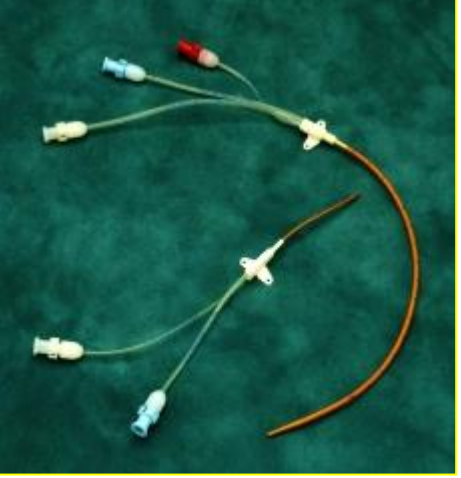
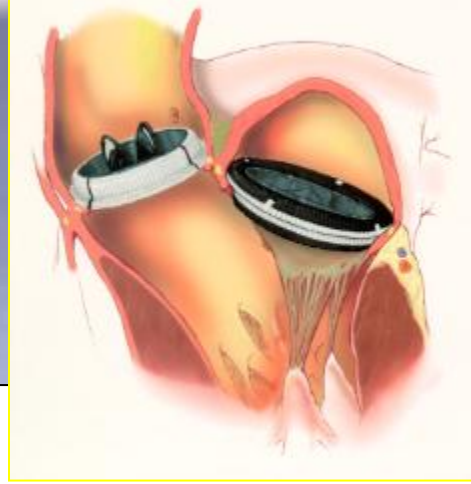
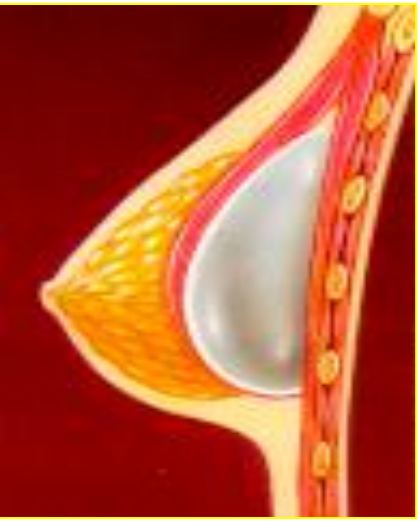
CLSI Biofilm Symposium

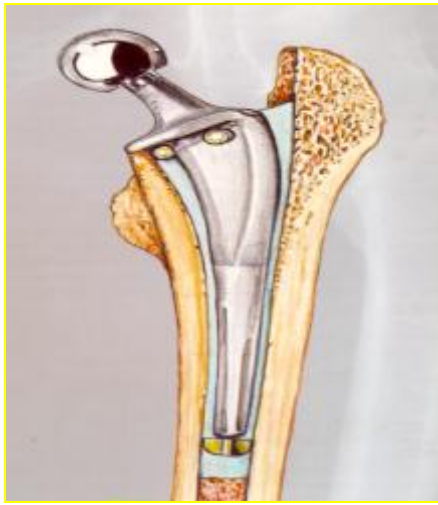
Relevance of Susceptibility Testing for Fungal Biofilms

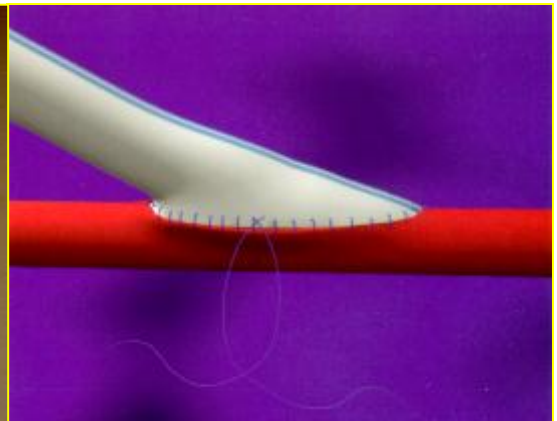
21 Jan 2012

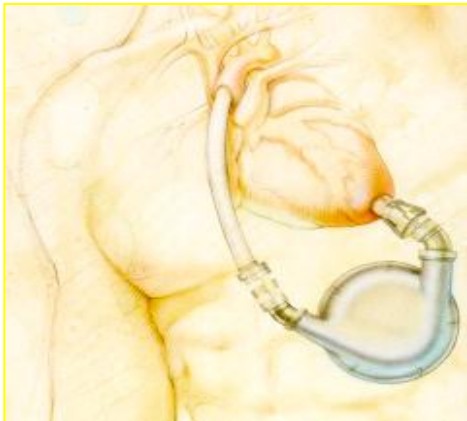


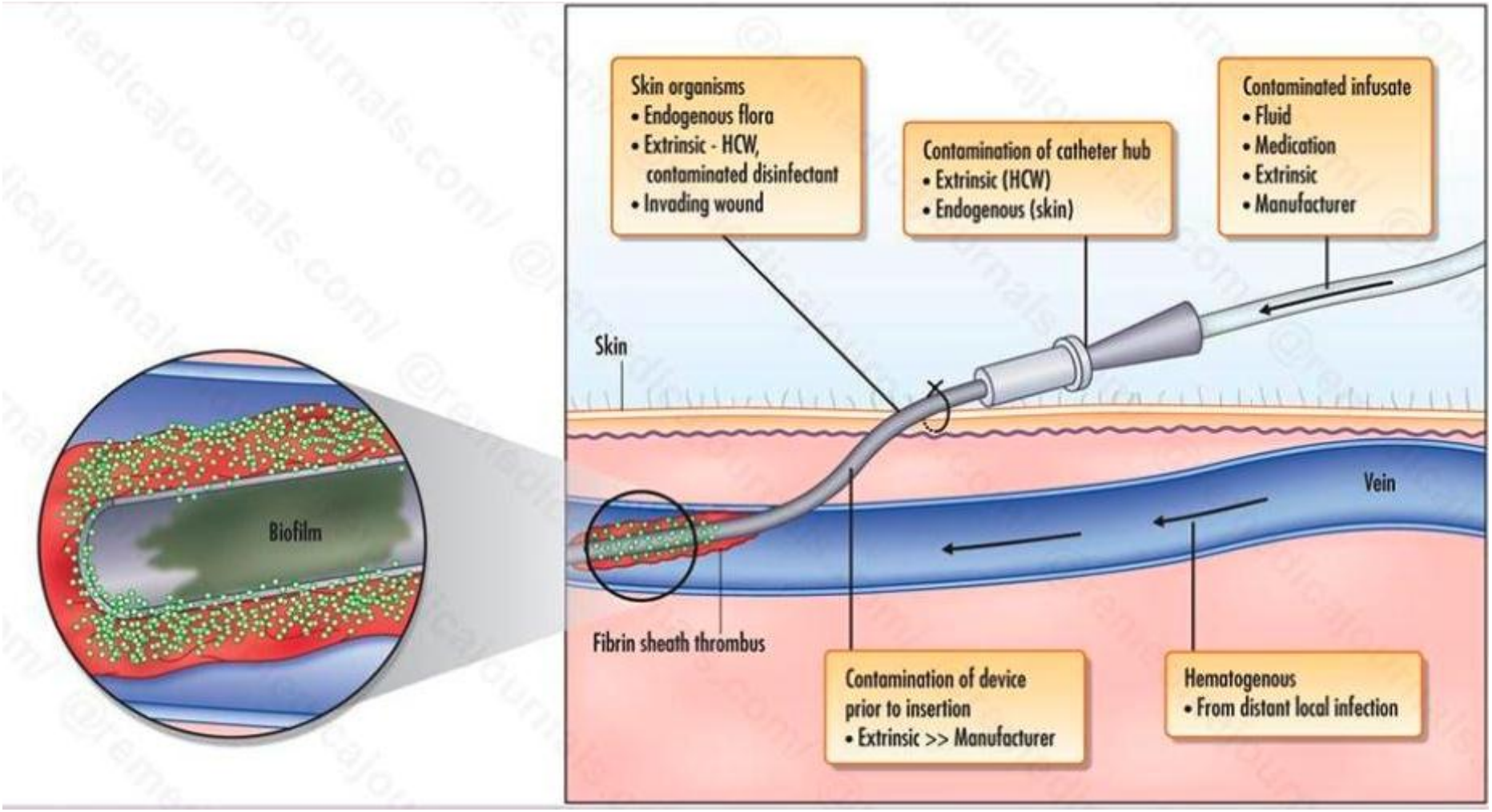
Possible surfaces for biofilm formation!







Biofilms and Infection



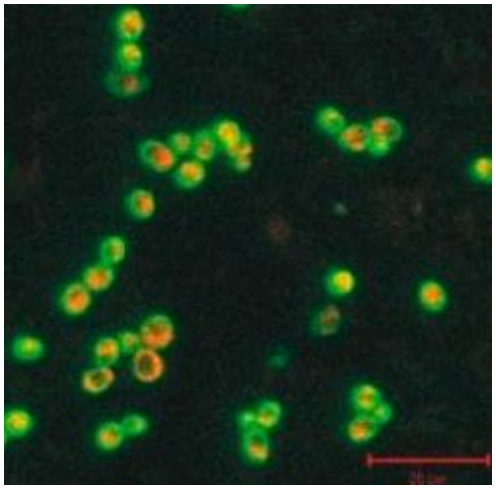
James et al. 2011. *J. Invas. Fung. Infec.* 5:37-42

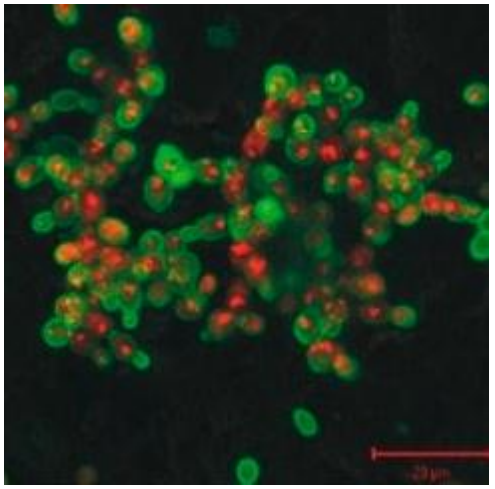
Optimal Conditions for Growing *C. albicans* Biofilms

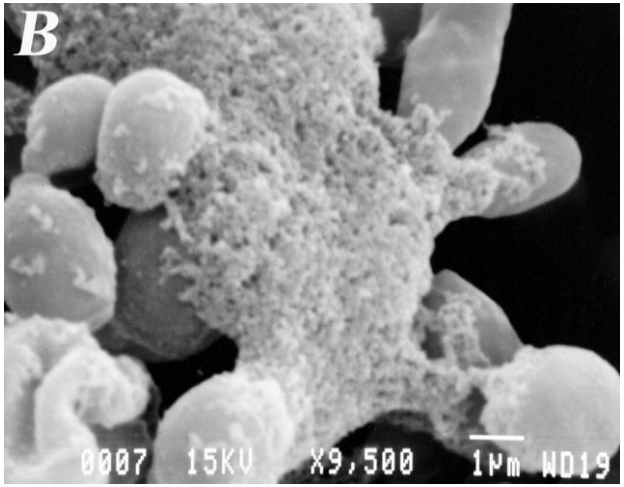
Variable	Denture[1]	Catheter[2]	Contact Lens[3]
Organism	*Candida*	*Candida*	*Candida*, *Fusarium*
Inoculum size	1 x 10^7 blastospores	1 x 10^7 blastospores	1 x 10^7 blastospores/conidia
Adhesion time	90 min	90 min	90 min
Incubation time	72 h	48 h	48 h
Pre-treatment	Saliva	Serum	Phosphate-buffered saline
Carbon Source	Glucose	Glucose	Glucose

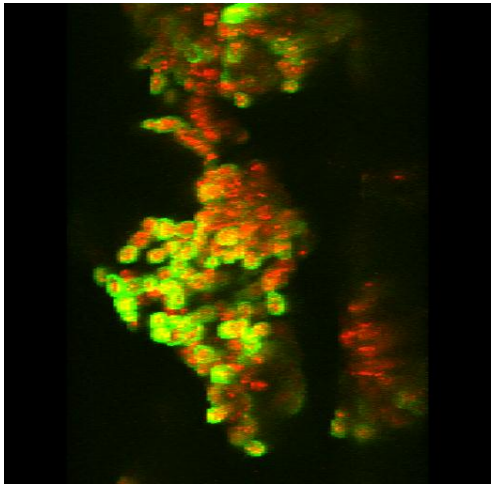
1. Chandra et al. 2001. J. Dental Res. 80(3): 903-908.
2. Chandra et al. 2001. J. Bacteriol. 183(18): 5385-5394.
3. Imamura et al. 2008. Antimicrob. Agents Chemother. 52(1): 171-182.

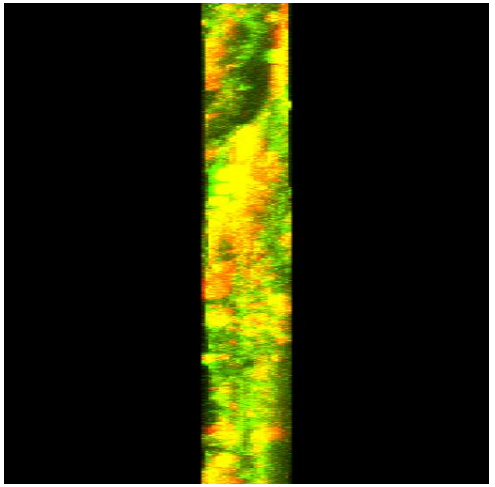
Microscopy Analyses



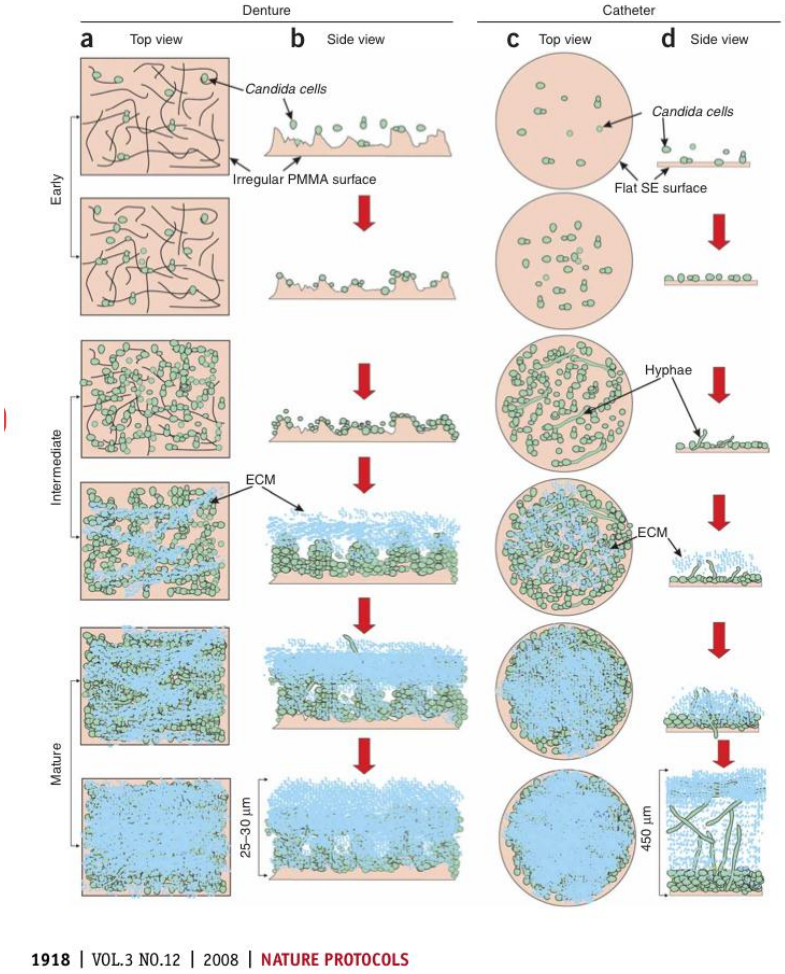








Schematic Representation of Biofilm Development in *C. albicans*



Chandra et al. 2008. *Nature Protocols* 3, 1909–1924

Resistance Against Antifungals

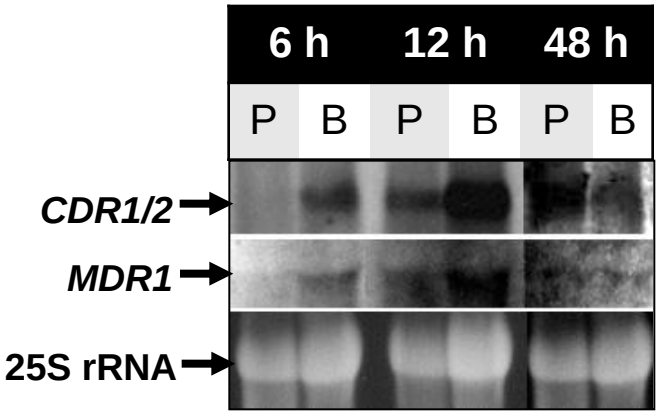
- Azoles were not active against biofilms
- Liposomal AmB formulations and echinocandins exhibited activity against biofilms

Drug	Planktonic MIC (µg/mL)	Biofilm MIC (µg/mL)
AmB	0.5	4
NYT	2	16
CHX	8	32
TERB	32	128
FLC	1	>256
VORI	0.5	>256
AmBisome	0.5	0.25
L-NYT	0.5	16
Abelcet	0.25	0.25
CASPO	0.125	0.5
MICA	0.001	0.5
ANIDULA*	0.125	≤ 0.03

Kuhn et al. (2002) Antimicrob. Agents Chemother. 46:1773-1780
*Jacobson et al. Antimicrob Agents Chemother 2008; 52: 2242-2243

Mechanisms of Antifungal Resistance

Gene Expression



Sterol Composition

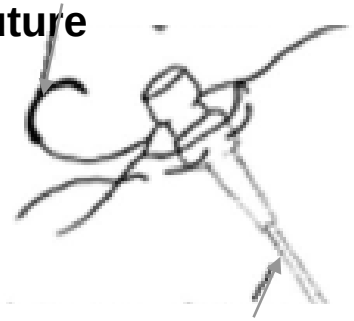
Sterol	6 h	12 h	48 h
Squalene	11.40	33.84	20.09
Breakdown product 1	8.35	8.48	13.32
Breakdown product 2	-	-	6.13
Zymosterol	27.50	12.63	10.53
Ergosterol	42.55	25.10	21.42
4,14-dimethylzymosterol	-	10.99	7.90
Obtusifoliol	10.20	8.96	19.86

- Efflux pumps confer resistance at early phase
- Alterations in sterol levels: intermediate and mature phases

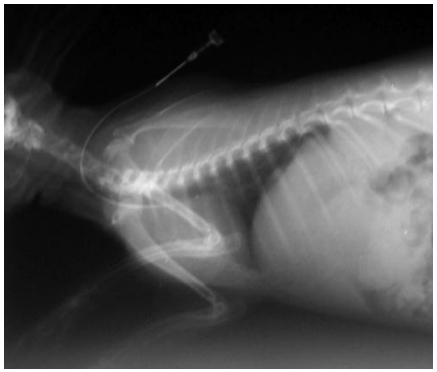
Mukherjee *et al*. (2003). *Infec. Immun.* 71(8):4333-4340

Development of Rabbit Catheter Biofilm Model

Purse string suture



Subcutaneous catheter



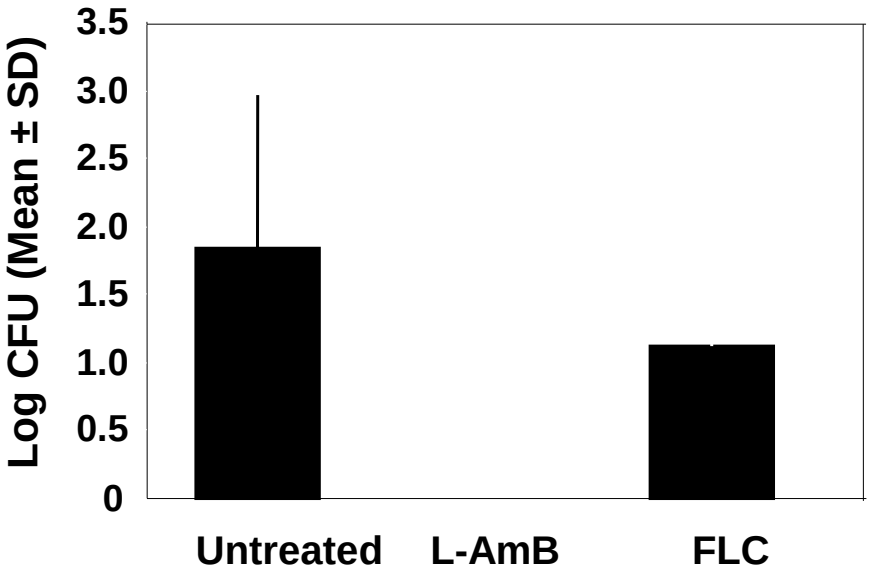
Biofilm Maturation

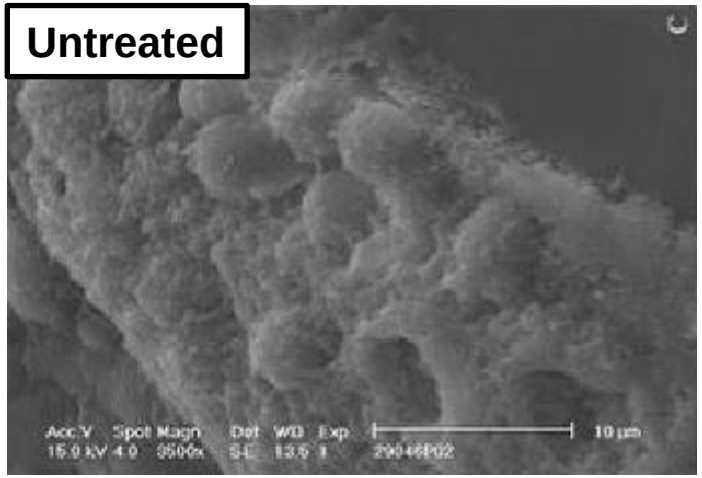
Day

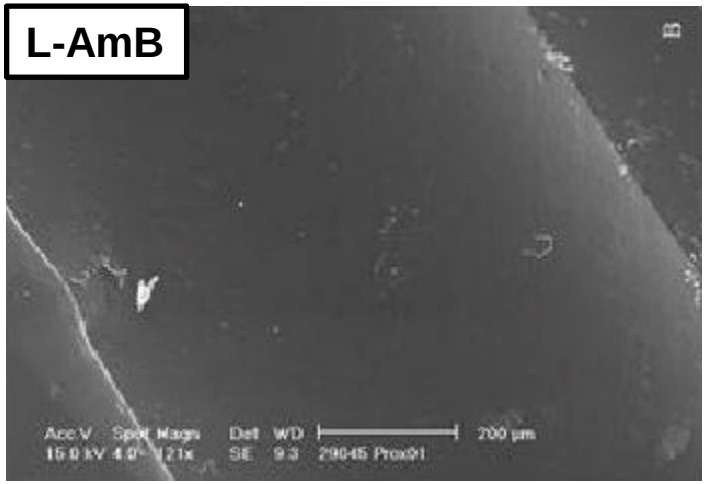
1	2	3	4	5	6	7	8	9	10	11
CVC insertion	Inoculation withdrawn		Blood from catheter							Blood from catheter & heart
C. albicans inoculum lock			AFLT for 7 days							Anaesthesia & euthanasia
	Daily heparin flushes									Catheter removal

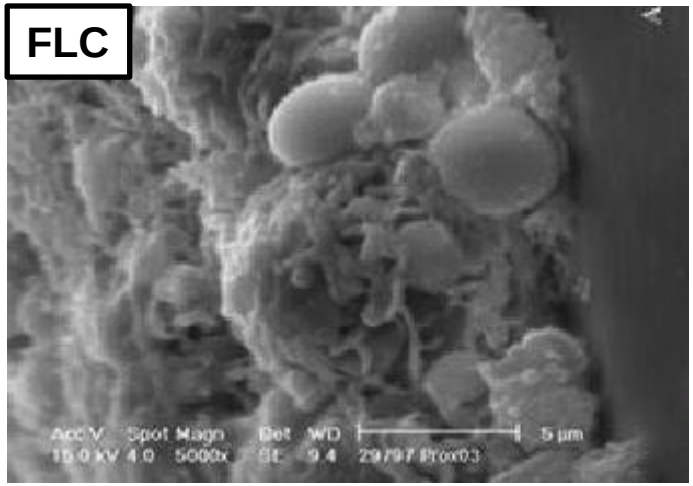
Schinabeck et al. (2004) Antimicrobial Agents and Chemotherapy 48, 1727-1732
Mukherjee et al. 2009. Int. J. Antimicrob. Agents 33:149-53

Effect of Antifungals on Biofilms Formed *In Vivo*









Schinabeck et al. (2004) Antimicrob. Agents Chemother. **48:** 1727-1732.

TOWARDS DEVELOPING A METHOD TO TEST ANTIFUNGAL SUSCEPTIBILITY OF FUNGAL BIOFILMS

Should CLSI try to develop a standardized biofilm-focused susceptibility testing method?

- Yes

- Why?
  - Aid management of patients with biofilm infections
  - Help industry in their R&D efforts to develop antibiofilm agents
  - Assist the FDA in evaluating the activity of potential antibiofilm products submitted for NDA
  - Investigators have performed a significant amount of work in establishing the optimal assay conditions

Current Models

- Current models used by different groups of investigators include
  - Catheter disks*
  - Acrylic (denture) strips*
  - Calgary biofilm device*
  - Fermentors/microfermentors
  - Cylindrical cellulose filters
  - Germanium substratum
  - Tissue culture flasks
  - Syringes
  - Modified Robbins devices
  - CDC reactor
- Tested under static and flow-through conditions
- Most of these models are complex, technically demanding and generally not amenable to high-throughput screening

Pierce et al. 2008. Nature Protocols **3:** 1494 – 1500

Monitoring Formation of Fungal Biofilms

- Formation of biofilms can be followed by:
  - Incorporation of [3H]-leucine into Candida cells in biofilm
  - Reduction of tetrazolium salts to colored formazan product
    - MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bro- mide (MTT)
    - XTT: 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5- [(phenyl amino) carbonyl]-2H tetrazolium hydroxide
    - WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphonyl)-2H-tetrazolium]
  - Crystal violet staining
  - Determination of the dry weight of biofilms
- Among these, metabolic activity assays based on XTT and dry weight determination have been more commonly used to quantitate the formation of *Candida albicans* biofilms
- Antifungal susceptibilities of biofilms formed by *Candida* species is commonly determined using a XTT-based assay
- The XTT-based assay has also been used previously to determine the susceptibility of planktonic *C. albicans* cells

Mukherjee and Chandra. 2004. Drug Resist. Updates 7, 301–309

Kuhn et al. 2003. J Clin Microbiol 41, 506–508

Summing Up … So Far

- Multiple methods exist
- Many are complex
- Disks and pins work pretty well
- XTT is a good readout system

Macrodilution vs. Microdilution Method

- Macrodilution-based XTT method requires:
  - large amounts of drug
  - multiple 12-well plates
  - bigger substrate disc (eg, catheter discs of 15-mm diameter)
  - long readout time (up to 4–5 days)
- These requirements render the macrodilution-based method unsuitable for routine use in clinical laboratories and drug screening
- Microdilution-based assay:
  - Requires 96-well plates, making it amenable to high-throughput screening of biofilms and their susceptibility to different agents
  - Smaller discs
  - Requires less drug
  - Shorter readout time
- Microdilution-based method would also be very useful in industry, by allowing screening of large number of potential biofilm compounds.
- A number of microdilution-based methods have been developed – with or without substrate discs/membranes

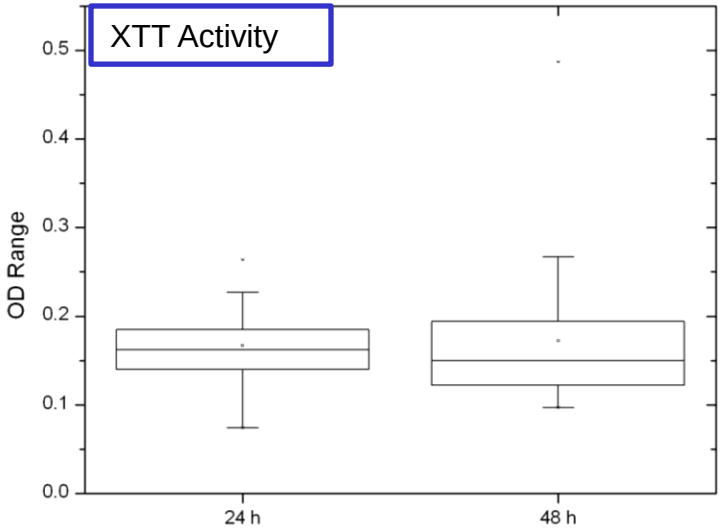
Chandra et al. 2010. Curr. Fungal Infect. Rep. **4(3):** 137-144
Chandra et al. 2008. Nature Protocols 3, 1909–1924
Pierce et al. 2008. Nature Protocols 3, 1494–1500

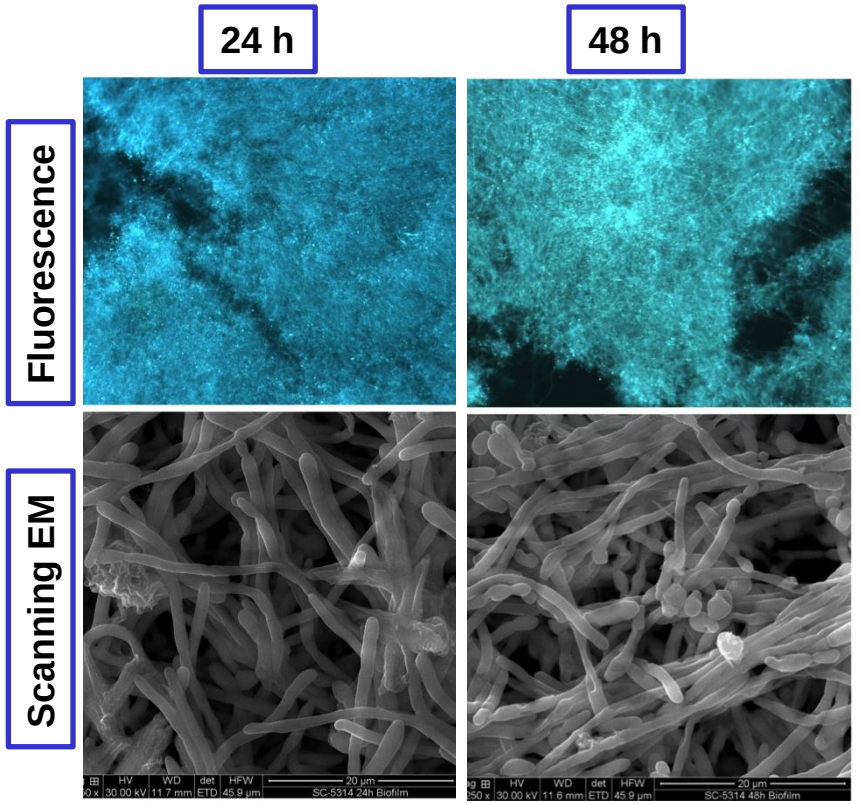
Microdilution Method – Using Substrate

- Biofilms were formed by *Candida* species on 5-mm catheter discs placed in microtiter (96-well) plates, and quantified by XTT assay
- Morphology, surface topography and three-dimensional architecture of these biofilms were evaluated by fluorescence, confocal scanning laser (CSLM) and scanning electron microscopy (SEM), respectively

Nweze et al. 2011. *J Antimicrob Chemother* **67**, 149–153

Microdilution Method – Using Substrate: Biofilm Growth Time and Ultrastructure





- Biofilms grown to 24 h or 48 h have similar metabolic activity
- Gross morphology and architecture of 24 versus 48 hour *Candida* biofilms were evaluated using fluorescence microscopy (with Calcofluor White) and scanning electron microscopy, respectively
- *C. albicans* biofilms exhibited typical fungal structural elements, with abundant hyphae enmeshed within a hazy extracellular matrix
- Confocal analyses confirmed these observations

Nweze et al. 2011. *J Antimicrob Chemother* **67**, 149–153

Drug Susceptibility Profile of Candida Biofilms Formed on Catheter Discs

Strain	Minimum inhibitory concentration (MIC, mg/L)			
	Fluconazole	Itraconazole	Voriconazole	Anidulafungin
C. albicans SC5314	>256	128 (32 - 128)	128 (64 - 256)	0.063*
C. albicans M61	>256	64*	64*	0.125*
C. parapsilosis A71	>256	64 (32-64)	64*	0.125*
C. parapsilosis 514	>256	64*	64*	0.125*

- Biofilms formed by all tested strains exhibited resistance to fluconazole (MIC >256 mg/L)
- Both *albicans* and non-*albicans* species of *Candida* exhibited reduced susceptibility to itraconazole and voriconazole (MIC ≥ 64 mg/L against all tested strains)
- In contrast, biofilms formed by all *Candida* strains tested were susceptible to anidulafungin (MIC ≥ 0.063 mg/L and 0.125 mg/L for strains SC5314 and M61, respectively); similar results were obtained for the two *C. parapsilosis* strains tested (MIC = 0.125 mg/L for both strains tested).
- These results showed that biofilms formed in our catheter-based microtiter assay were not susceptible to azoles, but were susceptible to anidulafungin, an echinocandin.

Nweze et al. 2011. *J Antimicrob Chemother* **67**, 149–153

Microdilution Method – Without Substrate

Strain	MIC (µg/ml)					
	Fluconazole			Amphotericin B		
	Planktonic	SMIC50	SMIC80	Planktonic	SMIC50	SMIC80
SC5314	4	>1,024	>1,024	0.25	0.25	1
3153A	4	>1,024	>1,024	0.5	0.5	1
ATCC 64550	16	>1,024	>1,024	0.25	0.25	8
ATCC 64558	0.5	>1,024	>1,024	0.25	0.5	1
ATCC 76615	0.25	>1,024	>1,024	0.25	0.5	2
ATCC 90028	0.5	>1,024	>1,024	0.5	0.5	1
ATCC 90029	0.5	>1,024	>1,024	0.5	0.5	2
SMIC - Sessile MIC						

- Microtiter (96-well) method for the formation of fungal biofilms and antifungal susceptibility testing
- The model is based on XTT activity of biofilms formed in the absence of substrate, in 96-well plates
- The antifungals tested in this study showed decreased activity against sessile cells of all *C. albicans* strains tested

Ramage et al. 2001. *Antimicrob. Agents Chemother.* 45, 2475–2479

Multi-well plate with pins in lid

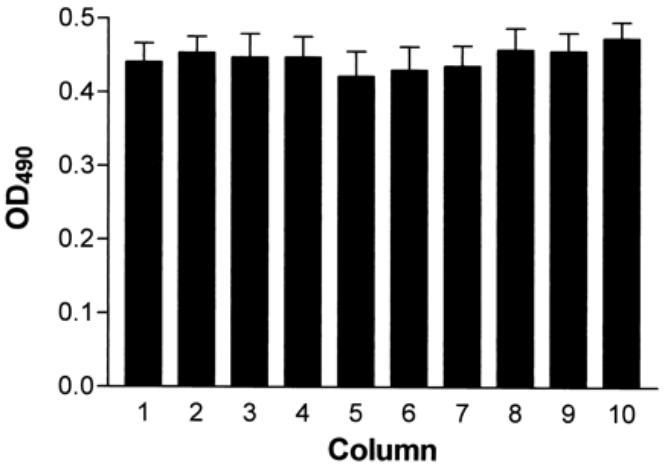
- Used to evaluate:
  - formation of *Candida* biofilms
  - susceptibility to antimicrobials

Parahitiyawa, N.B. et al. 2006. *APMIS* 114, 298–306
Harrison et al. 2007. FEMS Microbiol Lett. 272(2):172-81
Palmer 2010. Compend Contin Educ Dent. 31(2):104-8

Microdilution Method, Without Substrate: Assay Reproducibility

***C. albicans* biofilm formation on 70 independent wells of polystyrene microtiter plates grown for 24 h**	
Parameter	OD490
Mean	0.4466
Median	0.4435
Standard deviation	0.0297
Standard error	0.00355
Lower 95% confidence limit	0.4395
Upper 95% confidence limit	0.4536
Maximum	0.515
Minimum	0.386

Colorimetric readings (OD490s) of biofilms formed in wells of microtiter plates



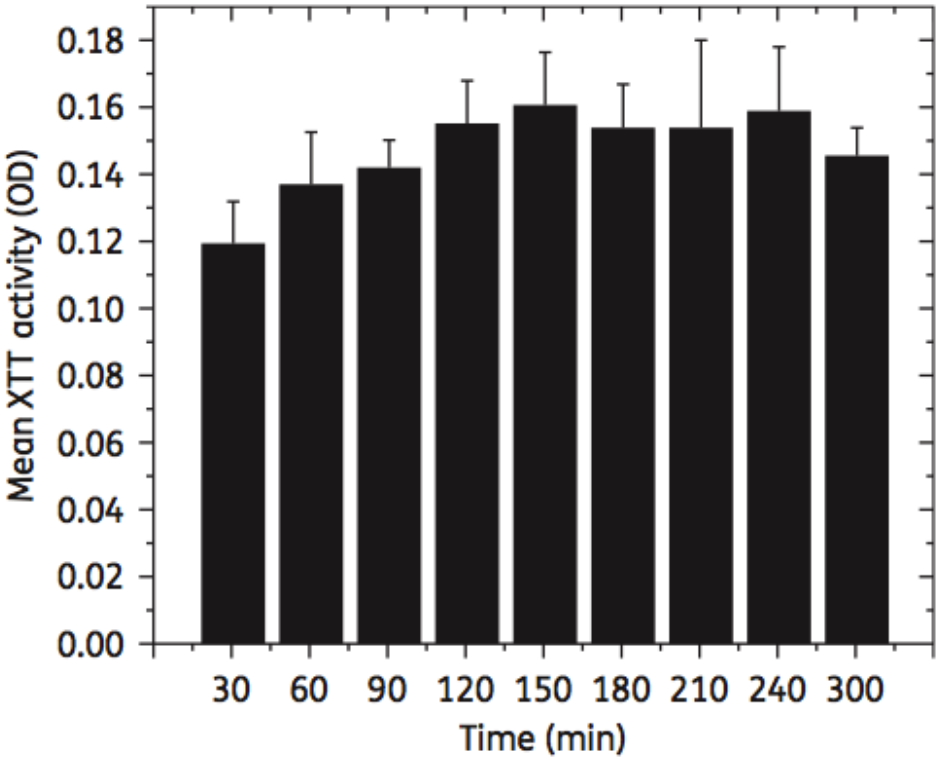
Values represent the mean and standard deviations of multiple independent biofilms formed in wells of each of 10 different columns of the same microtiter plate.

- No statistically significant difference was noted between C. albicans 3153A biofilms formed on multiple wells in each of 10 columns of the same microtiter plate ($P > 0.05$), a requisite for valid comparisons for susceptibility testing
- Furthermore, there were no significant differences in the XTT absorbance readings from 70 independent biofilms formed on the same microtiter plate

Ramage et al. 2001. *Antimicrob. Agents Chemother.* 45, 2475–2479

Microdilution Method (With Substrate): Incubation Time

- Metabolic activity (XTT OD) of biofilms after incubation for different times.
- N = 22 for each time point
- Maximum XTT activity was reached within 90 min (P = 0.022 versus biofilms grown to 30 min, ≥0.05 for comparisons with biofilms grown to other timepoints)
- Readout time can be reduced to 90 min



Nweze et al. 2011. *J Antimicrob Chemother* **67:** 149–153

Microdilution Method (Without Substrate): Impact of Subculture Medium and Temperature

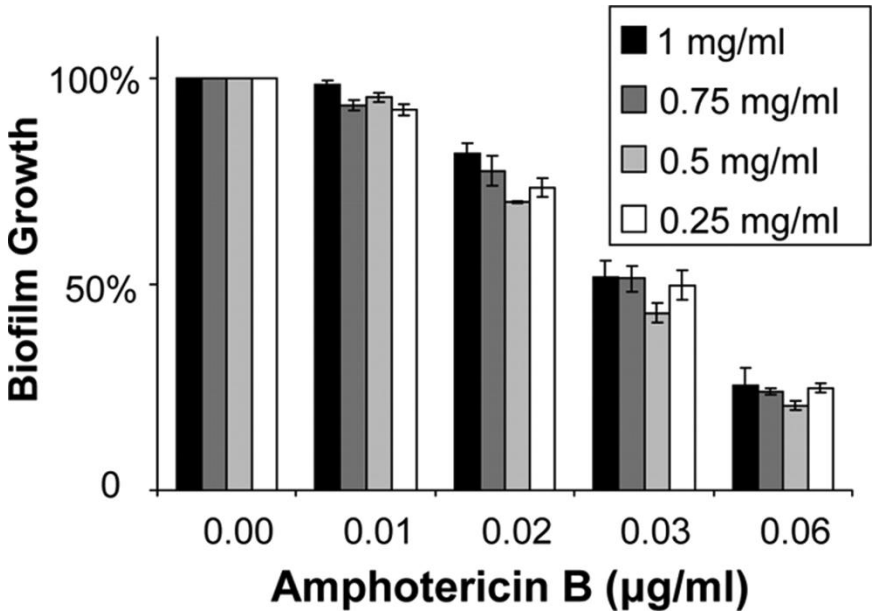
Strain	Subculture condition		EC50	
	Temp (°C)	Medium	Amphotericin B (µg/ml)	H_2O_2 (mM)
***C. albicans* DAY185**	30	YPD	0.06	250
	30	RPMI	0.06	250
	37	YPD	NA	NA
***C. albicans* K1**	30	YPD	0.06	125
	30	RPMI	0.13	125
	37	YPD	0.13	500
***C. glabrata* 5376**	30	YPD	0.13	500
	30	RPMI	0.06	1,000
	37	YPD	0.13	500
***C. parapsilosis* 5986**	30	YPD	0.13	250
	30	RPMI	0.13	500
	37	YPD	0.13	500

- Using an EC_{50} endpoint, no major differences were detected between the susceptibilities of the biofilms formed under the various subculture conditions

Nett et al. 2011. J Clin Microbiol. **49(4):**1426-33

Microdilution Method (Without Substrate): Impact of XTT Concentration

- After a 24-h biofilm formation period, biofilms were treated with serial dilutions of amphotericin B for 24 h
- Absorbance at 492 nm was measured following incubation with XTT for 1 h
- Susceptibility results were similar for all XTT concentrations tested



Nett et al. 2011. J Clin Microbiol. **49(4):**1426-33

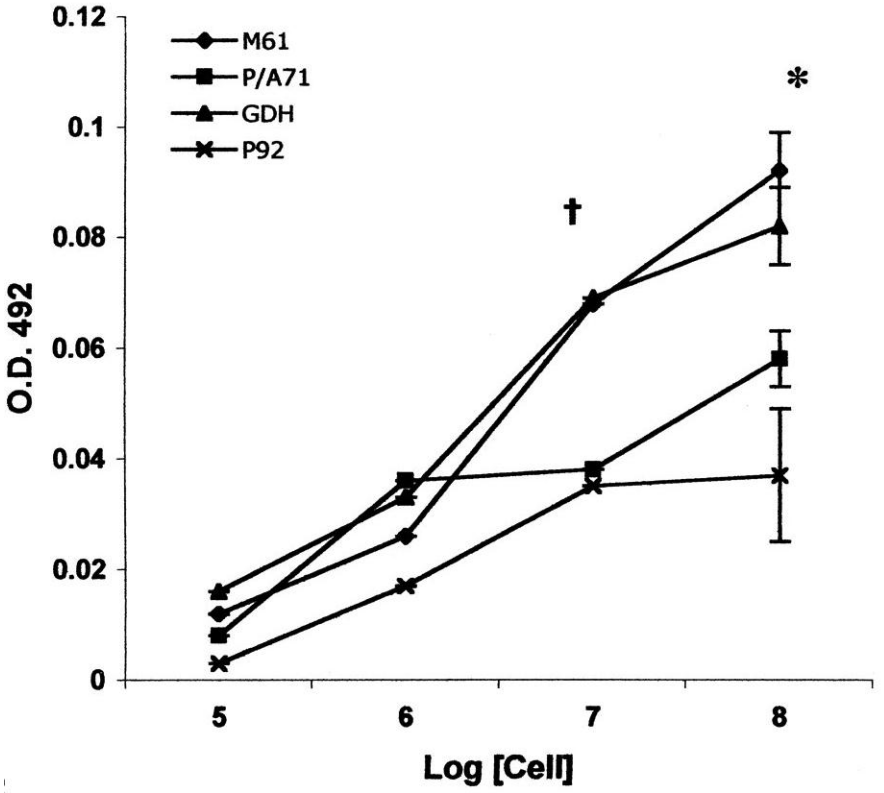
XTT Assay Variables
(Common to Microdilution Methods)

Objective	Proposed change in variables
Increase assay sensitivity	Shorten duration of biofilm formation
	Increase drug concn
	Increase antifungal treatment period
	Shorten interval between drug doses
	Shorten XTT incubation time
Increase biofilm formation	Try alternative subculture conditions (RPMI-MOPS or 37°C)
	Lengthen duration of biofilm formation
Decrease time and cost	Use cells directly from subculture without additional washes
	To avoid having to filter sterilize XTT, prepare it fresh daily
	Use XTT at 0.25 mg/ml
	Shorten XTT incubation time to 30 min
	Record absorbance directly in original 96-well plate to avoid transfer to new plate

Nett et al. 2011. J Clin Microbiol. **49(4):**1426-33

XTT Variables: Species Dependence

- Macrodilution method (12-well), with substrate
- Organisms tested:
  - *C. albicans* (M61 and GDH)
  - *C. parapsilosis* (P/A71, P92, and P177)
- Determined linear dose-response relationship between cell number and OD
- *C. albicans* exhibited a steeper response curve than did *C. parapsilosis*
- Conclusion: Results from XTT activity are species-dependent



Kuhn et al. 2003. J Clin Microbiol 41, 506–508

Going Forward

- Discussion Points
- Which microdilution method to select?
  - With or without substrate
  - Which medium
  - Growth temperature
  - Growth time (24 h)
  - XTT incubation time (1-4 h)
  - Reporting endpoint – 50% inhibition
  - Species-specificity
  - Cost
- Intra- and inter-laboratory multicenter study
- QC
- Positive and negative control (Fluconazole and Echinocandin)

CMM Team

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particularly Pranab Mukherjee

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