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Original Article

K3-EDTA differentially inhibits the growth of *Candida* strains according to their azole resistance status

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Abstract

The diagnosis of the life-threatening invasive *Candida* infections is mainly established using culture of specimens that might be collected on different devices including ethylene diamine tetraacetic acid (EDTA)-coated tubes. Despite the knowledge that EDTA inhibits bacterial cultures, and its use to treat oral fungal infections, its impact on *Candida* cultures has not been completely assessed. This study aimed at assessing it on azole-resistant and azole-susceptible strains. Clinical and American Type Culture Collection (ATCC) strains for *Candida albicans* (CA), *C. glabrata* (CGS), *C. krusei* (CK), azole-susceptible and azole-resistant strains of *C. glabrata* (CGS and CGR), *C. lipolytica* (CL), and *C. inconspicua* (CI) were characterized using MALDI-TOF MS and susceptibility testing and then incubated (1) with serial dilutions of tripotassic EDTA (0%–500% of the concentration in a sample tube) for 2 hours before plating onto ChromID Can2 agar; (2) for 0, 2, 4, 6, 7, or 8 hours at EDTA concentrations at 20% and 33% before seeding; and (3) with sodium citrate or lithium heparinate instead of EDTA for 2 hours before plating. After 48 hours at 35°C, colony-forming units were automatically quantified. An inhibitory effect of EDTA was observed, at different concentrations, for CA (20%), CGS (100%), and CGR (500%) ($P < .05$), but none was observed for CL, CI, and CK. The effect increased with incubation duration, at a faster rate for azole-susceptible strains. K3-EDTA inhibits *Candida* growth and EDTA-coated tubes should not be used for mycological culture-based analyses. The correlation between EDTA inhibition and *Candida* azole-resistance offers perspectives for the development of selective agar and new antifungal strategies.

Key words: *Candida*, fungal infections, EDTA, antimicrobial resistance.

Introduction

Candidiasis are a major public health problem. Invasive candidiasis are life-threatening infections with mortality as high as 40% despite appropriate antifungal therapy.¹ They represent more than 10% of nosocomial bloodstream infections leading to high mortality, increased length of stay and cost.^{2,3} These

infections include candidemia as well as deep-seated candidiasis resulting from hematogenous dissemination or direct inoculation into a sterile site.⁴ These diseases are frequently complicated by biofilm formation, allowing adherence of these pathogens to vascular endothelium and decreasing the effect of standard antifungal treatments.⁵ Their diagnosis is challenging and may require

sampling as many as possible infected sites in addition to fungal blood culture which remain the gold standard for candidemia. Different specimen transport devices may include different anticoagulant coated tubes, and among them tubes coated with ethylene diamine tetraacetic acid (EDTA). EDTA is a calcium binder used for anticoagulation of samples intended for hematology analysis, and for the preservation of samples for cytologic analyses and of puncture liquids.⁶ The microbiological impact of EDTA has already been demonstrated on bacteria.⁷

The impact of EDTA additive on the growth of *Candida* strains has not been completely investigated despite its use by odontologists to treat oral fungal infections via a microbiological effect and mechanical cleaning of the dental plaque, based on clinical or biological studies.^{8–10}

This biological effect is supposedly due to the destabilization of the fungal cell wall, inhibiting fungal growth by the chelation of membranous and extracellular cations (calcium, magnesium, zinc).^{11–13} On the contrary, protein and nucleic acid syntheses are not impacted by EDTA, even if it has been demonstrated that EDTA inhibits biofilm synthesis in *Cryptococcus neoformans*, in *Candida albicans* or *Aspergillus* sp.^{13–15} Doing so, some studies have suggested that associations of EDTA with some molecules (such as chitosan for example) with limited antifungal capacities would be very interesting, by synergism.⁷

With respect to these previous studies, this study aimed to evaluate the *in vitro* inhibitory effect of EDTA on the results of routine *Candida* cultures, when present in clinical samples to be analyzed.

Methods

Strain selection and preparation

Eighteen *Candida* strains (three strains of each of six species) were selected depending on their prevalence in medical pathologies. They were all fully characterized using MALDI-TOF MS (VITEK MS, bioMérieux, Marcy-l'Étoile, France) and their antifungal susceptibility tested (eTest[®], bioMérieux) as recommended by the manufacturer, to verify expected results regarding azoles susceptibility. Four were American Type Culture Collection (ATCC, Manassas, VA, USA) strains: azole-susceptible *C. albicans* ATCC 90028, azole-susceptible *C. albicans* ATCC 10231, azole-susceptible *C. glabrata* ATCC MYA2950, and fluconazole-resistant *C. krusei* ATCC 6258). The 14 others were isolated from clinical samples: one azole susceptible *C. albicans*, two azole susceptible *C. glabrata* (fluconazole = 2 µg/ml, voriconazole = 0.023 µg/ml), three azole-resistant *C. glabrata* (fluconazole >256 µg/ml; voriconazole >32 µg/ml), two naturally resistant *C. krusei* and three of each naturally resistant *C. lipolytica* and *C. inconspicua*.¹⁶ Before use, to work with strains in exponential growth phase, all were cultured for 24 hours on ChromID CAN2 (bioMérieux).

Preparation of EDTA solution

EDTA solution was prepared using commercially available tripotassic EDTA (Promega, Madison, WI, USA). Dilutions were freshly prepared in distilled water, to obtain a scale of dilutions of EDTA, corresponding respectively to 0%, 20%, 33%, 50%, 100%, 200%, 300%, and 500% of the concentration obtained by adding 5 ml in an EDTA sampling tube (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA). The 100% concentration was estimated as containing 7.2 g per liter. The same concentrations for sodium citrate (Sigma, Darmstadt, Germany) and lithium heparinate (Sigma) were also prepared for further analyses.

Candida inocula preparation

Harvested strains were resuspended in aqueous sodium chloride 0.9% (Sigma) to obtain a 0.5 McFarland suspension. Yeasts were then quantified using a KOVA slide (Dutscher, Issy-les-Moulineaux, France) to determine the dilution ratio needed to obtain a suspension containing 10 colony-forming units (cfu)/ml. This freshly obtained suspension was then put in contact with the same volume of either EDTA (or other anticoagulant solution) or saline, to obtain the final mix. After an appropriate time of incubation (2 hours for the first and third steps and variable times for the second step), 100 µl of each final mix was plated on ChromID Can2 (bioMérieux) before incubation at 35°C for 48 hours. The cfu grown on each plate were automatically counted at 24 and 48 hours, using Scan[®] 1200 (Interscience, Saint Nom, France), under the operator's supervision. All preparations were done three times, to allow the analysis of triplicates. Moreover, all experiments were performed on three to four different days (replicates) to ensure robust results.

Determination of the EDTA inhibitory impact and of its specificity

It was based on a three-step process.

1. The serial dilutions of EDTA and a negative control (0% EDTA) were incubated with the *Candida* suspensions obtained, that is, a standard incubation period of 2 hours, at room temperature representing the median time between sampling and culture. A negative control (0%) without EDTA was analysed similarly to measure differential effect.
2. The lower inhibiting EDTA concentrations (20% and 33%) and the negative control were then put in contact with the strain suspensions for 0, 2, 4, 6, 7, or 8 hours, at room temperature before being plated.
3. Finally, to determine the specificity of the EDTA effect, the effects of sodium citrate and lithium heparinate which are also used for sample conservation were then tested after a standard 2-hour incubation, at room temperature to allow for the comparison between EDTA and non-EDTA results.

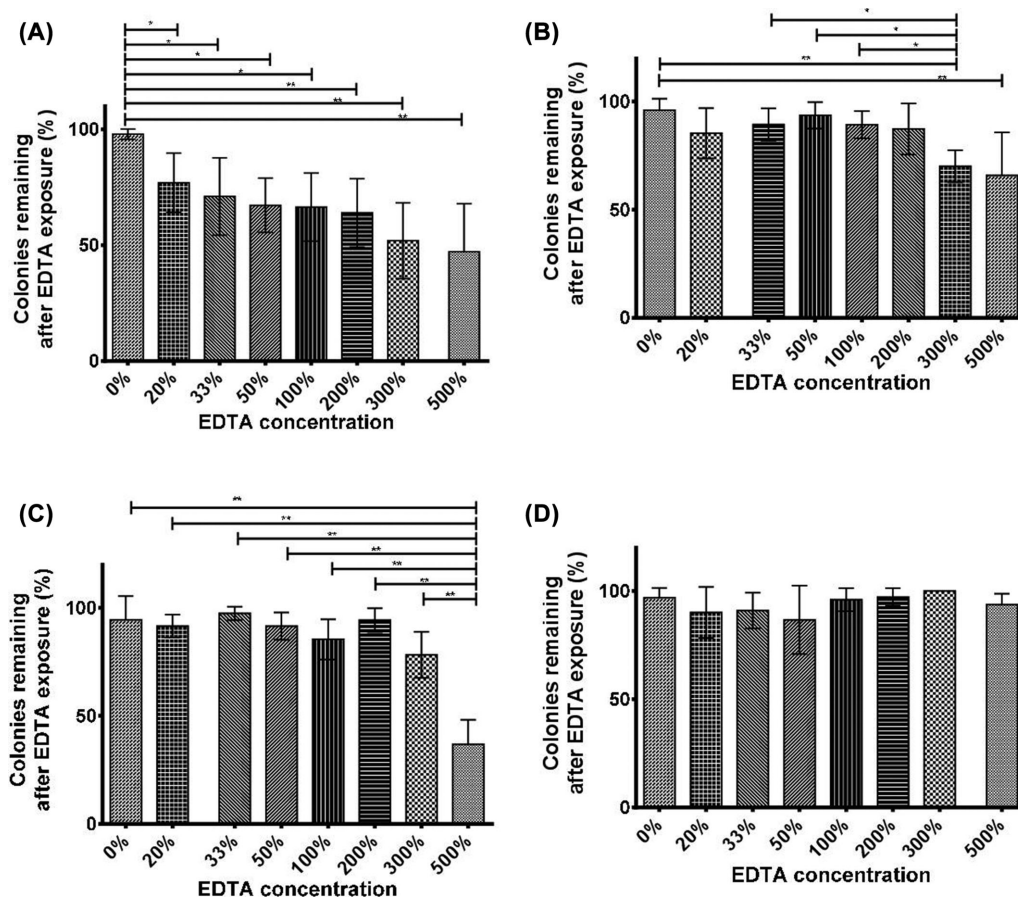


Figure 1. Impact of EDTA concentrations on yeast culture after 2 hours of contact. (A) Complete impact on *Candida albicans*. (B) Partial impact on azole-susceptible *Candida glabrata*. (C) Very partial impact on azole-resistant *Candida glabrata*. (D) Absence of impact on *Candida krusei*. *Represents significant association ($P < .05$) and **represents a significant association ($P < .01$) after multiple comparison test.

Statistical analysis

Student's *t* test, ANOVA, Bonferroni's post-tests and graphical representations were performed with GraphPad Prism software version 7.0. To limit the alpha risk inflation bias, appropriate statistical corrections were applied, if needed. A *P*-value of $< .05$ was considered as significant, and a *P*-value of $< .01$ as very significant.

Ethical statement

Clinical samples were collected for regular clinical management during hospital stay. No additional samples were taken for this study. Patient confidentiality was strictly protected. This study was approved by the ethical committee of Hospices Civils de Lyon, France, on August 1st, 2017.

Results

Differential EDTA inhibitory effect is associated with the level of azole-resistance

As summarized in Fig. 1, four levels of impact of EDTA on culture growth were observed depending on the azole-resistance

of the strains: (i) a full impact for *C. albicans* (mean decrease of 21.0%; $P < .01$ with a minor 20% EDTA concentration; Fig. 1A); (ii) a partial impact for the fluconazole-susceptible ATCC strain of *C. glabrata* (mean decrease of 29.0%; $P < .01$ at an EDTA concentration of at least 300%; Fig. 1B); (iii) a very partial effect for the fluconazole-resistant *C. glabrata* (mean decrease by 57.64%; $P < .01$ at the highest 500% EDTA concentration; Fig. 1C); and (iv) an absence of effect on the *C. krusei*, *C. lipolytica* and *C. inconspicua* strains ($P > .05$; Fig. 1D).

Increased EDTA effect with longer incubation period on susceptible strains

Three timings were observed and summarized in Fig. 2: (i) an immediate effect for *C. albicans* with a second decrease after 6 hours of contact (mean decrease by 26.1%; $P < .05$; Fig. 2A), (ii) a delayed effect on fluconazole-susceptible *C. glabrata* strain (mean decrease by 12.7% respectively; $P < .05$ after a period of 4 hours; Fig. 2B), and (iii) a very delayed effect for *Candida krusei* and fluconazole-resistant *C. glabrata* strains (mean decrease after a period of 7 hours by 50.1% and 27.1% respectively; $P < .05$; Fig. 2C and D).

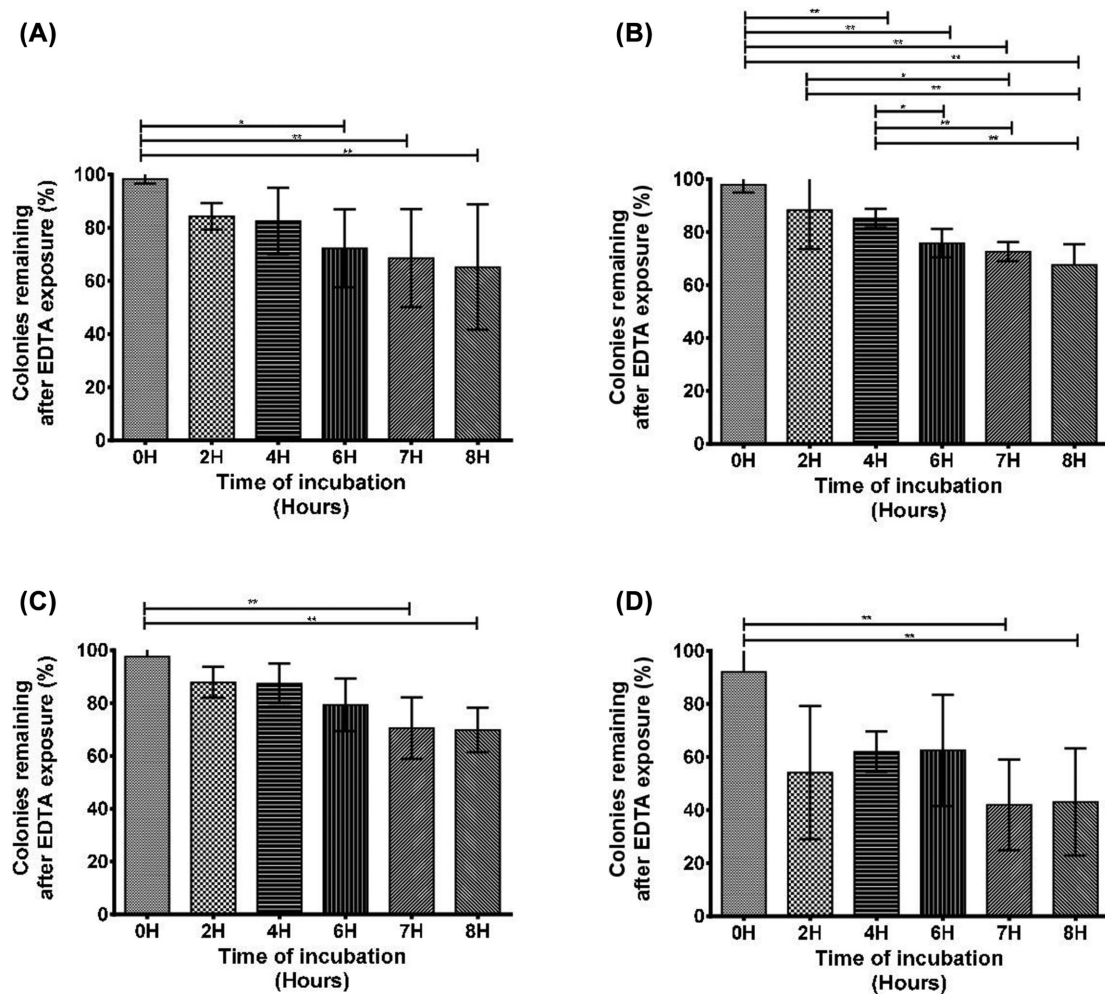


Figure 2. EDTA impact increases with incubation period. (A) Rapid impact on *Candida albicans*. (B) Delayed impact on azole-susceptible *Candida glabrata*. (C) Much delayed impact on *Candida krusei*. (D) Much delayed impact on azole-resistant *Candida glabrata*. *Represents significant association ($P < .05$) and **represents a very significant association ($P < .01$) after Bonferoni's multiple comparison test.

EDTA-like effect with sodium citrate but not with lithium heparinate

Candida cultures were inhibited by sodium citrate at similar EDTA concentrations as for *C. albicans* (mean decrease by 54.0% at a 20% concentration; $P < .01$) and for fluconazole-susceptible *C. glabrata* (mean decrease by 32.1% at a 100% concentration; $P < .01$; Fig. 3A). At high concentration (200%), an effect was observed for azole-resistant *C. glabrata* (mean decrease by 67.8%; $P < .05$; Fig. 3B). No effect was observed for *C. krusei* ($P > .05$; Fig. 3C) as for *C. inconspicua* or *C. lipolytica*.

In contrast with EDTA and sodium citrate, no association between lithium heparinate concentration and cultural growth was observed on any of all the tested strains ($P > .05$; Fig. 4).

Discussion

In this study, our own experience encouraged us to explore the impact of EDTA on yeast growth since several studies and on-

tology clinical practice showed impact of this compound on *Candida* or its subsequent infections.⁸ Moreover, microbiological evidence of impact of EDTA has already been demonstrated on bacteria.⁷

Herein we demonstrated that EDTA had an impact on *Candida* cultures that was dependent of the species. Indeed, effect was clear for CA whereas we observed only partial effect on CGS. Interestingly, in the tested strains, azole intrinsically resistant species showed no effect of EDTA on growth, whereas azole induced resistant species (CGR) showed a very partial effect restricted to very high EDTA concentrations (500%). The observed effect seems to display a plateau. Furthermore, the impact of EDTA seems to be determined by the incubation period: again, azole susceptible strains seem to be more impacted than resistant strains as CA is rapidly impacted (4 hours), whereas the impact on *C. krusei* is greatly delayed (7 hours).

Finally, to determine the specificity of the impact of EDTA on yeast growth, we studied two other compounds, sodium

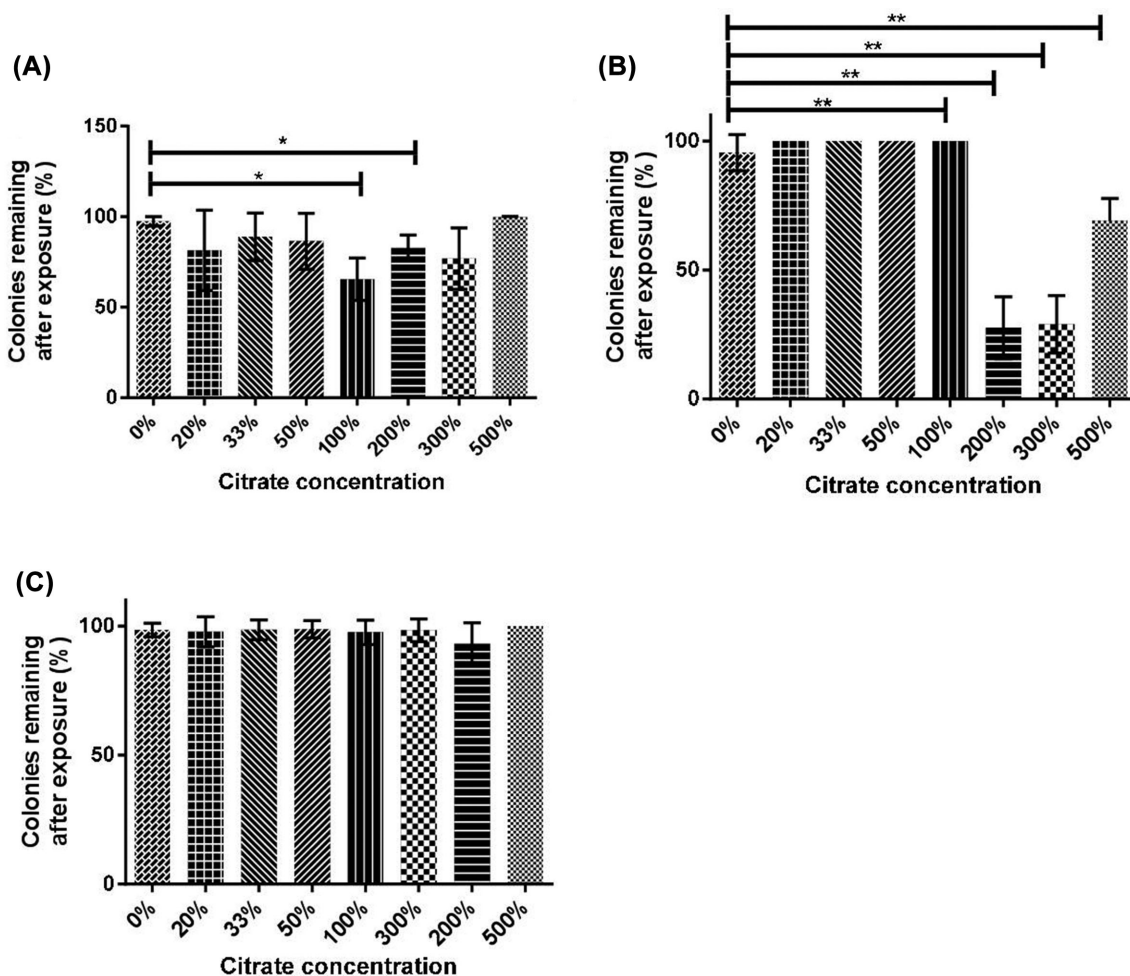


Figure 3. Inhibition effect existed with sodium citrate. (A) Azole-susceptible *Candida glabrata*; (B) azole-resistant *Candida glabrata*; (C) *Candida krusei*. * and ** represent significant associations ($P < .05$ and $P < .01$, respectively) after multiple comparison test.

citrate and lithium heparinate. Lithium heparinate had no significant effect whereas sodium citrate showed a species-dependent effect, being active against *C. albicans* and azole-susceptible *C. glabrata* at lower concentration but not against *C. krusei* nor azole-resistant *C. glabrata* (effect observed only at the highest concentration 500%).

Of interest is this correlation between fluconazole resistance and EDTA resistance. Partial or total azole resistance among *Candida* is intrinsic in several species, for example, *C. krusei*, *C. lipolytica*, *C. inconspicua*, *C. guilliermondii*.¹⁶ On the other hand, it is well-known that with the increased use of antifungal compounds, acquired resistance is on the rise alongside selection of naturally resistant species. Although rare and unlike bacteria, infrequently transferred from patient to patient, resistance in yeast remains a major problem since the therapeutic arsenal remains limited. Resistance to azoles is mainly due to the constitutive over-expression of two types of multidrug efflux pumps (encoded namely by the MDR1 and CDR1 genes). Furthermore, resistance (upregulation or altered binding site) might also be

due to mutation in the ERG11 gene encoding the azoles' target enzyme: the cytochrome P450 lanosterol 14 α -demethylase.¹⁷⁻¹⁹ In our case, the mechanisms of resistance to EDTA have not yet been identified. Consistent with these possible mechanisms of resistance, it can be hypothesized that efflux pumps might be responsible, but it has yet to be determined by targeted screening of these efflux pumps.

This study explored the quality of the mycological culture of a sample taken from a tube containing EDTA. Although certain analyses can be made using this type of sample, our study shows that the impact on cultures must be considered, to give reliable biological laboratory results. If the result of the culture is negative, it is important to consider the fungistatic effect and the potential fungicide effect from contact with EDTA, especially if the initial inoculum is low. Therefore, it is not advised to use EDTA tubes as containers for samples in mycological culture analyses.

Moreover, this correlation between EDTA- and azole-resistance could be used to help select resistant strains of yeast,

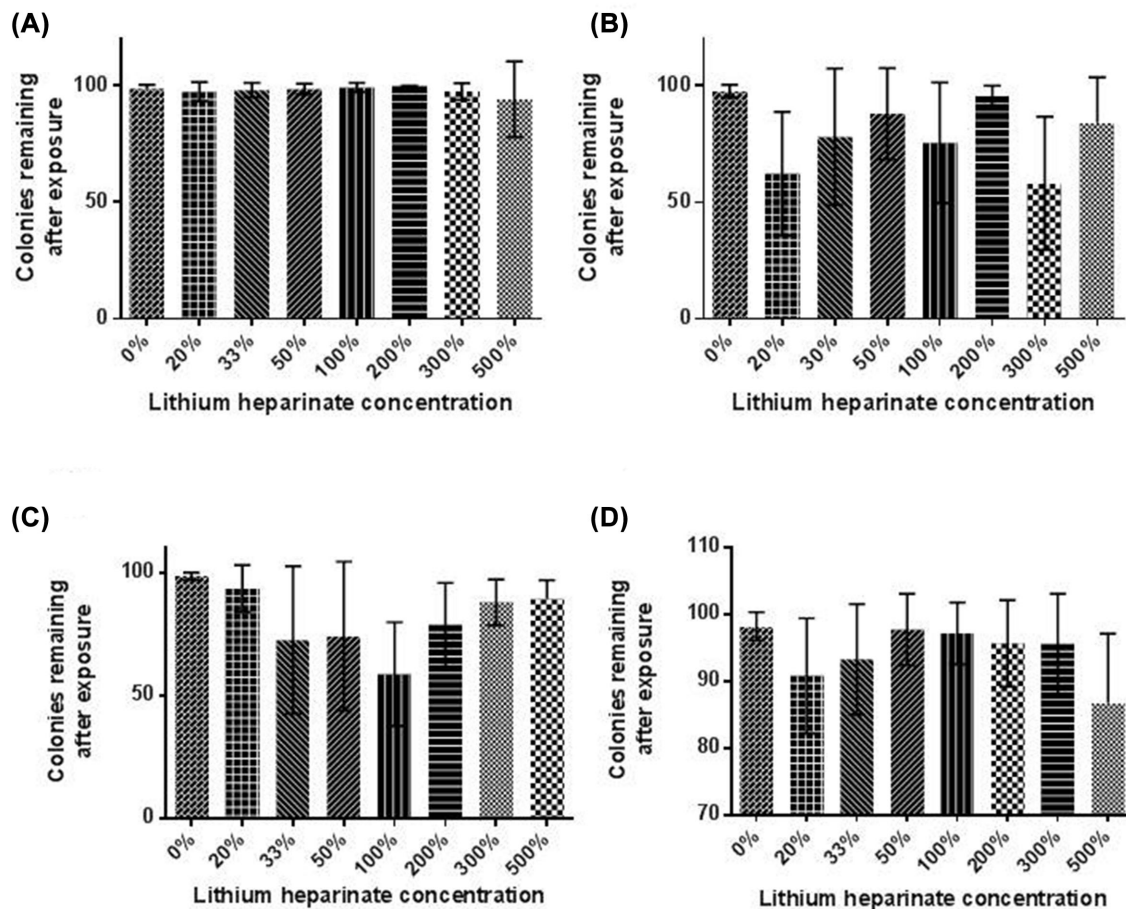


Figure 4. Lithium heparinate has no significant impact on culture. (A) *Candida albicans*; (B) azole-susceptible *Candida glabrata*; (C) azole-resistant *Candida glabrata*; (D) *Candida krusei*. No significant difference could be observed depending on the lithium heparinate concentration.

like the principle, currently used in bacteriology, of selective agar plates.

However, there are some limitations to this study. In fact, only three samples for each species were studied here. The results of this study were exploratory and further investigation is needed. It could be interesting to multiply these analyses by varying the source of the strains so that the implication of inter-individual variability may be limited.

When looking at the results of this study, it is important to consider other strains or species of *Candida*, as well as other species of pathogenic fungi, filamentous fungi especially (*Aspergillus*, Mucorales, Dermatophytes, *Histoplasma*, *Cryptococcus*, etc.). Different types of resistance mechanisms that have already been described (*Aspergillus fumigatus* and mutations of *cyp51*, TR34/L98H, etc.) must be kept in mind.

Apart from the implications on laboratory results, this path of research could improve clinical and therapeutic care of patients presenting candidemia and may provide solutions to infection of medical devices (prostheses, venous and urinary catheters, etc.) with suspected presence of biofilm, or any other infection that is resistant to usual treatment.

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Author contributions

D.D.: Study design, development and methodology; collection of data; data analysis and interpretation; writing all sections of the manuscript; manuscript revision. P.T.C.: Collection of data; manuscript revision. F.P.: Writing all sections of the manuscript; manuscript revision. J.M.: Data analysis and interpretation; writing all sections of the manuscript; manuscript revision. E.J.: Study design, development and methodology; collection of data. M.W.: Collection of data; manuscript revision. M.P.: Study design, development and methodology; collection of data; data analysis and interpretation; writing all sections of the manuscript; manuscript revision. All authors reviewed and approved the final version of the manuscript.

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Declaration of interest

J.M. has received a fee for speaking and a research grant outside the submitted work from MSD and helps for attending symposia from Gilead, MSD, Pfizer and Roche Diagnostics. The other authors (D.D., P.T.C. E.J., F.P., M.P., and M.W.) declare that there are no conflicts of interest regarding to this study.

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