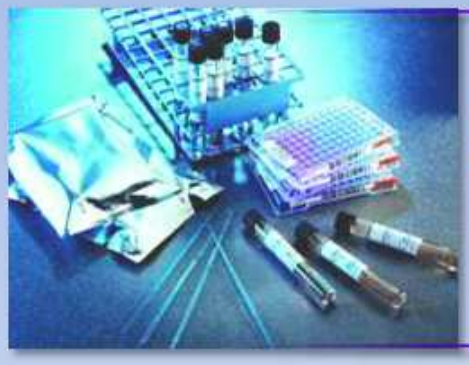
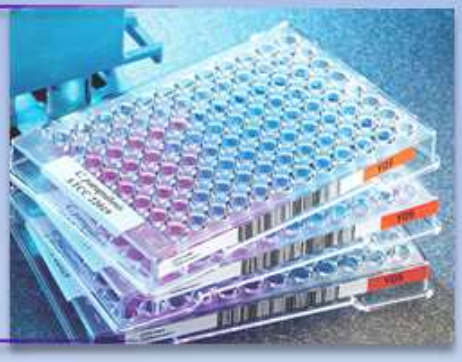
ANNUAL SCIENTIFIC MEETING ON EMERGING INFECTIOUS THREATS 2017



Antifungal susceptibility testing using Sensititre YeastOne



UNIVERSITI KEBANGSAAN MALAYSIA
National University of Malaysia

DING CHUAN HUN

MBBS (IMU), DrPath (Med Microbiol)(UKM), AM (Mal.)

Lecturer & Clinical Microbiologist

Department of Medical Microbiology & Immunology, Faculty of Medicine, Universiti Kebangsaan Malaysia

OUTLINE

- **Introduction to the kit**
- **Available kit formats**
- **Test principle & interpretation**
- **Advantages and disadvantages**
- **Comparison with reference method**
- **The UKMMC experience**

Introduction

- Commercial calorimetric broth microdilution test kit
- MIC readings for up to 10 antifungal agents for each test isolate

- Susceptibility testing of *Candida, Cryptococcus, Aspergillus*
  - not intended for slow growing/fastidious yeasts e.g. *Histoplasma*)

- Manufacturer: TREK diagnostic systems (USA)
- Local distributor: Thermo Scientific

- Packaging: 10 plates/box, with broth to be purchased separately

- Estimated 2017 price for each Sensititre YeastOne YO10 test: **RM 206** (plate + broth + 6% GST)

Current Kit Formats

- As listed on TREK's website
  http://www.trekds.com/products/sensititre/c_yo2.asp
- Available in **dried-plate** formats

- <u>Sensititre YeastOne YO7</u>
  - SEVEN antifungal agents

- <u>Sensititre YeastOne YO9 and YO10</u>
  - NINE antifungal agents (no ketoconazole but 2 extra echinocandins and one extra azole)
  - YO10 has the CE mark and is for sale *outside* the U.S.

YeastOne YO7

Antifungal agent	Dilution range (µg/ml)
Amphotericin B	0.008 - 16
Caspofungin	0.008 - 16
Fluconazole	0.125 - 256
Itraconazole	0.008 - 16
5-Flucytosine	0.03 - 64
Voriconazole	0.008 - 16
Ketoconazole	0.008 - 16

Table 1. Interpretive Guidelines for *In Vitro* Susceptibility Testing of *Candida* spp. and Echinocandins[1]

Antifungal Agent	Species	MIC Range (µg/mL)		
		S	I[a]	R
Anidulafungin[b]	*C. albicans*	≤0.25	0.5	≥1
	C. glabrata	≤0.12	0.25	≥0.5
	C. tropicalis	≤0.25	0.5	≥1
	C. krusei	≤0.25	0.5	≥1
	C. parapsilosis	≤2	4	≥8
	C. guilliermondii	≤2	4	≥8
Caspofungin[b,c]	*C. albicans*	≤0.25	0.5	≥1
	C. glabrata	≤0.12	0.25	≥0.5
	C. tropicalis	≤0.25	0.5	≥1
	C. krusei	≤0.25	0.5	≥1
	C. parapsilosis	≤2	4	≥8
	C. guilliermondii	≤2	4	≥8
Micafungin[b]	*C. albicans*	≤0.25	0.5	≥1
	C. glabrata	≤0.06	0.12	≥0.25
	C. tropicalis	≤0.25	0.5	≥1
	C. krusei	≤0.25	0.5	≥1
	C. parapsilosis	≤2	4	≥8
	C. guilliermondii	≤2	4	≥8

Abbreviations: I, intermediate; MIC, minimal inhibitory concentration; R, resistant; S, susceptible.

CLSI M27-S4

YeastOne YO10

Antifungal agent	Dilution range (µg/ml)
Amphotericin B	0.12 - 8
Caspofungin	0.008 - 8
Fluconazole	0.12 - 256
Itraconazole	0.015 - 16
5-Flucytosine	0.06 - 64
Voriconazole	0.008 - 8
Anidulafungin	0.015 - 8
Micafungin	0.008 - 8
Posaconazole	0.008 - 8

Table 2. Interpretive Guidelines for *In Vitro* Susceptibility Testing of *Candida* spp. and Selected Azoles After 24-hour Incubation

Antifungal Agent	Species	MIC Range (µg/mL)		
		S	SDD[a]	R
Fluconazole[b]	*C. albicans*	≤2	4	≥8
	C. glabrata	–	≤32	≥64
	C. krusei	–	–	–
	C. parapsilosis	≤2	4	≥8
	C. tropicalis	≤2	4	≥8
Voriconazole[c,d]	*C. albicans*	≤0.12	0.25–0.5	≥1
	C. glabrata[e]	–	–	–
	C. krusei	≤0.5	1	≥2
	C. parapsilosis	≤0.12	0.25–0.5	≥1
	C. tropicalis	≤0.12	0.25–0.5	≥1

Abbreviations: MIC, minimal inhibitory concentration; R, resistant; S; susceptible; SDD, susceptible-dose dependent.

Plate Layout

YeastOne **YO10**: 96 wells in each plate, as viewed from above

	1	2	3	4	5	6	7	8	9	10	11	12
A	POS	AND	AND	AND	AND	AND	AND	AND	AND	AND	AND	AB
		0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	0.12
B	MF	MF	MF	MF	MF	MF	MF	MF	MF	MF	MF	AB
	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	0.25
C	CAS	CAS	CAS	CAS	CAS	CAS	CAS	CAS	CAS	CAS	CAS	AB
	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	0.5
D	FC	FC	FC	FC	FC	FC	FC	FC	FC	FC	FC	AB
	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	1
E	PZ	PZ	PZ	PZ	PZ	PZ	PZ	PZ	PZ	PZ	PZ	AB
	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	2
F	VOR	VOR	VOR	VOR	VOR	VOR	VOR	VOR	VOR	VOR	VOR	AB
	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	4
G	IZ	IZ	IZ	IZ	IZ	IZ	IZ	IZ	IZ	IZ	IZ	AB
	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	8
H	FZ	FZ	FZ	FZ	FZ	FZ	FZ	FZ	FZ	FZ	FZ	FZ
	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256

ANTIMICROBICS

POS	Positive Control
AND	Anidulafungin
AB	Amphotericin B
MF	Micafungin
CAS	Caspofungin
FC	5-Flucytosine
PZ	Posaconazole
VOR	Voriconazole
IZ	Itraconazole
FZ	Fluconazole

[Antifungal] increase from L→R for all agents except for AmB (lowest con. on top, highest at the bottom)

For YO7, all readings are done L→R including AmB (layout not shown)

Test Principle

- Each microtitre plate is dosed with various antifungal agents at appropriate dilutions
- The calorimetric indicator (alamarBlue) measures the proliferation of cells (bacteria and fungi)
  - REDOX indicator that yields a colour change (blue → red) in response to metabolic activity
- Results are read by observing the lowest antifungal concentration with inhibition of growth (evidenced by no colour change)

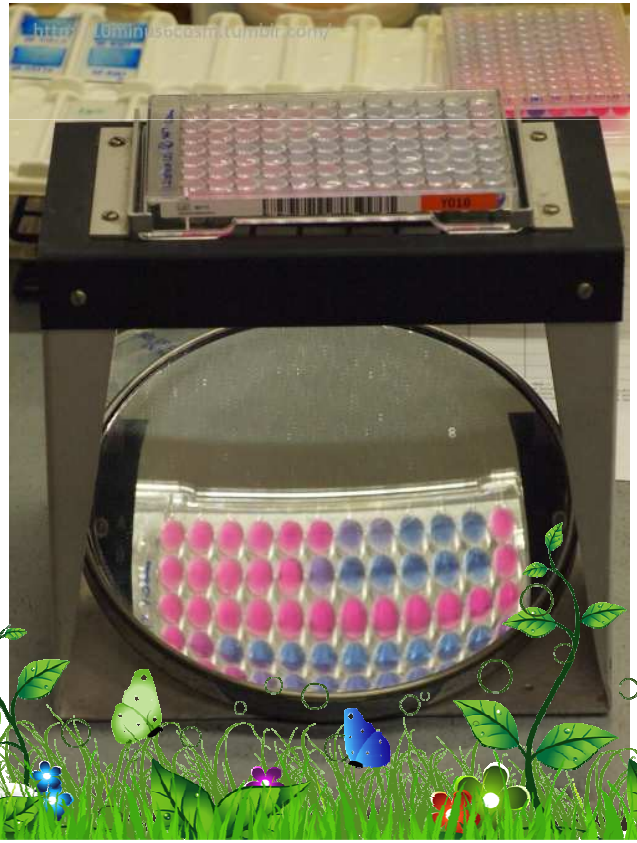
Test Interpretation

- Before reading the MICs, make sure
  - Inoculated plates have been adequately incubated at 35°C as follows:
    - *Candida* (and other rapid growing yeasts): 24 hrs
    - *Cryptococcus*: 72 hrs
    - *Aspergillus*: 48-72 hrs
  - The positive control well has turned **red**

- The MIC is the lowest [antifungal] that inhibits fungal growth. Therefore the *first well* which remains **blue** contains the MIC
  - **Purple** wells also indicate growth and should not be read as the well with the MIC. Exceptions to this rule are discussed below

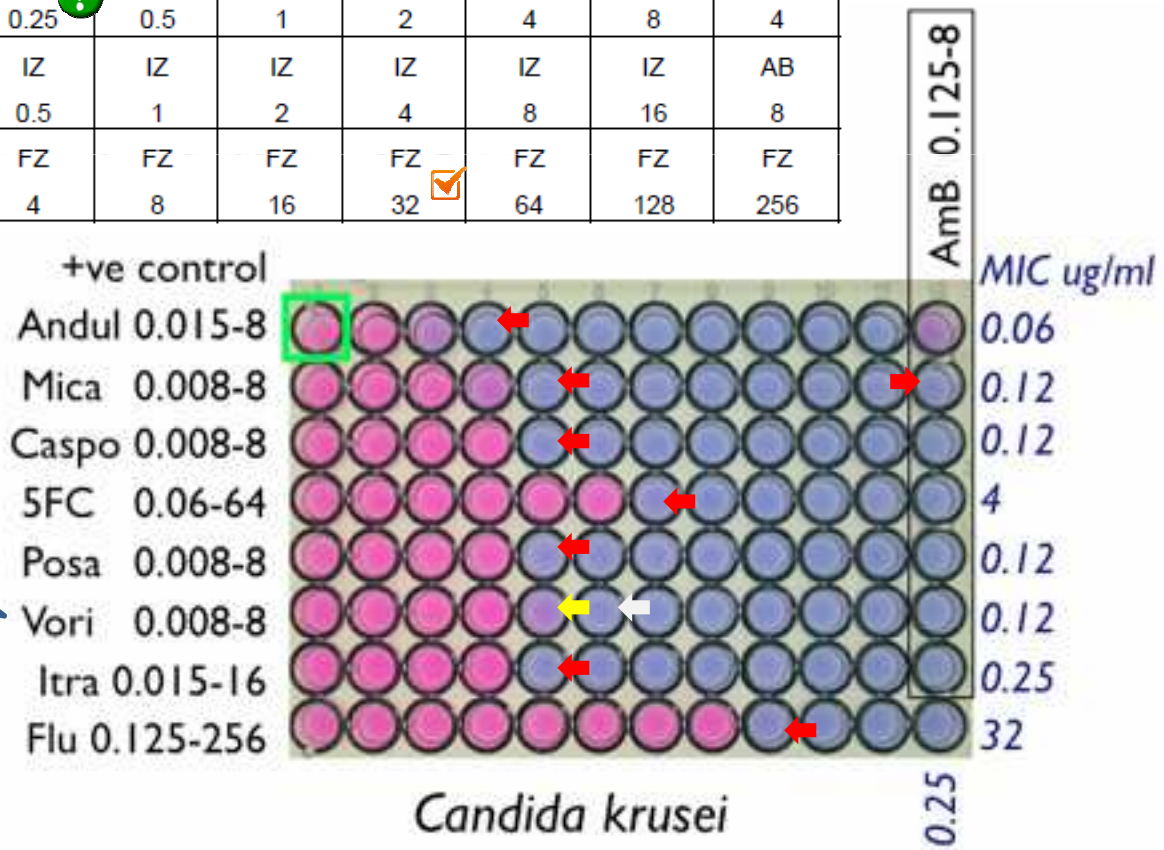
Test Interpretation

- Using a mirror placed below the plate helps with the visualization of colour changes



	1	2	3	4	5	6	7	8	9	10	11	12
A	POS	AND 0.015	AND 0.03	AND 0.06 ✓	AND 0.12	AND 0.25	AND 0.5	AND 1	AND 2	AND 4	AND 8	AB 0.12
B	MF 0.008	MF 0.015	MF 0.03	MF 0.06	MF 0.12 ✓	MF 0.25	MF 0.5	MF 1	MF 2	MF 4	MF 8	AB 0.25 ✓
C	CAS 0.008	CAS 0.015	CAS 0.03	CAS 0.06	CAS 0.12 ✓	CAS 0.25	CAS 0.5	CAS 1	CAS 2	CAS 4	CAS 8	AB 0.5
D	FC 0.06	FC 0.12	FC 0.25	FC 0.5	FC 1	FC 2	FC 4 ✓	FC 8	FC 16	FC 32	FC 64	AB 1
E	PZ 0.008	PZ 0.015	PZ 0.03	PZ 0.06	PZ 0.12 ✓	PZ 0.25	PZ 0.5	PZ 1	PZ 2	PZ 4	PZ 8	AB 2
F	VOR 0.008	VOR 0.015	VOR 0.03	VOR 0.06	VOR 0.12 ?	VOR 0.25 ?	VOR 0.5	VOR 1	VOR 2	VOR 4	VOR 8	AB 4
G	IZ 0.015	IZ 0.03	IZ 0.06	IZ 0.12	IZ 0.25 ✓	IZ 0.5	IZ 1	IZ 2	IZ 4	IZ 8	IZ 16	AB 8
H	FZ 0.12	FZ 0.25	FZ 0.5	FZ 1	FZ 2	FZ 4	FZ 8	FZ 16	FZ 32 ✓	FZ 64	FZ 128	FZ 256

Ambiguity for Vori endpoint: 0.12 or 0.25?



Test Interpretation

- For azoles and flucytosine, endpoints may be less sharp, due to **trailing growth**

- Trailing growth is due to incomplete inhibition of growth at higher [antifungal] above the MIC.
  - Colour change will be slight, and present over several (or all) concentrations above the MIC
  - Exact cause unknown /not fully understood

    (Possible causes: pH, temp, Glu content of medium; up-regulation of azole resistance genes etc)
  - **Does not indicate clinical/in-vivo resistance**

    [Revankar SG et al. Interpretation of trailing endpoints in antifungal susceptibility testing by the National Committee for Clinical Laboratory Standards method. J Clin Microbiol. 998;36(1):153–156]

Test Interpretation

- Hence, for azoles, when trailing growth occurs, the MIC is the concentration in the first well with a ***less intense*** colour change
  - i.e. the first **purple** well in a series of purple wells

Test Interpretation

- **Skipped wells** can sometimes be encountered.

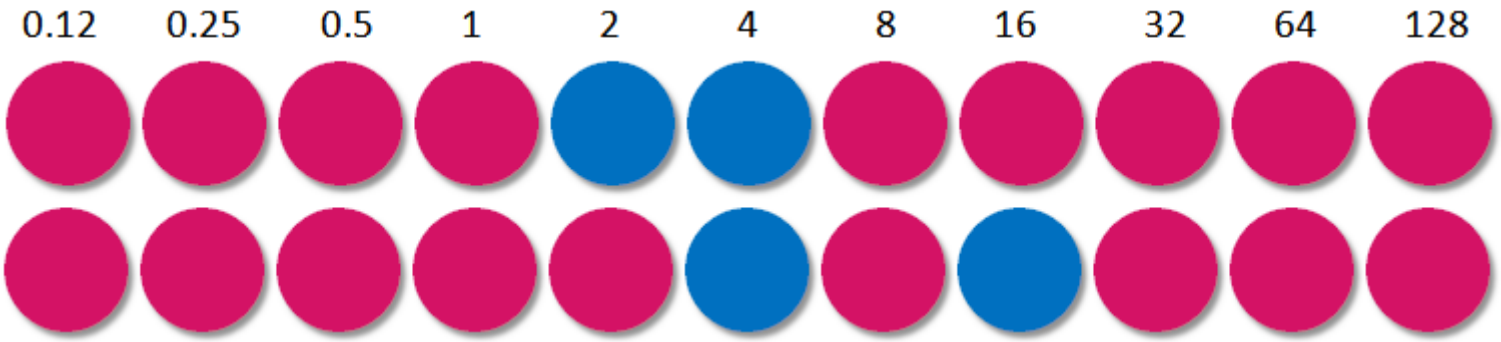
- Essentially, a skipped well happens when the well(s) on both sides of a **blue** well are **red**

In the above example, the MIC is **>128µg/mL** and the blue well with conc. of 16µg/mL is *disregarded*

Test Interpretation

A single skipped well does not invalidate the test but….

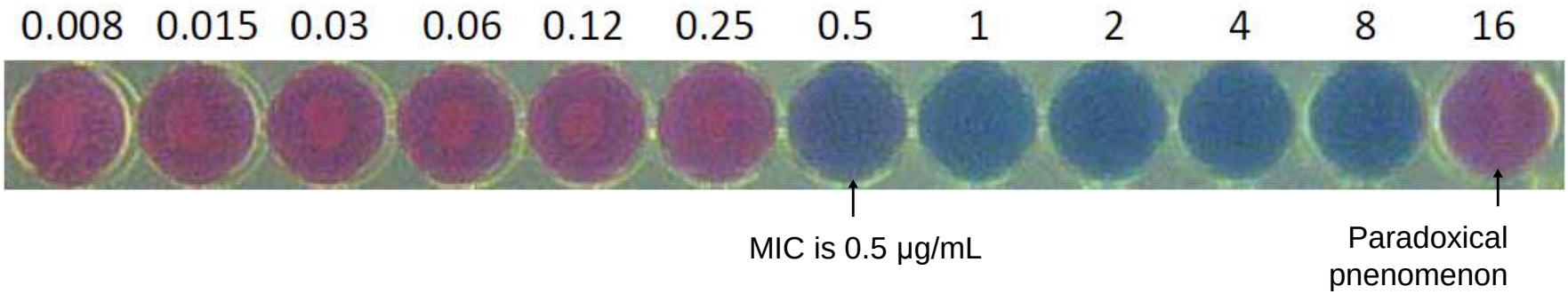
if **two** (or more) skipped wells are encountered, the test should be *repeated!*



Test Interpretation

To add to the confusion, itraconazole can display a **paradoxical phenomenon**, allowing growth in wells when the [drug] is very high

- Also known as the *Eagle phenomenon*, was originally seen with penicillin
- Postulated explanations: precipitation of the drug at high con., self-antagonizing of the binding receptor etc.
- Like trailing growth, paradoxical effect is also **not likely to be clinically relevant**



Advantages

- **All** the **antifungal agents** with published CLSI breakpoints can be tested in a single kit
- Provides **MIC** readings (unlike the disk diffusion method)
- Kits **stored at room temperature** and negates the need for physical space in chillers
- **Individual packaging** allows testing one plate at a time with no waste
- Software-facilitated visual reading (i.e. Vizion™ System) available (next slide)

Vizion™ Digital MIC Viewing System

Disadvantages

- **Costly!**
  - Paying for susceptibility testing of antifungals with no CLSI breakpoints (posaconazole) or for antifungals which are rarely used to treat *Candida* infections (itraconazole, flucytosine)

- **Overcalling of resistance** esp. to azoles (next slide)

- **Skipped wells** and **trailing growth** can complicate reading of MICs

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Comparative Evaluation of Etest and Sensititre YeastOne Panels against the Clinical and Laboratory Standards Institute M27-A2 Reference Broth Microdilution Method for Testing *Candida* Susceptibility to Seven Antifungal Agents▽

Barbara D. Alexander,[1]* Terry C. Byrne,[2] Kelly L. Smith,[1] Kimberly E. Hanson,[1] Kevin J. Anstrom,[3] John R. Perfect,[1] and L. Barth Reller[1,2]

Division of Infectious Diseases and International Health, Department of Medicine,[1] Clinical Microbiology Laboratory, Department of Pathology,[2] and Duke Clinical Research Institute,[3] Duke University Medical Center, Durham, North Carolina

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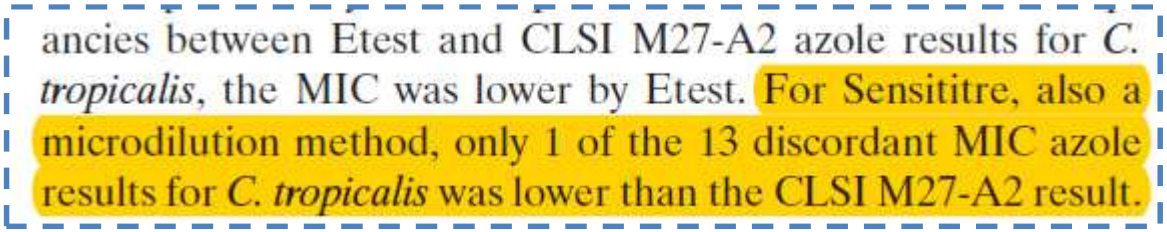
To assess their utility for antifungal susceptibility testing in our clinical laboratory, the Etest and Sensititre methods were compared with the Clinical and Laboratory Standards Institute (CLSI) M27-A2 reference broth microdilution method. Fluconazole (FL), itraconazole (I), voriconazole (V), posaconazole (P), flucytosine (FC), caspofungin (C), and amphotericin B (A) were tested with 212 *Candida* isolates. Reference MICs were determined after 48 h of incubation, and Etest and Sensititre MICs were determined after 24 h and 48 h of incubation. Overall, excellent essential agreement (EA) between the reference and test methods was observed for Etest (95%) and Sensititre (91%). Etest showed an ≥92% EA for MICs for all drugs tested; Sensititre showed a ≥92% EA for MICs for I, FC, A, and C but 82% for FL and 85% for V. The overall categorical agreement (CA) was 90% for Etest and 88% for Sensititre; minor errors accounted for the majority of all categorical errors for both systems. Categorical agreement was lowest for *Candida glabrata* and *Candida tropicalis* with both test systems. Etest and Sensititre provided better CA at 24 h compared to 48 h for *C. glabrata*; however, CA for *C. glabrata* was <80% for FL with both test systems despite MIC determination at 24 h. Agreement between technologists for both methods was ≥98% for each agent against all organisms tested. Overall, Etest and Sensititre methods compared favorably with the CLSI reference method for determining the susceptibility of *Candida*. However, further evaluation of their performance for determining the MICs of azoles, particularly for *C. glabrata*, is warranted.

Definitions
Essential agreement: test MIC is within **two dilutions** of the reference method MIC
Categorical agreement: when the test and reference MICs fell within the **same interpretive category** (i.e. susceptible, susceptible dose dependent, intermediate etc.)

Implications

- *C. tropicalis* is emerging as an important non-albicans *Candida* species around the world, even overtaking *C. albicans* as the perdominant *Candida* species in several reports.



ancies between Etest and CLSI M27-A2 azole results for *C. tropicalis*, the MIC was lower by Etest. For Sensititre, also a microdilution method, only 1 of the 13 discordant MIC azole results for *C. tropicalis* was lower than the CLSI M27-A2 result.

- Essentially 12/13 MICs were higher with Sentititre
- So will labs relying on Sensititre overcall azole resistance in their *C. tropicalis* isolates, leading to unnecessary usage of AmB?

Implications

the azoles. For all discrepant results for *C. glabrata* and an azole, the MIC was higher by both test systems compared to the reference MIC. This raises the possibility that *C. glabrata* may be associated with azole resistance detectable by the test

- Similarly there will be overcalling of azole resistance for *Candida glabrata* isolates

Implications

resistance. We tested a relatively large cohort of isolates with 56 instances of resistance to one or more drugs; the Etest system failed to detect resistance only twice. The Sensititre system did not fail to detect resistance.

On the bright side,

- Since an important reason of doing antifungal testing in the clinical laboratory is to detect resistance, using Sensititre would reduce the chances of missing resistant isolates

The UKMMC Experience…

- Sensititre has been used by UKMMC for ≥7 years
- Due to cost constraints, only done for *Candida* isolates from sterile specimens (tissue, body fluids and blood)

- Have isolated *C. tropicalis* from blood/CVL cultures with high (SDD or resistant) MICs to fluco and voriconazole
  - Frequency once in 2-3 months; unclear if these are truly resistant strains

- Frequently encounter trailing (approx 50% of tests) and occasionally skipped wells (≈10%)

- Posaconazole susceptibility not reported due to lack of CLSI interpretative criteria


Thank
You

Table 1. Interpretive Guidelines for *In Vitro* Susceptibility Testing of *Candida* spp.

Antifungal Agent	Susceptible (S)	Susceptible-dose dependent (S-DD)[a]	Intermediate (I)[b]	Resistant (R)	Nonsusceptible (NS)
Anidulafungin[c]	**≤2**	-	-	-	**>2**
Caspofungin[c]	**≤2**	-	-	-	**>2**
Fluconazole[d]	≤8	16-32	-	≥64	-
Flucytosine[e]	≤4	-	8-16	≥32	-
Itraconazole[f]	≤0.125	0.25-0.5	-	≥1	-
Micafungin[c]	**≤2**	-	-	-	**>2**
Voriconazole[c]	≤1	2	-	≥4	-

NOTE 1: Shown are the breakpoints (µg/mL) for *Candida* spp. against the indicated agents. If minimal inhibitory concentrations (MICs) are measured using a scale that yields results falling between categories, the next higher category is implied. Thus, an isolate with a fluconazole MIC of 12.5 µg/mL would be placed in the S-DD category.

NOTE 2: The MIC breakpoints in boldface type were adopted at a meeting of the subcommittee held on 9 June 2007 in Boston, MA. These breakpoints are considered tentative for one year and are open for comments. There is no Resistant category assigned for the echinocandin agents; isolates with higher MICs may be described as nonsusceptible.

7.7.1 Amphotericin B

Experience to date using the procedures described in this standard indicates that amphotericin B MICs for *Candida* spp. isolates are tightly clustered between 0.25 and 1.0 µg/mL. When isolates that appear resistant to amphotericin B in animal models are tested by M27 methods, MIC values greater than 1 µg/mL may be obtained. Unfortunately, the M27 methodology does not consistently permit detection of such isolates, and all that can at present be concluded is that if an amphotericin B MIC of >1 µg/mL is obtained for a *Candida* spp. isolate, then that isolate is likely resistant to amphotericin B. Some work has suggested that testing with Antibiotic Medium 3 supplemented with 2% glucose (dextrose) and reading MICs after 24 hours incubation permits more reliable detection of resistant isolates.[23,24] However, the reproducibility of this method has been questioned,[25] and laboratories that choose to do this testing must carefully compare their results with those obtained for isolates with known responses to amphotericin B. A collection of potentially useful reference isolates has been deposited in the American Type Culture Collection (ATCC®)[a]: *Candida lusitaniae* ATCC® 200950, ATCC® 200951, ATCC® 200952, ATCC® 200953, ATCC® 200954; *C. albicans* ATCC® 200955; and *Candida tropicalis* ATCC® 200956.

7.7.2 Flucytosine (5-FC)

Based largely on historical data and partially on the drug's pharmacokinetics, interpretive breakpoints for *Candida* spp. and flucytosine have been established (see Table 1, M27 Informational Supplement).[13]