Antifungals: resistant mechanisms, drug susceptibility testing, and emerging drug resistant fungi

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- Received research funds from IMMY diagnostics, Vela diagnostics
- Advisor, CLSI (Clinical and Laboratory Standards Institute) Antifungal Susceptibility Subcommittee

Objectives

- To review the common antifungal drug resistant mechanisms
- To describe antifungal drug susceptibility testing methods
- To recognize emerging antifungal drug resistant organisms

COMMON ANTIFUNGAL DRUG RESISTANT MECHANISMS

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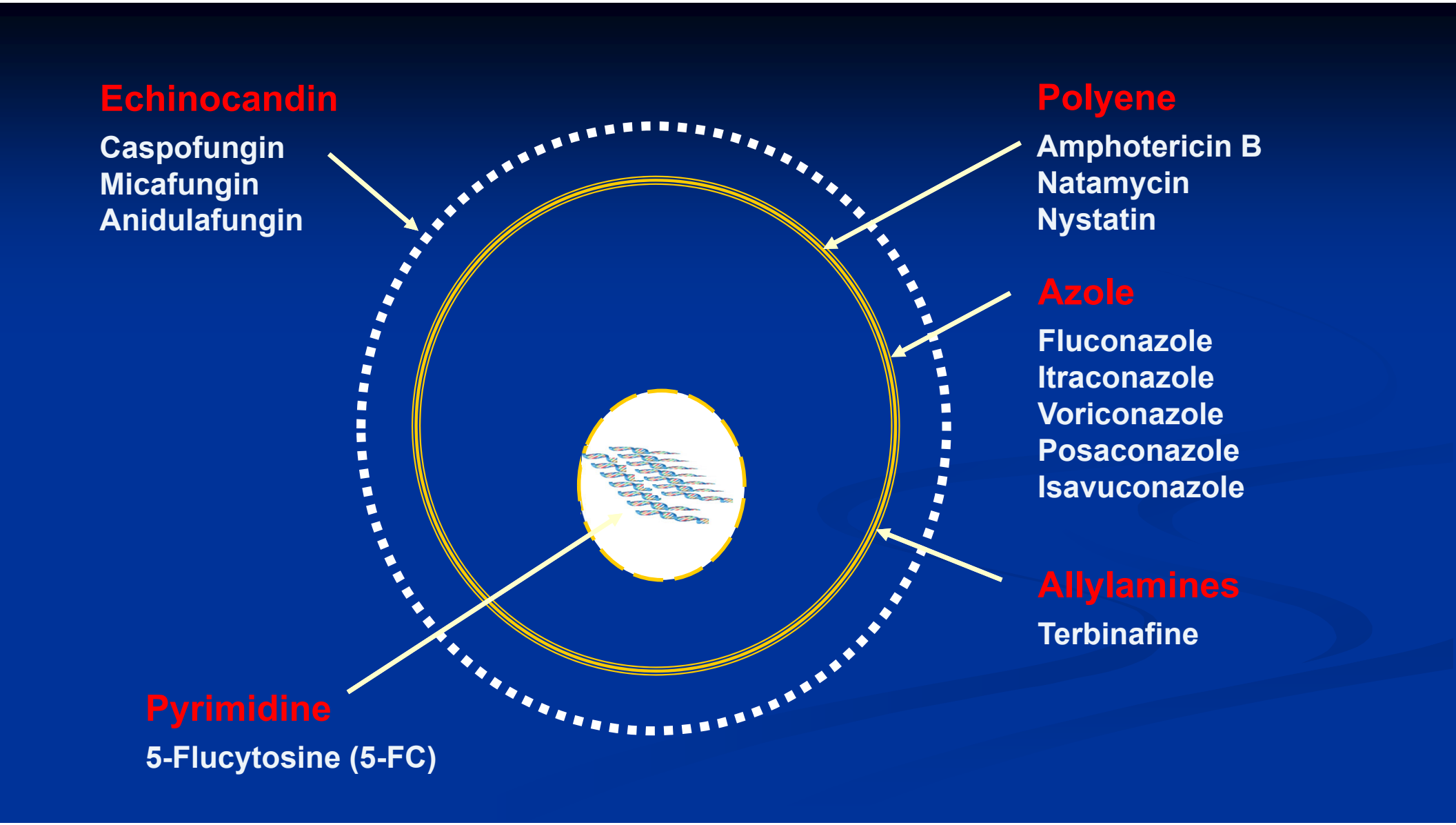
Echinocandin
Caspofungin
Micafungin
Anidulafungin
Polyene
Amphotericin B
Natamycin
Nystatin
Azole
Fluconazole
Itraconazole
Voriconazole
Posaconazole
Isavuconazole
Allylamines
Terbinafine
Pyrimidine
5-Flucytosine (5-FC)

Antifungal drug resistance

- Primary resistance (intrinsic resistance; inherent resistance)
  - Found naturally without prior drug exposure.
  - Underlying resistant mechanism is inherent and not acquired during therapy.

- Acquired resistance
  - Drug selection pressure
  - Horizontal transmission between patients (rarely)

Arendrup MC J Infect Dis 2017; 216:S445

Amphotericin B

- Fungicidal
- Mechanisms of action: binding to ergosterol to destroy fungal cell membrane
- Resistance (No CLSI breakpoints; ECV of 2 µg/ml for *Candida* sp, and 2 or 4 µg/ml for *Aspergillus* sp)
  - High intrinsic reduced-susceptibility found in *C. krusei, C. auris, Trichosporon* sp, *A. terreus, L. prolificans, Fusarium* sp
  - Acquired resistance rare
- Mechanisms of resistance
  - Reduced content of ergosterol in the cell membrane
  - Biofilms

Azoles (Fluconazole → Voriconazole, Itraconazole → Posaconazole, Isavuconazole)

- Fungistatic (e.g. Fluconazole)
- Mechanisms of action
  - Inhibiting ergosterol biosynthesis by interfering with the action of lanosterol 14α-demethylase (encoded by *ERG11, Cyp51A*)
- Resistance
  - Fluconazole has no activity against *Aspergillus* sp, *Fusarium* sp, Mucorales
  - Intrinsic resistance to fluconazole: *C. krusei*, *C. guilliermondii*
  - Acquired resistance to fluconazole seen in *C. albicans*, *C. tropicalis*, *C. glabrata*

Mechanisms of azoles resistance

- Drug-target modification (genetic modification of the target *Erg11, Cyp51A*)
- Increase the target abundance (mutation in *UPC2* leads overexpression of *ERG11*)
- Upregulation of efflux transporter genes (drug efflux pumps) resulting reduction of intracellular drug concentration
  - *C. albicans (MDR1)*
  - *C. glabrata* (*CDR1*, *CDR*2)
  - *C. krusei* (*ABC1*)
- Modification of other ergosterol biosynthesis pathway (*ERG3*)
- Biofilms

Echinocandins (Caspofungin, Micafungin, Anidulafungin)

- Fungicidal for *Candida* sp, but fungistatic for *Aspergillus* sp.
- Mechanisms of action
  - Targeting 1,3-beta-D-glucan synthase (bind to Fksp major subunit) to irreversibly inhibit fungal cell wall synthesis
- Resistance
  - No activities for *C. neoformans*, *Fusarium* sp, Mucorales
  - Intrinsic resistance seen in *C. parapsilosis* species complex, *C. guilliermondii*
  - Acquired resistance found in *C. glabrata*, *C. albicans*, *C. tropicalis*
- Mechanisms of resistance
  - *FKS1* gene mutations (*C. albicans*)
  - *FKS1* and *FKS2* mutations (*C. glabrata*)

ANTIFUNGAL DRUG SUSCEPTIBILITY TESTING (AFST)

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Antifungal susceptibility testing (AFST)

- To measure the lowest concentration of a drug that inhibits the growth of the organism, so called Minimum Inhibitory Concentration (MIC)

Why we perform susceptibility testing?

- To reliably estimate antimicrobial activities against pathogens
- To correlate with in vivo activity and to predict likelihood of outcome of therapy
- To survey/monitor resistance development
- To provide spectrum of activity of newly developed agents

Important factors about antifungal susceptibility testing (AFST)

- Host factors often more important than AST results in determining clinical outcomes
  - Underline conditions
  - PK and PD (Pharmacokinetics and pharmacodynamics)
- In vitro AST results do not 100% predict successful treatment
- Resistance described in vitro often predicts clinical failure

IDSA Candidiasis guideline

- Recent surveillance studies suggest that triazole resistance among *C. glabrata* isolates has increased to a degree that is it difficult to rely upon these agents for therapy in the absence of susceptibility testing
- A similar trend has begun to emerge for a smaller proportion of *C. glabrata* isolates and the echinocandins
- Because of these trends, susceptibility testing is increasingly used to guide the management of candidemia and invasive candidiasis

Pappas et al. *Clin Infect Dis* 2015 doi: 10.1093/cid/civ933.

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IDSA Candidiasis guideline

- Recommended to test for azole susceptibility for all bloodstream and other clinically relevant *Candida* isolates
  - Those from sterile sites
  - Non-sterile may be clinically relevant
    - Neutropenic patients
- Consider echinocandin susceptibility testing
  - *C. glabrata* or *C. parapsilosis* infections
  - Prior echinocandin exposure

Pappas et al. *Clin Infect Dis* 2016.

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Who sets susceptibility testing standards in the US?

- Clinical and Laboratory Standards Institute (CLSI)

	Method	Standards
Yeast	M27-A4 (broth dilution)	M60 2nd
	M44-A3 (disk diffusion)	
Mold (filamentous fungi)	M38-A3 (broth dilution)	M61 2nd
	M51-A (disk diffusion)	

Antifungal drug susceptibility testing methods

- Visual measurement (CLSI broth microdilution method M27-A4, M38-A3)
- Disk diffusion (CLSI M44-A3, M51A)
- Colorimetrical (YeastOne Sensititre)
- Gradient diffusion (E-test)
- Automated (Vitek 2, bioMerieux)
- Optical density (EUCAST)

Test methods used in the Clinical Labs in the USA as per recent CAP survey (2015 – 2018)

Test methods	Participant response (%)			
	2016	2017	2018	2019
Vitek 2	152 (42%)	172 (42%)	198 (44%)	214 (46%)
YeastOne colorimetric microdilution	155 (42%)	162 (40%)	171 (38%)	182 (39%)
Gradient diffusion strips (e.g. E-test, MTS)	28 (8%)	36 (9%)	36 (8%)	34 (7%)
Broth microdilution	21 (6%)	21 (5)	26 (6%)	19 (4%)
Disk Diffusion	6 (2%)	6 (1%)	8 (2%)	9 (2%)
Other	4 (1%)	8 (2%)	8 (2%)	8 (2%)
Total	***366***	***405***	***447***	***466***

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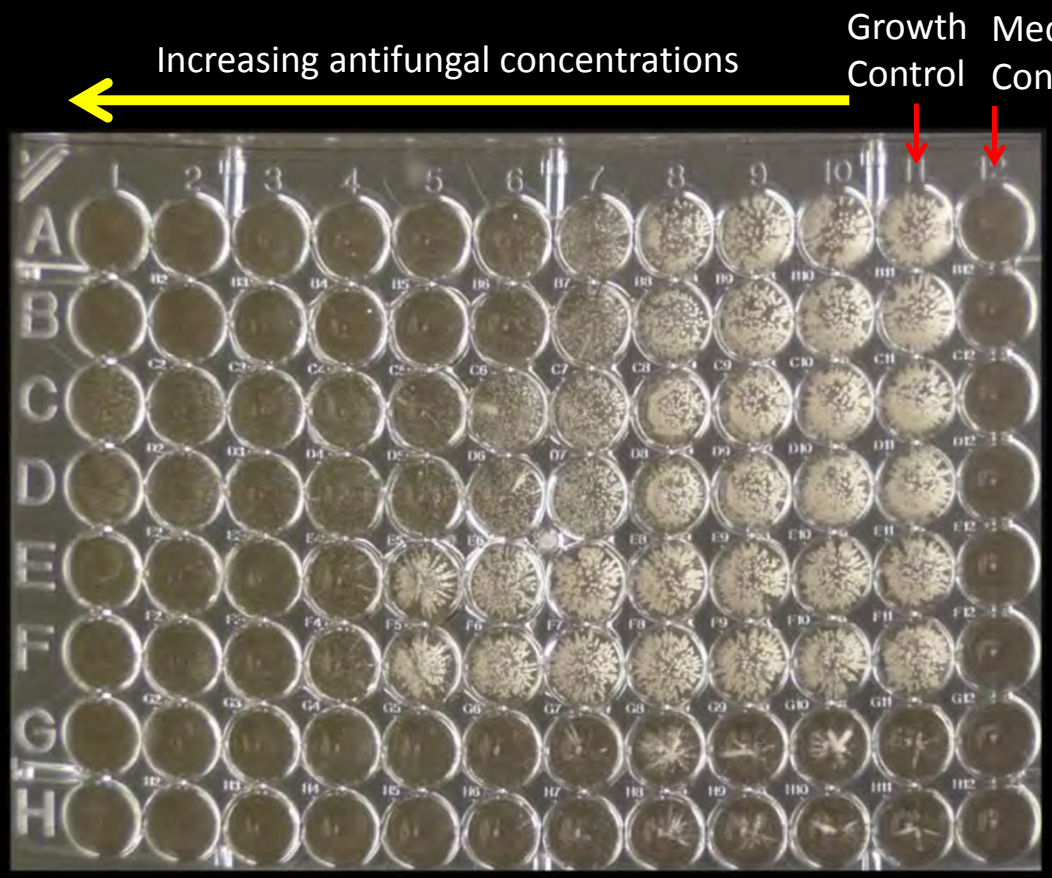
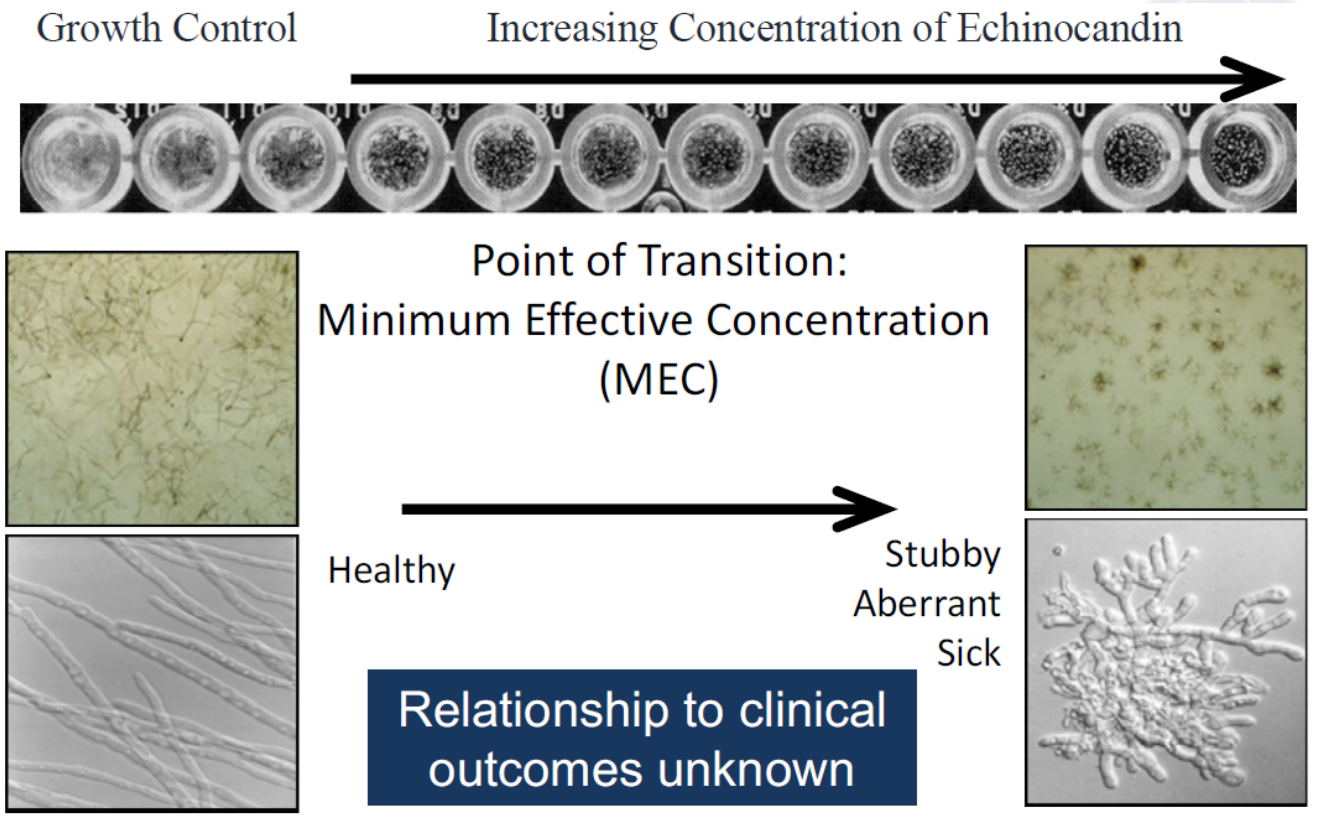
CLSI broth microdilution susceptibility testing



Endpoints

- Amphotericin B: 100% inhibition
- Azoles: 50% inhibition for yeast; 100% inhibition for mold
- Echinocandins: 50% inhibition for yeast; MEC for mold

Echinocandins: MEC (Minimum Effective Concentration) for filamentous fungi



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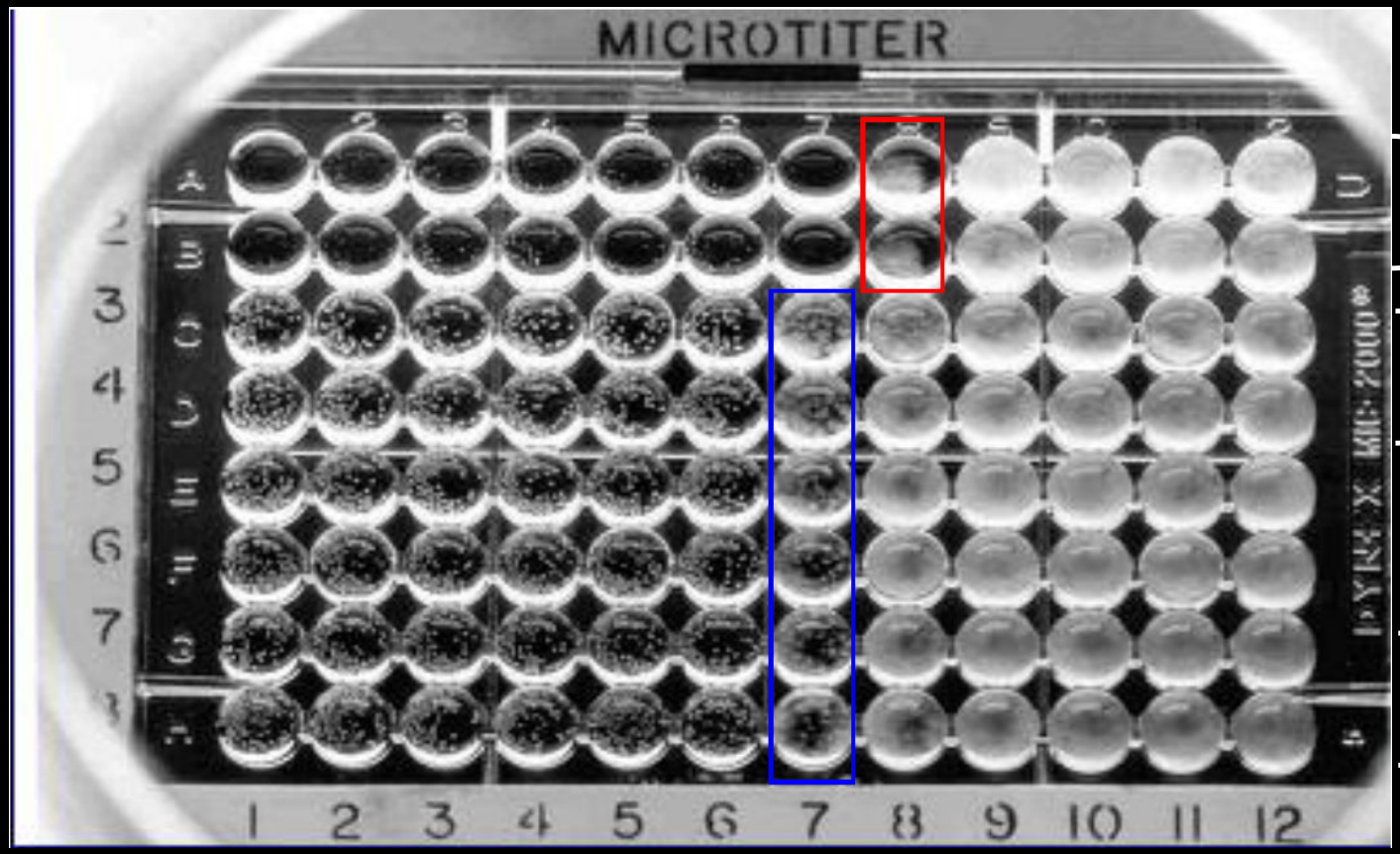
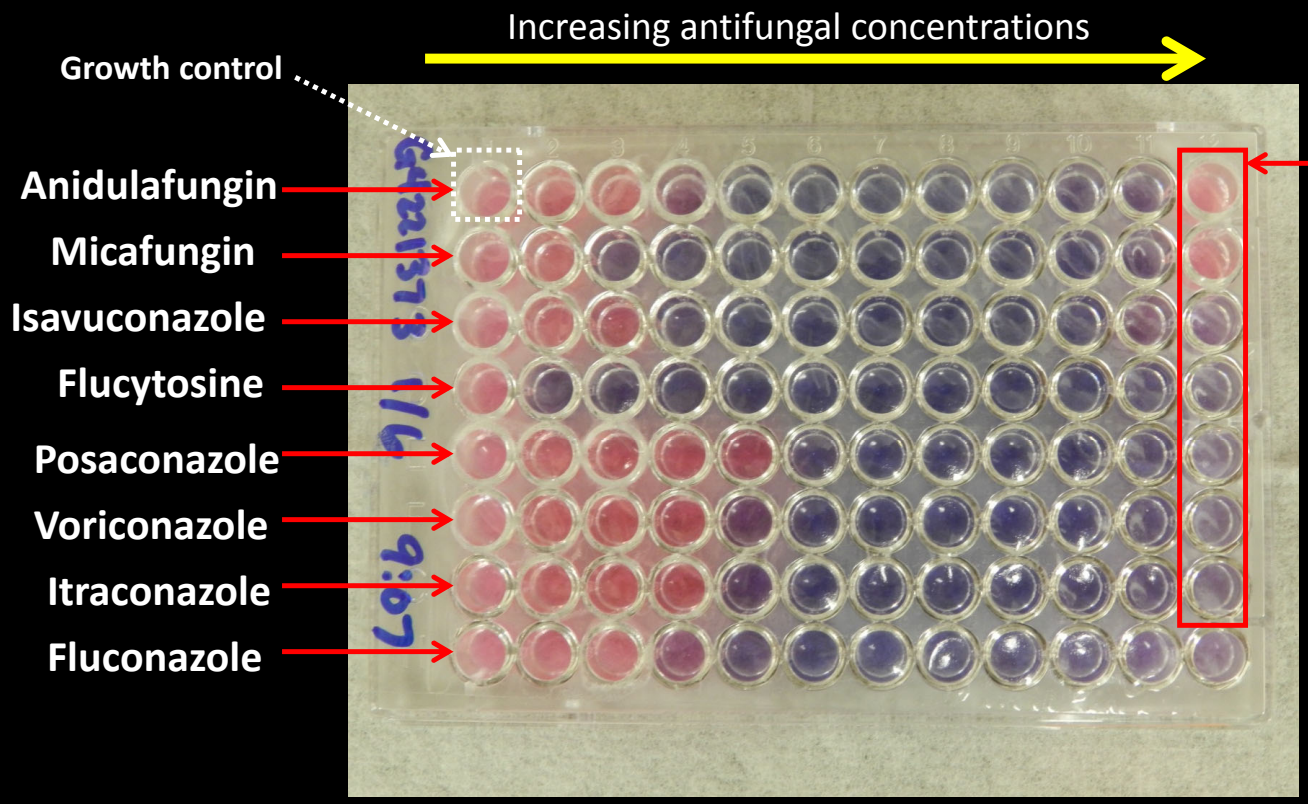
Minimal Effective Concentration (MEC) of Caspofungin (CLSI M38-A3)



Limitations for broth microdilution

- Time consuming to prepare in house
- Availability of pure antifungal compounds
- Lack of standards in materials/reagents used
- Human errors

YeastOne Colorimetric assay (Thermo Scientific TREK Diagnostics)

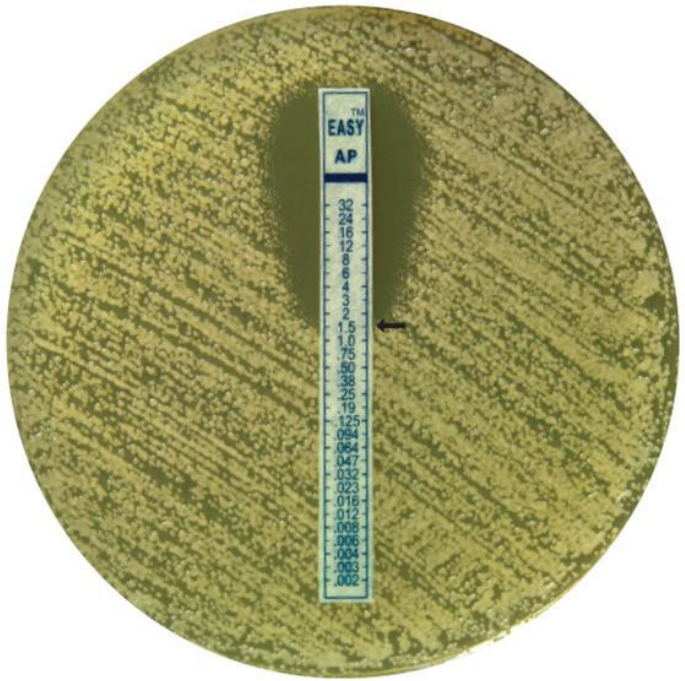


Endpoints

- 1st **blue** well

AlamarBlue - viable cells able to reduce resazurin (*blue – no growth*) to resorufin (*pink/red – growth*)

E-test (agar diffusion method with strips containing pre-defined antifungal concentration gradient): Amphotericin B



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Clinical Breakpoint (CBPs)

- Threshold MIC value established to classify microbes as susceptible or resistant to a drug based on
  - MIC distribution
  - Pharmacokinetics/pharmacodynamics
  - Clinical outcomes
- In the US, CBPs are set up by
  - Clinical and Laboratory Standards Institute (CLSI) antifungal subcommittee
    - CLSI M60 2nd (yeast); CLSI M61 2nd (mold)
  - FDA

CLSI M60 2nd (2020): CBPs for *In vitro* susceptibility testing of *Candida* sp. and selected **azoles** after 24h incubation

Antifungal Agent[a]	Species	MIC Breakpoints and Interpretive Categories, µg/mL			
		S	I[b]	SDD[c]	R
Fluconazole[2,c]	*C. albicans*	≤2	–	4	≥8
	C. glabrata[g]	–	–	<32	>64
	C. krusei[h]	–	–	–	–
	C. parapsilosis[e]	≤2	–	4	≥8
	C. tropicalis	≤2	–	4	≥8
Voriconazole[3,d]	*C. albicans*	≤0.12	0.25–0.5	–	≥1
	C. glabrata[i]	–	–	–	–
	C. krusei	≤0.5	1	–	≥2
	C. parapsilosis[e]	≤0.12	0.25–0.5	–	≥1
	C. tropicalis	≤0.12	0.25–0.5	–	≥1

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CLSI M60 2nd (2020): CBPs for *In vitro* susceptibility testing of *Candida* sp. and selected **echinocandins** after 24h incubation

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

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CLSI M61 2nd (2020): CBPs for *Aspergillus fumigatus*

Antifungal Agent	Species	MIC Breakpoints and Interpretive Categories, µg/mL		
		S	I	R
Voriconazole[a]	*A. fumigatus*	≤0.5	1	≥2

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Epidemiology cut-off value (ECV)

- ECV are MIC values that separate organism into those with wild-type (WT) and non-wild-type (NWT) population based on in vitro MIC data only.
  - WT: MIC result is consistent with no acquired mutational resistance
  - NWT: MIC result is consistent with either acquired or mutational resistance
  - Method: CLSI M57 (Principles and procedures for the development of ECVs for AFST)
  - Standards: CLSI M59 3rd ED (ECVs for AFST)
- ECVs do not predict clinical outcome to therapy as clinical breakpoints do

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CLSI M59 3rd ED (2020): ECVs for In vitro susceptibility testing of *Candida* sp. with no CBPs

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Antifungal Agent	Species	ECV, µg/mL[a,b,c]
Amphotericin B	*C. albicans*	2
	C. dubliniensis	**0.5**
	C. glabrata	2
	C. guilliermondii	**2**
	C. parapsilosis	**1**
	C. tropicalis	2
Fluconazole	*C. dubliniensis*	0.5
	C. duobushaemulonii	**32**
	C. guilliermondii	8
	C. kefyr	**1**
	C. lusitaniae	1
	C. metapsilosis	**4**
	C. orthopsilosis	**2**

When CBPs are available for the fungal species and antifungal agents being evaluated, the ECVs should not be used in clinical pratice

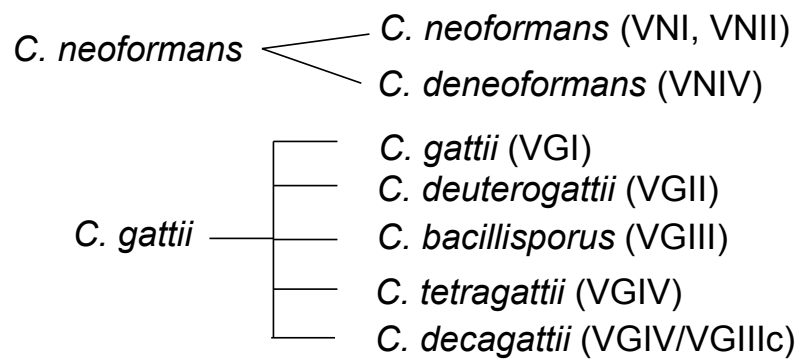
CLSI M59 3rd ED (2020): ECVs for In vitro susceptibility testing of *Candida* sp. with no CBPs

- *C. parapsilosis* complex (*C. parapsilosis*, *C. orthopsilosis*, *C. metapsilosis*)
  - If the clinical labs are now able to identify the subspecies within the complex (mostly by MALDI), then *C. parapsilosis* CBP should not be applied to *C. orthorpsilosis* and *C. metapsilosis* (CLSI M60Ed2). Instead, ECVs should be applied (CLSI M59Ed3)
  - However, identification of sub-species is not possible, *C. parapsilosis* CBP may still be applied since the prevalence of *C. orthropsilosis* or *C. metapsilosis* is still very low (may vary in different institutions).
- More antifungal agent ECVs are added to *Candida* species without CBP (CLSI M59Ed3)

CLSI M59 3rd ED (2020): ECVs for In vitro susceptibility testing of *Cryptococcus* spp.

- No CBP for *Cryptococcus*, but ECVs are available but interpretation based on genotype and has recent nomenclature change (CLSI M59Ed3)
- If the lab identified *C. neoformans*, it may still apply ECVs established for *C. neoformans* VNI since VNI is the most common molecular genotype of *C. neoformans* (may include a note when report).
- If the lab identified *C. gattii*, it may be challenging in deciding which ECVs to apply since most clinical labs don't have capacity to get down to genotype VGI vs VGII (new name *C. deuterogattii*)

Antifungal Agent	Species (Genotype)	ECV, µg/mL[b,c]
Amphotericin B	*C. gattii* (VGI)	0.5
	*C. **deuterogattii*** **(formerly *C. gattii*)** (VGII)	1
	C. neoformans (VNI)	0.5
Fluconazole	*C. gattii* (VGI)	16
	*C. **deuterogattii*** **(formerly *C. gattii*)** (VGII)	32
	C. neoformans (VNI)	8
Flucytosine	*C. gattii* (VGI)	4
	*C. **deuterogattii*** **(formerly *C. gattii*)** (VGII)	32
	C. neoformans (VNI)	8
Itraconazole	*C. gattii* (VGI)	0.5
	*C. **deuterogattii*** **(formerly *C. gattii*)** (VGII)	1
	C. neoformans (VNI)	0.25
Posaconazole	*C. neoformans* (VNI)	0.25
Voriconazole	*C. gattii* (VGI)	0.5
	*C. **deuterogattii*** **(formerly *C. gattii*)** (VGII)	0.5
	C. neoformans (VNI)	0.25



Hagen F. et al. Fungal Gen Biololgy 2015; 78:16

CLSI M59 3rd ED(2020): ECVs for In vitro susceptibility testing of *Aspergillus* spp.

Antifungal Agent	Species	ECV (µg/mL)[‡§]
Amphotericin B	*A. flavus*	4
	A. fumigatus	2
	A. niger	2
	A. terreus	4
	A. versicolor	2
Caspofungin[¶]	*A. flavus*	0.5
	A. fumigatus	0.5
	A. niger	0.25
	A. terreus	0.12
Isavuconazole	*A. flavus*	1
	A. fumigatus	1
	A. niger	4
	A. terreus	1
Itraconazole	*A. flavus*	1
	A. fumigatus	1
	A. niger	4
	A. terreus	2
Posaconazole	*A. flavus*	0.5
	A. niger	2
	A. terreus	1
Voriconazole	*A. flavus*	2
	A. fumigatus	1
	A. niger	2
	A. terreus	2

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New CAP Checklist Questions

Question	Description
MIC.42700	**Inconsistent Antifungal Susceptibility Reports (PHASE I)** A policy addresses unusual or inconsistent antifungal testing results. Results from testing of patient isolates should be reviewed, and unusual or inconsistent results should be investigated. Each laboratory should have a policy for confirming unusual or inconsistent results. For yeasts and moulds, the time of endpoint reading (particularly for the echinocandins) and the effect of trailing growth (particularly for the azoles and flucytosine) can be significant factors impacting susceptibility results. In some cases, it may be necessary to repeat susceptibility testing and/or identification procedures to confirm initial results. This may involve using alternative testing methods or sending the isolate to a reference lab.

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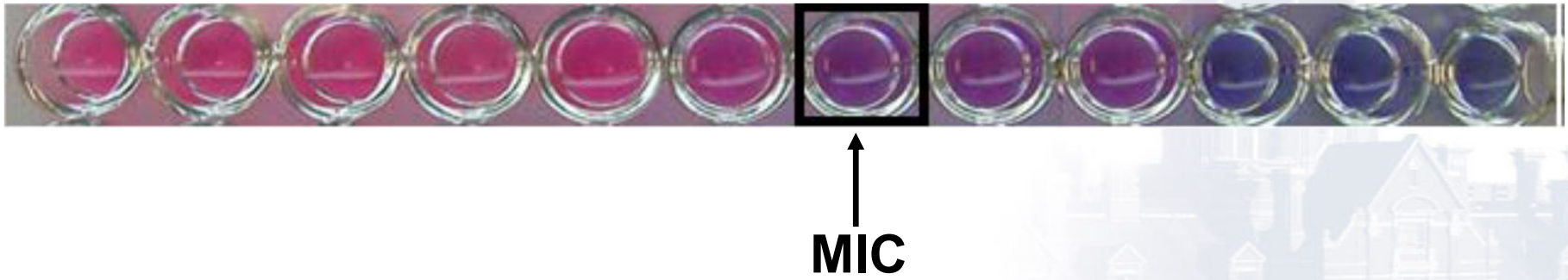
Unusual or inconsistent AFST results

- *Candida albicans* R to all azoles
- *Candida albicans* R to echinocandins
- *Candida glabrata* S to azoles but R to echinocandins

Trailing growth seenin YeastOne Sensititre plate



- A slight color change persists above the MIC and it is often identical for several or all drug concentrations above the MIC.
- The MIC should be read as the first well showing a less intense color change compared to the more positive growth wells of the lower concentrations.
- Commonly seen in azoles
- Overcall R or false R

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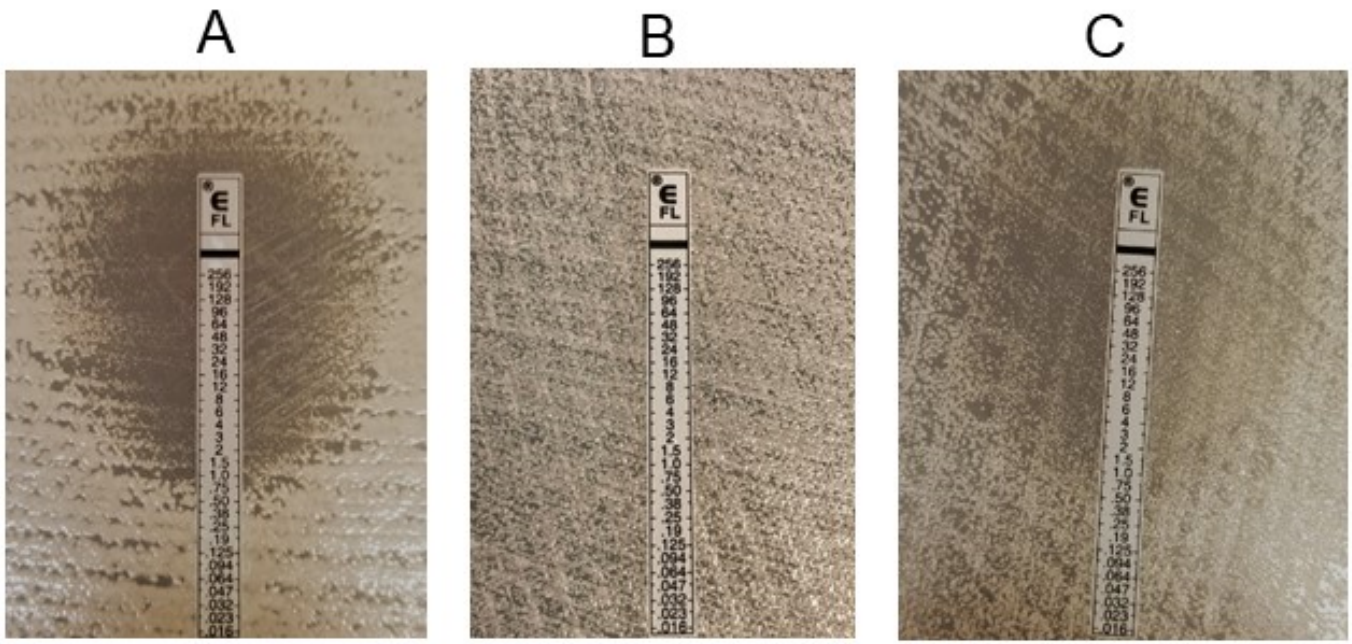
Trailing growth seen in E-Test



Lockhart S. Clin Micro Newsletter 2019

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Should the clinical lab be testing Caspofungin for *Candida* spp.?

- Significant inter-laboratory variability
- Overall resistant
- EUCAST does not recommend testing it
- Anidulafungin & Micafungin can serve as surrogate markers for caspofungin

Espinel-Ingroff et al. AAC 2013; 57:5836. Pfaller et al. JCM 2014; 52:3223

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Intrinsic resistance (IR) organisms (CLIS M59 3rd Ed)

- *Candida krusei:* IR to fluconazole
- *Cryptococcus neoformans/C. gattii*: IR to echinocandins (anidulafungin, caspofungin, micafungin)
- *Aspergillus* spp.: IR to fluconazole, flucytosine
- These antifungal drugs should not be tested and need to be reported as R

EMERGING ANTIFUNGAL DRUG RESISTANT ORGANISMS

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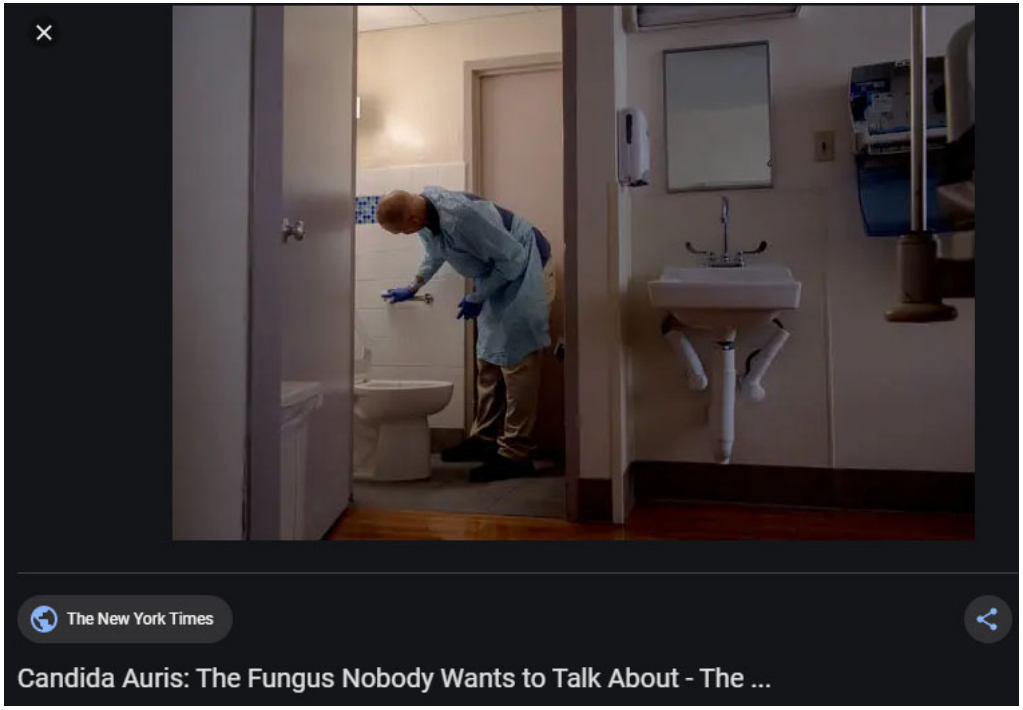
Candida auris: Here's what you should know about the superbug fungus spreading worldwide

There are three main reasons we should be worried about C. auris infection

BY **BAILEY KING**
PhillyVoice Staff

HEALTH NEWS Superbugs

SOURCE/CENTERS FOR DISEASE CONTROL AND PREVENTION



Multi-drug resistant *Candida auris*

- Resistant ≥2 antifungal classes
- Cause invasive infections with high mortality
  - Up to 60% patients infected with *C. auris* died (CID,2017;64:134)
- Spread in healthcare settings and cause healthcare-associated outbreaks
  - Colonize patients' skin and other body sites indefinitely; patients can continue to be colonized with *C. auris* despite daily chlorhexidine bathing (Antimicrob Resist Infect Control 2016;5:35)
  - Persist in the healthcare environment for very long time (JCM 2017 Jul 26)
- Difficulty to identify (close to *C. haemulonii*, *C. duobushaemulonii*)

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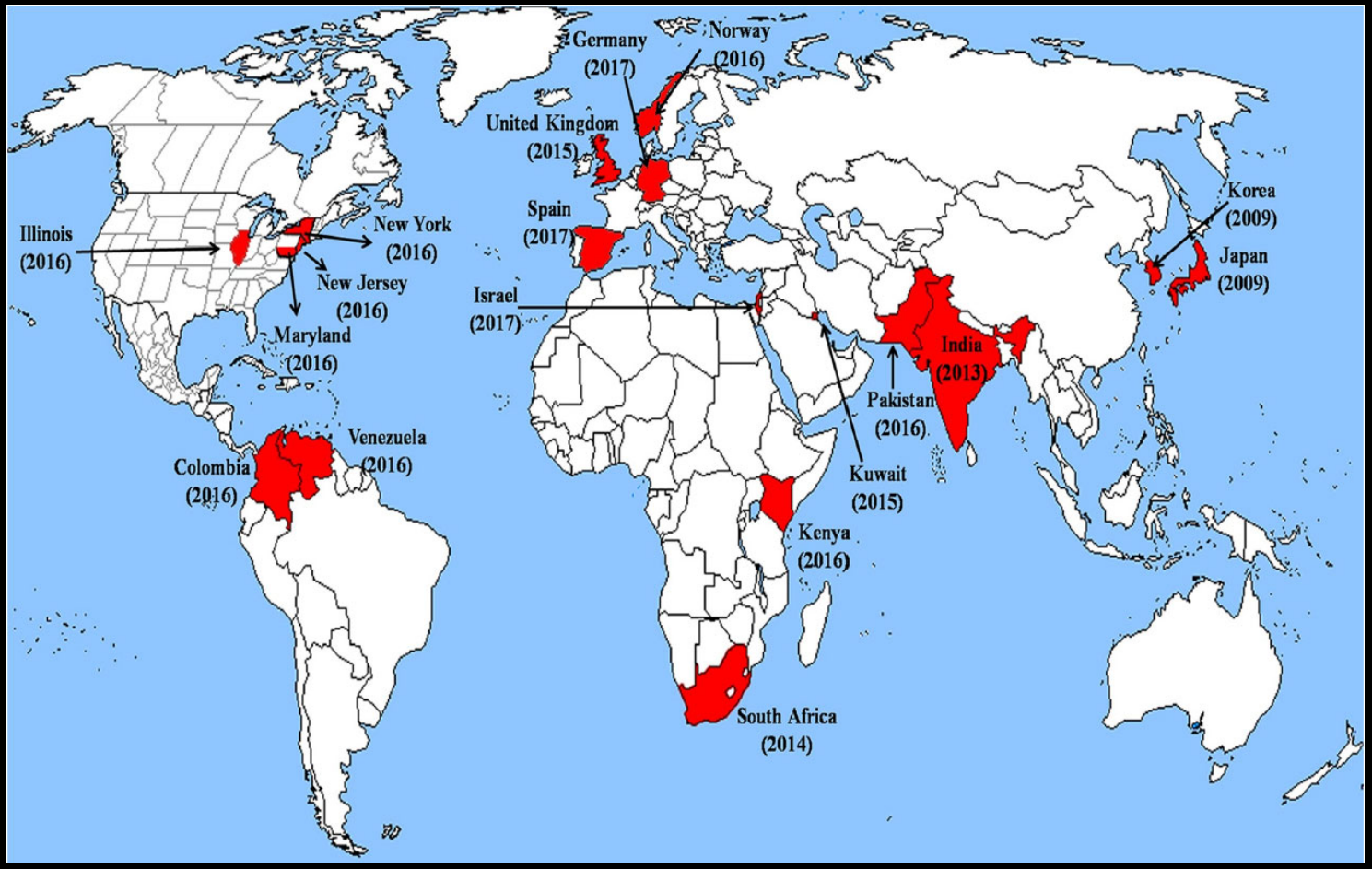
C. auris (Auris: Latin word for ear): history and epidemiology

- First recognized in 2009 from an ear canal specimen in Japan; reservoir is unknown.
  - Earliest strain actually dated back to 1996 from a BSI in a child in Korean
- No single strain for the widespread is identified; whole-genome sequencing revealed four distinct geographical clades
  - South Asia (Clade I), East Asia (Clade II), Africa (Clade III), South America (Clade IV)
- A nosocomial pathogen
  - First outbreak in healthcare settings was reported in an ICU in UK in 2015
  - In a span of only 7 years, it has become widespread across a dozen countries causing a severe healthcare-associated invasive fungal infections
    - The transmission of *C. auris* in one hospital outbreak was found to be linked to reusable axillary temperature probes, indicating that this emerging pathogen can persist in the environment and be transmitted in health care settings (N. Eng J. Med 2018; 379:1322)

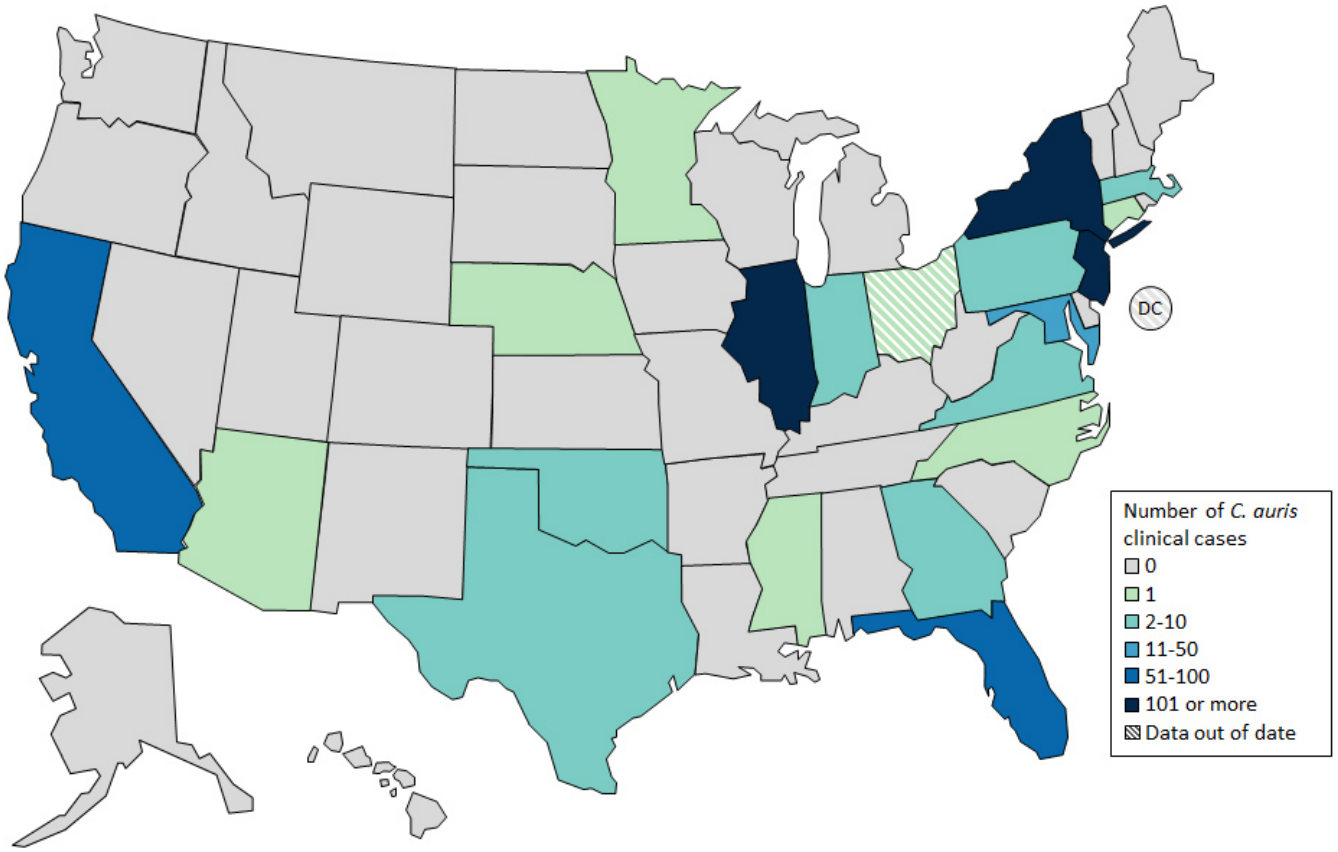
Bradley SF. JAMA 2019; Antimicrob Resist Infect Control 2016;5:35; Microbiol Immunol 2009;53:41

A global map depicting rapid emergence of multidrug-resistant clinical *Candida auris* strains in 33 countries across 5 continents



Bradley SF. JAMA 2019
Chowdhary A et al. PLOS Pathogens 2017 13(5): e1006290

U.S. Map: Clinical cases of *Candida auris* reported by U.S. states, as of August 31, 2020



State	Confirmed cases
California	74
Connecticut	1
Florida	86
Illinois	364
Indiana	10
Maryland	11
Massachusetts	8
New Jersey	185
New York	605
Oklahoma	2
Texas	***6***
TOTAL	***1,364***

Most US isolates are closely related to isolates from South Asia (India, Pakistan), East Asia (South Korea, Japan), South African and South America (Venezuela) by whole-genome sequencing analysis (www.cdc.gov/fungal/diseases/candidiasis/)

C. auris antifungal resistance

- Tentative MIC breakpoints (µg/mL) (CDC recommendation)
  - Fluconazole: ≥32
  - Amphotericin B: ≥2
  - Caspofungin: ≥2
  - Micafungin and Anidulafungin: ≥4

	54 isolates from 3 continents		123 isolates in India	
Antifungals	MIC Range	MIC_{90}	MIC Range	MIC_{90}
Amphotericin B	0.38 - 4	2	0.125 - 8	2
Fluconazole	4 - 256	256	4 - ≥64	≥64
Voriconazole	0.03 - 16	8	0.03 - 16	4
Posaconazole	0.06 - 1	1	0.016 - 8	0.125
Micafungin	0.06 - 4	2	0.015 - 8	0.25
Anidulafungin	0.125 - 16	1	0.015 - 8	0.5
Caspofungin	0.03 - 16	1		
Flucytosine	0.125 - 128	0.5		

Lockhart et al. CID 2017; 64:134 Arendrup MC et al. Antimicrob. Agents Chemother. 2017;61:e00485-17

C. auris antifungal resistance

- ~80% R to fluconazole; >50% R to voriconazole; 30-40% R to amphotericin B; 1-7% R to echinocandins; ~40% R to 2 antifungal classes; R to all three classes has been observed.
- Resistant mechanisms
  - *Erg11* mutation (Y132F, K143R)
  - *ABC, CDR1* efflux transporter (deletion of *CDR1* abrogates resistance)
  - *FKS1* HS1 (S639F)
- Most *C. auris* isolates susceptible to echinocandins, but acquired R to echinocandin could become more common
  - In one patient, R to echinocandin drugs developed while being treated with echinocandins

CID 2017; 64:134; Perlin DS Lancet Infect Dis. 2017 July 31); MMWR 2017; 66:514; Rybak et al. AAC 2019 63(4)

Azole resistant *Aspergillus*

- Surveillance
  - In the US, 1.4% (19/1356) *A. fumigatus* isolates from 2015 to 2017 showed elevated MIC against azoles, of them 5 harbored *Cyp51A* mutations (TR34/L98H)
  - In the Netherlands, 11% (508/4496) of *A. fumigatus* isolates from 2013 – 2018 showed azole resiatance; resistant rate increased from 7.6% in 2013 to 14.7% in 2018.
- Source
  - Prolong azole therapy
  - Environmental derived azole resistant ones (due to agricultural usage of azoles)
- The actual azole R *Aspergillus* in the US may be underestimated since most clinical labs do not routinely testing azoles in *Aspergillus* clinical isolates

Berkow EL, et al. AAC 2018, 26;62(5):e02240-17; Lestrade PPA et al. Emerg Infect Dis. 2020 Jul;26(7):1447-1455.

VIP (Voriconazole-Itraconazole-Posaconazole) plate: screen azole resistant *A. fumigatus* ≤ 48h

(Mediaproducts BV)

Arendrup MC, et al. J Antimicrob Chemother. 2018 Aug 1;73(8):2274.

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Summary

- Amphotericin B resistance is still rare; echinocandin resistance is mostly associated with target mutation and is emerging. Azole resistant mechanism is multimodal and more commonly seen.
- Clinical labs should embrace AFST capacity and utilize clinical breakpoints and ECVs to aid clinicians to choose appropriate antifungal drugs for treatment
- Clinical labs should be aware of emerging antifungal drug resistant fungal pathogens and be able to detect them