

Comparison of three methods for *in vitro* susceptibility testing of *Candida* species with flucytosine

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Optimal methods for susceptibility testing of *Candida* spp. with flucytosine have not been determined. Breakpoints were recommended in 1984, but never validated. In this study, we compared the 1984 recommended macrodilution broth method (using an 80% endpoint) with a modification of the more recent NCCLS-recommended microdilution broth method with three endpoints—spectrophotometric 50% and 80% and a no growth endpoint determined by eye. NCCLS and British Society for Medical Mycology (BSMM) breakpoints were also compared. One hundred and fifty isolates comprised of *Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Candida glabrata*, *Candida parapsilosis* and *Candida lusitanae* were tested. Reproducibility was excellent. For *C. albicans* ($n = 65$), the correlation between tests was excellent ($>75\%$), with few major discrepancies ($<5\%$). For *C. tropicalis* ($n = 27$), correlation was good (59%), but there were a small number of major discrepancies (up to 11%, depending on breakpoint used). Results by the broth macrodilution method were generally higher than both microdilution methods for *C. glabrata* ($n = 16$; correlation of 18.8%), but only one major discrepancy was seen. Ten of the 11 *C. parapsilosis* isolates tested were susceptible by all methods, regardless of breakpoint chosen, with a correlation of 18.2%, but no major discrepancies were seen. A correlation between all methods (50%) was seen with *C. lusitanae* ($n = 10$), with many isolates resistant or intermediate. In contrast, correlation between methods for *C. krusei* was poor ($<5\%$); NCCLS microtitre modification produced results that were classified as intermediate or resistant, regardless of the breakpoint used. The methodology for susceptibility testing *C. albicans* is robust. Additional work to optimize susceptibility testing with flucytosine is necessary for non-*albicans Candida* species, especially *C. krusei*.

Introduction

Serious infections caused by yeasts are an increasing problem due to the immunosuppressive nature of surgery, human immunodeficiency virus (HIV) infection, organ transplantation and the treatment of malignancy.¹ Used as monotherapy, flucytosine (5FC) is often effective, but the development of resistance during therapy is well recognized.^{2,3} For this reason, it is usually used in combination with amphotericin B or occasionally with either fluconazole or itraconazole. A reliable susceptibility testing method is required. At present, there are two recognized methods for *in vitro* testing of flucytosine: the broth macrodilution method using Yeast Nitrogen

Base with 1% glucose (YNBG), as detailed previously by the British Society for Mycopathology⁴ [now the British Society for Medical Mycology (BSMM)] and the NCCLS M27-A method.⁵ Several modifications of the original NCCLS M27-A broth macrodilution testing method have been adopted in various laboratories.⁶ These include the addition of glucose to RPMI-1640 medium to enhance yeast growth and the use of flat-bottomed microtitre plates. The Working Group Report⁴ suggested that most yeast isolates, including the standard susceptible strain, have MICs ≤ 1 mg/L and strains with MICs ≥ 16 mg/L may be considered resistant. Breakpoints have also been proposed for the NCCLS M27-A method.^{5,6} However, these are based only on historical and pharmacokinetic data

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rather than any *in vitro*–*in vivo* correlation work. Animal studies have previously shown a relationship between susceptibility testing, using a microtitre method, and *in vivo* outcome in murine haematogenous candidiasis.⁷

In this study, we compared three methods: the broth macrodilution method⁴ and two microtitre modifications of the NCCLS M27-A method⁵ using either RPMI supplemented with 1.8% glucose (RPMI-G) or YNBG as the test medium, to attempt to correlate results between methods. MICs were also compared according to different endpoint definitions; either no growth or optical density reduction by 50% or 80% compared with the drug-free control. Few inter-species comparisons have been carried out; therefore, we have compared results for six pathogenic *Candida* species.

Materials and methods

Yeast strains

The three methods were compared using 150 isolates of *Candida* belonging to six different species. These consisted of 144 clinical isolates from a variety of patient types and six American Type Culture Collection (ATCC) isolates: *Candida albicans* ATCC 24433, *C. albicans* ATCC 90028, *Candida tropicalis* ATCC 750, *Candida krusei* ATCC 6258, *Candida glabrata* ATCC 90030 and *Candida parapsilosis* ATCC 22019. The group comprised 65 *C. albicans*, 27 *C. tropicalis*, 21 *C. krusei*, 16 *C. glabrata*, 11 *C. parapsilosis* and 10 *Candida lusitanae*. Stored isolates were subcultured on blood agar plates (Oxoid Ltd, Basingstoke, UK) at 37°C for 48 h to ensure purity before testing.

Flucytosine

Flucytosine was provided by Roche Products Limited, Hertfordshire, UK. The powder was dissolved in sterile distilled water to produce a stock solution of 1280 mg/L. This was then dispensed into aliquots and stored at –20°C in the dark until required.

Media

For the macrodilution susceptibility testing method and one of the microtitre methods, YNBG (Difco, Surrey, UK) was used. RPMI-1640 (Sigma, Dorset, UK) supplemented with 1.8% glucose, buffered with MOPS (Sigma) and adjusted to pH 7.0 (RPMI-G) was used for the other microtitre method.

Susceptibility testing

All three methods were carried out simultaneously.

Macrodilution method. The broth macrodilution method employed was that of the 'Report of a Working Group of the

British Society for Mycopathology'⁴ using 1 mL volumes of YNBG broth in 5 mL glass, loose-capped tubes. The final drug range was 0.03–32 mg/L. Yeast suspensions were prepared by suspending single colonies in sterile distilled water. The final inoculum in the assay was $\sim 5 \times 10^4$ cfu/mL. A flucytosine-susceptible strain *Candida kefyr* San Antonio (SA) was included with each batch of test organisms as a control. In addition, a negative control was included to ensure the sterility of the medium and one tube for each isolate was left drug-free to act as a positive control. The tubes were incubated at 37°C for 48 h in air. The MIC was read visually, with the aid of an 80% inhibition standard tube,⁵ and was taken as the lowest drug concentration to inhibit 80% of the growth compared with the positive control.

Microtitre methods. The method used was a microtitre modification of the NCCLS M27-A method⁵ in flat-bottomed microtitre plates with either YNBG or RPMI-G broth. The final drug range was 0.03–32 mg/L. The yeast suspensions used for the macrodilution method were then adjusted further, using the appropriate medium, to give a final inoculum of 0.5 – 2.5×10^3 cfu/mL. The *C. kefyr* SA strain was used as a control. Positive and negative controls were also included. The microtitre plates were incubated in a moist chamber at 37°C for 48 h. After incubation, the microtitre plates were shaken for 5 min to obtain a uniform suspension before reading. The growth in each well was measured by determining the optical density at 490 nm by spectrophotometry. Three different endpoints were then recorded; no growth by eye, and the drug concentrations that reduced the OD₄₉₀ by either 50% or 80% when compared with the drug-free control.

Reproducibility

Twenty per cent of the isolates (30/150) were randomly selected and re-tested to establish the reproducibility of each method and each endpoint.

Results

Table 1 presents the results obtained for the six ATCC isolates tested, together with the expected MIC range by the NCCLS M27-A method.⁵ All ATCC isolates gave results within the recommended limits when tested using the modified NCCLS method with RPMI-G and an 80% endpoint.

Table 2 shows the *in vitro* susceptibilities of all 150 *Candida* isolates with all three methods. However, in presenting the results, we shall use the modified NCCLS microtitre method with RPMI-G and an 80% endpoint as our reference point, since it gives comparable results to that of the NCCLS M27-A method,⁵ and the other two methods will be compared to this. Results will be presented by species, because there are substantial inter-species variations (Table 2). The classifica-

Table 1. MICs (mg/L) obtained for ATCC control organisms

ATCC number	Species	Recommended MIC limits ^{5,8}	Macrodilution method YNBG 80% endpoint	Microtitre method					
				YNBG			RPMI-G		
				50% endpoint	80% endpoint	no growth endpoint	50% endpoint	80% endpoint	no growth endpoint
24433	<i>C. albicans</i>	1–4	>32	2	>32	>32	1	4	>32
90028	<i>C. albicans</i>	0.5–2	1	0.25	0.25	1	0.5	0.5	1
750	<i>C. tropicalis</i>	≤0.125–0.25	0.25	0.06	0.06	0.125	0.06	0.06	0.25
6258	<i>C. krusei</i>	4–16	1	0.5	2	8	4	8	16
90030	<i>C. glabrata</i>	≤0.125–0.25	0.25	≤0.03	≤0.03	≤0.03	0.06	0.06	0.125
22019	<i>C. parapsilosis</i>	0.125–0.5	0.25	≤0.03	0.06	0.125	0.125	0.25	0.5

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Table 2. In vitro susceptibilities (mg/L) of 150 *Candida* isolates to flucytosine using three different MIC methods

Species (n)	Macrobroth method YNBG 80% endpoint			Microtitre method																	
				YNBG									RPMI-G								
				50% endpoint			80% endpoint			no growth endpoint			50% endpoint			80% endpoint			no growth endpoint		
				MIC ₅₀	MIC ₉₀	range	MIC ₅₀	MIC ₉₀	range	MIC ₅₀	MIC ₉₀	range	MIC ₅₀	MIC ₉₀	range	MIC ₅₀	MIC ₉₀	range	MIC ₅₀	MIC ₉₀	range
<i>C. albicans</i> , n = 65	0.25	>32	0.06–>32	0.06	16	≤0.03–>32	0.06	>32	≤0.03–>32	0.06	>32	≤0.03–>32	0.25	>32	≤0.03–>32	0.25	>32	≤0.03–>32	0.25	>32	≤0.03–>32
<i>C. tropicalis</i> , n = 27	1	>32	0.25–>32	0.25	1	≤0.03–>32	0.25	2	0.06–>32	0.5	4	0.125–>32	0.25	16	≤0.03–>32	0.25	32	0.06–>32	0.5	>32	0.125–>32
<i>C. krusei</i> , n = 21	1	4	0.5–8	0.25	0.5	≤0.03–1	0.5	1	0.25–2	4	8	0.5–8	4	8	0.5–8	8	16	8–16	16	32	8–32
<i>C. glabrata</i> , n = 16	0.5	2	0.125–16	≤0.03	0.125	≤0.03–0.5	≤0.03	0.25	≤0.03–1	≤0.03	0.25	≤0.03–2	0.06	1	≤0.03–2	0.06	2	≤0.03–4	0.06	4	≤0.03–4
<i>C. parapsilosis</i> , n = 11	0.25	0.5	0.125–0.5	≤0.03	≤0.03	≤0.03–1	≤0.03	0.06	≤0.03–2	0.06	0.125	≤0.03–8	0.06	0.125	≤0.03–0.12	0.125	0.25	0.06–0.5	0.5	0.5	0.06–2
<i>C. lusitanae</i> , n = 10	2	16	0.125–>32	0.5	2	≤0.03–32	0.5	16	≤0.03–32	2	>32	≤0.03–>32	16	>32	≤0.03–>32	32	>32	0.06–>32	32	>32	0.06–>32
All species, n = 150	0.5	16	0.06–>32	0.125	1	≤0.03–>32	0.125	2	≤0.03–>32	0.125	32	≤0.03–>32	0.25	16	≤0.03–>32	0.25	32	≤0.03–>32	0.5	32	≤0.03–>32

tion of susceptible, intermediate or resistant will be based on the RPMI-G microtitre method using an 80% endpoint. The two published breakpoints will be compared: (i) NCCLS M27-A⁵ with MICs (mg/L) classed as: ≤ 4 susceptible, 8–16 intermediate and ≥ 32 resistant; and (ii) BSMM⁴ with MICs (mg/L) classed as: ≤ 1 susceptible, 2–8 intermediate and ≥ 16 resistant. When comparing results *within* each method, single dilution differences between results are considered identical, and when comparing results *between* methods, a range encompassing one dilution difference either way (i.e. within two doubling dilutions) will be classed the same. When analysing different endpoints, the 50% and no growth endpoints will each be compared with the 80% endpoint. The 80% endpoint will also be used when comparing the results obtained between the three methods, and a major discrepancy is classed as susceptible in one test and resistant in another.

C. albicans (n = 65)

With NCCLS breakpoints,⁵ seven isolates were resistant, one intermediate and the remainder susceptible. With BSMM breakpoints,⁴ seven isolates were resistant, five intermediate and the remainder susceptible. The seven isolates were classed as resistant regardless of breakpoint used.

Comparisons within microtitre methods. When comparing the different endpoints, only two (3.1%) and five (7.7%) isolates showed differences of more than one doubling dilution with RPMI-G and YNBG microtitre methods, respectively.

Comparison between all methods. Correlation between all three methods was good with over 75% of isolates having MICs within two doubling dilutions (Table 3). A small percentage of major discrepancies were found when employing either NCCLS (4.6%) or BSMM (1.5%) breakpoints, and these showed varied differences (Table 4).

C. tropicalis (n = 27)

With either NCCLS⁵ or BSMM⁴ breakpoints four isolates were resistant, two intermediate and the remainder susceptible.

Comparisons within microtitre methods. Comparison of the three endpoints showed that seven (25.9%) and eight (29.6%) isolates differed by more than one doubling dilution with RPMI-G and YNBG microtitre methods, respectively.

Comparison between all methods. Overall, the correlation between the different tests was good, with almost 60% of isolates having MICs within two doubling dilutions (Table 3). Again, a small number of major discrepancies were seen when using either NCCLS (11.1%) or BSMM (3.7%) breakpoints, and these showed varied differences (Table 4).

C. krusei (n = 21)

With NCCLS breakpoints,⁵ all isolates were intermediate. With BSMM breakpoints,⁴ nine isolates were resistant and 12 intermediate. No isolate was classed as susceptible regardless of breakpoint chosen.

Comparisons within microtitre methods. Comparison of the three endpoints found that 10 (47.6%) and 16 (76.2%) isolates showed differences greater than one doubling dilution for RPMI-G and YNBG microtitre methods, respectively.

Comparison between all methods. Correlation between the three methods was very poor, with <5% of isolates having MICs within two doubling dilutions. Over 70% of isolates showed differences of four or five dilutions (Table 3). RPMI-G results were significantly higher for most isolates compared with the other two methods. Perhaps surprisingly, there were no major discrepancies when NCCLS breakpoints were

Table 3. Discrepancies between MICs obtained using three different MIC methods

Species (n)	MICs ± 2 dilutions (%)	MICs ± 3 dilutions (%)	MICs ± 4 dilutions (%)	MICs ± 5 dilutions (%)	MICs more than ± 5 dilutions (%)
<i>C. albicans</i> , n = 65	49 (75.4%)	11 (16.9%)	4 (6.2%)	0	1 (1.5%)
<i>C. tropicalis</i> , n = 27	16 (59.3%)	7 (25.9%)	2 (7.4%)	1 (3.7%)	1 (3.7%)
<i>C. krusei</i> , n = 21	1 (4.8%)	5 (23.8%)	8 (38.1%)	7 (33.3%)	0
<i>C. glabrata</i> , n = 16	3 (18.8%)	4 (25.0%)	6 (37.5%)	1 (6.2%)	2 (12.5%)
<i>C. parapsilosis</i> , n = 11	2 (18.2%)	8 (72.7%)	1 (9.1%)	0	0
<i>C. lusitanae</i> , n = 10	5 (50.0%)	2 (20.0%)	0	1 (10.0%)	2 (20.0%)
All species, n = 150	76 (50.7%)	37 (24.7%)	21 (14.0%)	10 (6.7%)	6 (4.0%)

Comparisons were made between a broth macrodilution method using YNBG, and two microtitre methods (using either YNBG or RPMI-G). All MICs were obtained using an 80% endpoint.

Table 4. Major discrepancies resulting in a change of susceptibility category

Species (n)	NCCLS breakpoints ⁵						BSMM breakpoints ⁴					
	no. of major discrepancies (%)			change in susceptibility category ^a			no. of major discrepancies (%)			change in susceptibility category ^a		
	S/S/R	S/R/R	R/S/S	R/S/I	R/S/R	R/S/I	S/S/R	I/S/R	R/S/S	R/S/I	R/S/S	R/S/I
<i>C. albicans</i> , n = 65	3 (4.6%)	2	1	1	1	1	1 (1.5%)					1
<i>C. tropicalis</i> , n = 27	3 (11.1%)	1	1	1	1	1	1 (3.7%)	1				5
<i>C. krusei</i> , n = 21	0						9 (42.8%)				4	5
<i>C. glabrata</i> , n = 16	0						1 (6.2%)		1			
<i>C. parapsilosis</i> , n = 11	0						0					
<i>C. lusitaniae</i> , n = 10	3 (30.0%)				3	3	3 (30.0%)					3
All species, n = 150	9 (6.0%)	1	2	3	2	1	15 (10.0%)	1	1	1	4	9

^aSusceptibility categories reflect the interpretation of the MIC obtained from the three methods tested in the order of microtitre (RPMI-G) method/microtitre (YNBG) method/macrodilution (YNBG) method, e.g. with *C. albicans*, one major discrepancy was found using BSMM breakpoints, and it was resistant by the RPMI-G microtitre method, susceptible with the YNMG microtitre method and intermediate with the macrobroth (YNBG) method.

utilized. However, when using BSMM breakpoints, nine (42.8%) isolates showed discrepancies, all of which were resistant in RPMI-G, susceptible in YNMG microtitre and either susceptible or intermediate with the macrodilution broth method (Table 4).

C. glabrata (n = 16)

With NCCLS breakpoints,⁵ all isolates were susceptible. With BSMM breakpoints,⁴ three isolates were intermediate and the remainder susceptible. No isolate was classed as resistant regardless of breakpoint used.

Comparisons within microtitre methods. The different endpoints showed a difference of more than one doubling dilution for only one isolate (6.2%) with the YNMG microtitre method. The RPMI-G microtitre method showed no differences between endpoints.

Comparison between all methods. Correlation between methods was poor, with only 18.8% of isolates having MICs within two doubling dilutions (Table 3). The poor correlation resulted mainly from the broth macrodilution method since these results were usually higher than those of either of the microtitre methods. However, since most isolates were susceptible with either breakpoint or any methods, only one major discrepancy was found with BSMM breakpoints (Table 4). This isolate was susceptible or intermediate by either microtitre method, but resistant by the broth macrobroth dilution method. No major discrepancies were found when using NCCLS breakpoints.

C. parapsilosis (n = 11)

With either NCCLS⁵ or BSMM⁴ breakpoints all isolates were susceptible.

Comparisons within microtitre methods. Comparison between the different endpoints showed differences of greater than one doubling dilution for six (54.5%) and one (9.1%) isolate with RPMI-G and YNMG microtitre methods, respectively.

Comparison between all methods. The three methods exhibited a poor correlation, with only 18.2% of strains having MICs within two doubling dilutions. The majority of isolates (72.7%) had MICs that differed by three dilutions (Table 3). The YNMG microtitre results were notably lower than those of the other methods; however, no major discrepancies were seen with either breakpoint.

C. lusitaniae (n = 10)

With NCCLS breakpoints,⁵ six isolates were resistant and the remainder susceptible. With BSMM breakpoints,⁴ six isolates

were resistant, one intermediate and the remainder susceptible.

Comparisons within microtitre methods. When analysing the different endpoints, two (20%) and four (40%) isolates were different by more than one doubling dilution with RPMI-G and YNB microtitre methods, respectively.

Large endpoint variations were noted with this species, with some MICs increasing dramatically as the stringency of the endpoint increased.

Comparison between all methods. Correlation between the three methods showed that 50% of isolates had MICs within two doubling dilutions (Table 3). Three major discrepancies (30%) were seen with both NCCLS and BSMM breakpoints (Table 4). These three isolates were resistant by RPMI-G microtitre, susceptible by YNB microtitre and either susceptible (NCCLS breakpoints) or intermediate (BSMM breakpoints) in the broth macrodilution test.

Reproducibility

Of the 20% of isolates (30/150) re-tested with all seven endpoints (210 results), 15 (7.1%) results in nine isolates differed by more than one dilution and one (0.5%) by more than two dilutions. This was an isolate of *C. albicans* tested using the YNB microtitre method, with a 50% endpoint that rose from 4 to >32 mg/L.

Discussion

Historically, most susceptibility testing with flucytosine has been carried out with YNB broth.⁹ Since the drug's introduction, early experiments had established that media containing complex nitrogen sources antagonized its activity.⁹ In 1984, a Report of a Working Group of the British Society for Mycopathology was published.⁴ Its main concern was to recommend appropriate laboratory methods for flucytosine susceptibility testing. The Group found that the *in vitro* activity of flucytosine is very dependent on the conditions of testing: medium, incubation time, inoculum and reading. These factors were reflected in disagreement between numerical results for individual strains among members of the Group.⁴

In 1982, the first subcommittee of the NCCLS on antifungal susceptibility testing was formed. This subcommittee was charged with developing a reference method that might improve inter-laboratory reproducibility. A comparison of macrodilution and microdilution tests with flucytosine with RPMI-1640 showed equivalence or lower MICs with microdilution, especially with *C. tropicalis*.^{10,11} Other collaborative work indicated better inter-laboratory agreement (65–90%) with flucytosine using a smaller inoculum, a 2 day incubation period and a 1+ turbidity growth endpoint.¹² Agreement with

C. lusitaniae was worse than other species and *C. krusei* was not included. Selection of quality control isolates involved repetitive testing of 10 candidate strains and three *Candida* isolates were selected with a range of flucytosine MICs.^{8,13,14} Agreement for *C. albicans* with the supplementation of the RPMI-1640 with 1.8% glucose was 98.1% in a microtitre format.¹⁵ Alteration of pH barely affected the results.¹⁶

In this study, we identified a number of resistant isolates of *C. albicans* (10.8%), *C. tropicalis* (14.8%) and *C. lusitaniae* (60%). In addition, we found all *C. krusei* isolates to be intermediate to flucytosine. This figure is three times higher than that previously published.⁶ These rates of reduced susceptibility were in contrast to that found by others,⁶ where much lower levels of resistance were observed. However, the previous study was carried out in Spain where flucytosine is rarely used; therefore our results may reflect greater use of this drug in the UK. Interestingly though, *C. glabrata* was always found to be susceptible in our study, compared with over 25% of isolates showing intermediate resistance in the Spanish study.⁶ *C. parapsilosis* was also fully susceptible in the present study, thus agreeing with the findings of others.⁶

Most published studies of flucytosine have compared the NCCLS method⁵ with commercial tests, such as Etest,¹⁷ Sensititre YeastOne Colorimetric Antifungal Panel¹⁸ and Fungitest,¹⁹ with varying degrees of success. None of these tests utilizes YNB broth as the test medium; however, YNB broth has previously been used to carry out flucytosine susceptibility testing against *Candida* species.^{20,21} Galgiani *et al.*²¹ showed that variations in the formulation of YNB did not affect the susceptibility testing of flucytosine to any significant extent. In addition, YNB broth has previously been used to test flucytosine against *Cryptococcus neoformans*,²² where it was found to give the widest range of MICs compared with other media, which included RPMI-1640 with and without additional glucose. It is also noted in the NCCLS document⁵ that YNB broth may improve clinical relevance when testing *C. neoformans*. Nevertheless, no comparisons between the methods of the NCCLS⁵ and BSMM⁴ with any *Candida* species have been published to date.

A few studies have shown a correlation with flucytosine susceptibility testing and outcome *in vivo*. Stiller *et al.*²³ found a correlation between a broth macrodilution method and *in vivo* response in *C. albicans*. Isolates with an MIC of <12.5 mg/L responded much better to treatment than those isolates with an MIC of 12.5 mg/L or greater. In 1986, Radetsky *et al.*²⁴ showed agreement with animal and clinical outcome with a broth microdilution method similar to the NCCLS microtitre method. They tested an isolate of *C. albicans* that was considered susceptible to flucytosine by both laboratory (MIC 2 mg/L) and clinical criteria, and an isolate of *C. tropicalis* that was considered resistant to flucytosine by the same criteria (MIC > 16 mg/L). Mice were infected and treated with 150 mg/kg per day flucytosine. All of the mice infected with

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the *C. albicans* isolate were still alive after 30 days, whereas all the mice infected with the *C. tropicalis* isolate had died within 3 days. Similar and more comprehensive results were obtained by Anaissie *et al.*⁷ with multiple isolates of *C. albicans*, *C. krusei* and *C. lusitaniae*.

For *C. albicans* and, to a slightly lesser extent, *C. tropicalis*, many different testing formats appeared to give comparable results. Variation was limited in *C. glabrata* and *C. parapsilosis*, but few isolates had elevated MICs. Considerable variation in MICs was seen with *C. krusei* and *C. lusitaniae*.

In summary, substantial *in vitro/in vivo* correlation work needs to be undertaken to establish optimum susceptibility testing procedures with flucytosine for non-*albicans* *Candida*, particularly *C. krusei*.

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