Echinocandin susceptibility testing of *Candida* spp. using the EUCAST EDef 7.1 and CLSI M27-A3 standard procedures: Analysis of the influence of Bovine Serum Albumin Supplementation, Storage Time and Drug Lots.

Running Title: Echinocandin microdilution susceptibility testing

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Abstract

The MICs of the echinocandins against *Candida* isolates with *fks* mutations are higher than those of wild type (WT) isolates. However, the MIC ranges of susceptible and mutant populations overlap or are poorly separated. It was recently reported that a greater separation could be achieved in the presence of serum. To more fully explore this possibility, we compared the performance of the reference microdilution methods using standard and bovine serum albumin (BSA) supplemented growth medium.

Anidulafungin, caspofungin and micafungin MICs were determined according to the EUCAST and CLSI methods and with 50% BSA in the medium for 93 clinical isolates (no./no. of mutants): *C. albicans* (20/10), *C. glabrata* (19/10), *C. dubliniensis* (2/1), *C. krusei* (16/3), *C. parapsilosis* (19), and *C. tropicalis* (19/4). Stability of the plates was tested after storage at -80°C for two and six months, respectively, and performance of two different lots of caspofungin was investigated.

The addition of BSA to the medium resulted in higher MICs (1-9 2-fold dilution steps) for all isolates and compounds. The increase was greatest for anidulafungin and micafungin, and among WT isolates for *C. parapsilosis*. The number of very major errors (VME) was reduced (24% (20/84) vs. ≤7% (6/84)) using BSA supplemented EUCAST medium but not for the CLSI method (6% vs. 9%). MIC results were unchanged after six months storage of test plates. The two lots of caspofungin yielded identical results.

Addition of BSA to the EUCAST medium increases the ability to differentiate between WT isolates and isolates harbouring resistance mutations.

Introduction

Three echinocandin class drugs anidulafungin, caspofungin and micafungin are licensed for the treatment of invasive candidiasis. Following increased use, sporadic cases of failures associated with elevated MICs have been reported. In the majority of cases, these failures have been associated with mutations in two hot spot regions of *FKS* genes, which encode the target and major subunit of 1,3-ß-D-glucan synthase complex (4,6,18,21,22,28,29). Consequently, close monitoring and robust susceptibility testing methods have become increasingly important. For both CLSI and EUCAST reference methods the MICs of the three echinocandins against isolates with *fks* mutations are higher than those relative to wild type (WT) isolates, but the ranges of these susceptible and mutant populations either overlap one another or are separated by only 1-2 dilution steps making correct identification of hot spot mutant isolates challenging (5). As reproducibility as well as appropriate classification of susceptibility endpoints into S, I and R categories are highly correlated to the distance between MIC ranges for WT and mutant isolates, a modification of the reference methods achieving a better separation would be a major step forward.

Earlier studies demonstrated that the addition of human serum in MIC assays neutralizes differences between the in vitro properties of the echinocandin drugs (24,26,38). A recent preliminary study (14) reported that the addition of 50 mg/ml BSA to the growth medium leads to a better separation between WT and *fks* mutant isolates. We therefore undertook the present study to investigate in a systematic manner if the addition of 50 mg/ml BSA to the growth medium would alter the discriminatory potential of the CLSI and EUCAST microdilution methods. Furthermore, we evaluated the robustness of the assay by examining the stability of EUCAST susceptibility plates stored up to half a year at -80°C.

We previously reported that while microdilution testing for anidulafungin and caspofungin performed equally well with respect to separation between WT and *fks* mutant isolates, caspofungin MIC distributions appear to be variable due to unknown factors (4). For this reason,

we included two different lots of caspofungin pure substance in the study and retrospectively retrieved repeated MIC values for eight control strains representing different species.

Materials and Methods

Isolates: A well characterised set of WT and *fks* hot spot mutant isolates was used (5) including 93 clinical isolates and two reference strains (*C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258). Clinical isolates included 10 *FKS* WT and 10 *fks* hot spot mutant *C. albicans* isolates, 9 *FKS* WT and 10 *fks* hot spot mutant *C. glabrata* isolates, 1 *FKS* WT and 1 *fks* hot spot mutant *C. dubliniensis* isolates, 13 *FKS* hot spot WT and 3 *fks* hot spot mutant *C. krusei* isolates, 19 *FKS* WT *C. parapsilosis* isolates, and 15 *FKS* hot spot WT and 4 *fks* hot spot mutant *C. tropicalis* isolates. Three isolates were found to harbour mutations outside the resistance hot spots and were regarded as WT concerning echinocandin susceptibility because of their normal kinetic inhibition properties (David S Perlin personal communication). Thus, a total of 28 isolates with characteristic echinocandin resistance mutations in the *FKS* hot spot regions were included. All isolates were coded and tests performed blinded for susceptibility patterns.

Compounds: Pure substances were provided by the manufacturers (one lot of anidulafungin by Pfizer, two lots of caspofungin by Merck (TEK0010 and VEK0090, respectively) and one lot of micafungin by Astellas). Stock solutions were prepared in water (for CLSI testing) or in DMSO (Sigma) (for EUCAST testing) taking into account the potencies of the powders.

Retrospective comparison of caspofungin pure substance batches: Caspofungin MIC results were retrieved retrospectively for four different caspofungin lots (NEK0040, TEK0010, LEK0030 and an unnamed batch received from Merck) and the following reference strains used routinely as quality controls: *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019, *C. albicans* ATCC 64548, *C. tropicalis* CL-3412REX, *C. glabrata* ATCC 90030, *Saccharomyces cerevisiae* ATCC 9763 and *C. lusitaniae* CL-3408REX. The number of repetitions performed for these strains ranged between 7 and 21 times per caspofungin lot.

EUCAST microdilution: was performed strictly according to the standard (EDef 7.1 (35) and additionally using medium supplemented with BSA 50 mg/ml (BAH66-0500, Equitech Bio, Inc, Kerrville, Texas). Three hundred-fifty plates were prepared in one batch, sealed in aluminium foil

and stored at -80° C for two weeks, 2 month and 6 months before susceptibility testing was performed. Microtiter plates were read spectrophotometrically at 490 nm after 24 hours and MIC determined using 50% growth inhibition. *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality control strains through all experiments. In addition to the wild type upper limit values (see paragraph below) generated in the current study the following tentative EUCAST anidulafungin breakpoints were applied: *C. albicans* S ≤0.03 µg/ml, *C. glabrata, C. krusei* and *C. tropicalis* S ≤0.06 µg/ml.

CLSI microdilution was performed strictly according to the CLSI M27-A3 standard (9) and additionally using medium supplemented with BSA 50 mg/ml. Plates were stored at -86˚C for a maximum of 15 days before use. Micro-titre plates were read visually and the MIC determined using prominent inhibition (corresponding to 50%) as endpoint. *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality control strains through all experiments. In addition to upper limit values generated in the current study, the following revised echinocandin breakpoints for *C. albicans, C. glabrata, C. krusei* and *C. tropicalis* were applied: S: ≤0.25 µg/ml except for micafungin and *C. glabrata*: S: ≤0.06 µg/ml (CLSI meeting in Atlanta, the USA, June 2010, manuscript submitted).

***FKS* gene sequence analysis** was performed previously for all isolates as described (5).

Evaluation of test performance. For each of the drug-bug combinations, the following parameters were used to evaluate and compare the test performance: Distance between endpoint ranges (MIC) for *fks* hotspot mutant isolates and WT isolates was calculated as the number of 2 fold dilution steps. Negative values indicate the degree of overlap expressed as the number of dilution steps involved in the overlap. Overlap: defined as number of endpoints for the *fks* hotspot mutant isolates that overlapped with the endpoint range of the WT populations; WT-UL (WT upper limit values): defined as two 2-fold dilution steps higher than the $MIC_{50.}$ If the population was truncated with all MIC values at or below the lowest concentration tested, the WT-UL was defined as two times the lowest dilution tested; VME (very major errors): Number of *fks* hotspot mutant

126 isolates with MIC lower or equal to the WT-UL. Finally, the number of very major errors was
127 evaluated applying the tentative EUCAST and revised CLSI breakpoints for susceptibility as
128 mentioned above.

Results

EUCAST microdilution testing performed according to the EUCAST Edef 7.1 but using medium supplemented with 50 mg/ml BSA resulted in higher MIC values for all drug bug combinations (Fig 1). Overall, WT and *fks* hot spot mutant populations were separated by 1 to 8 dilution steps and no overlap were observed between WT and *fks* hot spot mutant isolates for any of the drug- *Candida* spp. combinations (micafungin MICs for *C. krusei* extended above the tested range for WT as well as mutant populations, and thus could not be evaluated for potential overlap). Using the WT-UL as a breakpoint for susceptibility one (4%) VME was observed for anidulafungin (1/3 *C. krusei* with a heterozygous F655F/C substitution in Fks1p), none for caspofungin and 2 (7%) VMEs were observed for micafungin (2/11 *C. glabrata* with a D632G and a D666E in Fks1p and Fks2p substitutions, respectively) (Table 1).

In comparison, WT and *fks* hot spot mutant MIC populations were separated by -1 to 6 dilution steps using the reference EUCAST method with the MIC range of *C. krusei* overlapping with the WT population for anidulafungin and caspofungin (in both cases involving the isolate with a F655F/C substitution at Fks1p) (Fig 1). For 4 drug-bug combinations, potential overlap could not be excluded as MICs for the WT as well as *fks* mutant isolates were below the lowest dilution tested (anidulafungin and *C. albicans* and micafungin and *C. albicans, C. glabrata* and *C. tropicalis*, respectively). Using the WT-UL as breakpoint for susceptibility, four VMEs were observed for the reference method: all three echinocandins and 1/3 *C. krusei* with the F655F/C substitution at Fks1p and caspofungin and 1/4 *C. tropicalis* with a F76S substitution at Fks1p (Table 1). Finally, applying the tentative EUCAST breakpoints for anidulafungin 3/28 (11%) VMEs were observed.

CLSI microdilution testing. The influence of using BSA supplemented growth medium was less uniform for the CLSI method. For *C. albicans* no overlap or misclassifications applying the WT-ULs were observed and WT and *fks* mutant populations were separated by four, four and two 2-fold dilution steps for anidulafungin, caspofungin and micafungin, respectively, in contrast to two, at least four and two dilution steps for the reference method (Fig 2). For the other species, overlap

155 was observed for anidulafungin (1 *C. krusei*, F655F/C) and micafungin (2 *C. glabrata*, Fks1p-
156 D632G and Fks2p-F659V, 1 *C. krusei*, F655F/C and 1 *C. tropicalis*, F76S, respectively) using BSA
157 supplemented medium, and for anidulafungin (1 *C. glabrata*, Fks2p-P667T) and micafungin (4 *C.*
158 *glabrata* isolates, Fks2p-P667T, D632G, Fks2p-F659V, and Fks2p-D666G, respectively) using the
159 reference medium. Using the WT-UL as breakpoint for susceptibility, seven VMEs were observed
160 for the BSA modified test as compared to five for the reference method (for two *C. krusei* with MIC
161 of >16 µg/ml, potential VME could not be evaluated, Table 1). Most VMEs involved micafungin (no.
162 5) and *C. glabrata* (no. 3). Notably, no VMEs were observed for caspofungin and any of the
163 species or CLSI based methods (Table 1). Finally, applying the recently revised CLSI breakpoints
164 for echinocandins, six VMEs were observed for anidulafungin (22%), three for caspofungin (11%)
165 and eight for micafungin (30%) (Table 1).

166 ***C. parapsilosis.*** The EUCAST MIC_{50} (1 µg/ml) and WT-ULs (4 µg/ml) for *C. parapsilosis* and all
167 three echinocandins were considerably higher than for the other species. Addition of 50 mg/ml of
168 BSA elevated the MIC ranges particularly for anidulafungin and micafungin (MIC_{50} ≥ 32 µg/ml) (Fig
169 1). The same was true for the CLSI based methods as MIC_{50} values for anidulafungin, caspofungin
170 and micafungin were 1, 0.25 and 1 µg/ml for the standard method and 16, 2 and >32 µg/ml, for the
171 BSA modified method, respectively (Fig. 2).

172 **Stability of plates stored at -80 °C.** Storage of EUCAST plates with and without BSA for up to 6
173 months did not affect performance of the susceptibility plates for any of the three echinocandins or
174 any of the two media (Table 2). Overall, 95.4% of the results were either identical (69.2%) or within
175 +/- one dilution step after 2 months of storage and the same was true for 94.6% (65.5%) after 6
176 months, and at both time points with an equal distribution above and below the mean of the few
177 isolates differing two or more 2-fold dilutions (Table 2).

178 **Batch to batch variation of caspofungin.** A retrospective compilation of MIC values for eight
179 reference strains and four lots of caspofungin is shown in Fig 3. One lot, TEK0010, consistently
180 yielded higher modal MICs for all eight reference strains. The difference was less pronounced for

181 *C. parapsilosis* ATCC22019 and *C. krusei* ATCC6258 than for the two *C. albicans* control strains.
182 The 93 isolates were tested against the two available and most recently produced caspofungin
183 batches TEK0010 and VEK0090. The MICs were in 100% agreement within ±2 MIC steps and with
184 identical MICs for the vast majority of the isolates (Table 2).
185

Discussion

A direct comparison MIC values obtained by EUCAST and CLSI methods with and without supplementation of BSA was used to evaluate the discriminatory potential for identifying *fks* hot spot mutant from WT isolates and then to compare the performance of the individual tests with respect to this parameter expressed as distance between the populations, number of overlaps and VMEs according to WT upper limits and proposed breakpoints.

BSA supplementation of the growth medium improved the discriminatory potential of the EUCAST test in terms of better separation of the WT and *fks* mutant populations leading to fewer overlaps and *fks* mutants classified as susceptible when applying a WT upper limit as breakpoint for susceptibility. The better separation was due to a more pronounced increase in MICs for *fks* mutants than for WT isolates. The distance between the WT population of each species and the individual mutants depended on the location of the mutation. Thus, the echinocandin MICs were consistently lower for one *C. krusei* mutant leading to this isolate being misclassified by the majority of tests. This isolate had a heterozygous substitution at Fks1p at the position F655 and may represent a less resistant phenotype. For *C. albicans* substitutions at Fks1p-D648 and Fks1p-P649 were associated with the lowest MICs and similarly *C. glabrata* isolates with amino acid substitutions at position Fks1p-D632 and Fks2p-S663 and higher were the least resistant mutants *in vitro* when tested with and without BSA in the medium. This is in agreement with difference in susceptibility of the glucan synthase enzyme complex it self dependent on the position of the amino acid substitution (13). It remains to be assessed if these *in vitro* observations translate into a differential susceptibility *in vivo* and thus if that the challenges associated with the discrimination of these isolates from the wild type isolates by in vitro susceptibility testing is less crucial.

The poorest separation between WT and *fks* hot spot mutants was observed for micafungin and *C. glabrata* and although EUCAST testing with BSA eliminated overlap between the two populations, VMEs were still observed. Hypothetically, the more discrete micafungin MIC elevation may reflect a better activity compared to that of the other two echinocandins against *fks* hot spot mutants of *C.*

212 *glabrata*. However, *C. glabrata* breakthrough infection during micafungin therapy was recently
213 been described involving isolates with mutations at Fks1p-S629 or at Fks2p-S663 (34). In that
214 study the CLSI MICs of mutant isolates were 4-8 µg/ml and thus higher than those seen for similar
215 *fks1* and *fks2* mutants in our study. Thus, the *in vivo* activity of micafungin in comparison with the
216 others for the treatment of *C. glabrata fks* hot spot mutants deserves further study.

217 CLSI testing with BSA supplemented medium performed less well than expected for the species
218 other than *C. albicans.* This is in contrast with the initial findings in the previous pilot study that
219 prompted this investigation (14). The reason behind the apparent differential impact on the different
220 methods and species is not understood. But it may partially reflect the fact that the tests were
221 performed in different laboratories. One limitation associated with this study is, however, that the
222 echinocandin concentration range in this study resulted in truncation of some of the distributions.
223 Future studies with wider concentration ranges are needed to explore if the performance of CLSI
224 testing with BSA supplemented medium is underestimated in our study.

225 The MICs of all three candins were higher for *C. parapsilosis* than for the other species, as
226 previously described (10,25,31,33). *C. parapsilosis* has a naturally occurring mutation at *FKS1* hot
227 spot 1 accounting for the elevated MIC levels (12). However, the overall clinical response for
228 invasive *C. parapsilosis* infections treated with echinocandins is comparable to that for the other
229 species (19,23,27,33), which may in part be due to *C. parapsilosis* being less virulent (2,3,15-
230 17,37). Interestingly, the MIC increase for anidulafungin and micafungin when tested in presence
231 of BSA was more pronounced than for caspofungin leading to MIC values notably higher than the
232 peak concentrations obtained during treatment. This observation might suggest these two
233 compounds could be less active against *C. parapsilosis* infections. So far, one study has compared
234 the clinical outcome of two echinocandins head to head without demonstrating a difference in
235 outcome between micafungin and caspofungin for *C. parapsilosis* (27). Thus, the potential
236 implications of the observed differences in MICs in presence of BSA remain uncertain.

We have previously reported variability associated with caspofungin susceptibility testing despite tests being performed in reference laboratories and correcting for potency, and that caspofungin values obtained following the EUCAST methodology are typically higher than when following the CLSI standard (4,5). Here we demonstrate that the variability is more pronounced for the *C. albicans* reference strains than for the most frequently used control strains *C. krusei* ATCC6258 and *C. parapsilosis* ATCC22019. This observation is in line with the fact that variability between MIC ranges is most pronounced for species other than *C. parapsilosis* when comparing MIC ranges reported in different studies (1,4,7,8,20,30-33) and indicate that variability may go un-noticed when *C. krusei* ATCC6258 or *C. parapsilosis* ATCC22019 are used as quality control strains. One hypothesis has been that the higher glucose concentration in the EUCAST medium might affect potency particularly if the plates were not used immediately. We found no change in MIC results after up to 6 months of storage and a head to head comparison with 0.2 and 2% glucose in the growth medium was performed recently without detecting any difference between the two (36). Another possibility is that potency may be affected by the choice of solvent for stock solutions of caspofungin. Water is recommended by both EUCAST and CLSI standards. Yet, from a chemical point of view, the substance is more soluble in DMSO. In this study, we substituted water with DMSO for the stock preparation of caspofungin for the EUCAST plates and obtained systematically lower MIC values than when running the same strain collection in a previous study using water as solvent (5).

In conclusion, this study demonstrates for the first time that addition of BSA to the test medium improves the ability of the EUCAST reference method to separate *fks* hot spot mutants from WT isolates, that these susceptibility plates are stable up to 6 month of storage and that variability of caspofungin MIC values is observed when systematically comparing different lots of caspofungin. For the CLSI method, however, a benefit of adding BSA to the medium was only seen for *C. albicans*, and correct classification of *fks* hot spot mutants still is a challenging even after the revision of the CLSI echinocandin breakpoints. Therefore, mutational analysis is currently the most

precise way to detect echinocandin resistance. However, this approach may not yet be applicable for every isolate and routine laboratory and has the limitation of not detecting resistance due to other resistance mechanisms should such exist.

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Potential conflicts of interest:

M.C.A. has been a consultant for Astellas, Merck, Pfizer, and SpePharm, been an invited speaker for Astellas, Cephalon, Merck Sharp & Dohme, Pfizer, Schering-Plough, and Swedish Orphan and has received research funding for this particular study from Astellas, Merck and Pfizer.

D.S.P. is shareholder in Merck, has acted as a consultant for Merck, Pfizer, and Astellas, is advisory board member for Merck, Pfizer, Astellas, and Myconostica, have the following patent: Assays for resistance to echinocandin-class drugs Application: 07763-O69WO1, have received research funding although not for this particular study from Merck, Pfizer, Astellas, and Myconostica, and has been invited speaker Merck, Pfizer, Astellas, and Myconostica.

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447

448 **Legends for Figures**

449 **Fig. 1.** MIC distributions obtained following EUCAST methodology but supplementing the medium
450 with 50% BSA (above the x-axis) in comparison with the standard procedure (below the x-axis) for
451 susceptible isolates (white bars) and *FKS* mutant isolates (black bars). (*C. dubliniensis* isolates are
452 indicated in dotted bars). MICs for anidulafungin is shown in panel), for caspofungin in panel b and
453 for micafungin in panel c.

454

455 **Fig. 2.** MIC distributions obtained following CLSI methodology but supplementing the medium with
456 50% BSA (above the x-axis) in comparison with the standard procedure (below the x-axis) for
457 susceptible isolates (white bars) and *FKS* mutant isolates (black bars). (*C. dubliniensis* isolates are
458 indicated in dotted bars). MICs for anidulafungin is shown in panel), for caspofungin in panel b and
459 for micafungin in panel c.

460

461 **Fig 3.** MIC variation among four different lots of caspofungin. Eight reference strains were tested
462 according to the EUCAST methodology and using the following four different lots of caspofungin:
463 NEK0040 (white bars, seven repetitions), TEK0010 (black bars, 6 repetitions, the batch also used
464 in this study), LEK0030 (stripped bars, 18 repetitions) and Unknown (checkerboard, 21 repetitions).

465

466

Table 1. Upper limit values for WT and hot spot mutant isolates and no. of very major errors (VMEs) by antifungal compound and species using the EUCAST and CLSI methods with and without BSA.

	EUCAST					CLSI				
	WT-UL-Ref	WT-UL-BSA	VME-UL-Ref	VME-UL-BSA	VME-EUCAST	WT-UL-Ref	WT-UL-BSA	VME-UL-Ref	VME-UL-BSA	VME-CLSI
Anidulafungin	na	na	4/28	1/28	3/28	na	na	1/27	2/27	6/27
C. albicans	0.06	0.25	3/10	0/10	2/10	0.03	0.25	0/10	0/10	2/10
C. dubliniensis	0.06	2	0/1	0/1	na	0.03	0.5	0/1	0/1	0/1
C. glabrata	0.06	4	0/10	0/10	0/10	0.125	2	1/9	1/9	3/9
C. krusei	0.125	8	1/3	1/3	1/3	0.25	4	0/3	1/3	0/3
C. parapsilosis	4	>64	na	na	na	4	64	na	na	na
C. tropicalis	0.06	2	0/4	0/4	0/4	0.03	0.5	0/4	0/4	1/4
Caspofungin	na	na	2/28	0/28	na	na	na	0/27	0/27	3/27
C. albicans	0.25	0.25	0/10	0/10	na	0.03	0.25	0/10	0/10	1/10
C. dubliniensis	0.25	1	0/1	0/1	na	0.03	0.25	0/1	0/1	0/1
C. glabrata	0.25	1	0/10	0/11	na	0.125	1	0/9	0/9	1/9
C. krusei	1	4	1/3	0/3	na	0.5	4	0/3	0/3	0/3
C. parapsilosis	4	≥64	na	na	na	1	8	na	na	na
C. tropicalis	0.5	1	1/4	0/4	na	0.06	0.5	0/4	0/4	1/4
Micafungin	na	na	14/28	2-5/28	na	na	na	4/27	≥5/27	7/27
C. albicans	0.06	4	4/10	0/10	na	0.03	2	0/10	0/10	2/10
C. dubliniensis	0.06	16	0/1	0/1	na	0.25	2	0/1	0/1	0/1
C. glabrata	0.06	4	8/10	2/11	na	0.06	4	4/9	3/9	4/9
C. krusei	0.125	64	1/3	≤3/3	na	0.5	32	0/3	≥1/3	0/3
C. parapsilosis	4	≥64	na	na	na	4	>32	na	na	na
C. tropicalis	0.06	16	1/4	0/4	na	0.125	8	0/4	1/4	1/4
In total	na	na	24%	4-7%	11%	na	na	6%	9%	20%

WT-UL: wild-type upper limit values, Ref: reference method, BSA: testing with BSA supplemented medium, VME: Very Major Errors, na: not applicable/available, VME-EUCAST: very major errors applying tentative EUCAST breakpoints (*C. albicans* S: ≤0.03 µg/ml, *C. glabrata, C. krusei* and *C. tropicalis* S: ≤0.06 µg/ml), VME-CLSI: very major errors applying revised CLSI breakpoints.

Table 2. The influence of storage of prepared susceptibility plates on susceptibility results and a comparison of two different lots of caspofungin pure substance. Susceptibility plates were stored for 2 and 6 months, respectively, and MIC results compared with results obtained after less that one week of freezing. For each test and compound the number of isolates with identical, lower or higher MIC results is given.

Method	**MIC change (no. of 2-fold dilutions)**						
	-3	-2	-1	Identical	1	2	3
Two months storage							
EUCAST Edef 7.1							
Anidulafungin			7	69	12		
Caspofungin TEK0010		4	20	51	18	1	
Caspofungin VEK 0090		4	20	50	13	1	
Micafungin		4	5	68	9	5	
BSA modified EUCAST							
Anidulafungin			10	70	9		
Caspofungin TEK0010		2	11	60	13	2	1
Caspofungin VEK 0090		2	9	63	11	3	1
Micafungin	1	1	11	66	10	1	
Six months storage							
EUCAST Edef 7.1							
Anidulafungin			11	68	8	1	
Caspofungin TEK0010		3	19	50	20	3	
Caspofungin VEK 0090		5	16	40	26	2	
Micafungin		1	8	66	12	2	
BSA modified EUCAST							
Anidulafungin		2	6	62	16	2	1
Caspofungin TEK0010	3	2	18	57	8	1	
Caspofungin VEK 0090	2	4	10	61	10	2	
Micafungin	1	1	11	66	10	1	
Two lots of caspofungin							
EUCAST Edef 7.1		1	11	64	13		
BSA modified EUCAST			8	47	7		

Fig. 1. MIC distributions obtained following EUCAST methodology but supplementing the medium with 50% BSA (above the x-axis) in comparison with the standard procedure (below the x-axis) for susceptible isolates (white bars) and *FKS* mutant isolates (black bars). (*C. dubliniensis* isolates are indicated in dotted bars). MICs for anidulafungin is shown in panel), for caspofungin in panel b and for micafungin in panel c.

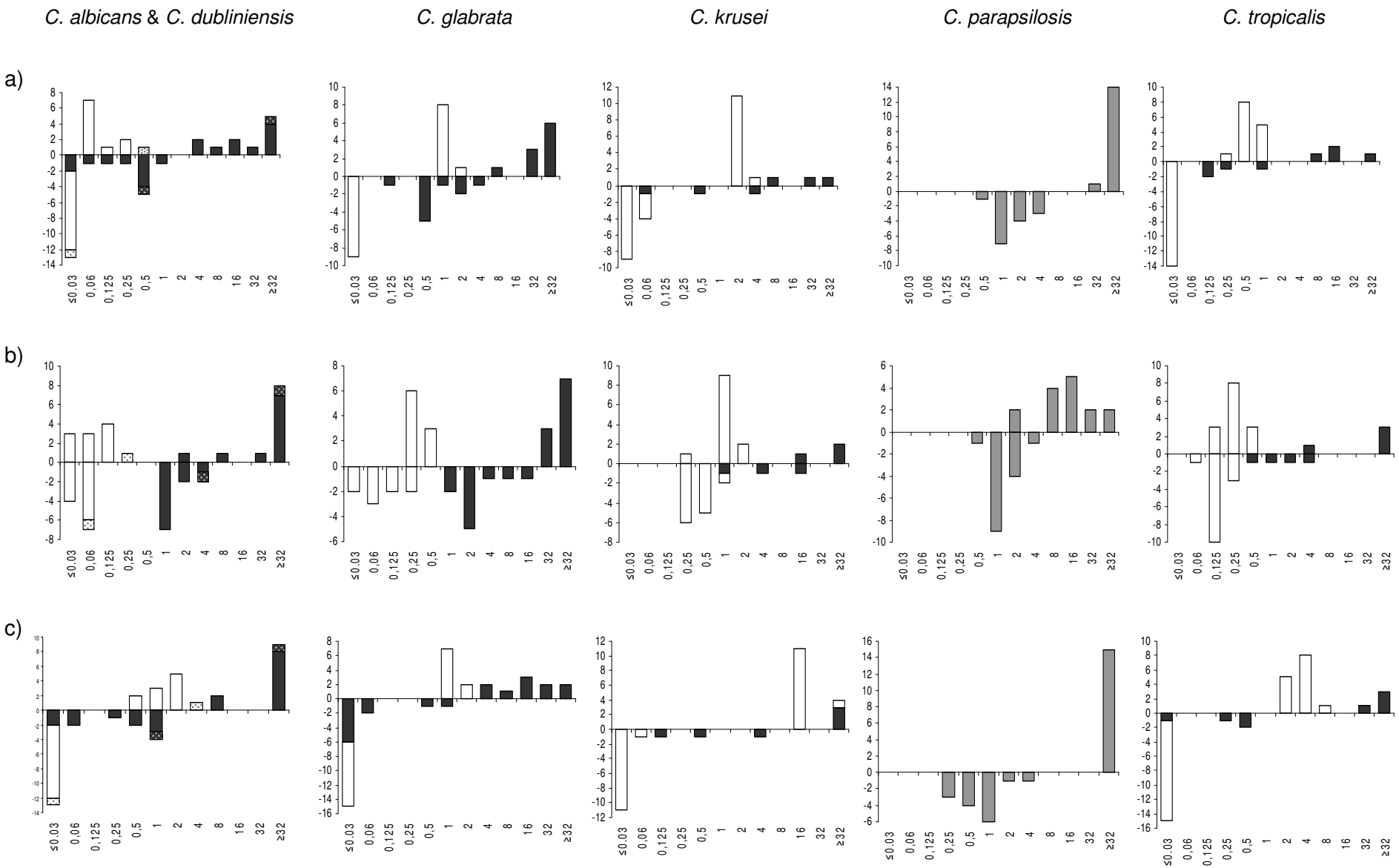

Fig. 2. MIC distributions obtained following CLSI methodology but supplementing the medium with 50% BSA (above the x-axis) in comparison with the standard procedure (below the x-axis) for susceptible isolates (white bars) and *FKS* mutant isolates (black bars). (*C. dubliniensis* isolates are indicated in dotted bars). MICs for anidulafungin is shown in panel), for caspofungin in panel b and for micafungin in panel c.

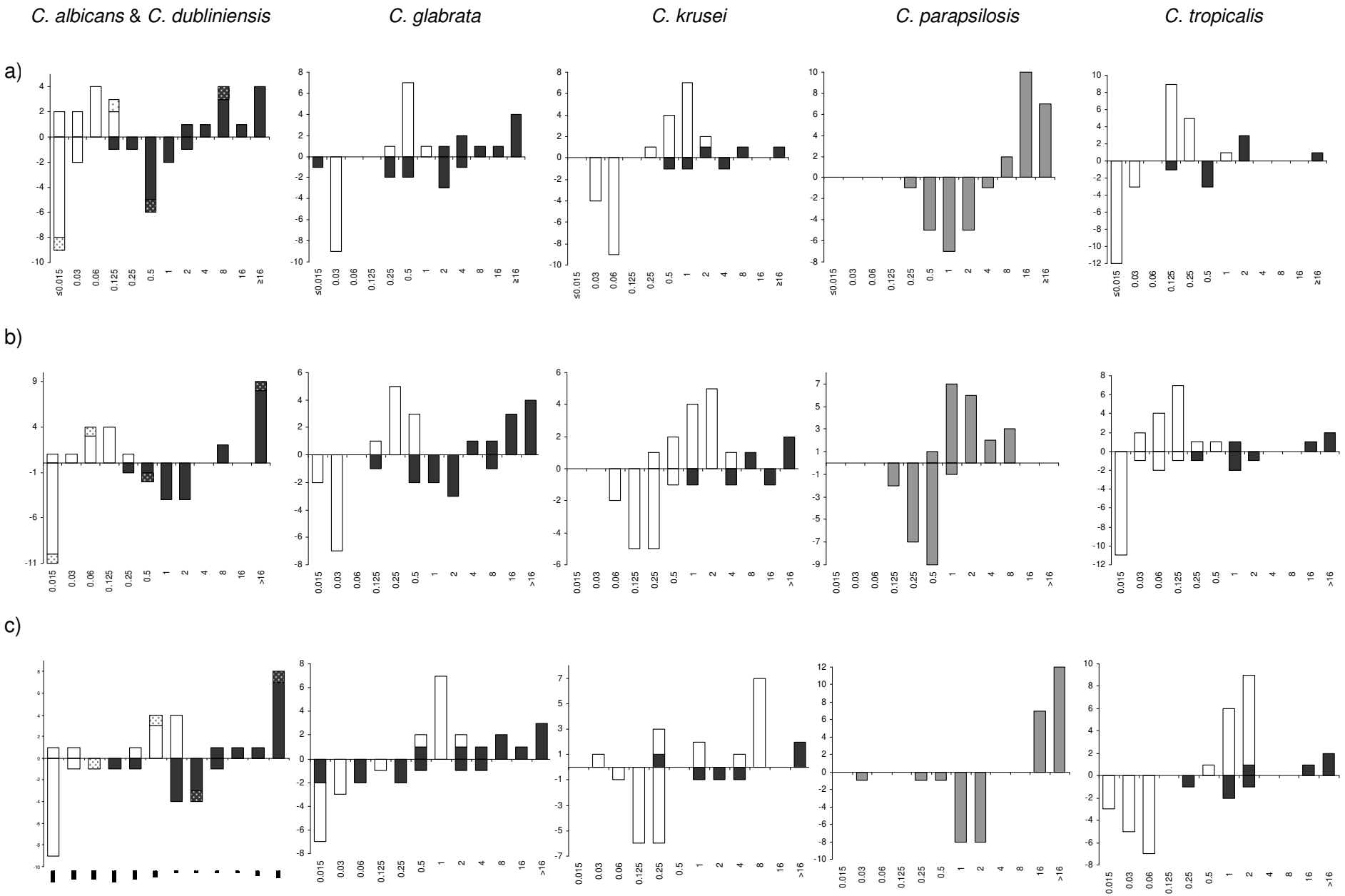

Fig 3. MIC variation among four different lots of caspofungin. Eight reference strains were tested according to the EUCAST methodology and using the following four different lots of caspofungin: NEK0040 (white bars, seven repetitions), TEK0010 (black bars, 6 repetitions, the batch also used in this study), LEK0030 (stripped bars, 18 repetitions) and Unknown (checkerboard, 21 repetitions).