast extract, 2% peptone and 2% dextrose). Then the strains were incubated overnight in 5 mL of YPD broth at 30 °C with rotation (150 rpm).

Antifungal susceptibility testing against C. albicans in vitro

Antifungal susceptibility testing of five drugs (fluconazole, itraconazole, voriconazole, caspofungin and amphotericin B) was performed using a broth microdilution method according to the CLSI (Clinical and Laboratory Standards Institute) in document M27–A3. The MIC was defined visually as the lowest concentration of drug that caused a prominent decrease when compared to the growth control. All tests were performed in triplicate and were repeated at least three times.

Hyphal formation assay

A 10 µL of cell suspension (5×10^3 CFU/mL) was spread onto Spider agar (10 g/liter nutrient broth, 10 g/liter mannitol, 2 g/liter K2HPO4, 20 g/liter agar, pH 7.2) and photographed after 3 days growth at 37 °C. After culturing, we transferred a colony with 1mm diameter into 5ml physiological saline. After shaking, 20 µl of the mixture was dropped onto a glass slide and observed under inverted microscope. In concert with this, filamentation was tested in liquid Spider medium. Yeast suspensions (3×10^6 CFU/mL) were incubated in liquid Spider medium at 37 °C and 200 r/minute for 24 hours. 10 µL portion of the suspension was dropped on a blood cell counting plate to count the number of hyphae and yeasts of the various strains. The proportion of hyphal cells was calculated as [number of hyphae/ (number of yeasts + number of hyphae)] × 100. Each leg of this portion of the experiment was repeated at least 3 times.

Cell surface hydrophobicity assay

The Cell surface hydrophobicity (CSH) assay of *C. albicans* was performed as previously described.[9] Briefly, *C. albicans* cultures were adjusted to a cell suspension with an OD600 (optical density at 600 nm) of 1.0 in YPD medium. A total of 2.5 mL of the suspension from each group was drawn into a clean glass tube, mixed with 0.5 mL of xylene at 37 °C for 10 minutes, and shaken for 30 seconds. Then these tubes were left for 30 minutes at 37 °C in order to facilitate separation of the phases. The turbidity of the aqueous phase was read at 600 nm. The OD600 for the group before xylene treatment was used as the negative control. The HI (hydrophobicity index) was calculated as [(OD600 of the control – OD600 after xylene overlay)/OD600 of the control] × 100. Three repeats were performed for each group.

Biofilm Formation

500ul standardized *C. albicans* cell suspensions (OD600 of 0.5 in liquid Spider medium) were introduced into the wells of 24-well tissue culture plates (Corning Inc., Corning, NY) and incubated at 37 °C.

Inverted microscopy

The medium was aspirated at 2, 4, 8, 12 and 24 hours of the incubation period. Biofilms were harvested and washed three times in sterile phosphate-buffered saline (PBS) [10 mmol/L phosphate buffer, 2.7 mmol/L potassium chloride, 137 mmol/L sodium chloride (pH 7.4)] and observed by inverted microscopy.

Confocal laser scanning microscopy (CLSM)

After 24 hours of biofilm growth in 24-well plate, biofilms were washed three times with PBS. Two hundreds microliters of 25 µmol/L FUN-1(1 g dextrose, 1.1915 g 10 mmol/L Na-Hepes, 50 mL H_2O, pH 7.2) was added to each well and biofilms were then cultured at room temperature in the dark. After 30 minutes, biofilms were washed gently with PBS and observed under confocal laser scanning microscope. The emission wavelength was 488 nm.

XTT Reduction Assay

Cells were washed three times with PBS before adding 500 µL XTT/VitK3 mixture prepared by mixing 1 µL 10 mmol/L VitK3 with 10 mL 0.5 mg/mL XTT ([2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide]) solution, and the resulting mixture was then incubated in the dark at 37 °C for 3 hours. The metabolically active cells reduced the substrate to orange-colored formazan which was measured spectrophotometrically at 490 nm. Results are given as mean ± standard error in the mean for three independent experiments.

Statistical analysis

Data analysis was performed using SPSS (IBM Corp., Armonk, NY, USA). For hyphal growth rate, CSH value and XTT assays, multiple comparison was performed by one-way ANOVA and Turkey HSD was used for post-hoc

multiple pair-wise comparisons. $P \leq 0.05$ were considered statistically significant.

Results

Antifungal effects against planktonic cells of C. albicans in vitro

Antifungal susceptibility profiles of the tested isolates are shown in Table 1. The MIC of fluconazole against six oral isolates range from 0.5 μg/mL to 128 μg/mL. The series clearly shows progressive increases in MICs against itraconazole and voriconazole as well, perhaps due to cross-resistance mechanism of azoles. In addition, all the *C. albicans* strains tested in this study are susceptible to amphotericin B and caspofungin.

Hyphal growth and CSH values

Hyphal growth on Spider agar revealed that Ca1 and -17 formed creamy colonies with smooth surfaces, whereas Ca2, -5, -8 and -14 exhibited a more wrinkled morphology (Fig. 1A). Moreover, under light microscopy, Ca1 and -17 formed colonies predominantly composed of yeast cells, but appeared to be morphologically distinct from Ca2, -5, -8 and -14 in that considerably more hyphae were observed (Fig. 1B). From Figure 1C, similar results were obtained from the liquid Spider medium test as well ($F = 85.641$, $P < 0.01$). In addition, CSH values of the other four strains were significantly higher than that of Ca1 or -17 ($F = 256.322$, $P < 0.01$) (Fig. 1D), which was consistent with the results attained form hyphal formation assays.

Visualization of biofilm formation under inverted microscopy

We then further characterized the architecture of the biofilms by inverted microscopy. When cultured for 2 hours, the strains form a biofilm with a monolayer of round budding yeast cells (Fig. 2A). After 2 hours, pseudohyphal and hyphal cells begin to form from these dividing yeast cells and microcolonies appear interspersed to form a membrane architecture at 4 hours (Fig. 2B). When incubated for 12 hours, a multilayer membrane structure is formed with yeast cells and hyphal cells (Fig. 2C). Biofilms become thicker, with more extracellular matrix encompassing all cells after 24 hours, presenting with a dense multilayer membrane structure (Fig. 2D). In general, Ca2, -5, -8 and -14 formed thicker biofilms, consisting of denser hyphal networks and smaller pore spaces than Ca1 or -17.

Table 1

MICs of five antifungal drugs against *C. albicans* planktonic cells.

C. albicans strains	MICs (μg/mL)				
	FLC	ITR	VOC	AMB	CAS
Ca1	0.5	0.0313	0.0313	0.25	0.0313
Ca2	2.0	0.0313	0.0313	0.25	0.0625
Ca5	8.0	0.0313	0.0313	0.50	0.0625
Ca8	8.0	0.0313	0.0313	0.50	0.1250
Ca14	32.0	0.0625	0.1250	0.25	0.1250
Ca17	128.0	0.5000	1.0000	0.50	0.1250

FLC, fluconazole; ITR, itraconazole; VOC, voriconazole; AMB, amphotericin B; CAS, caspofungin.

CLSM image and XTT reduction assay

C. albicans biofilms were considered mature at 24 hours and assessed by confocal laser scanning microscopy (CLSM). In contrast to the less developed biofilms of Ca1 and Ca17, the CLSM images show strains Ca2, -5, -8 and -14 with multiple layers of hyphal cells and a compact architecture of biofilm matrices (Fig. 3A). In addition, from Figure 3b, XTT reduction assay used to quantify the biofilm activity of experimental strains showed the following ranking (from most complex to least complex): Ca2 > Ca8 > Ca5 > Ca14 > Ca1 > Ca17 ($F = 149.122$, $P < 0.01$).

Discussion

Polyenes, azoles and echinocandins are three major classes of antifungal drugs used in invasive fungal infections.[1,3,10] Among them, azoles, especially fluconazole, are the most commonly therapeutic drugs for *C. albicans* infections due to their high efficiency and low toxicity. However, they lack fungicidal activity and prolonged usage of fluconazole results in the emergence of resistant strains of *C. albicans* during treatment.[10-11] Ca1 was a fluconazole-susceptible strain (0.5 μg/mL) initially isolated in the series through treatment with low doses of fluconazole (100 mg/d). Ca17 was a final isolate of this series with the highest resistance to fluconazole (greater than 128 μg/mL), which was collected when the patient was receiving 800 mg of fluconazole per day. The remaining four strains gathered in this study (Ca2, -5, -8 and -14) and the concomitant increases in MIC values (from 2 μg/mL to 32 μg/mL) occurred in a step-wise pattern, which matched the step-wise increases in the dosage of fluconazole (from 100 mg/d to 400 mg/d) prescribed for the patient.

As a resident of the commensal microbial flora, *C. albicans* can form biofilms on host mucosal tissue primarily in the oral cavity causing oropharyngeal candidiasis.[4] Additionally, cells in *C. albicans* biofilms can evade the host immune responses, combat conventional drugs, and seed new infections, making them a troublesome clinical problem.[1,3] Overall, *C. albicans* biofilm formation is a systematic, highly controlled process partitioned into four basic stages: adherence, initiation, maturation and dispersion.[1,2,12] Microbial adhesion is considered the crucial first step for biofilm formation process and cell surface hydrophobicity (CSH) plays an important role in the adhesion of pathogenic microorganisms to abiotic and biotic surfaces.[1,13] In the case of *C. albicans*, a positive association between CSH and adhesion has been reported.[14] One consensus is that *C. albicans* filamentous forms are naturally invasive, able to penetrate tissues and cause damage in the host.[15] Indeed, hyphal formation in *C. albicans* is also closely related to biofilm maturation, and mutants defective in hyphal formation demonstrated defects in biofilm formation as well.[16] The ability of *C. albicans* to switch from yeast to filaments plays a crucial role in enhancing virulence.[17] Many factors can induce hyphal formation, namely, serum, neutral PH, 37 °C, Spider medium,

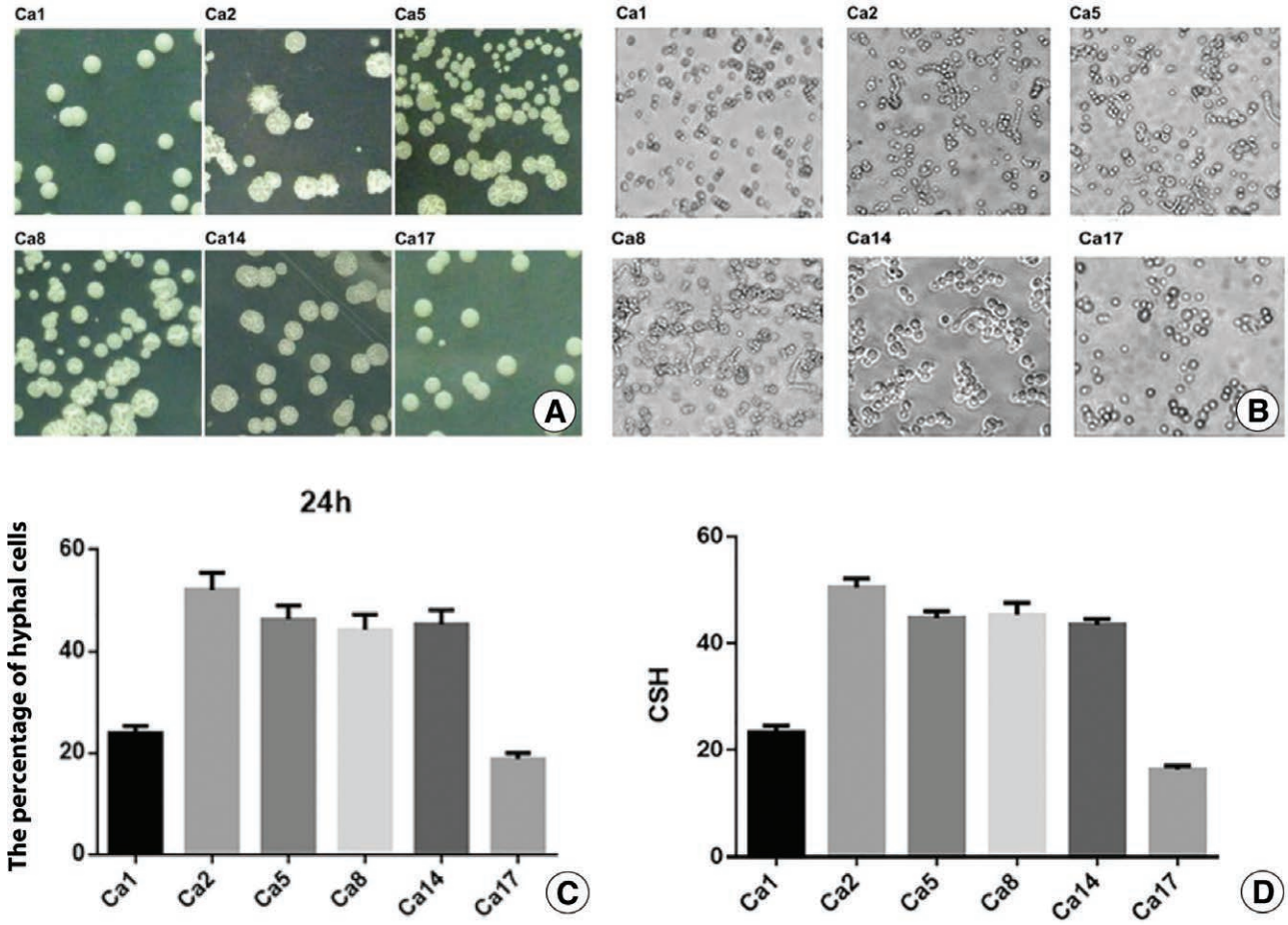

Figure 1. Hyphal formation assay and CSH assay. (A) Morphologies of colonies on Spider agars were photographed under a light microscopy after incubation at 37°C for 3 days. (B) Single colony mixed with physiological saline was observed by an inverted microscope. (C) Yeast suspensions were incubated in liquid Spider medium for 24h to evaluate the percentage of hyphal cells ($F = 85.641$, $P < 0.01$). (D) CSH was evaluated by using the water- hydrocarbon two-phase assay ($F = 256.322$, $P < 0.01$). The filamentation rate and CSH values of strains Ca5, -8 and -14 were not significantly different ($P > 0.05$) but significantly higher than that of Ca1 or -17 ($P < 0.01$). CSH: cell surface hydrophobicity.

and Roswell Park Memorial Institute-1640 medium.[18] Because biofilm formation in *C. albicans* has been associated with hyphal formation and adherence ability,[14] we assessed the ability of the six strains to form hyphae on Spider medium and recorded CSH values (referred to adherence) during the biofilm development. Overall, with the exception of Ca17, strains Ca2, -5, -8 and -14 presented a stronger capacity for hyphal growth and CSH values than Ca1. Since *C. albicans* biofilms begins with the adhesion of yeast cells to a solid surface, growth to form an anchoring layer, and yeast-to-hypha transition.[19] We preliminarily inferred that the biofilm formation ability of *C. albicans* strains Ca2, -5, -8 and -14 could be stronger than that of Ca1 and Ca17, which was absolutely verified in the biofilm assays.

A correlation between biofilm formation and antibiotic resistance has been noted recently in some bacteria. For example, majority of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates showed stronger biofilm formation than methicillin-sensitive *S. aureus* (MSSA) isolates in two different studies.[20-21] On the other hand, the weak biofilm-forming *Enterococcus faecalis* isolates were uniformly resistant to ampicillin and vancomycin, compared to their strong biofilm-forming counterparts.[22] Interestingly, in the eukaryotic microorganism *C. albicans,* our study was the first to observe an unexpected phenomenon between biofilm formation and drug resistance of *C. albicans*. As described in more detail below, all tested strains were divided into two groups based on their biofilm-producing ability: one group with weak biofilm formation ability (the initially sensitive strain Ca1 and the final resistant strain Ca17) and the other group with strong biofilm formation ability (the four drug-intermediate strains Ca2, -5, -8 and -14). It seems that intermediate fluconazole resistance in Ca2, -5, -8 and -14 promote biofilm formation *in vitro*, while the high fluconazole resistance (200 times that of the original isolate Ca1) of Ca17 inhibits the biofilm formation. However, an alternative explanation for this last data point could be that length of time and the high dosage of fluconazole treatment *in vivo* somehow restricted some critical biofilm-related cellular process or processes in Ca17 during co-evolution with that strain's drug resistance. As is mentioned in one study, mutations in genes related to cell adhesion (agglutinin-like protein 3,5 and 7 and *HYR3*), filamentous growth (filamentous growth regulator 14, filamentous growth regulator 28, and transcriptional regulator EFH1) and biofilm formation (biofilm and cell wall regulator 1 and serine/threonine protein kinase 1)

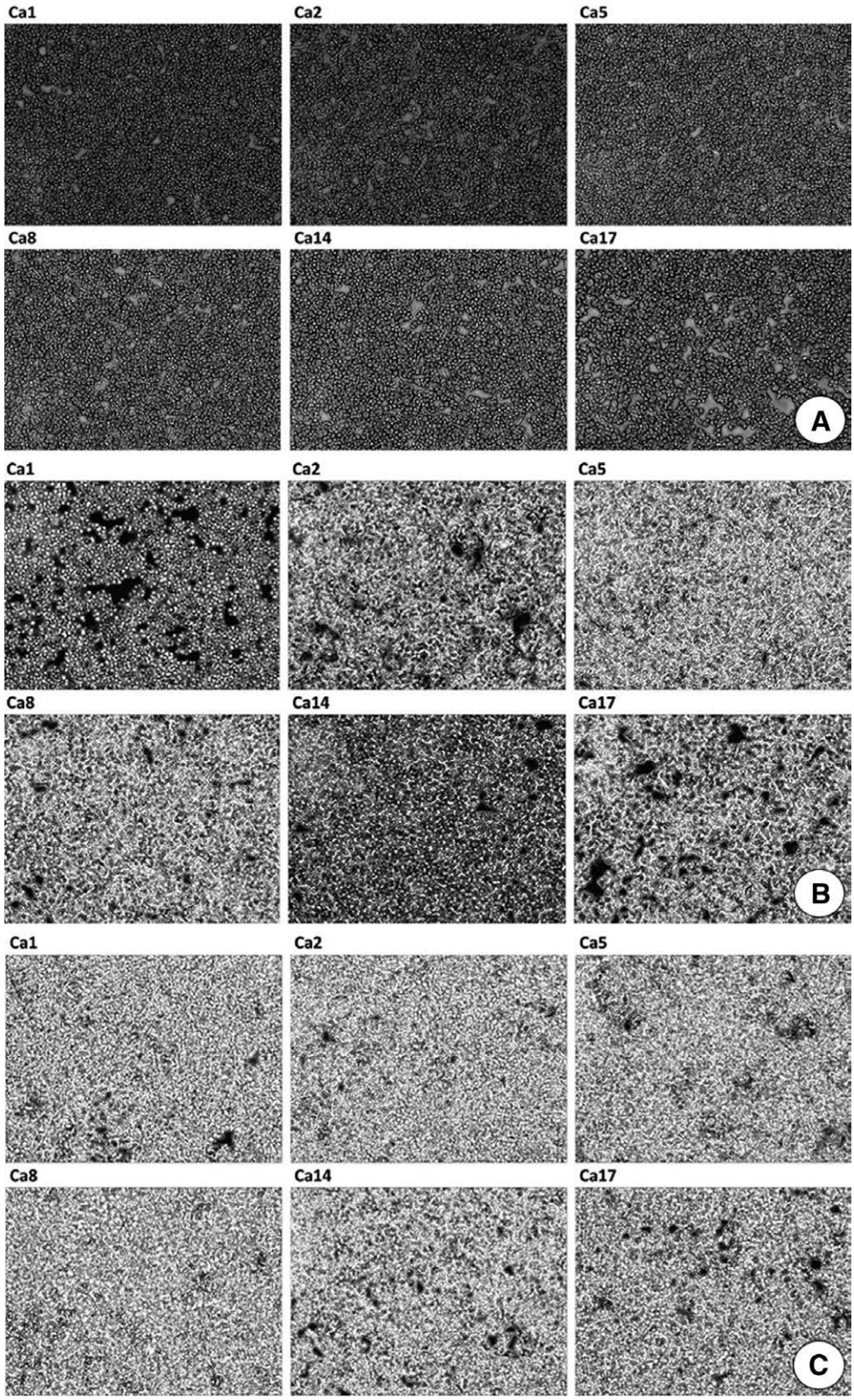

Figure 2. Biofilms under an inverted microscope. *C. albicans* biofilms were cultured and photographed under an inverted microscope at 2 hours (A), 4 hours (B), 12 hours (C) and 24 hours (D), respectively.

occurred in several sequential isolates, suggesting a co-evolution between virulence and drug resistance.[11] Since the fungistatic (growth inhibiting but not necessarily lethal) nature of azoles imposes strong directional selection for the evolution of resistance, only the cells with acquired resistance-related alterations could survive in the presence of the drug.[10] Over the past 20 years, many studies have addressed the same series of isolates we used in this study

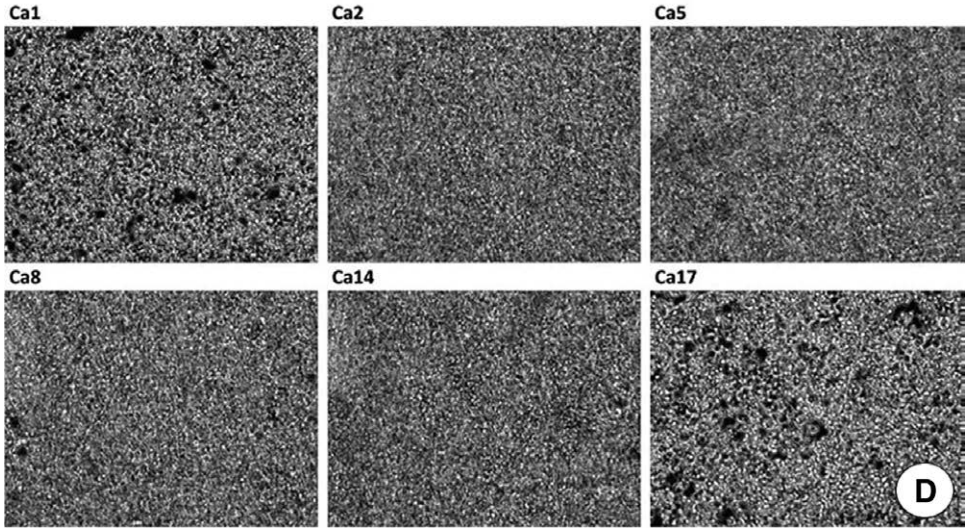

Figure 2. Continued.

to understand the evolutionary emergence of drug resistance in *C. albicans in vivo*. They found gain of function (GOF) mutations in the transcriptional regulator sterol uptake control protein 2 (*UPC2)* caused the overexpression of lanosterol 14-alpha demethylase, the gene encoding the fluconazole target.[23-25] Moreover, GOF mutations in transcriptional activator (*TAC1)* mediated overexpression of the multidrug efflux pumps pleiotropic ABC efflux transporter of multiple drugs1 and CDR2.[26] Similarly, the observed overexpression *of* multidrug resistance protein 1 efflux pumps was also detected, which was associated with GOF mutations in *MRR1*.[24,27] Noteworthy, it was proposed that the development of *C. albicans* resistance to azoles occur in a stepwise manner in which loss-of-heterozygosity (LOH) events commonly followed the acquisition of mutations in drug resistance genes.[26,28] Not surprisingly, as reported in previous studies, Ca17 contains hyperactive forms of all three zinc cluster transcription factors (ZnTFs) Mrr1, Tac1, and Upc2 that are well associated with high resistance to antifungal drugs.[27]

As biofilms are able to dynamically respond to various stresses and act as a "protective net" where fungus are afforded a stable environment and tolerate high antifungal concentrations, they usually result in the induction of resistance genes when exposed to antimicrobial agents.[3,29] However, Mukherjee *et al.* found the expression of drug efflux pump genes (CDR and MDR) decreased as the biofilms aged, suggesting that drug efflux pumps do not play a significant role in biofilm resistance to azoles.[30]

Indeed, acquired resistance *in vivo* is a long-term gradual development process involving a multi-level network regulatory system.[31] If the intermediate process is ignored, it is possible to draw a one-sided conclusion that biofilm-related virulence is irrelevant to drug resistance and events that play an important role would be omitted, which is not helpful in the search for the basis of this adaptive evolution. From our perspective, *C. albicans* combats drug pressures by several efficient means, such as the upregulation of genes encoding efflux pumps and the enhanced expression of genes encoding the drug target enzyme or mutations.[32] In order to reduce the "cost" of gene mutations, the organism exhibits a preference for suppressing other cellular processes including the biofilm formation we studied here.[29,33] During long-term fluconazole treatment or under an escalating dosage schedule, the capacity to form biofilm as a "protective net" gradually increase until it reaches a plateau and only declines later once the MIC passes a second threshold.

In summary, our research broadens knowledge on the relationship between biofilm formation and the evolution of fluconazole resistance in *C. albicans*, which appears to be more complicated than we had first thought. However, our study has some limitations: (1) We only focused on changes of *C. albicans in vitro*. As the cause of most infections in humans, biofilm formation *in vivo* embraces conditions considerably different from those in standard *in vitro* assays, such as liquid flow, host factors, and different components of the host's immune response.[34] (2) The durations of the early phase and maturation stage of biofilm formation *in vivo* seem to be shorter.[35] Therefore, the potential of these findings needs to be confirmed by results with models *in vivo*. (3) due to the small number of samples examined, additional studies will be needed to examine the relationship between biofilm formation and resistance in larger cohorts of patients.

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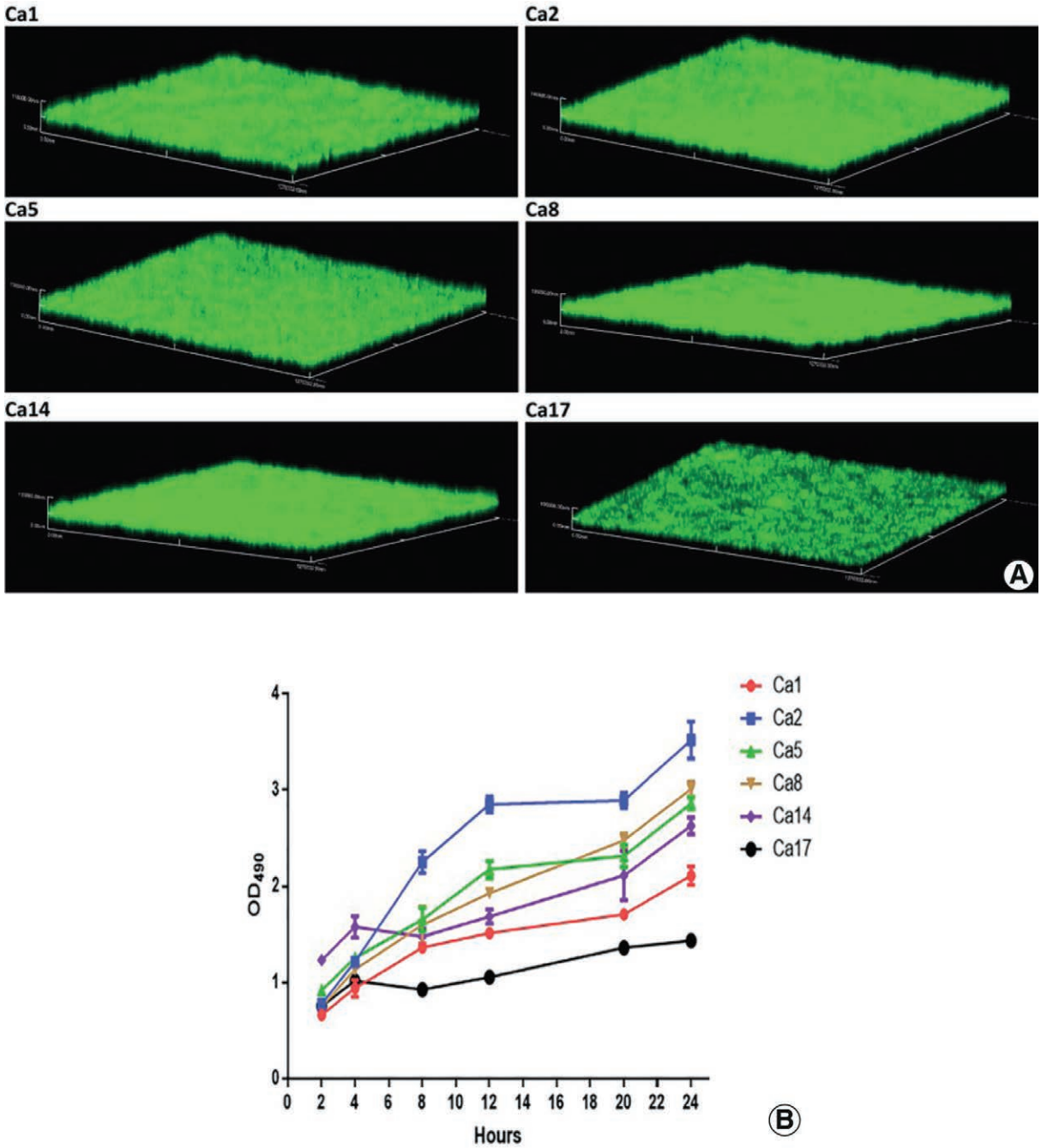

Figure 3. Biofilm assays. (A) Confocal laser scanning microscopy showing the *C. albicans* biofilm formation after grown for 24 hours (three dimension); (B) Metabolic activity of *C. albicans* biofilm was quantified by the XTT reduction assay (OD 490 nm): Ca2 > Ca8 > Ca5 > Ca14 > Ca1 > Ca17 ($F = 149.122$, $P < 0.01$).

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