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Improved Detection of *Candida* sp. *fks* Hot Spot Mutants by Using the Method of the CLSI M27-A3 Document with the Addition of Bovine Serum Albumin▽†

AQ: A

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Echinocandins are highly bound to serum proteins, altering their antifungal properties. The addition of 50% human serum to the MIC assay improves the identification of echinocandin-resistant *Candida* spp. harboring *fks* hot spot mutations. However, this modification cannot readily be applied to the method of the CLSI M27-A3 document due to safety and standardization difficulties. The aim of this study was to evaluate commercial bovine serum albumin (BSA) as a safe and standardized alternative to human serum. A collection of 28 echinocandin-susceptible strains, 10 *Candida parapsilosis* sensu lato strains (with naturally reduced echinocandin susceptibility), and 40 *FKS* hot spot mutants was used in this work. When RPMI 1640 was used for susceptibility testing, wild-type strains and *fks* mutants showed MIC range overlaps (−2, −1, and −3 2-fold-dilution steps separated these populations for anidulafungin, caspofungin, and micafungin, respectively). On the other hand, the addition of BSA to RPMI 1640 differentially increased echinocandin MIC values for these groups of strains, allowing better separation between populations, with no MIC range overlaps for any of the echinocandin drugs tested. Moreover, the use of RPMI-BSA reduced the number of *fks* hot spot mutant isolates for which MIC values were less than or equal to the upper limit for the wild type (very major errors) from 9, 2, and 7 with RPMI alone to 3, 0, and 3 for anidulafungin, caspofungin, and micafungin, respectively. When RPMI-BSA was used to study the susceptibility of *C. parapsilosis* sensu lato species to echinocandins, the strains behaved as anidulafungin- and micafungin-resistant isolates (MIC, ≥8 μg/ml). These data support the need for a revision of the CLSI protocol for *in vitro* testing of echinocandin susceptibility in order to identify all or most of the *fks* hot spot mutants. Also, caspofungin could be used as a surrogate marker of reduced susceptibility to echinocandins.

Fn2

Echinocandin drugs inhibit the 1,3-β-D-glucan synthase complex (EC 2.4.1.34) (GS), which catalyzes the biosynthesis of 1,3-β-D-glucan, the major glucan component of fungal cell walls. GS is an enzyme complex with at least two subunits, Fksp and Rho1p. Fksp, encoded by three related genes, *FKS1*, *FKS2*, and *FKS3*, is the catalytic subunit, and it is the target of the echinocandin drugs. Echinocandin resistance resulting in clinical failure has been linked to dominant mutations in the Fksp subunit of GS (27). These amino acid substitutions have been mapped onto two conserved regions of Fks1p (*Candida* spp.) and Fks2p (in *Candida glabrata* only) (13–16, 19, 27) named hot spot regions (e.g., *C. albicans* Fks1p hot spot 1 [Phe-641 to Pro-649] and hot spot 2 [Asp-1357 to Leu-1364]) (26). Recently, the CLSI Antifungal Subcommittee established a MIC of ≤2 μg/ml as an interpretative MIC breakpoint for the susceptibility of *Candida* spp. to the three echinocandin drugs (7, 33). However, it has been demonstrated that the CLSI breakpoints are not able to distinguish all the echinocandin-resistant *fks* mutants from wild-type (WT) isolates (2, 15, 16), which led us to propose new interpretative MIC breakpoints for the echinocandins against *Candida* species (for echinocandins against *C. albicans*, *C. tropicalis*, and *C. krusei*, ≤0.25 μg/ml for susceptibility, 0.5 μg/ml for intermediacy, and ≥1.00 μg/ml for resistance; for anidulafungin [ANF] and caspofungin [CSF] against *C. glabrata*, ≤0.25 μg/ml for susceptibility, 0.50 μg/ml for intermediacy, and ≥1.00 μg/ml for resistance; for micafungin [MCF] against *C. glabrata*, ≤0.06 μg/ml for susceptibility, 0.12 μg/ml for intermediacy, and ≥0.25 μg/ml for resistance; and for echinocandins against *C. parapsilosis* and *C. guilliermondii*, ≤2.00 μg/ml for susceptibility, 4.00 μg/ml for intermediacy, and ≥8.00 μg/ml for resistance) (27). In order to improve the detection of *fks* hot spot mutants with the CLSI susceptibility breakpoint, different options have been proposed: (i) reduce the ANF and MCF susceptibility breakpoints for *C. albicans* and *C. glabrata* (15, 16), (ii) use CSF as a surrogate marker for echinocandin cross-resistance and as the agent for the detection of *fks* hot spot mutations (5, 15, 16), (iii) identify *fks* hot spot mutants by testing for susceptibility to ANF with a susceptibility breakpoint defined as two 2-fold dilutions higher than the MIC_{50} (MIC at which 50% of isolates are inhibited) for the wild-type population of each *Candida* species (2), or (iv) add 50% human serum to the MIC assay medium in order to improve the identification of echinocandin-resistant *Candida* spp. harboring *FKS* mutations (15, 16). The last proposal more closely simulates the physiochemical properties of the active drug following intravenous administration, but it is difficult to apply in a clinical assay due to safety and standardization difficulties associated with human serum.

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The aim of this study was to overcome this issue by evaluating commercial fatty-acid-free bovine serum albumin (BSA) as a safe and standardized alternative to human serum for better discrimination between susceptible strains and resistant clinical isolates harboring *fks* mutations.

MATERIALS AND METHODS

Organisms. A collection of 78 *Candida* sp. strains was used in this study, including: 28 echinocandin-susceptible strains (14 *C. albicans*, 6 *C. glabrata*, 4 *C. tropicalis*, and 4 *C. krusei* strains), 10 isolates showing a naturally occurring amino acid substitution in Fks1p linked with reduced echinocandin susceptibility (RES) (13) (6 *C. parapsilosis*, 2 *C. orthopsilosis*, and 2 *C. metapsilosis* isolates), and 40 *FKS* hot spot mutants isolated after or during echinocandin therapy (20 *C. albicans*, 14 *C. glabrata*, 4 *C. tropicalis*, and 2 *C. krusei* isolates). The echinocandin-susceptible and RES strains used in this work were control strains ($n = 7$) (*C. albicans* ATCC 90028, ATCC 36082, and SC5314; *C. glabrata* ATCC 90030; *C. tropicalis* ATCC 750; *C. krusei* ATCC 6258; and *C. parapsilosis* ATCC 22019) or clinical isolates used previously by our group ($n = 21$) (2, 6, 12–16). The *FKS* hot spot mutants included in this work have been studied in several previous reports (2, 6, 13–16, 35, 41). AQ: B

The isogenicity of the *C. albicans* and *C. glabrata* strains was determined using the multilocus sequence typing (MLST) methods described by Robles et al. (37), Tavanti et al. (39), and Dodgson et al. (10).

***FKS* gene sequence analysis.** *Candida* sp. genomic DNA was extracted from yeast cells grown overnight in YPD (2% yeast extract, 4% Bacto peptone, 4% dextrose) broth with a Qbiogene (Irvine, CA) FastDNA kit. PCR and sequencing primers were designed based on the sequences of the following genes: *C. albicans FKS1* (GenBank accession no. XM_716336), *C. glabrata FKS1* and *FKS2* (GenBank accession no. XM_446406 and XM_448401, respectively), *C. krusei FKS1* (accession no. EF426563), *C. metapsilosis FKS1* (accession no. EU350514), *C. orthopsilosis FKS1* (accession no. EU350513), *C. parapsilosis FKS1* (accession no. EU221325), and *C. tropicalis FKS1* (accession no. EU676168). Primer sequences are listed in Table S1 in the supplemental material. DNA sequencing was performed with a CEQ Dye Terminator Cycle Sequencing Quick Start kit (Beckman Coulter, Fullerton, CA) according to the manufacturer's recommendations. Sequence analysis was performed with CEQ 8000 Genetic Analysis system software (Beckman Coulter, Fullerton, CA) and the BioEdit sequence alignment editor (Ibis Therapeutics, Carlsbad, CA).

Echinocandin susceptibility testing and compounds. Echinocandin susceptibility testing was performed in triplicate using the broth microdilution method of CLSI document M27-A3 (7) with or without 50% human serum (from human male blood, type AB; Sigma-Aldrich) or 50 mg/ml BSA. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as control strains for antifungal susceptibility testing. The drugs used were CSF (Merck & Co. Inc., Rahway, NJ), ANF (Pfizer, New York, NY), and MCF (Astellas Pharma USA, Inc., Deerfield, IL). The drugs were obtained as standard powders from their manufacturers. CSF and MCF were dissolved in sterile distilled water, and ANF was dissolved in 100% dimethyl sulfoxide (Sigma-Aldrich). Stock solutions of each drug were kept at −86°C.

Standardization of RPMI 1640 plus BSA. In order to establish the BSA concentration needed to mimic the effect of 50% serum on echinocandin MIC values, MIC testing was carried out according to the method of the CLSI M27-A3 document (7) with 50% (wt/vol) serum or different BSA concentrations (from 2.5 to 100 mg/ml). Once the BSA concentration was established, the effects of three fatty-acid-free BSA preparations obtained from three different companies (Affymetrix/USB, Sigma Aldrich, and Equitech Bio, Inc.) on echinocandin MIC values were assessed. Thirteen strains were used in the standardization experiments, including 5 echinocandin-susceptible strains (2 *C. albicans*, 1 *C. glabrata*, 1 *C. krusei*, and 1 *C. tropicalis* strain), 3 isolates showing a naturally occurring amino acid substitution in Fks1p with a RES phenotype (1 *C. parapsilosis*, 1 *C. metapsilosis*, and 1 *C. orthopsilosis* strain), and 5 echinocandin-resistant strains (1 *C. albicans fks1*-S645P, 1 *C. glabrata fks1*-S629P, 1 *C. glabrata fks2*-S663P, 1 *C. krusei fks1*-F655F/C, and 1 *C. tropicalis fks1*-F76S strain), representing all the species used in this study. AQ: C

Isolation of the 1,3-β-D-glucan synthase complex, characterization of the 1,3-β-D-glucan product, and assay. Eight 1,3-β-D-glucan synthase complexes were isolated from one strain each of the *Candida* species included in this study. Cell growth and disruption, membrane protein extraction, entrapment of the by-product of partial GS purification, and polymerization assays were performed as described previously (15, 16). The product of the reaction mixtures was characterized as 1,3-β-D-glucan using the endpoint assay Glucatell kit (13). AQ: D

1,3-β-D-Glucan synthase complex inhibition curves. Inhibition curves and 50% inhibitory concentrations (IC_{50}s) were determined using a sigmoidal response (variable-slope) curve and a two-site competition fitting algorithm with GraphPad Prism software, version 4.0 (13). The IC_{50}s were obtained with and without 50% serum or 50 mg/ml BSA.

Definitions. The isolates were classified into three groups depending on the *FKS* hot spot sequence. Each of the *Candida* strains included in the wild-type (WT) group harbors the same *FKS* hot spot sequence as that described in the GenBank accession number for the species given under "*FKS* gene sequence analysis" above. The species showing an intrinsic RES phenotype (13) were included in the IRES group. The echinocandin-resistant (ER) group contained characteristic *fks* hot spot mutants isolated during or after echinocandin therapy. AQ: E

To evaluate the abilities of the different medium supplements to identify *fks* hot spot mutants, the definitions given by Arendrup et al. (2) were utilized. Briefly, the following terms were used: (i) distance between endpoint ranges, defined as the number of 2-fold dilution steps between the MIC values of each group of strains (negative values represent overlaps); (ii) overlap, defined as the number of endpoint ranges of one group that overlapped with the endpoint ranges of another group of isolates; (iii) wild-type upper-limit (WT-UL) values, defined as two 2-fold dilution steps higher than the MIC_{50} for the WT group; (iv) very major errors (VME), defined as *fks* hot spot mutant isolates with MIC values less than or equal to the WT-UL; (v) major errors (ME), defined as WT isolates with MIC values above the WT-UL; (vi) MIC_{50} shift, defined as the number of 2-fold dilution steps by which the MIC_{50} for a particular group changed when a test condition was modified (from RPMI 1640 alone to RPMI-serum or RPMI-BSA). AQ: F AQ: G

Data analysis. MIC and IC_{50} data are the results of experiments performed in triplicate and on three separate days. Geometric means were used to compare MIC results statistically. Arithmetic means and standard deviations were used to analyze IC_{50}s (continuous variables) statistically. The significance levels of MIC differences were determined by Student's t test (unpaired, unequal variance); a P value of 0.01 was considered significant. In order to approximate a normal distribution, the MICs were transformed to $\log_2$ values for the establishment of susceptibility differences between strains. Both on-scale and off-scale results were included in the analysis. The off-scale MICs were converted to the next concentration up or down. Statistical analyses were performed with the Statistical Package for the Social Sciences software (version 13.0) (SPSS Inc., Chicago, IL).

RESULTS

BSA modifies echinocandin MICs and IC_{50}s, mimicking 50% serum. The antifungal efficacies of all the echinocandin drugs were significantly reduced in the presence of 50% human serum ($P < 0.001$). However, the effects of serum on the MIC_{50} and MIC_{90} were more pronounced for ANF and MCF than for CSF (Table 1). Similarly, the presence of BSA decreased the *in vitro* potencies of echinocandin drugs in a concentration-dependent manner, starting with 10 mg/ml BSA and saturating at 50 mg/ml. This effect was observed for the 13 strains (5 WT, 3 IRES, and 5 RES isolates) used in the standardization experiments and for all the echinocandin drugs used. (Examples of the effects of BSA on echinocandin MIC values for the *C. albicans* WT strain SC5314 and the *C. albicans* ER strain 205 are displayed in Fig. 1.) The effect of BSA at 50 mg/ml on echinocandin MIC values mimicked the behavior of 50% human serum (higher MIC values). For these reasons, 50 mg/ml of BSA was chosen as the BSA concentration to be used in this study. T1/AQ:H F1

The reduced antifungal properties of the echinocandin drugs in the presence of BSA were also observed in glucan synthase (GS) inhibition assays. Again, 50 mg/ml of BSA mimicked the effect of decreased drug effectiveness observed with 50% human serum (higher IC_{50}s) (Table 2 and Fig. 2). As reported by Paderu et al. for 50% serum (24), 50 mg/ml of BSA had a stronger effect on ANF and MCF than on CSF. T2, F2 AQ: I

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TABLE 1. Effects of 50% serum and 50 mg/ml of BSA on echinocandin MIC distributions among *Candida* spp.[a]

Species	Group[b] (no. of isolates)	Antifungal	MIC (μg/ml)[c] with the following additive:								
			None			50% Serum			50 mg/ml BSA		
			GM	50%	90%	GM	50%	90%	GM	50%	90%
C. albicans	WT (14)	ANF	0.02	0.015	0.03	0.16	0.12	0.25	0.37	0.25	0.50
		CSF	0.04	0.06	0.12	0.16	0.12	0.25	0.17	0.12	0.25
		MCF	0.03	0.03	0.06	0.21	0.25	0.25	0.25	0.25	0.25
	ER (20)	ANF	0.53	0.50	2.00	5.86	2.00	>8.00	9.85	>8.00	>8.00
		CSF	2.83	2.00	8.00	5.10	4.00	>8.00	5.28	4.00	>8.00
		MCF	0.42	0.50	2.00	5.46	4.00	>8.00	6.50	4.00	>8.00
C. glabrata	WT (6)[d]	ANF	0.02	0.015	0.06	0.25	0.25	0.50	0.50	0.50	0.50
		CSF	0.05	0.06	0.12	0.50	0.50	1.00	0.03	0.50	1.00
		MCF	0.03	0.03	0.03	0.28	0.25	0.50	0.63	0.50	0.50
	ER (14)	ANF	0.52	0.50	8.00	NDA	NDA	NDA	12.93	>8.00	>8.00
		CSF	4.00	4.00	>8.00	NDA	NDA	NDA	14.38	>8.00	>8.00
		MCF	1.80	2.00	>8.00	NDA	NDA	NDA	15.17	>8.00	>8.00
C. tropicalis	WT (4)	ANF	0.02	0.015	0.03	0.42	0.50	0.50	0.50	0.50	0.50
		CSF	0.07	0.06	0.12	0.17	0.12	0.25	0.21	0.25	0.25
		MCF	0.03	0.015	0.06	0.29	0.25	0.50	0.42	0.50	0.50
	ER (4)	ANF	0.35	0.25	0.50	11.31	>8.00	>8.00	16.00	>8.00	>8.00
		CSF	1.19	2.00	2.00	9.51	8.00	8.00	9.51	>8.00	>8.00
		MCF	0.12	0.12	0.25	11.31	>8.00	>8.00	11.31	>8.00	>8.00
C. krusei	WT (4)	ANF	0.07	0.06	0.12	0.30	0.25	0.50	0.35	0.25	0.50
		CSF	0.14	0.12	0.25	0.84	1.00	1.00	1.00	1.00	1.00
		MCF	0.05	0.06	0.06	0.84	1.00	1.00	1.00	1.00	1.00
	ER (2)	ANF	1.41	ND	ND	11.31	ND	ND	16.00	ND	ND
		CSF	2.83	ND	ND	8.00	ND	ND	8.00	ND	ND
		MCF	0.69	ND	ND	8.00	ND	ND	11.31	ND	ND
C. parapsilosis	IRES (6)	ANF	0.89	0.50	8.00	8.00	8.00	>8.00	16.00	>8.00	>8.00
		CSF	0.44	0.25	4.00	2.50	2.00	>8.00	4.00	2.00	>8.00
		MCF	1.41	1.00	4.00	14.25	>8.00	>8.00	16.00	>8.00	>8.00
C. orthopsilosis	IRES (2)	ANF	0.25	ND	ND	5.66	ND	ND	16.00	ND	ND
		CSF	0.17	ND	ND	0.50	ND	ND	1.41	ND	ND
		MCF	0.25	ND	ND	16.00	ND	ND	16.00	ND	ND
C. metapsilosis	IRES (2)	ANF	0.50	ND	ND	2.83	ND	ND	11.31	ND	ND
		CSF	0.25	ND	ND	0.22	ND	ND	1.00	ND	ND
		MCF	0.35	ND	ND	2.83	ND	ND	5.66	ND	ND

[a] Susceptibility testing was carried out according to the guidelines of the CLSI M27A3 document.
[b] Classification depends on the *FKS* hot spot sequence. WT isolates have the same *FKS* hot spot sequence as that of the GenBank accession number for the species given in Materials and Methods. IRES isolates show a phenotype of intrinsically reduced echinocandin susceptibility. ER strains show *FKS* hot spot mutations and were isolated during or after echinocandin therapy.
[c] GM, geometric mean; 50% and 90%, MICs at which 50% and 90% of isolates, respectively, were inhibited; NDA, no data available (13 out of 14 *C. glabrata* ER strains did not grow due to the addition of 50% serum to RPMI 1640 medium); ND, not done (low number of strains).
[d] There were 5 isolates when 50% serum was added.

Fifty percent human serum inhibits the growth of some *C. glabrata* strains. The presence of 50% human serum in MIC plates inhibited the growth of 14 out of 20 (70%) *C. glabrata* strains tested in this study. Of these strains, 13 harbored mutations in the hot spot regions of *FKS1* or *FKS2*. The only WT *C. glabrata* strain that did not grow in RPMI-50% serum was isogenic with an *FKS2* mutant that showed the same behavior in this medium. On the other hand, the only *C. glabrata* mutant (Fks1p-D632E) that grew in the presence of 50% serum was isogenic with a WT strain that grew under the same conditions (6). These data indicate that the ability of *C. glabrata* to grow in the presence of 50% serum is independent of its *FKS* sequence. One *C. glabrata* mutant strain (Fks2p-F659S) did not grow in any of the broths used, with or without human serum or BSA. However, the same strain grew when it was cultured in a richer medium, such as YPD broth (data not included in the analysis). The inhibitory properties of serum against *C. glabrata* were not observed in our previous study using a different lot of serum from the same company (14). Moreover, when the same *C. glabrata* strains were retested using the newest serum, 62.5% (10 out of 16) failed to grow. The MIC testing was repeated with 2 other lots of serum, and the number of *C. glabrata* strains that did not grow differed for each lot of serum tested (data not shown). On the other hand, when different highly purified fatty-acid-free BSA products were used, growth was uninhibited, and the same results were obtained consistently for all *Candida* spp. and echinocandin drugs. This result suggests that the albumin fraction of serum is not responsible for the inhibitory activity of serum against *C. glabrata*.

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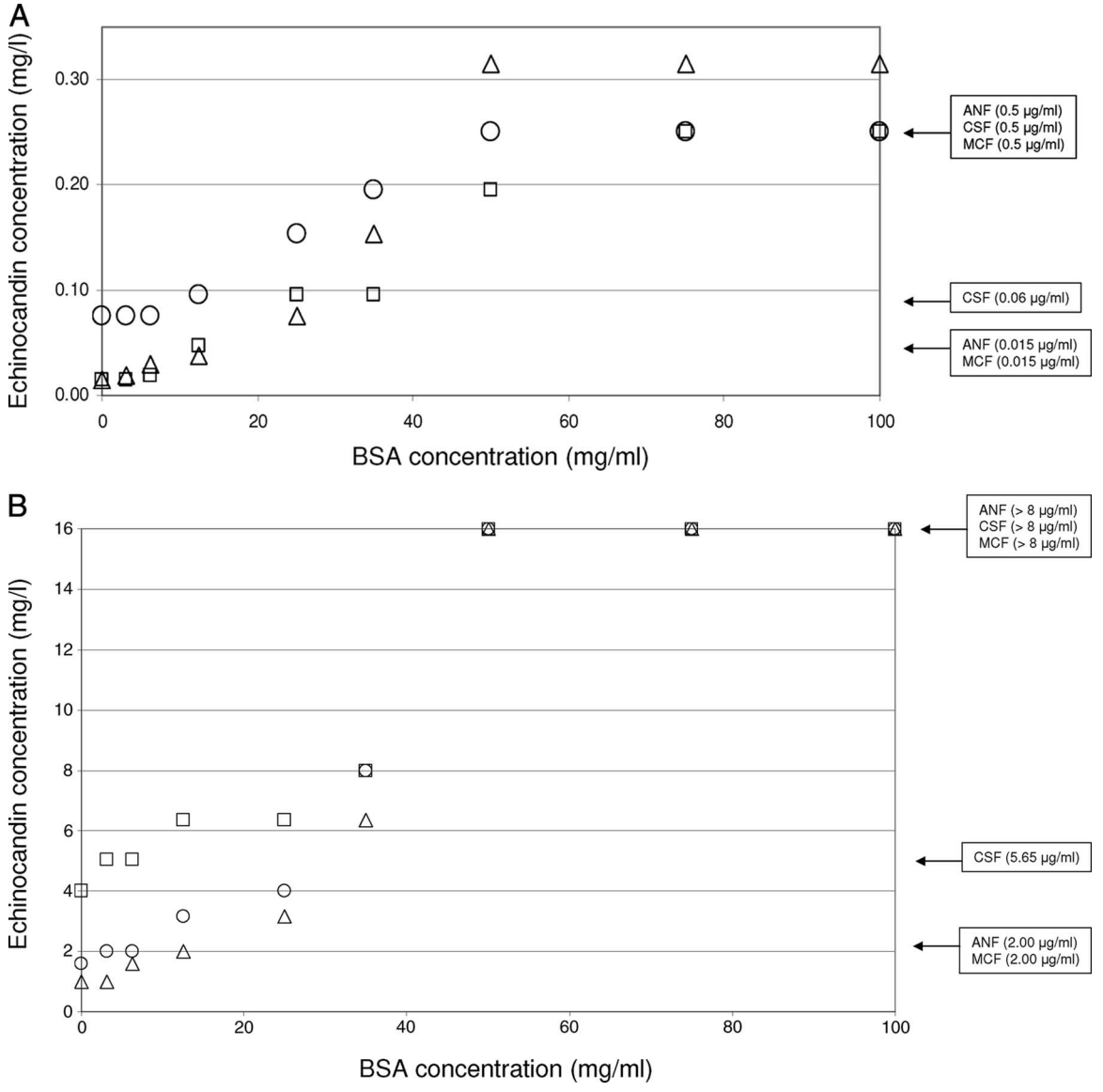

FIG. 1. Effects of different BSA concentrations on echinocandin MIC values for the *C. albicans* WT strain SC5314 (A) and the *C. albicans* ER strain 205 (Fks1p-S645P) (B). Squares, circles, and triangles represent the MIC values of ANF, CSF, and MCF, respectively (geometric means from three repetitions), obtained with BSA concentrations of 3.12, 6.25, 12.5, 25, 35, 50, 75, and 100 mg/ml. Arrows represent MIC values obtained with no additive (black arrows) or with the addition of 50% serum (gray arrows) for the three echinocandin drugs. AQ: Q

RPMI 1640 with 50 mg/ml BSA distinguishes wild-type from *fks* mutant populations. The MIC values obtained using RPMI 1640, RPMI 1640 with 50% serum, and RPMI 1640 with 50 mg/ml BSA were compared. WT and ER populations were separated by −2, −1, and −3 2-fold-dilution steps when RPMI alone was used for testing susceptibility to ANF, CSF, and MCF, respectively (Fig. 3A, D, and G). In other words, the MIC ranges of all the echinocandins overlapped for the WT and ER populations when RPMI 1640 alone was used. Moreover, when the WT-UL values were used as susceptibility breakpoints (≤0.12 μg/ml for ANF and MCF and ≤0.25 μg/ml for CSF), 9, 2, and 7 VME were found for ANF, CSF, and MCF, respectively. On the other hand, no ME were found for any of the echinocandin drugs when RPMI alone was used. F3

When RPMI 1640 with 50% serum was used, the WT and ER groups were separated by no 2-fold dilutions with ANF, while −1 2-fold dilution step separated these populations when CSF and MCF were tested (Fig. 3B, E, and H). For RPMI-serum, when the WT-UL was used as the susceptibility breakpoint, the numbers of VME were 2, 2, and 7 for ANF, CSF, and MCF, respectively. Thus, *in vitro* ANF susceptibility testing using RPMI-serum showed better discriminatory power than RPMI 1640 alone in separating the WT and ER populations ($P < 0.01$). On the other hand, RPMI alone and RPMI-serum showed no difference in the ability to distinguish between the WT and ER groups when CSF and MCF MICs were compared ($P = 0.2$). AQ: J

When RPMI-BSA was used, it became clear that this was the best option for differentiating between WT and ER populations. This medium showed the lowest number of VME when

TABLE 2. 1,3-β-D-Glucan synthase inhibition profiles[a] of echinocandin drugs for the strains included in the study

Organism	IC_{50} (ng/ml)[b] of the following drug with the indicated medium additive:								
	ANF			CSF			MCF		
	No additive	50% human serum	50 mg/ml BSA	No additive	50% human serum	50 mg/ml BSA	No additive	50% human serum	50 mg/ml BSA
C. albicans[c]	7.0 ± 1.2	100.9 ± 4.6	104.5 ± 3.8	13.7 ± 1.2	60.5 ± 5.6	58.6 ± 6.1	26.9 ± 2.4	90.1 ± 4.3	94.3 ± 2.3
C. albicans[c]	2.7 ± 0.5	60.7 ± 5.4	65.6 ± 4.6	7.3 ± 2.4	39.7 ± 3.5	35.3 ± 5.6	15.6 ± 1.2	79.0 ± 3.4	75.6 ± 4.5
C. glabrata[d]	1.2 ± 0.2	55.2 ± 4.4	59.4 ± 3.3	3.1 ± 0.8	28.9 ± 1.1	30.2 ± 2.9	0.6 ± 0.2	71.6 ± 9.1	62.6 ± 1.7
C. krusei[c]	3.6 ± 0.1	88.1 ± 7.6	92.6 ± 5.5	9.2 ± 1.6	51.7 ± 2.4	48.1 ± 5.8	6.7 ± 1.6	99.1 ± 5.5	107.6 ± 7.0
C. tropicalis[c]	0.6 ± 0.1	44.7 ± 3.9	52.7 ± 6.2	0.4 ± 0.1	22.6 ± 2.0	26.4 ± 1.5	0.4 ± 0.1	29.5 ± 2.2	33.7 ± 3.1
C. parapsilosis[e]	410.0 ± 5.5	1,216.1 ± 88.1	1,154.0 ± 121.0	21.2 ± 3.2	70.4 ± 9.0	65.6 ± 3.2	245.3 ± 4.7	671.8 ± 8.8	656.2 ± 12.4
C. metapsilosis[e]	119.5 ± 9.0	421.5 ± 29.8	444.4 ± 57.0	40.3 ± 4.1	111.6 ± 5.9	120.1 ± 4.2	70.3 ± 3.5	222.6 ± 9.1	231.7 ± 11.6
C. orthopsilosis[e]	133.9 ± 12.3	399.1 ± 38.5	402.0 ± 29.6	58.2 ± 4.5	142.8 ± 8.1	138.5 ± 10.5	152.7 ± 6.6	415.1 ± 5.0	401.6 ± 5.2

[a] Expressed as IC_{50}s.
[b] Arithmetic means ± standard deviations (three repetitions on three separate days). IC_{50}s were obtained using trapped 1,3-β-D-glucan synthase enzyme. Pearson's coefficients of variation are between 1.2 and 32.9%.
[c] Wild type at *FKS1* hot spot regions.
[d] Wild type at *FKS1* and *FKS2* hot spot regions.
[e] Naturally occurring proline-to-alanine amino acid substitution at the *FKS1* hot spot 1 region.

WT-UL values were used as the susceptibility breakpoint. RPMI-BSA showed no MIC overlap for any of the echinocandin drugs tested and showed no VME when the WT-UL value was used as the breakpoint for CSF (≤1 μg/ml) (Fig. 3C, F, and I). For the other echinocandin drugs, 3 VME were observed when WT-UL values were used as breakpoints (≤1 μg/ml for ANF and ≤2 μg/ml for MCF).

When the MIC_{50} values obtained with RPMI, RPMI-serum, and RPMI-BSA were compared, it was evident that the use of serum or BSA facilitated discrimination between WT and ER populations (Fig. 3). RPMI alone splits WT and ER groups by 16 to 32 2-fold dilution steps, while RPMI-serum and RPMI-BSA separated these populations by 32 and 64 2-fold dilution steps for all the echinocandin drugs (Fig. 3). These phenomena can be explained by analyzing how serum and BSA increased the echinocandin MIC_{50}s in the different populations. When RPMI-serum or RPMI-BSA was used, the MIC_{50} shifts for the WT population were lower (8, 4, and 8 2-fold dilution steps for ANF, CSF, and MCF, respectively) than those for the ER group (32, 8, and 32 2-fold dilutions for ANF, CSF, and MCF, respectively) ($P < 0.01$), separating the populations. On the other hand, there were no differences between the MIC_{50} shifts obtained with RPMI-serum and RPMI-BSA ($P = 0.8$).

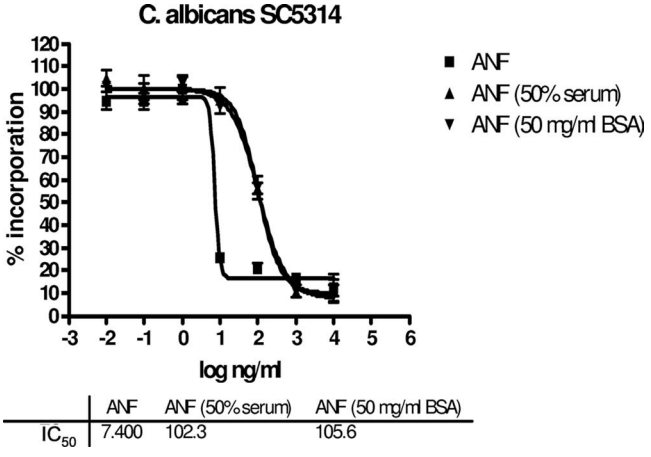

FIG. 2. Effect of 50 mg of BSA/ml or 50% human serum on the inhibition kinetics (IC_{50}) of ANF for the 1,3-β-D-glucan synthase complex isolated by trapping from *C. albicans* SC5314. IC_{50}s were determined by monitoring the incorporation of [^{3}H]uridine diphosphoglucose as a function of the ANF concentration. The calculated IC_{50}s are the results of triplicate values.

RPMI 1640 with 50 mg/ml of BSA makes ANF and MCF behave differently from CSF against the IRES population. Overall, 9, 9, and 7 IRES strains were considered susceptible to ANF, CSF, and MCF when the CLSI susceptibility breakpoint (≤2 μg/ml) was used. With RPMI 1640 alone, the WT and IRES populations were separated by 1, −1, and 1 2-fold-dilution step for ANF, CSF, and MCF, respectively (Fig. 3A, D, and G). In addition, the IRES and ER populations were not separated when this medium was used for ANF and MCF susceptibility testing (all the IRES strains overlapped with the ER population) (*P*, 0.25 and 0.26, respectively). On the other hand, RPMI with no additives was the only medium able to show significant CSF MIC differences between the WT and IRES populations ($P < 0.0001$).

The MIC_{50}s obtained for the IRES population with RPMI alone versus RPMI-serum were compared. Serum shifted the MIC_{50}s for this population 16 (from 0.5 μg/ml to 8 μg/ml), 2 (from 0.25 μg/ml to 0.5 μg/ml), and 32 (from 0.5 μg/ml to 16 μg/ml) 2-fold dilution steps for ANF, CSF, and MCF, respectively (Fig. 3A, B, D, E, G, and H). The same analysis was performed to compare the MIC_{50}s obtained for the IRES population with RPMI alone versus RPMI-BSA, and 32, 8, and 32 2-fold dilution step shifts were observed for ANF, CSF, and MCF, respectively. Interestingly, the ANF and MCF MIC ranges for the IRES population were displaced to higher values for RPMI-BSA than for RPMI-serum (ANF and MCF MICs were 8 or 16 μg/ml for all the IRES strains when RPMI-BSA was used) ($P < 0.01$).

DISCUSSION

Echinocandin drugs bind to the albumin fraction of serum *in vitro*. It is well established that echinocandin drugs bind to serum proteins at very high levels (98% for ANF, 96.5% for

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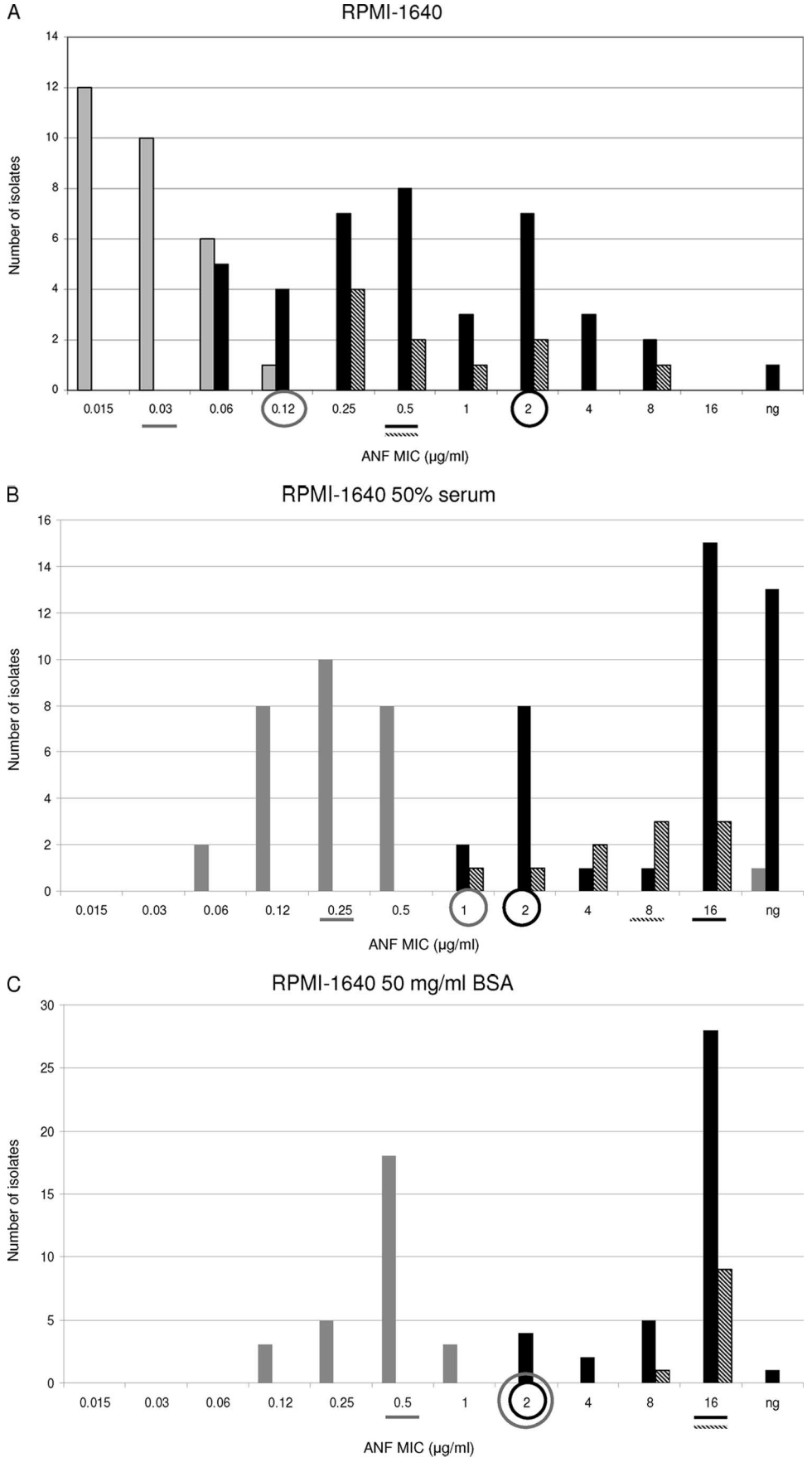

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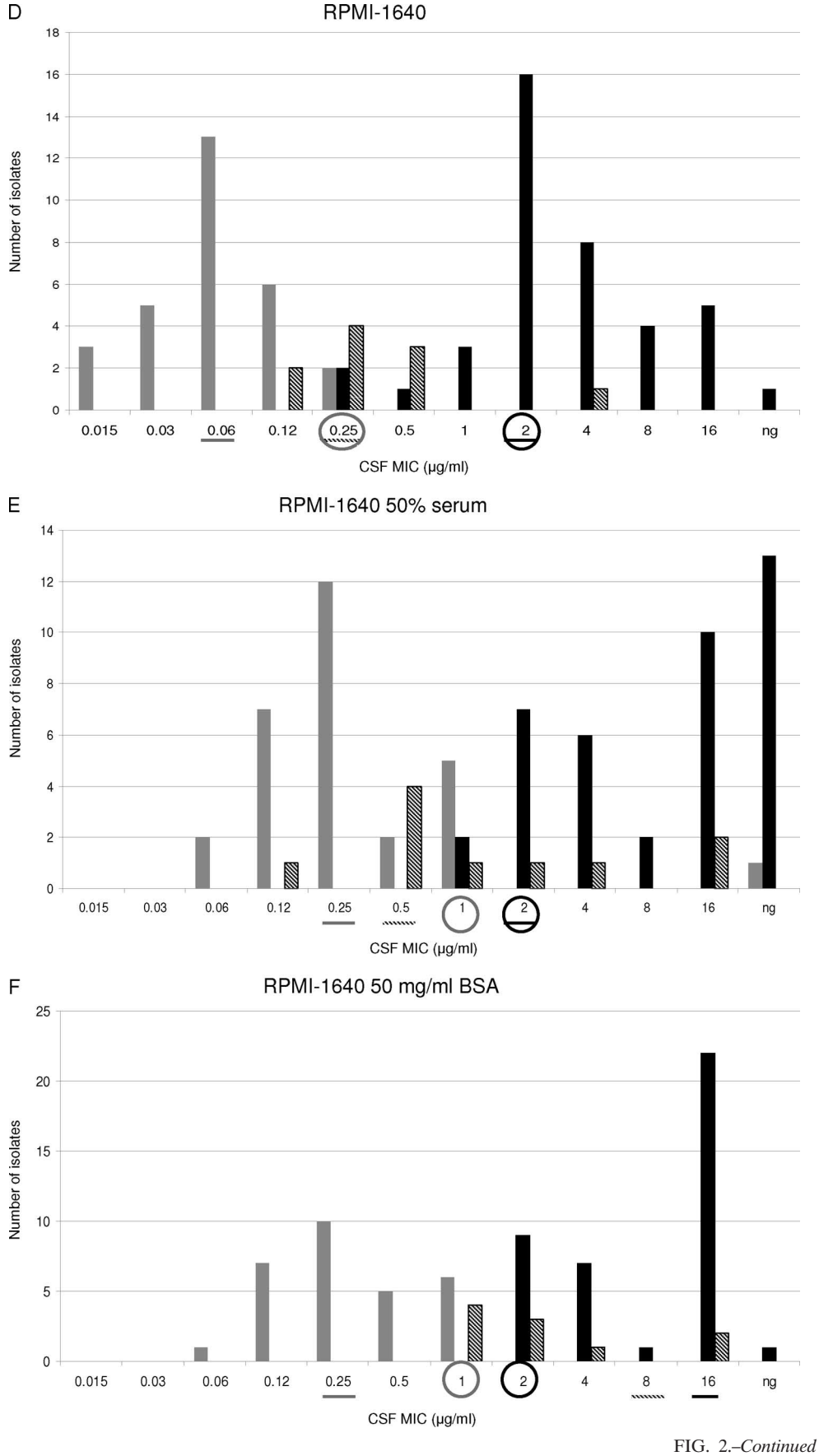

FIG. 2.–*Continued on following page*

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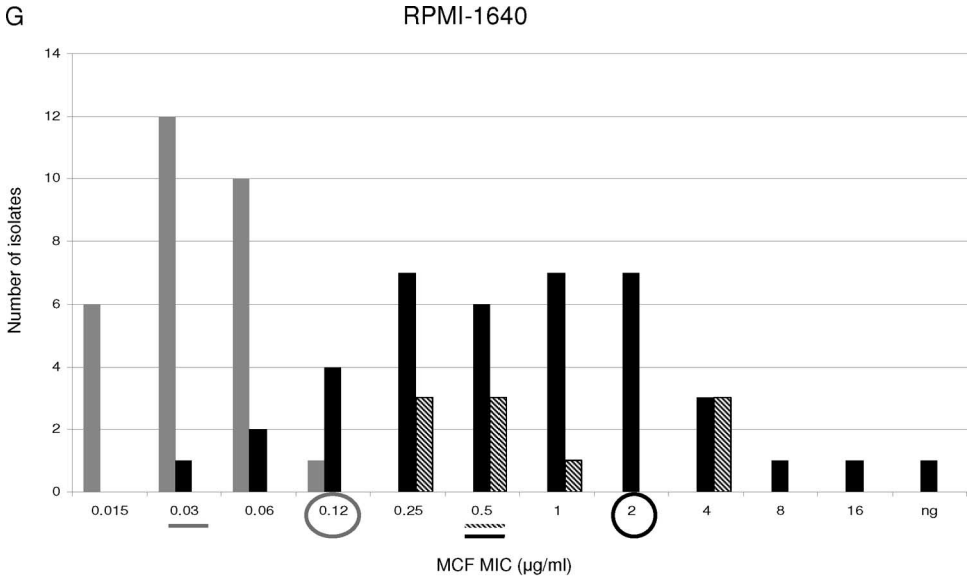

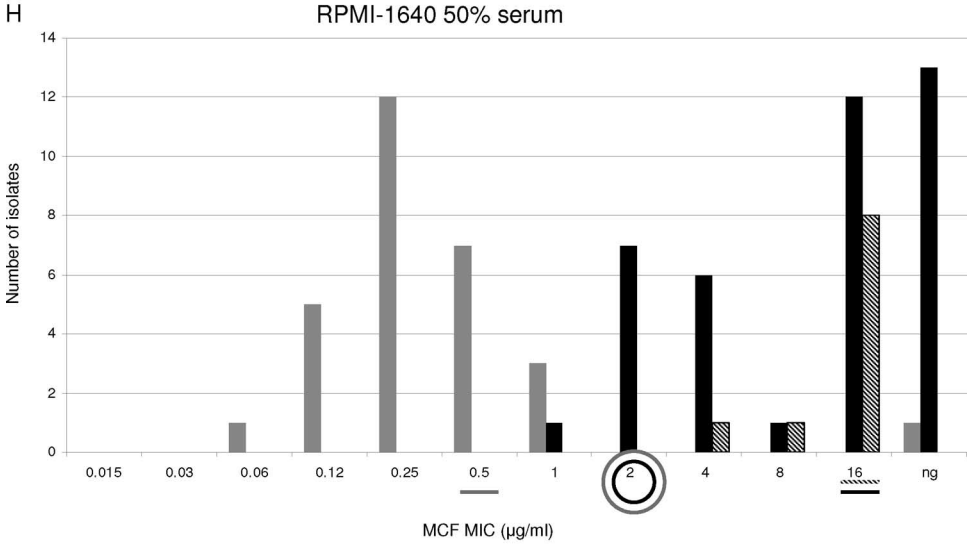

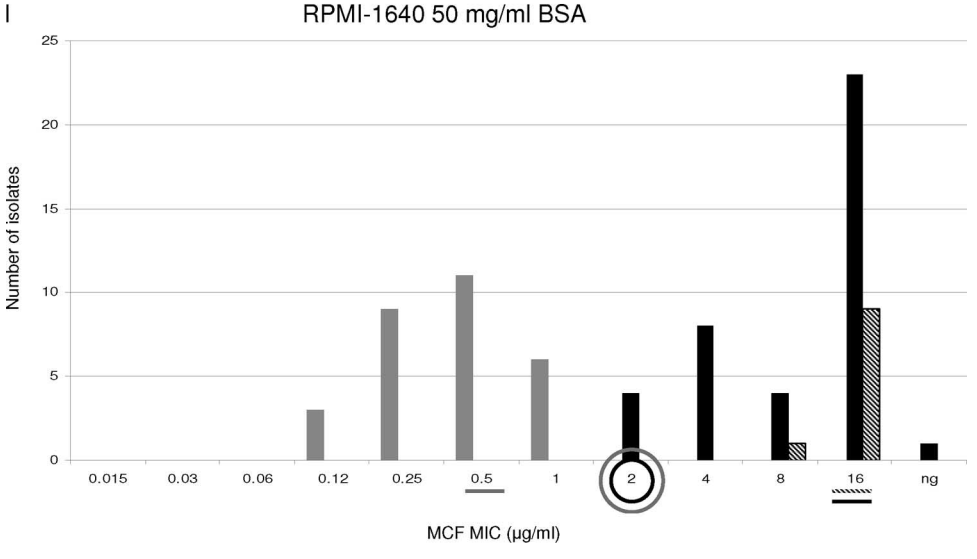

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CSF, and 99.8% for MCF) (17, 24, 38, 42). However, it is not clear whether echinocandins are active only as unbound free drug or whether these drugs are also active when complexed with protein. According to the free-drug hypothesis, only 2%, 3.5%, and 0.2% of the ANF, CSF, and MCF present in RPMI-serum would have pharmacological activity, respectively. Thus, the presence of serum should increase the MIC values between 200- and 500-fold. Our *in vitro* data suggest that the increases in echinocandin MIC_{50}s (2 to 32 2-fold dilution steps) are not totally consistent with the free-drug hypothesis and confirm older *in vitro* and *in vivo* data (23, 24, 42, 43). In a recent report, Ishikawa et al. demonstrated that the free-drug hypothesis was unsuitable for MCF (17). Thus, we can infer that at least part of the protein-bound drug is active against *Candida* spp.

The data presented in this study support the contention that the addition of 50 mg/ml of BSA to the medium for the standard CLSI testing methodology mimics the *in vitro* activity of 50% serum with echinocandin drugs (Tables 1 and 2). The fact that BSA mimics the effect of serum is consistent with the notion that echinocandin drugs bind primarily to albumin and that the other serum fractions have minimal or no effect on the *in vitro* potency of these drugs. On the surface, this conclusion would appear to contradict that of Abe et al. (1), who demonstrated, using an analbuminemic rat model, that MCF pharmacodynamics were not affected by albumin. These authors concluded that MCF must be bound *in vivo* to other serum fractions besides albumin. However, it is likely that the echinocandin drugs bind albumin and other serum components, with the latter being more significant in the absence of albumin. These differences between the *in vitro* and *in vivo* activity effects of serum on echinocandin drugs need to be studied further.

RPMI 1640 with 50 mg/ml of BSA is better than RPMI 1640 alone at revealing *Candida* sp. *FKS* mutants. Multiple reports have demonstrated the excellent *in vitro* activity of echinocandin drugs against clinical *Candida* sp. isolates (25, 26). However, these studies also demonstrated a bimodal echinocandin MIC distribution, with strains from highly susceptible species exhibiting elevated MIC values. Furthermore, they highlighted the presence of *Candida* spp. with RES phenotypes (8, 11, 21, 29, 32, 34). However, there are no convincing data linking high echinocandin MIC values and clinical failure, leading to skepticism regarding the value of routine echinocandin MIC testing for resistance (3, 14, 18, 22, 31). Moreover, it has been suggested that there is a better correlation between the presence of certain *FKS* hot spot mutations and echinocandin clinical failure than between high MIC values alone and echinocandin clinical failure (27). The CLSI published the echinocandin susceptibility breakpoint (≤2 μg/ml) (7), but multiple reports have shown that this breakpoint may misclassify as echinocandin susceptible many of the *Candida* sp. *fks* hot spot mutants isolated during or after echinocandin therapy (2, 14–16, 35, 40). The CLSI committee is revisiting the issue of breakpoints for echinocandins to reflect these concerns in the literature (30).

Lately, a variety of suggestions have been made to improve the ability of *in vitro* susceptibility testing to detect *FKS* mutants (2, 9, 15, 16), including the use of RPMI 1640 with 50% human serum to distinguish *C. albicans* and *C. glabrata fks* mutants from WT strains and to normalize the behavior of all three echinocandin drugs (15, 16, 24). Yet the standardization of human serum is too difficult to allow its incorporation into a standard assay. To overcome this issue, we have used highly pure fatty-acid-free BSA as a surrogate for human serum albumin. It was evident that the use of RPMI-BSA differentially increased echinocandin MIC values, clearly separating the WT and ER populations (Fig. 3). Also, the addition of 50 mg/ml of BSA to RPMI 1640 showed no MIC range overlaps and the lowest number of VME. Moreover, this work reinforces our group's previous suggestion that CSF should be used as a marker of the RES phenotype (2, 15, 16) and that the original CLSI breakpoint for echinocandins requires revision in order to identify all or most of the *fks* hot spot mutants. On the other hand, the ANF and MCF WT-UL values (0.12 μg/ml) were similar to the MIC and IC_{50} breakpoints (0.25 and 0.5 μg/ml) proposed previously by our group for *C. albicans* and *C. glabrata* (15, 16).

***C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* strains showed MIC values similar to those for the ER population.** *C. parapsilosis* sensu lato species showed a MIC_{90} value more than 16-fold higher than the MIC_{90}s of other *Candida* spp. (11, 28). Moreover, none of the echinocandin drugs showed fungicidal activity against *C. parapsilosis* (4). This lower activity of echinocandin drugs against these species has been linked to a naturally occurring amino acid substitution in hot spot 1 of *FKS1* (13, 27). Despite all these *in vitro* and genetic data, most infections caused by *C. parapsilosis* sensu lato species respond to echinocandin therapy (20, 25, 36). The data presented in this work demonstrated that *C. parapsilosis* sensu lato species showed echinocandin MIC values similar to those for other *Candida* sp. *fks* mutants regardless of the medium used. However, 50 mg/ml of BSA or 50% human serum made MCF and ANF behave differently from CSF with these species. BSA reduced the ANF and MCF MIC range for the IRES population and displaced it to higher MIC values (≥8 μg/ml). On the other hand, CSF MIC values were not reduced, and the MIC_{50} shift was lower for this drug. These results suggest that CSF is more active than ANF and MCF against *C. parapsilosis* sensu lato species *in vitro*. Our group has shown that the glucan synthase complexes of these species are 20-fold more sensitive to CSF than to the other two echinocandin drugs (13). More-

FIG. 3. Distributions of anidulafungin (ANF), caspofungin (CSF), and micafungin (MCF) MICs for WT (shaded bars), IRES (diagonally striped bars), and ER *FKS* hot spot mutant (filled bars) populations. MIC susceptibility tests were performed by the method presented in the CLSI M27A3 document by using either RPMI 1640 alone (A, D, and G), RPMI plus 50% serum (B, E, and H), or RPMI plus 50 mg of BSA/ml (C, F, and I). A line under a MIC value indicates the MIC_{50} for the population represented by a bar with the corresponding shading. Circles outlined in gray indicate the wild-type upper-limit breakpoint. Circles outlined in black represent the CLSI echinocandin susceptibility breakpoint. ng, no growth.

over, a recently published paper showed that 83% of the *C. parapsilosis* strains not responding to MCF therapy had MCF MICs of ≥4 μg/ml and that those isolates had CSF MICs ranging from 0.5 to 1 μg/ml (35). The results of that work reinforce the notion that *C. parapsilosis*, *C. metapsilosis*, and *C. orthopsilosis* behave as weakly echinocandin resistant organisms *in vitro* but that other factors influence their behavior as weak pathogens. One possibility is a potential fitness cost to the *Candida* sp. cells harboring *FKS* hot spot mutations (or polymorphisms) due to a decreased V_{max} of the GS complex (13, 15, 16).

Overall, it is apparent that 50 mg/ml of BSA mimicked the effect of human serum, altering the antifungal properties of the echinocandin drugs. RPMI-BSA is a viable alternative to human serum for *in vitro* susceptibility testing standardization. Most importantly, RPMI-BSA is able to distinguish between WT and ER populations when one is using the existing susceptibility breakpoint of <2 μg/ml (or a lower susceptibility breakpoint, once it is redefined by the CLSI). In conclusion, the addition of BSA to RPMI 1640 could be considered a modification of CLSI echinocandin susceptibility testing. However, the optimum testing conditions have to be established, and multilaboratory reproducibility studies are essential for proposing such a modification. Collaborative studies will be arranged in the near future.

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AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES 1

AQA—Please check running title at top of p. 3. It has been slightly modified to add "method" per ASM style while still fitting into allotted space—maximum of 54 characters and spaces, with spaces counting toward the total.

AQB—ATCC 750 as meant?

AQC—"mimic" as meant?

AQD—If "entrapment of the by-product of partial GS purification" not as meant, please clarify.

AQE—If GenBank accession numbers not identified correctly, please clarify which GenBank accession numbers are meant.

AQF—"each group of strains" as meant? or "each pair of groups of strains"?

AQG—"endpoint ranges" as meant? If not, please clarify.

AQH—Please check caption for Table 1 and correct if necessary. Explanation of underlined and boldface numbers deleted because there is no underlining or boldface in Table 1.

AQI—In Table 2, please specify the difference between *C. albicans* in the first row and *C. albicans* in the second row (for instance, by giving the strain number for each or by characterizing each according to group [WT, ER, RES, or IRES] in parentheses).

AQJ—"no 2-fold dilutions" instead of "0 2-fold dilution" OK?

AQK—Deletion of BSA before WT-UL (twice in this paragraph) OK, since the MIC is an echinocandin MIC, not a BSA MIC?

AQL—If "than between high MIC values alone and echinocandin clinical failure" not as meant, please clarify between what and high MIC values alone.

AQM—"the RES phenotype" as meant?

AQN—"identify" as meant?

AQO—"that work" as meant—reference 35, not the present study?

AQP—"or a lower susceptibility breakpoint" as meant, since once it is redefined, it will no longer be the existing breakpoint?

AQQ—Please check that the gray arrows in Fig. 1 can be distinguished from the black arrows.

AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES 2

AQR—Please clarify what kind of results the calculated IC_{50}s are—means of triplicate values?