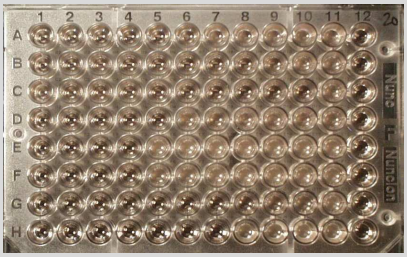
Susceptibility testing of Fungi: Techniques, Breakpoints & Interpretation



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Research grants & Speaker: Astellas, Gilead, MSD & Pfizer; Advisory board: MSD, Pcovery;
Acted as consultant for: Alcimed, Astellas, Gilead & Pfizer
Chair(wo)man for EUCAST-AFST
Advisor for CLSI-AFST

Available documents

- CLSI
  - M27-A3 Yeast broth dilution 120 $
  - M27-S3 QC and BPs 35 $
  - M38-A2 Mould broth dilution 120 $
  - M44-A2 Yeast disk diffusion 200 $
  - M44-S3 QC and BPs 35 $
  - M51-A Mould disk diffusion 170 $
  - M51-S1 QC and ECVs 200 $

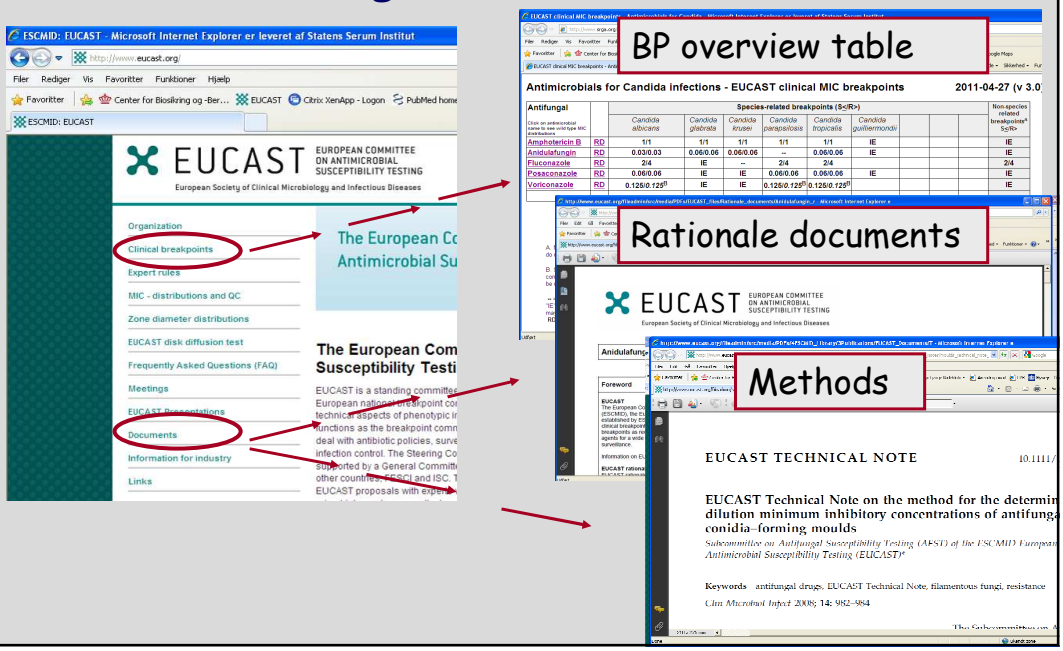
  } 880 $

- EUCAST
  - Methods EDef 7.1 (yeast) and EDef 9.1 (mould)
  - Rationale documents (breakpoints)
    - *Candida*: amphotericin, anidulafungin, flu-, vori- and posaconazole
    - *Aspergillus*: amphotericin, itraconazole, posaconazole
  - Technical notes in Clin Microbiol Infect

  } Free

www.clsi.org, www.eucast.org

www.eucast.org



Factors influencing the endpoint

- Medium (type, brand, batch)
  - CLSI vs. EUCAST: glucose conc. 0.2% vs. 2%
- Inoculum size
  - the higher → the higher the MIC
- Inoculum growth phase
  - the shorter the lag phase → the higher the MIC
- Incubation temperature
  - affects growth rate, expression of res mechanisms
- Incubation time
  - the longer → the higher the MIC

↑ Growth → ↑ MIC

- Definition of endpoint (50%, 80%, 100% inhibition)
  - the more stringent → the higher the MIC
- Reading variation
  - visual vs. spectrophotometric
  - trailing

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CLSI M27-A→A3 & EUCAST

	M27-A	M27-A3	EUCAST
Glucose /Inoculum size	0.2%, 0.5-2.5 x 10^3		2%, 0.5-2.5 x 10^5
Plates & Reading	Round bottom & Visual		Flat & Spec.
Incubation time	48 h	24 h	24 h
End point	80% inhib.	50% inhib.	50% inhib
Fluconazole QC			
C. parapsilosis ATCC 22019	2-8 µg/ml	0.5-4 µg/ml	0.5-2 µg/ml
C. krusei ATCC 6258	16-64 µg/ml	8-64 µg/ml	16-64 µg/ml

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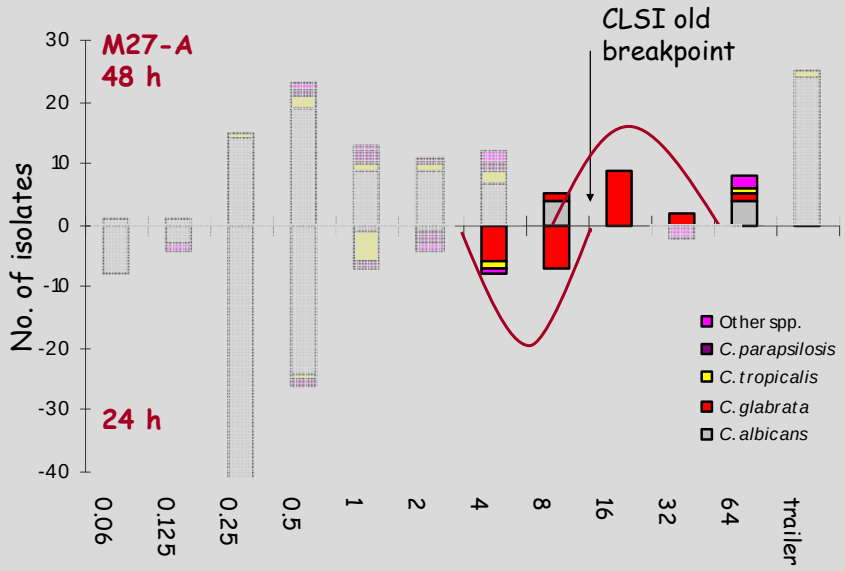
Incubation time: Fluconazole CLSI



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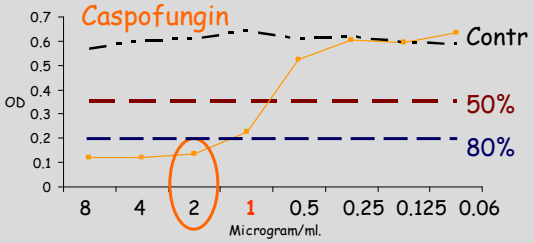
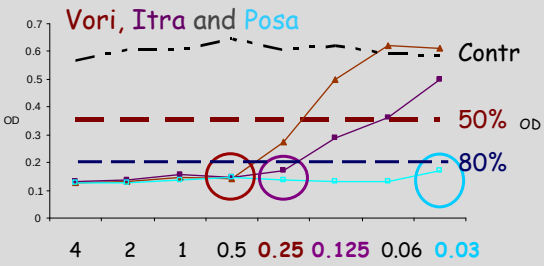
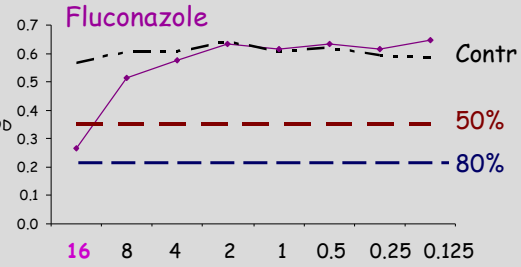
Influence of endpoint: % inhibition



	80%	50%
Caspofungin	2	1
Voriconazole	0.5	0.25
Itraconazole	0.25	0.125
Posaconazole	≤0.03	≤0.03
Fluconazole	>16	16





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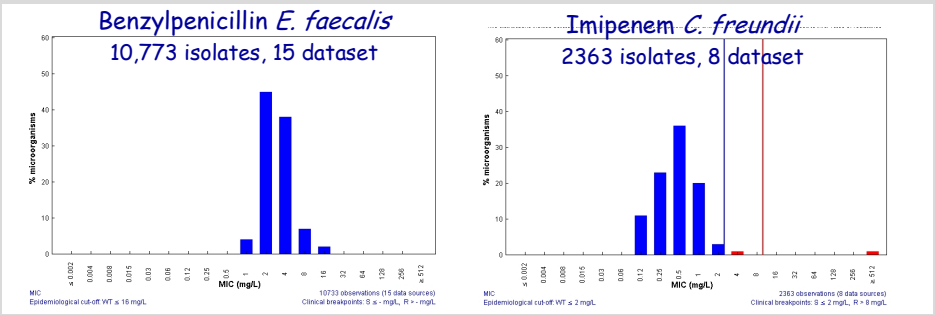
How precise is the MIC value?

- Well standardised antibacterial test can at best provide MICs at
  - ± 1 dilution 95% of the times
  - ± 2 dilutions 99% of the times



www.eucast.org

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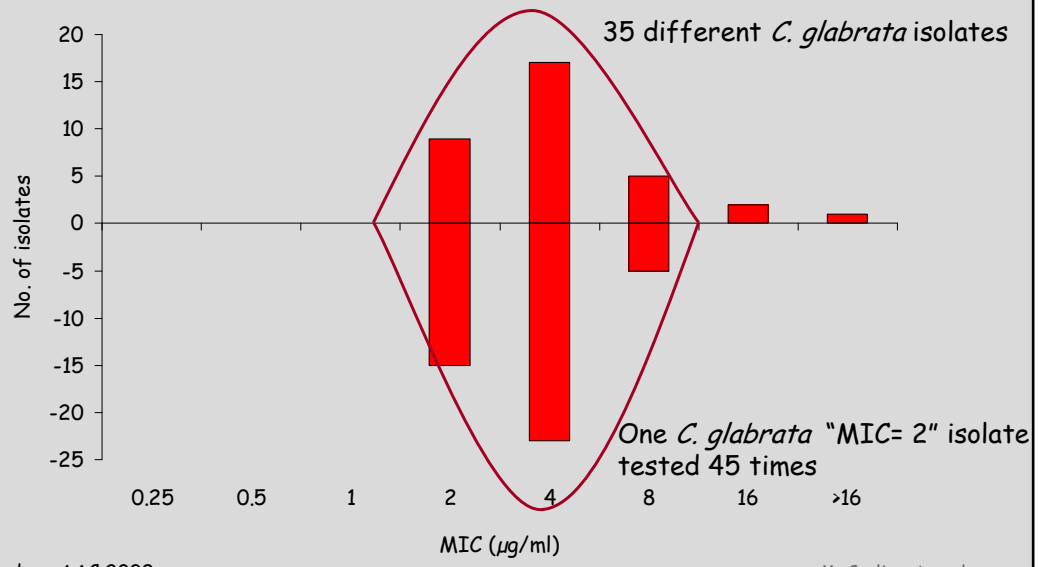
Wild-types EUCAST MICs



Arendrup AAC 2009

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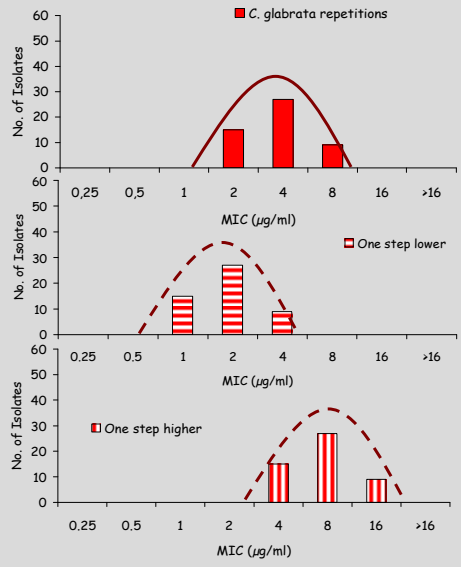
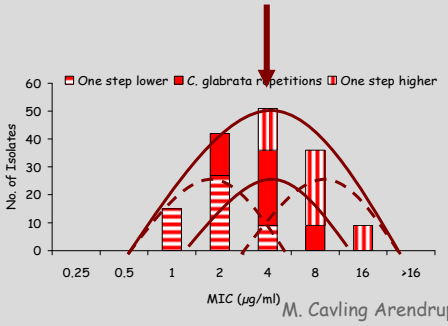
Wild-type distributions incl. variability



Mode MIC / MIC_{50}

Reflects the inherent susceptibility of the WT population



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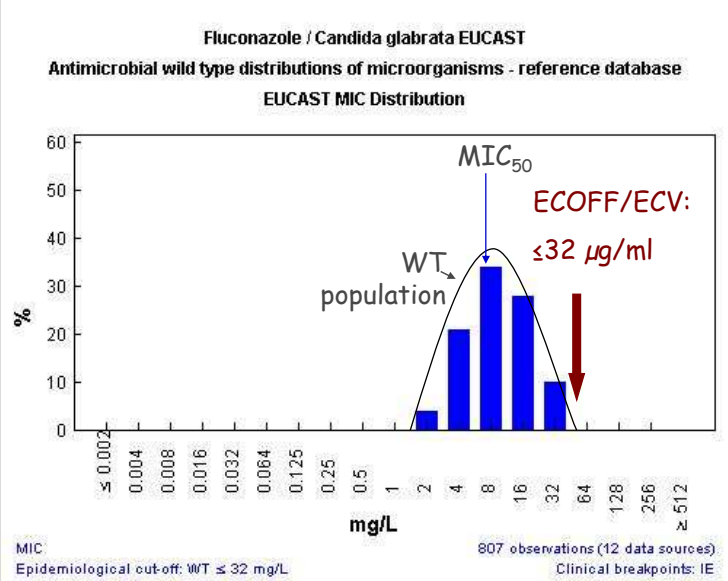
EUCAST fluconazole MIC *C. glabrata*



Variation within the WT population can be explained solely by test variation

MIC_{50} reflects the susceptibility of the entire WT population

http://www.eucast.org/mic_distributions_of_wild_type_microorganisms/

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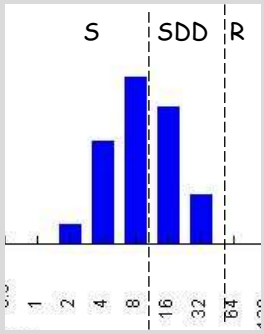
M27-S3 CLSI breakpoints for *Candida*

Breakpoints given as: S: ≤X; R: >Y

	CLSI
Amphotericin	≤1
Anidulafungin	≤2
Caspofungin	≤2
Micafungin	≤2
Fluconazole	**≤8; >32**
Itraconazole	≤0.125; >0.5
Posaconazole	-
Voriconazole	≤1; >2



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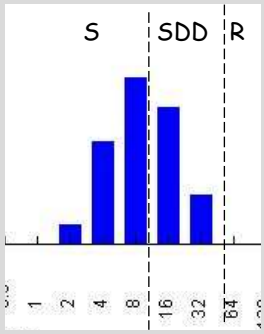
Correlation MIC and outcome

177 candidaemia cases in Taiwan 1999-2005

Table 3
In vitro susceptibilities of *Candida glabrata* isolates to five antifungal agents.

Antifungal	MIC (μg/mL)			Susceptible (%)[a]
	Range	MIC_{50}	MIC_{90}	
Fluconazole	2 to >64	8	32	63
Voriconazole	0.06–64	0.25	1	93
Caspofungin	0.25 to >64	0.5	1	96
Flucytosine	0.06 to >64	0.06	0.12	99
Amphotericin B	0.25–2	1	1	98



. The different levels of susceptibility to fluconazole (susceptible, susceptible-dose dependent and resistant) were not significantly associated with 30-day mortality ($P = 0.09$).

Ryan Int J Antimicrob Agents 2009;
http://www.eucast.org/mic_distributions_of_wild_type_microorganisms/

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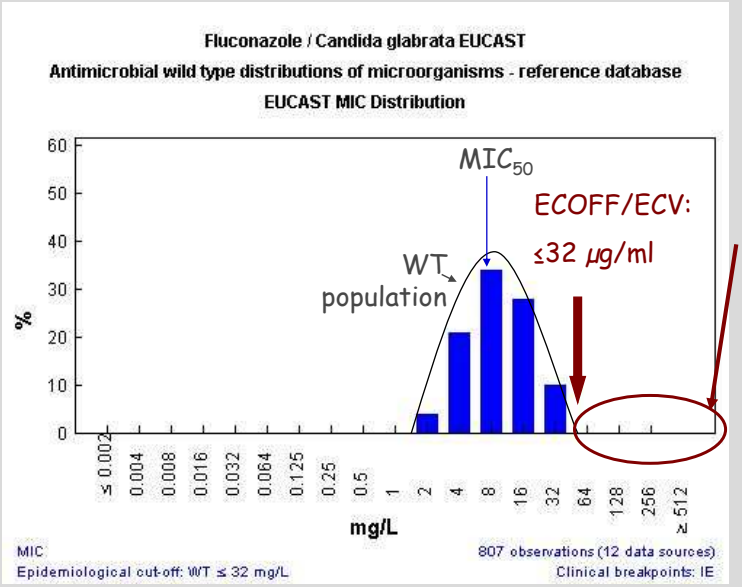
EUCAST fluconazole MIC *C. glabrata*



Non-WT

- MICs outside the WT population **cannot** be explained by test variation
- Microbiological resistance
- Outcome may be different than for WT isolates

http://www.eucast.org/mic_distributions_of_wild_type_microorganisms/

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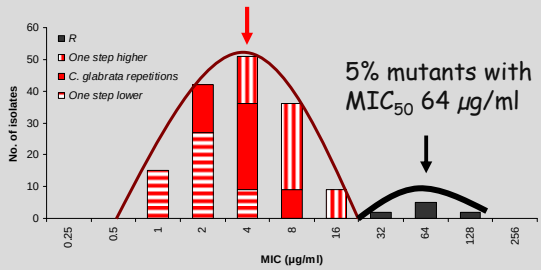
Population with WT and non-WT



Non-WT

Microbiological resistance

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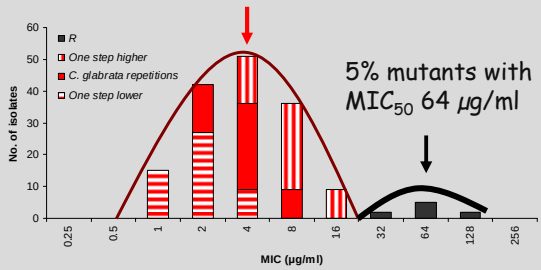
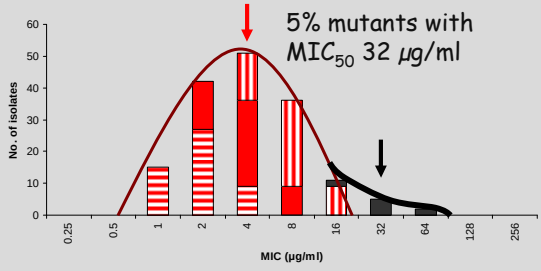
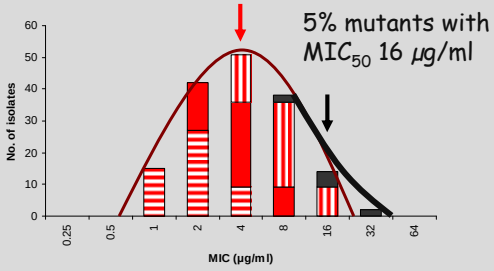
Population with WT and non-WT







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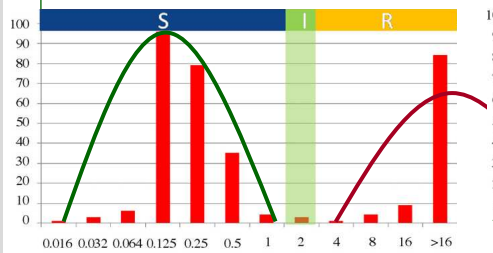
True life example

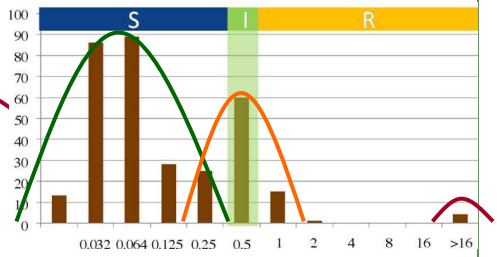
A. fumigatus

Itraconazole



Clear separation WT and non-WT

Posaconazole



Overlap WT and non-WT ➔ further analysis needed to identify all non-WT

Verweij Drug Res Updates 2009

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Strengths and limitations

- Precise within ± 2 dilutions
  - consider the MIC_{50} for the species
- ECOFF / ECV
  - upper limit of WT isolates
  - identical susceptibility
  - no resistance mechanisms
- Non-WT isolates
  - harbour resistance mechanisms
  - may or may not respond depending on the host & drug exposure
- If WT and non-WT overlap (& are clinically relevant…)
  - additional analysis is needed
- Clinical breakpoints should not be set higher than the ECOFF / ECV
  - unless clinical evidence demonstrate such isolates are clinically susceptible

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AMB & Azole breakpoints for *Candida* spp

Breakpoints (BPs): S: ≤X; R: >Y **Revised BPs**

	CLSI M27-S3	CLSI Revised 2010/11	EUCAST
AMB	≤1	≤1	≤1; >1
Fluco	≤8; >32	≤2; >4 (*alb, para, trop*) SDD ≤32; R >32 (*glab*) (*krus* poor target)	≤2; >4 (*albi, trop, para*) (*glab* IE) (*krus* poor target)
Itra	≤0.125; >0.5	≤0.125; >0.5	-
Posa	-	-	≤0.06; >0.06 (*alb, trop, para*) (*glab/krus* IE)
Vori	≤1; >2	≤0.125; >0.5(*alb, para, trop*) ≤0.5; >1 (*krus*) (*glab* IE)	≤0.125; >0.125 (*alb, trop, para*) (*glab/krus* IE)

www.eucast.org; Pfaller Drug Resist Updat. 2010 & 2011; www.clsi.org

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EUCAST Fluconazole MIC & outcome

Fluconazole 258 cases
(128 candidaemia 58% *C. albicans*; 133 OPC all *C. albicans*)

MIC in mg/L	Candidaemia		OPC ≥ 100 mg/d		**All**	
	No. cure/Total	% response	No. cure/Total	% response	**% response**	
< 0.5	98/107	92	26/26	100	**93**	
1	6/6	100	4/4	100	**100**	S
2	1/1	100	1/1	100	**100**	
4	3/3	100	5/9	56	**67**	I/SDD
8	2/5	40	7/32	22	**24**	R
≥16	3/4	75	0/60	0	**5**	

Rodriguez-Tudela AAC 2007

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AFST: good vs. poor correlation

- Unfortunately, …… current AST methods fall woefully short of accurately predicting outcomes for many patients with infection.

TABLE 1. Correlation of disease outcome with the results of MIC determinations in patients with infection who were treated with cefotaxime[a] (16)

Cefotaxime MIC (μg/ml)	Category[b]	Number of patients	% Cured or improved	% Eradication
≤4	S	1003	94	91
8	S	273	90	86
16	I	151	77	75
32	I	70	84	71
64	R	19	64	50

[a] All patients had defined monomicrobic infections and were treated with intravenous cefotaxime alone, typically at a dosage of 2 g q8h.
[b] Susceptibility categories: S, susceptible; I, intermediate; R, resistant.

90-60 rule

90% pts with S isolates respond

60% pts with R isolate respond

Doern JCM Sept. 2011 Proceedings of the Camp Clin Microbiol 2011

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Outcome Triangle

FUNGUS

- Virulence
- Susceptibility

HOST

- Immune status
- Severity/duration of infection at the start of therapy

DRUG PK/PD

- C_{max}, $T_{\frac{1}{2}}$, AUC
- Concentration at site

S → higher likelihood of success
Some pts will fail despite appropriate treatment

R → higher likelihood for failure
Some pts will get well despite inappropriate treatment

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Echinocandin breakpoints for *Candida* spp

Breakpoints (BPs): S: ≤X; R: >Y **Revised BPs**

	CLSI M27-S3	CLSI Revised 2011	EUCAST
ANF	≤2	≤0.25; >0.5 (*alb, krus, trop*) ≤0.125; >0.25 (*glab*) ≤2; >4 (*para, guillier*)	≤0.032; >0.032 (*alb*) ≤0.06; >0.06 (*glab, krus, trop*) (*para* not a good target) (*guillier* IE)
CSF	≤2	≤2; >4 (*para, guillier*)	-
MFG	≤2	≤0.25; >0.5 (*alb, krus, trop*) ≤0.06; >0.125 (*glab*) ≤2; >4 (*para, guillier*)	-

www.eucast.org; Pfaller Drug Resist Updat. 2010 & 2011; www.clsi.org

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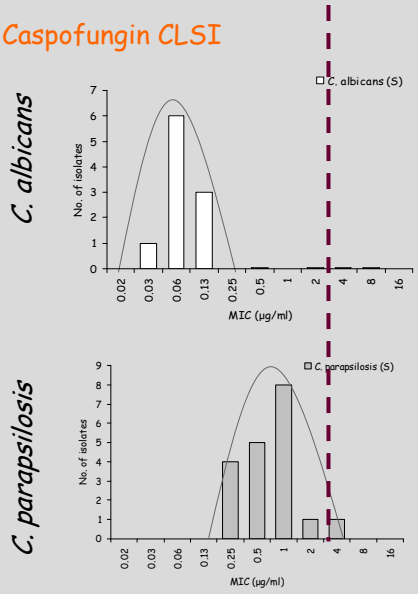
Why Species Specific BPs ?

Caspofungin CLSI



Clinical success rates

Agent/Study	*C. albicans*	*C. parapsilosis*
ANF/Reboli	81%	64%
MFG/Pappas	77%	76%
CFG/Pappas	74%	64%
MFG/Kuse	88%	89%

Arendrup AAC 2010

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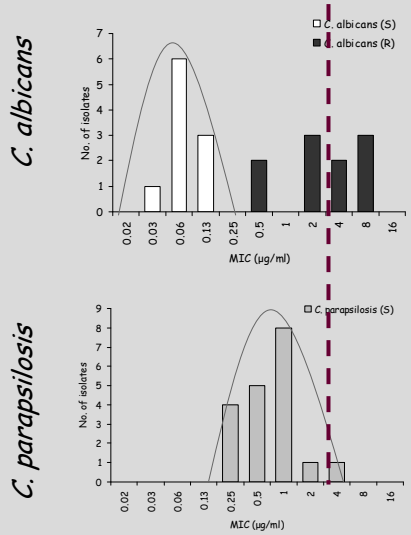
Species Specific BPs ?

Caspofungin CLSI



Virulence in a mouse model

Parameter	*C. albicans*	*C. parapsilosis*
Survival		
Inoculum 10^5	83%	100%
Inoculum 10^7	0%	100%
Cleared inf. d 7		
Inoculum 10^5	0%	58%
Inoculum 10^7	-	31%

Arendrup AAC 2010, Arendrup Infection 2002

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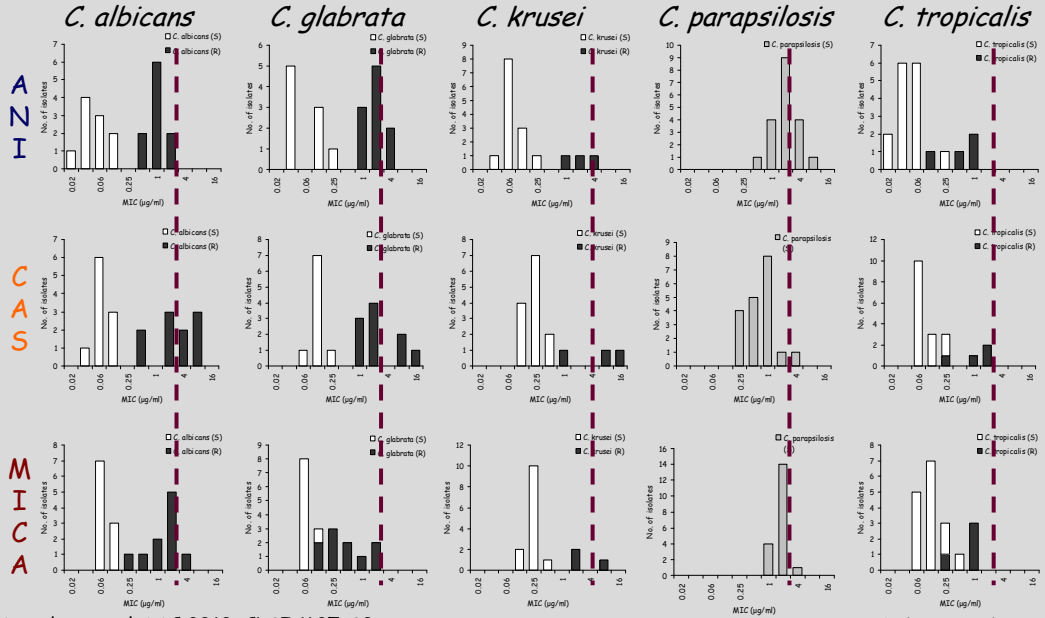
CLSI echinocandin MICs "M27-S3 BP"



Arendrup et al AAC 2010, CLSI M27-S3

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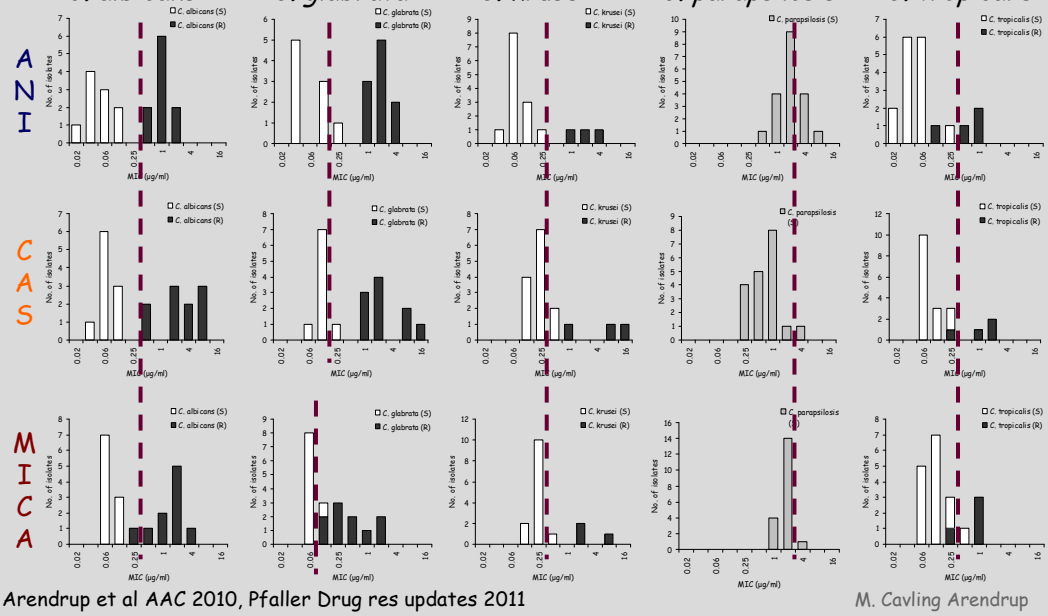
CLSI echinocandin testing – revised BP



Arendrup et al AAC 2010, Pfaller Drug res updates 2011

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EUCAST & CLSI Anidulafungin MICs

Candida sp	0.002	0.004	0.008	0.016	0.032	0.064	0.125	0.25	0.5	1	2	4	8	>=16	ECOFF	BP
albicans EUCAST	284	576	314	427*	703*	14	25	4	3	4	0	0	0	4	0.03	**0.03**
albicans CLSI			338	1278	1542	896	216	12		1					0.125	**0.25**
glabrata EUCAST	55	43	64	177*	441*	111	28	11	5	3	3	1	2	0	0.06	**0.06**
glabrata CLSI				7	161	715	320	26	2	2	2	1			0.25	**0.125**
krusei EUCAST	2	1	13	26*	83*	34	8	4	0	0	0	0	0	0	0.06	**0.06**
krusei CLSI				4	159	91	14	1	1						0.125	**0.25**
parapsilosis EUCAST	0	3	1	0	4	6	2	36	78	171	96	13	7	2	4	-
parapsilosis CLSI				1	2	1	1	14	49	319	765	86			4	**2**
tropicalis EUCAST	18	34	17	47*	175*	24	7	6	3	1	0	0	0	2	0.06	**0.06**
tropicalis CLSI			41	254	493	173	24	7	1		3				0.125	**0.25**

www.eucast.org and www.clsi.org

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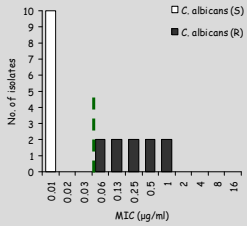
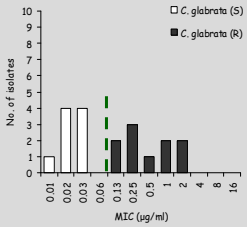
EUCAST Anidulafungin WT vs. resistant

C. albicans



C. glabrata

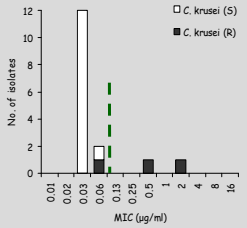


Wild type isolates

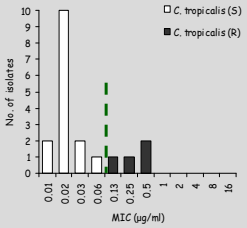
Resistant isolates

EUCAST BP

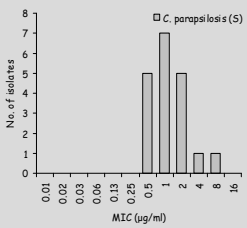
C. krusei



C. tropicalis



C. parapsilosis



Arendrup et al AAC 2010 and EUCAST-AFST

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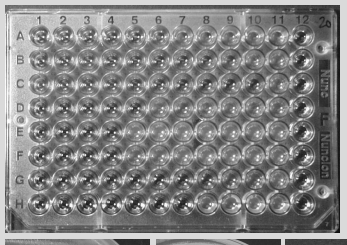
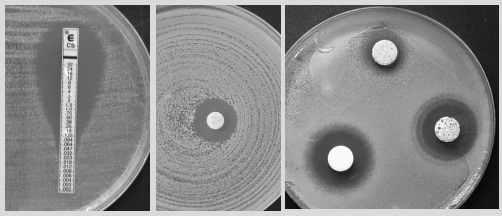
Techniques

- Reference methods
  - Micro-format
    - CLSI Reference methods
    - EUCAST Reference methods
- Commercial systems
  - Etest/disc/tablet diffusion
  - Sensititre, Micronaut, Vitek
- Agar dilution
  - Azole agars & control





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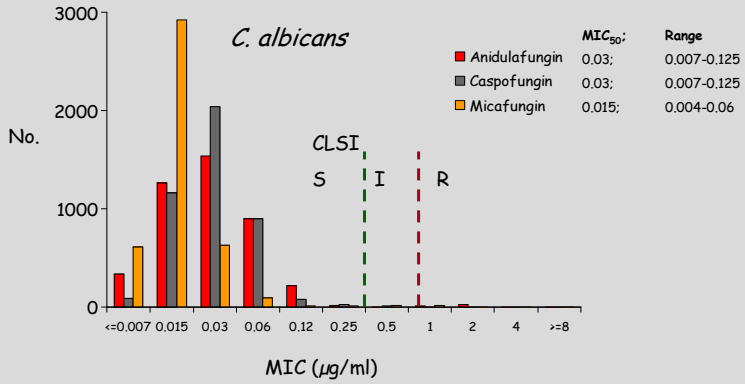
Commercial methods

- Before you adopt CLSI/EUCAST BPs
  - Test MIC_{50}s for each species = reference MIC?



Pfaller JCM 2010

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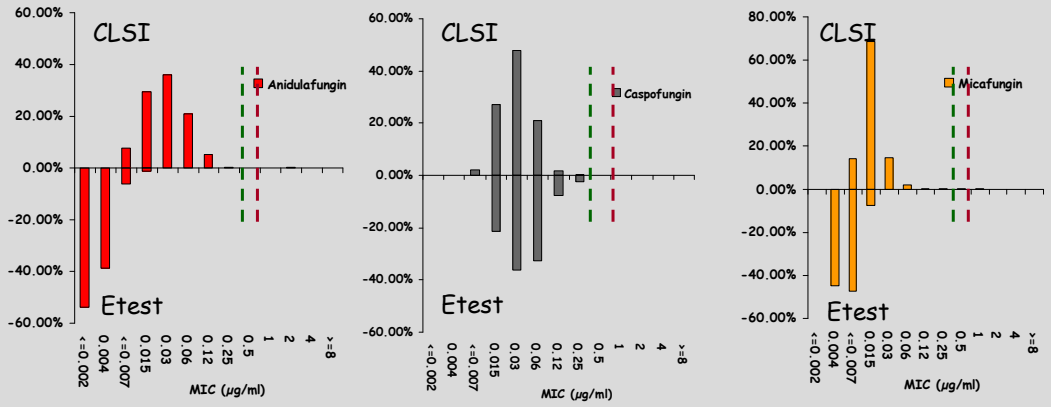
Commercial methods

- Are Test MIC_{50}s for each species = reference MIC?
  - example: *C. albicans*



Pfaller JCM 2010 and Axner-Elings JCM 2011

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Echinocandin Etest MICs and CLSI BP

Axner-Elings JCM 2011

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Susceptibility testing of moulds

- Reference microdilution methods
  - CLSI M-38A
  - EUCAST E.DEF 9.1
  - Visual reading of growth
    - Ampho & azoles: MIC no growth
    - Candins: MEC aberrant growth
    -
- Alternatives
  - Etest & disk diffusion CLSI M51A
    - Visual reading: Azoles and Ampho prominent growth inhibition (80%)
      - slight trailing should be ignored for azoles,
      - trailing growth and microcolonies should be ignored for caspofungin
  - Agar-dilution

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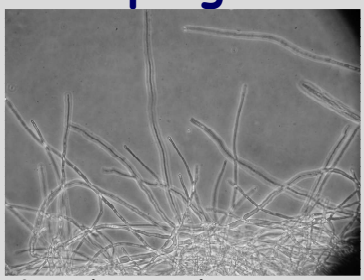
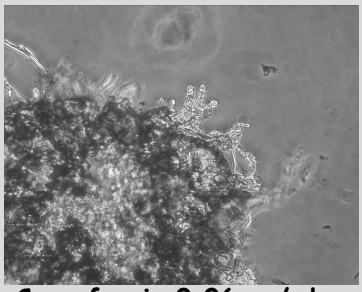
Microdilution MIC / MEC Aspergillus



Growth control



Caspofungin 0.06 µg/ml

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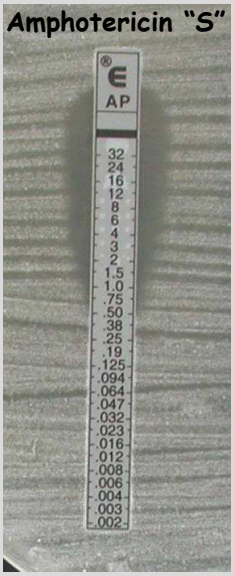
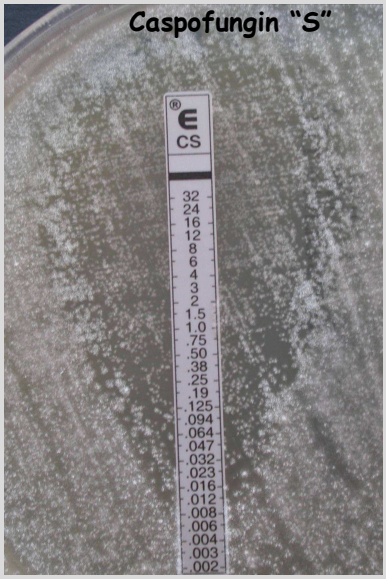
Aspergillus susceptibility testing Etest

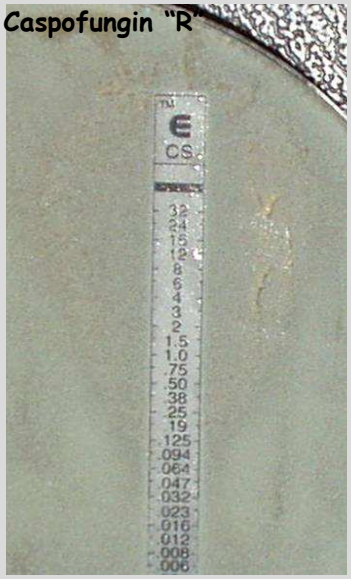
Amphotericin "S"



Caspofungin "S"



Caspofungin "R"



Arendrup AAC 2008

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Agar dilution- screening agar

Azole S

Azole R

Azole agar: Van der Linden TIMM 2009 O1.1;

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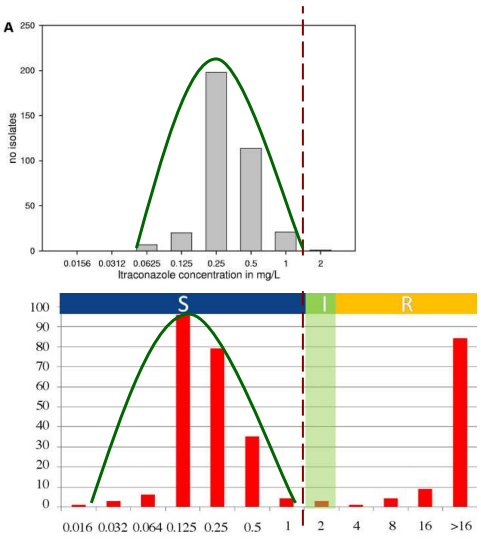
A. fumigatus Itraconazole MIC distribution

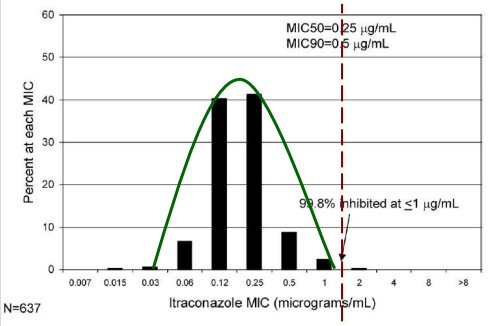
EUCAST



MIC_{50} at 0.25 µg/ml

ECOFF: ≤ 1 µg/ml

CLSI



Rodriguez-Tudela AAC 2008, Verweij Drug Res Updates 2009, Pfaller JCM 2009

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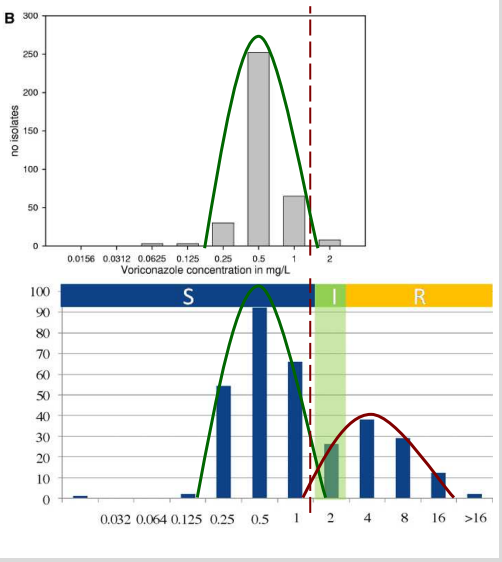
A. fumigatus Voriconazole MIC distribution

EUCAST

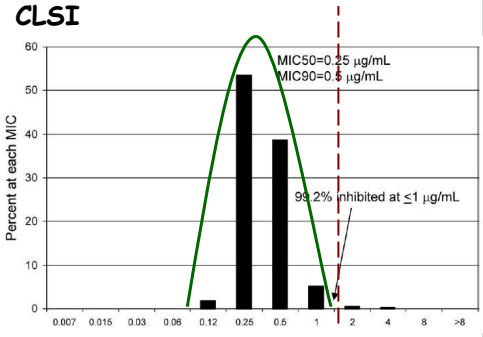


MIC_{50} EUCAST at 0.5 µg/ml

MIC_{50} CLSI at 0.25 µg/ml

ECOFF: ≤ 1 µg/ml

CLSI



Rodriguez-Tudela AAC 2008, Verweij Drug Res Updates 2009, Pfaller JCM 2009

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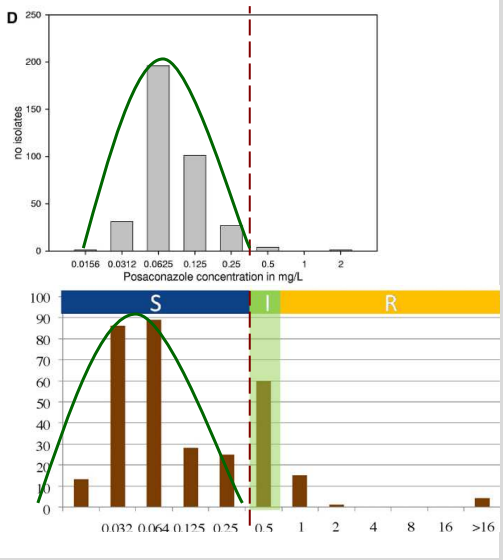
A. fumigatus Posaconazole MIC distribution

EUCAST

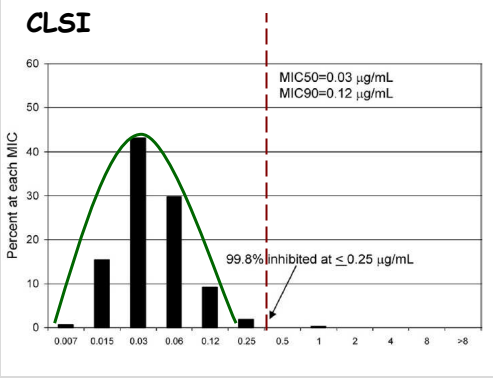


MIC_{50} EUCAST at 0.064 µg/ml

MIC_{50} CLSI at 0.032 µg/ml

ECOFF: ≤ 0.25 µg/ml

CLSI



Rodriguez-Tudela AAC 2008, Verweij Drug Res Updates 2009, Pfaller JCM 2009

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Published ECOFFs and BP suggestions

A. fumigatus only

	Susceptible	Intermediate	Resistant
Itraconazole	≤ 1 µg/ml	2	>2
Voriconazole	≤ 1 µg/ml	2	>2
Posaconazole	≤ 0.25 µg/ml 0.5	0.5 1	>0.5 >1

Rodriguez-Tudela AAC 2008, Verweij DRU 2009, Pfaller JCM 2009, Espinel-Ingroff JCM 2010 M. Cavling Arendrup

EUCAST multicentre ECOFFs

MICs from 8-12 European data set

Species (No.)	Amphotericin	Itraconazole	Posaconazole
A. flavus (257-389)	4	1	0.5
A. fumigatus (1821-2389)	1	1	0.25
A. nidulans (81-116)	ND	1	0.5
A. niger (192-307)	1	4	0.5
A. terreus (256-636)	4	1	0.25

Rationale documents available soon for consultation at www.eucast.org M. Cavling Arendrup

EUCAST multicentre BP suggestions

BPs indicated as S ≤x / R >y

Species (No.)	Amphotericin	Itraconazole	Posaconazole
A. flavus (257-389)	IE*	1/2	IE*
A. fumigatus (1821-2389)	1/2	1/2	0.125/0.25**
A. nidulans (81-116)	IE	1/2	IE*
A. niger (192-307)	1/2	IE*	IE*
A. terreus (256-636)	Poor Target	1/2	IE

ECOFF!

* MICs are higher than for *A. fumigatus*

** provided sufficient levels can be achieved

Available soon for consultation at www.eucast.org

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Poor separation WT vs. non-WT

Individual data set

	MIC (mg/L)														
	0.002	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32
EUCAST				7	70	**384**	**205**	55	12	3	3	3	4		
EUCAST					5	**28**	**50**	11	4		1		1		
Eucast 24h							1	3	15	1					
CLSI	0	0	0	0	21	1	73	87	1	1	0	0	0	0	0
EUCAST			3	1	7	5	6	8	3	1			3		
EUCAST					113	**116**	**128**	100	46	16	2		5		
EUCAST	0	0	0	0	6	49	**163**	57	7	6	2	0	1	14	0
EUCAST	0	0	0	5	12	**98**	**77**	48	44	69	18	1	2	1	6

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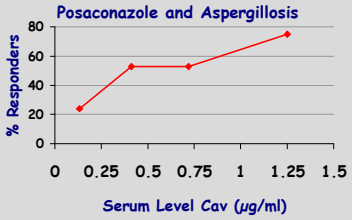
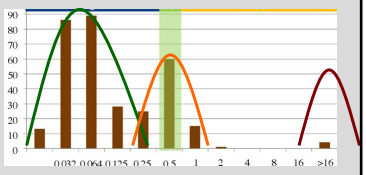
Concerns using ECOFF of 0.25 as BP

- Borderline exposure with current formulation
  - Cav of 1.25 mg/L → better outcome
  - Cav of 1.25 mg/L ~ AUC of ~30 mg.h/L



- Mouse models
  - AUC:MIC of 167 → ED_{50} (include 0.125)
    - GM as endpoint, neutropenic model
  - AUC:MIC of 498 → ED_{50} (include 0.06)
    - Survival as endpoint, non-neutropenic model
  - Poorer outcome for *cyp51A* mutants
    - some of which may have an MIC of 0.25 mg/L



Walsh CID 2007; Howard JID 2011; Mavridou AAC 2010; Verweij DRU 2009

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Conservative Posaconazole BP

- *Aspergillus fumigatus*
  - S ≤0.125, R >0.25 mg/L
    - Provided adequate drug exposure has been confirmed using therapeutic drug monitoring
  - TDM targets:
    - > 1 mg/L at steady state for salvage treatment
    - > 0.7 mg/L for prophylaxis
- App. 15% WT may be classified as non-S
- Itraconazole "S" → probably also posaconazole "S"

Jang Clin Pharmacol Ther. 2010; FDA; soon at www.EUCAST.org

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Aspergillus CLSI Caspofungin ECOFFs

	Statistical ECOFF	% above the ECOFF
A. fumigatus	0.5	2
A. flavus	0.25	5.1
A. nidulans	0.5	14.1
A. niger	0.25	5
A. terreus	0.25	8.3
A. versicolor	0.25	6.7

Species	No. of isolates	MEC (μg/ml) of:										
		≤0.016	0.03	0.06	0.125	0.25	0.5	1.0	2.0	4.0	8.0	16	32
A. fumigatus	1,691	8	81	166	394	866[a]	143[b,d]	19[c]	9	2	0	1	2
A. flavus	432	4	36	158[a]	137	76[b]	12[c,d]	4	3	1	0	0	1
A. nidulans	192	0	6	17	77[a]	57	8[b,c]	3	11	2	4[d]	7	0
A. niger	440	1	45	180[a]	137	55[b,c,d]	18	3	1	0	0	0	0
A. terreus	385	1	31	168[a]	105	48[b]	20[c,d]	10	2	0	0	0	0
A. versicolor	75	4	19	20	21[a]	6[b]	2[c,d]	0	3	0	0	0	0

Espinel-Ingroff AAC 2011

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A. fumigatus and Caspofungin testing

Method	Reference	MIC range (μg/ml)
EUCAST	Arendrup	0.125-2
CLSI	Fuller	0.125-1
CLSI	Johnson	0.06-1
CLSI	Arabatzis	0.125-0.5
CLSI	Pfaller	0.007-0.06
CLSI	Fiori	≤0.03
Etest	Fuller	0.016-0.25
Etest	Arendrup	0.016-0.25

Arendrup & Johnson unpublished data, Fuller JCM 2010, Pfaller JCM 2008, Arabatzis AAC 2011

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In conclusion

- Antifungal Susceptibility testing
  - Requires training
  - Precise within ± 2 dilutions
  - The MIC_{50} reflects the susceptibility of the species
  - Correlates with the likelihood of success
  - BPs should be carefully selected
    - CLSI M27-S3 >< revised CLSI >< **EUCAST**
    - check your "commersial" MIC mirrors those of the ref method
    - incorrect breakpoints → random or incorrect categorization S, SDD/I, R
  - Available BPs
    - *Candida*: amphotericin, anidulafungin, flu-, vori- and posaconazole
    - *Aspergillus*: amphotericin, itraconazole, posaconazole

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Acknowledgements
(in alphabetic order):

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C Lass-Flörl
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M Pfaller
P Verweij

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Thank you for your attention