Biofilm in fungal infections and therapeutic approaches for infections caused by biofilm forming fungi

Invasive Fungal Infections

- *Candida sp* : 70% to 90% of all invasive mycoses
- In patients with BSIs
  - *Candida* sp are the 4th commonly isolated pathogens in the United States and 7th in Europe
- In ICU patients
  - *Candida* sp are the 3rd cause of nosocomial BSIs
- Increased use of invasive procedures
  - Risk factors for candidemia (IV catheters and IV alimentation)
- Candidemia is associated with
  - High crude and attributable mortality rates
  - Increased costs
  - Prolonged hospitalization

Leroy O, et al.*Crit Care Med.* 2009;37(5):1612-1618, Bougnoux ME, et al *Intensive Care Med* 2008;34:292–299, Horn DL, et al *Clin Infect Dis.* 2009;48:1695-1703.

Infections Associated with Medical Devices

- Incidence: 2 million cases each year
- Mortality: 88,000 deaths
- Resources: 8 million excess hospital days
- Cost: $4.5 billion
- Pathogens: 70% due to organisms resistant to at least one antibiotic
- Association: 50% of cases are device-related

Martone, et al. In: Hospital Infections. 1992:577-596

The NIH estimates that 80% of human infections result from pathogenic biofilms

Morphological growth forms of *Candida*

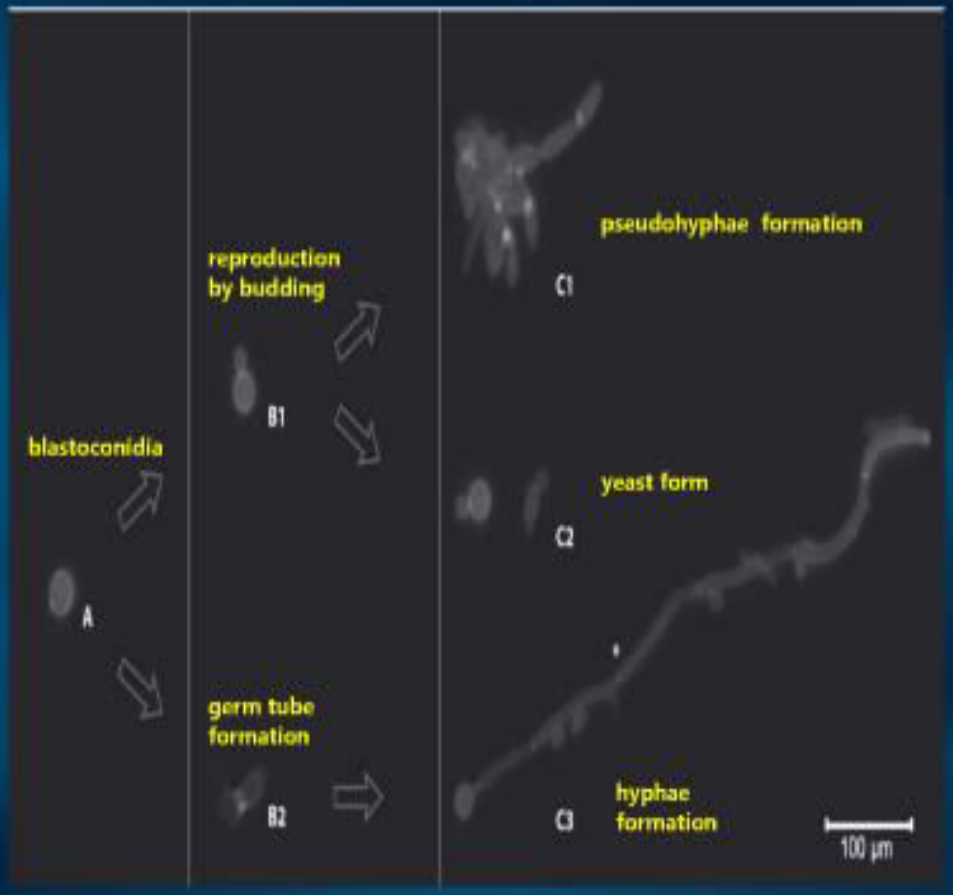
***C. albicans* = polymorphic fungus**

- **This polymorphism** between yeast, hyphae, pseudohyphae forms is critical for virulence and response to environmental changes

This morphological switch depends on

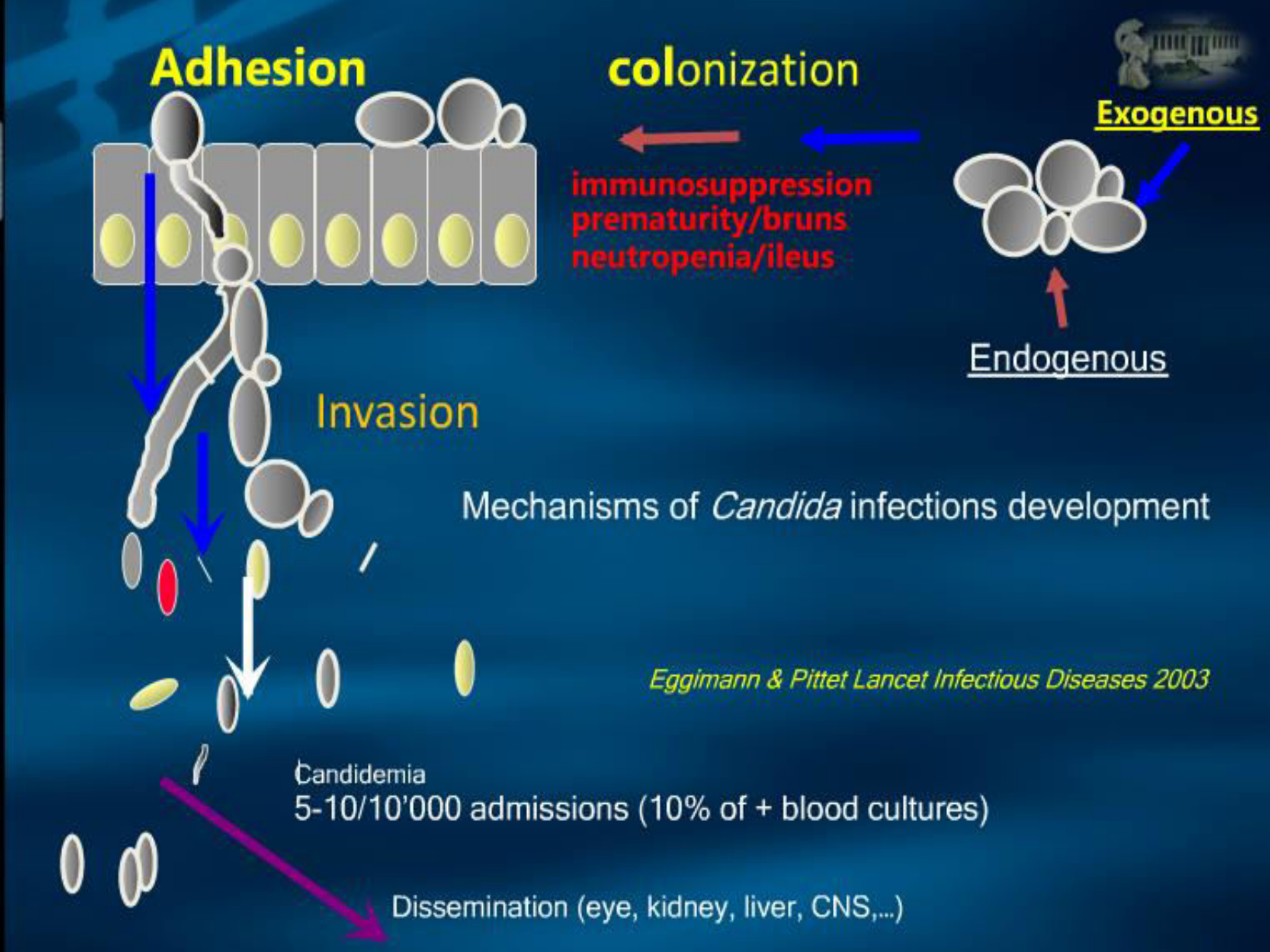
- ✓ temperature and pH alterations
- ✓ CO_2 concentration
- ✓ mammalian serum

In densities <10 6 cells/m *C. albicans* cells develop into filamentous forms at higher densities grow as budding yeasts



Epifluorescence photocomposition stained with calcofluor white

Silva S et al, FEMS Microbiol Rev 36 (2012) 288–305


Adhesion
colonization
Exogenous
immunosuppression
prematurity/bruns
neutropenia/ileus
Endogenous
Invasion
Mechanisms of *Candida* infections development
Eggimann & Pittet Lancet Infectious Diseases 2003
Candidemia
5-10/10'000 admissions (10% of + blood cultures)
Dissemination (eye, kidney, liver, CNS,...)

What is a biofilm ?

A structural community of microorganisms encased in a self-produced extracellular matrix that is adherent to biotic/abiotic surface.

Possible surfaces for biofilm formation!

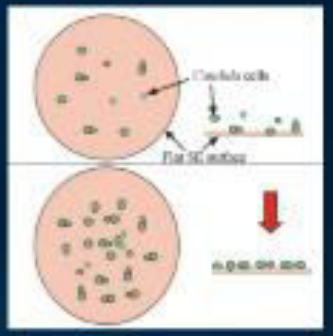
- Clinically, biofilm infections represent an overwhelming problem due to the highly recalcitrant nature of the embedded microbes, which are resistant to both antimicrobial drugs as well as host defenses

- Biofilms are a protected niche for microorganisms, where they are safe from antimicrobial treatment and can create a source of persistent infection

In vitro, biofilm formation

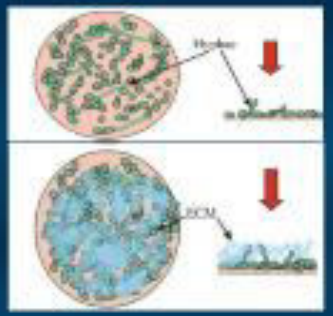
Early Phase

Yeast cells adhere to an appropriate surface and undergo morphogenesis, which is essential for normal biofilm formation



Intermediate Phase

Continued hyphal growth and Extracellular Matrix (ECM) production, which consists of cell wall polysaccharides and protein



Mature Phase

Consist of a yeast base, with hyphal elements forming a complex network encased in ECM extending away from the surface.

Harriott et al, Trends in Microbiology, November 2011;19(11): 557-63

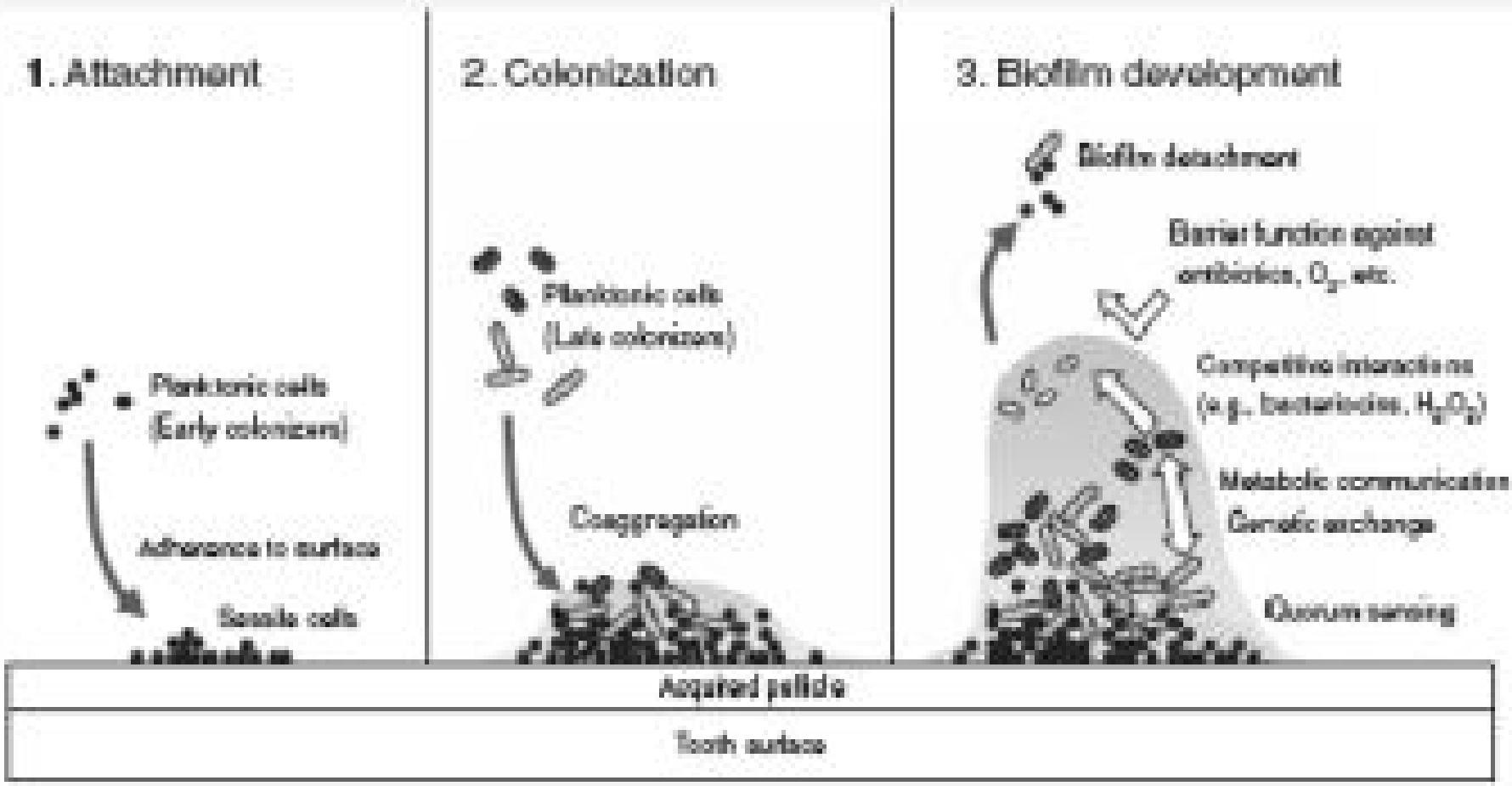
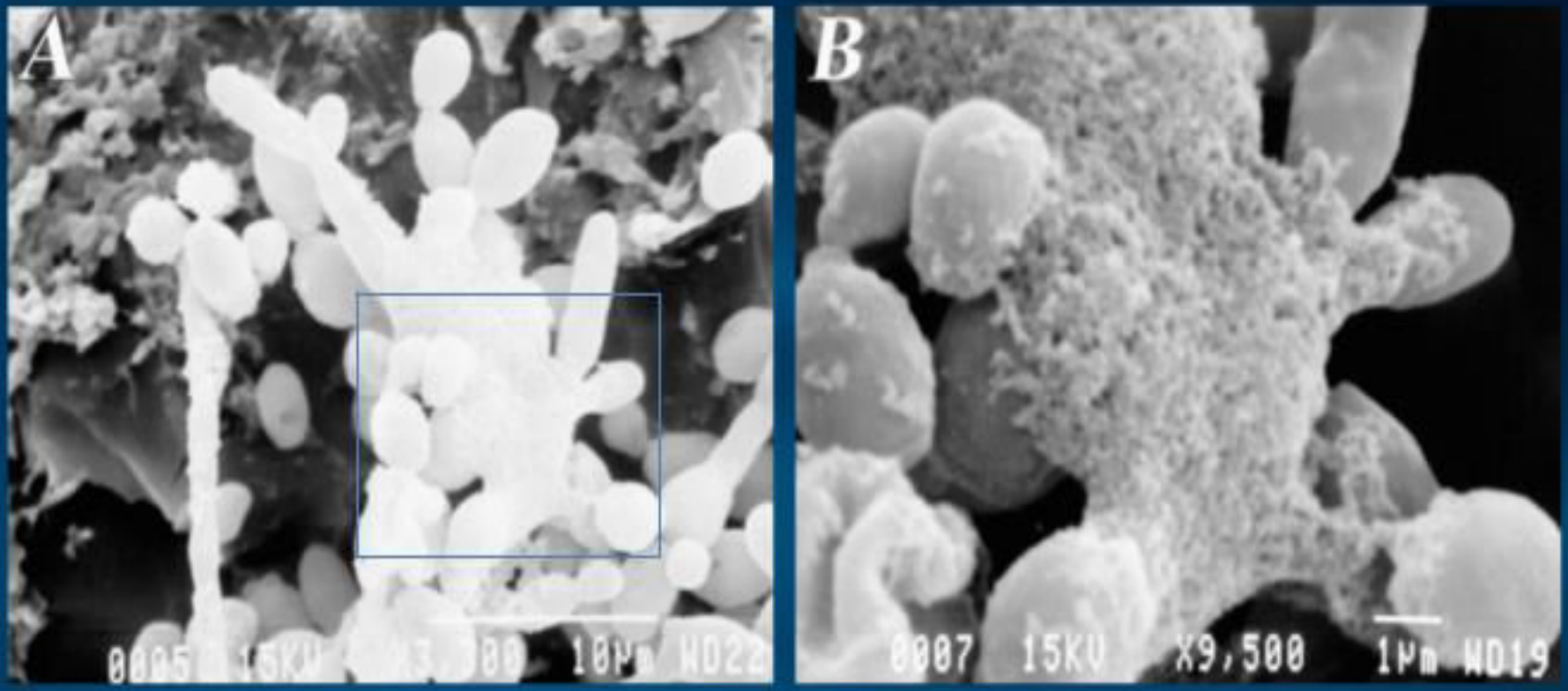


Figure 1. A diagrammatic representation of biofilm formation on the tooth surface and the potential roles of bacterial interactions. The tooth pellicle is generally colonized by early colonizers. Co-aggregation contributes to sequential binding and colonization. Bacterial interactions include metabolic communication and genetic exchange. The development of a biofilm having a high bacterial cell density increases the concentration of signaling molecules. Dental biofilms function as a barrier against deleterious factors such as antibiotics and oxygen.

Visualization of Biofilm by SEM



Dense layers of co-aggregating yeast and some hyphal forms

Fungi embedded in extracellular polymeric material

Extracellular material has amorphous granular appearance

CSLM Confirms 3 Distinct Phases of Biofilm Development

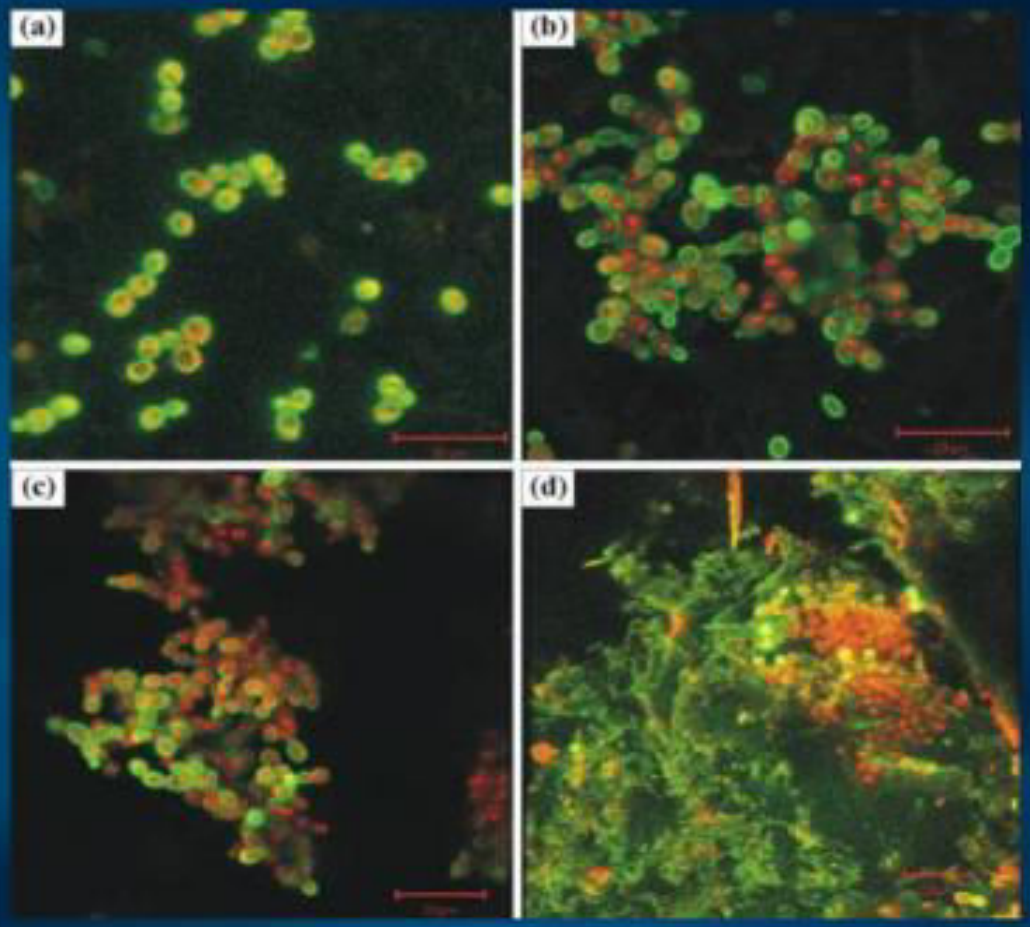
0 h, Adhesion

8 h, Proliferation

11 h, Micro-colonies

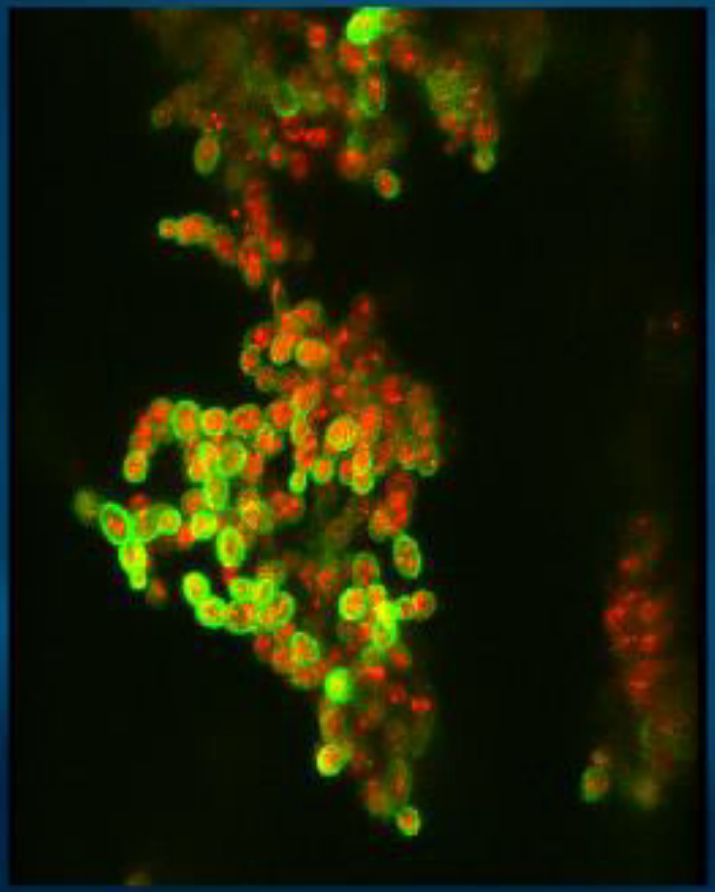
48 h, Cells within matrix

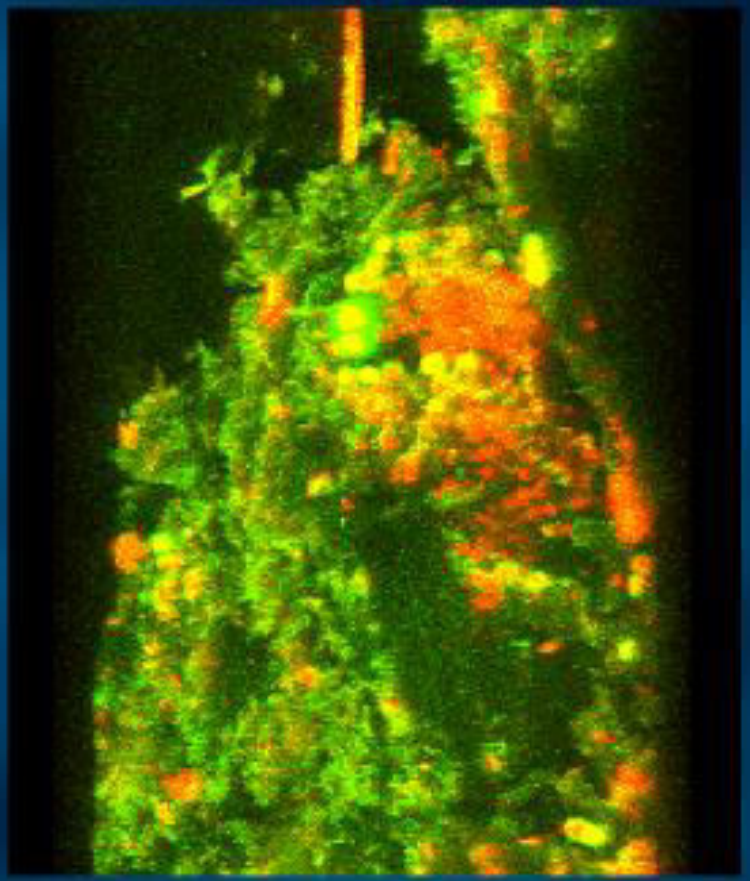
- **Green fluorescence** - ConA binding to polysaccharides
- **Red fluorescence** – FUN1 staining of metabolically active cells



Chandra et al. (2001) *J. Bacteriology* 183 (18).

Candida Biofilm – A Closer Look at the Intermediate and Mature Phases





QS and QSMs

QS is the synchronization of the expression of virulence factors by the microbial population density to regulate the developed virulence in order to overcome host defenses.

QS is a microbial communication

- occurs by the continuous release and monitoring of hormone-like molecules called auto-inducers or quorumsensing molecules (QSM)

QSMs (the most important)

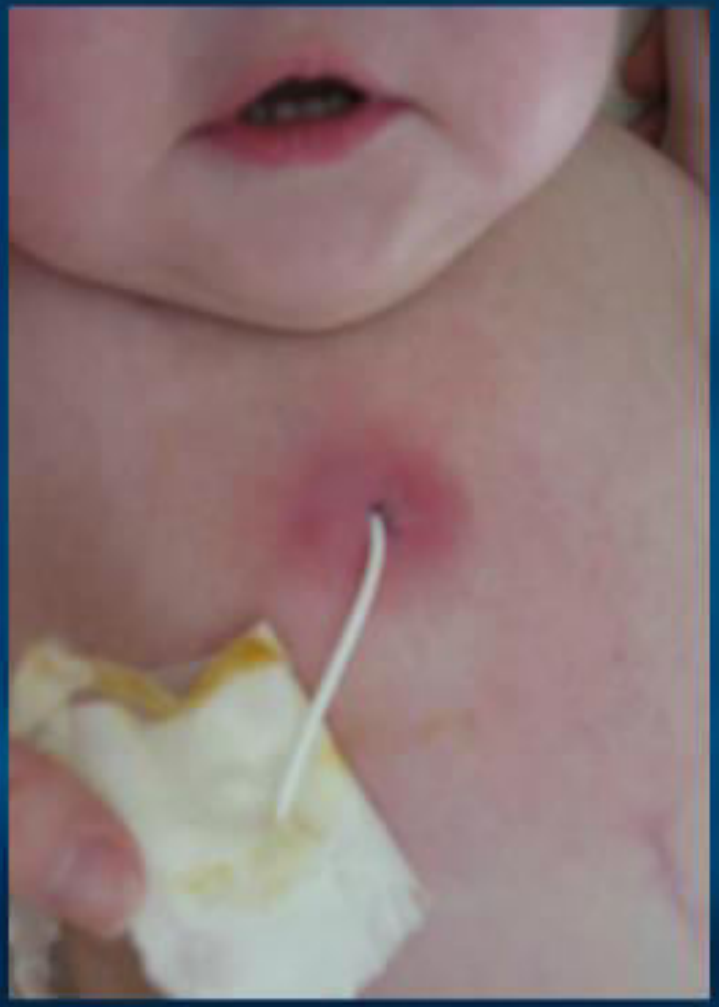
- Farnesol
- Tyrosol

Cao YY *et al, Antim Agents Chem 2005; 49 : 584 – 589*, Blankenship J et al, *Curr Opin Microbiol 2006; 9 : 588 -94*, Nadell CD et al, . *PLoS biology 2008; 6 : e14*, Ramage G, et al, *Appl Environ Microbiol 2002; 68 : 5459 -63*

Quorum Sensing

- Allows bacteria /fungus to monitor the environment for other bacteria/fungi and to alter behavior on a population-wide scale in response to changes in the number and/or species present in a community.
- Quorum sensing confuses the distinction between prokaryotes and eukaryotes because it enables microbes to act as multicellular organisms

Candida biofilm



- Resistant to antifungal agents and host immune factors
- Highly dependent on **QS**
- Is controlled by **QSMs**
  - ✓ Isoprenoid, farnesol and farnesoic acid acting as autoregulatory substance (ARS)

QS = Quorum Sensing
QSM=Quorum Sensing Molecules

Gow N, *Curr Opin Microbiol 2002;5:366 –71*,Sudbery Pet al,*Trends Microbiol 2004;12:317–24*,Saville S,*Eukaryot Cell 2003; 2: 1053 –60*, Oh K et al,*Proc Natl Acad Sci USA 2001;98 :4664– 68*, Nickerson KW et al,*Appl Envir Microb 2006;72* : 3805 –13

Farnesol and Tyrosol

- **Farnesol**
  - *C. albicans* morphology
  - Inhibits biofilm formatiom
    - ✓ **the rate of inhibition is dependent on how much time the cells had to adhere**
  - **Once the cells started to filament farnesol had no effect on thedevelopment of biofilm**

- **Tyrosol**
  - correlates with the increase of biomass of both planktonic cells and biofilms of *C. albicans*
  - at early stages of biofilm formation stimulates hyphal growth

Cao YY *et al, Antim Agents Chem 2005; 49 : 584 – 589,* Blankenship J et al, *Curr Opin Microbiol 2006; 9 : 588 –94,* Nadell CD et al, . *PLoS biology 2008; 6 : e14,* Ramage G, et al, *Appl Environ Microbiol 2002; 68 : 5459 -63*

QS signaling pathways and receptors

1. The signaling cascades controlling the expression of genes under QS regulation in *C. albicans* remain poorly understood

2. Ras-cAMP-PKA pathway, general repressor TUP1
   - farnesol inhibits Ras-cAMP-Efg1 activity resulting in hyphal growth
   - oxidative stress induced by farnesol is dependent on Ras1-adenylate cyclase signaling pathway

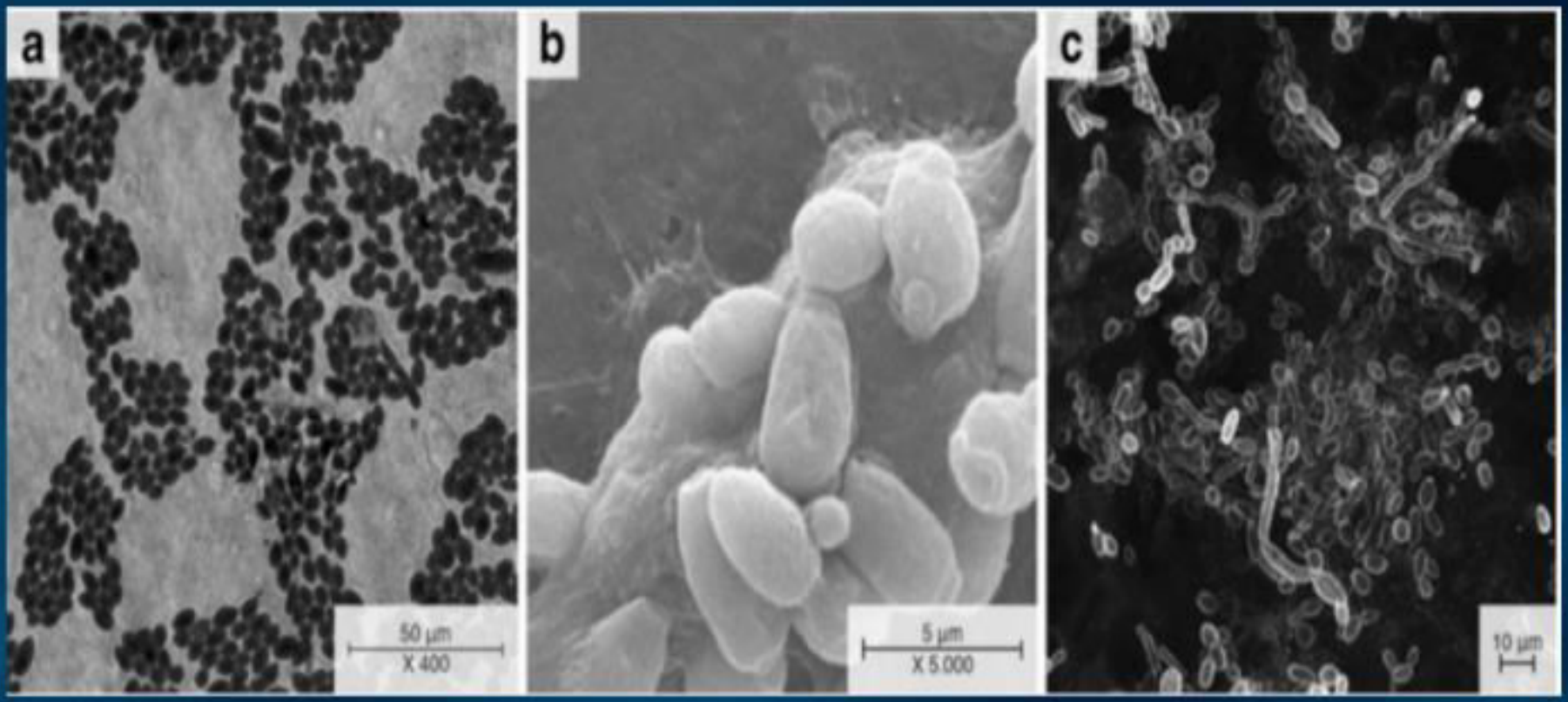
Langford ML et al, *Future Microbiol 2009;* ***4 : 1353 – 62,*** Hall RA et al, *Adv Appl Microbiol 2009;* ***67: 191– 2 12,*** Kebaara BW *et al, Eukaryot Cell 2008;* ***7 : 980 – 87,*** Sato T et al *Biol Pharm Bull 2004;* ***27*** :751 – 52, Davis-Hanna A, *Mol Microbiol 2008;* **67 : 47 – 62,** Deveau A et al, *Eukaryot Cell 2010;* ***9*** :569 – 77, Hall RA *et al, Eukaryotic Cell 2011;* ***10 : 1034– 42***

Aspartyl proteinase, phospholipase, hemolytic activities and biofilm production

- *Candida albicans* : bronchial aspirates of ICU pts
  - protease and hemolytic activities
    - aspartyl protease (facilitate invasion by degrading proteins at the site of the infection)
    - Phospholipase
    - Hemolytic activities

Sacrist α n B, *Medical Mycology January 2011, 49, 94–97*

Candida tropicalis
remarkable capacity to create biofims



Optical micrograph of *C. tropicalis* on silicone coupons

Scanning electron micrograph of *C. tropicalis* adhered to a human epithelial urinary bladder cell line

Confocal laser scanning microscopy image of *C. tropicalis* adhered to a reconstituted human oral epithelium

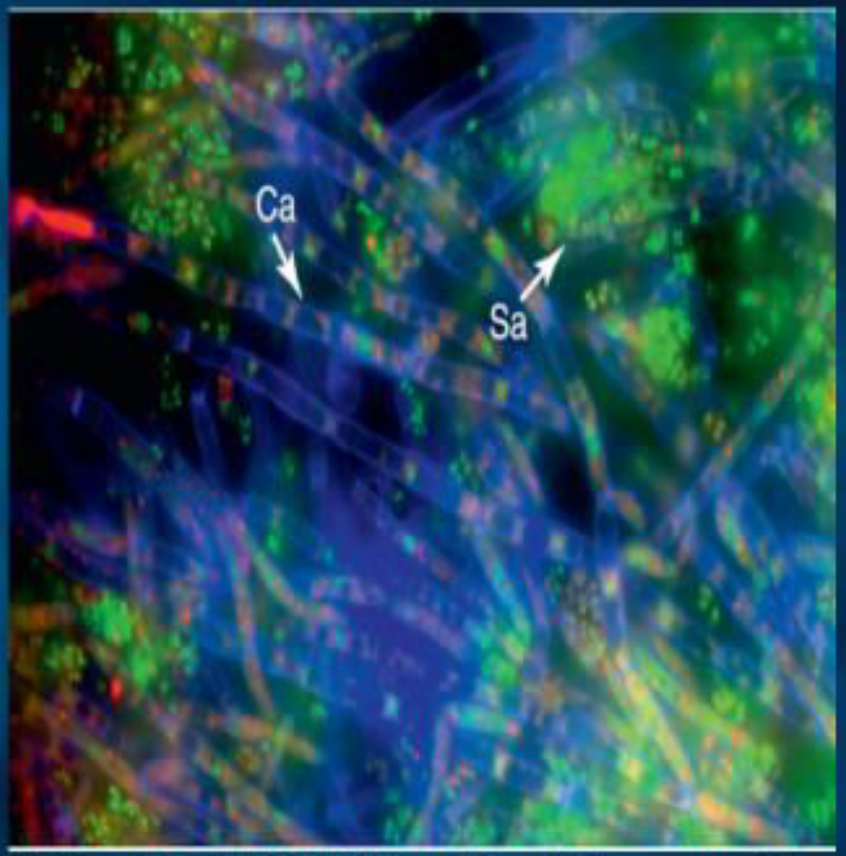
Negri et al, Eur J Clin Microbiol Infect Dis (2012) 31:1399–1412

Candida albicans monomicrobial and polymicrobial biofilms

The National Institutes of Health estimates that 80% of human infections result from pathogenic biofilms
(http://grants.nih.gov/grants/ guide/pa-files/PA-99-084.html)

An estimated 27–56% of nosocomial *C. albicans* bloodstream infections are polymicrobial

Staphylococcus epidermidis, Enterococcus spp. Staphylococcus aureus



Polymicrobial biofilm of *C. albicans* and *S. aureus*.

Original Research

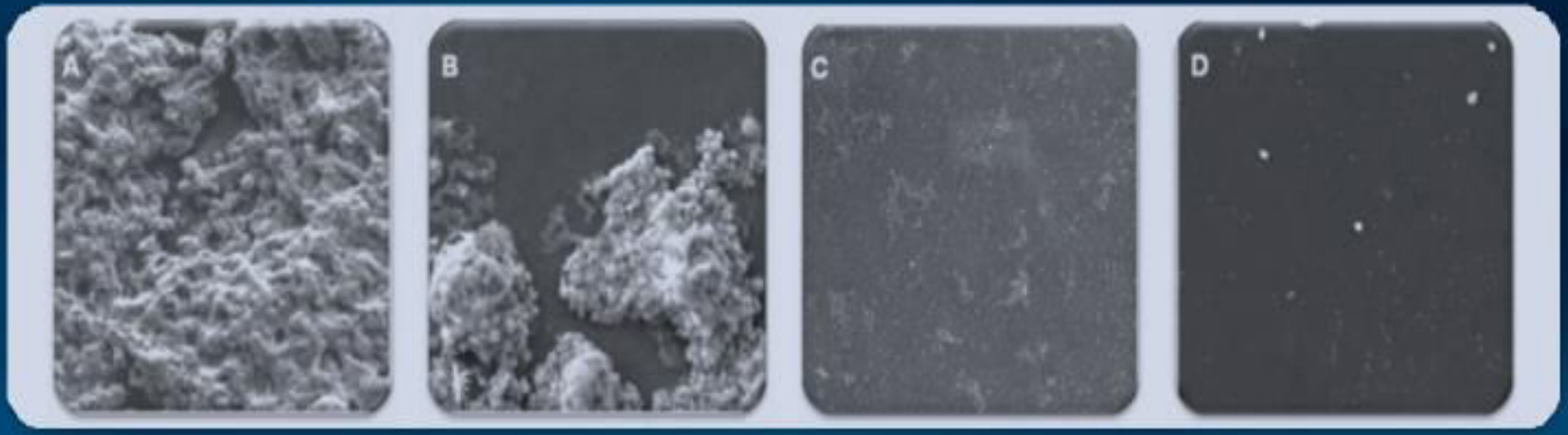
RESPIRATORY INFECTION

Candida Colonization of the Respiratory Tract and Subsequent Pseudomonas Ventilator-Associated Pneumonia*

Elie Azoulay, MD, PhD; Jean-François Timsit, MD, PhD; Muriel Tafflet; Arnaud de Lassence, MD; Michael Darmon, MD; Jean-Ralph Zahar, MD; Christophe Adrie, MD, PhD; Maité Garrouste-Orgeas, MD; Yves Cohen, MD; Bruno Mourvillier, MD; and Benoît Schlemmer, MD; for the Outcomerea Study Group†

(CHEST 2006; 129:110–117)

Treatment of biofilm



Scanning electron micrographs of catheter surfaces following 7 days of therapy

Untreated	Systemic	Lock therapy	Lock and systemic therapy

TABLE 1. MICs of antifungal agents against planktonic and biofilm-associated *C. albicans* (M61 and GDH) and *C. parapsilosis* (P/A71 and P92) strains[a]

Drug	MIC (μg/ml) in:							
	Planktonically grown cells for strain:				Biofilm at 48 h for strain:			
	M61	GDH	P/A71	P92	M61	GDH	P/A71	P92
AMB	0.5	0.25	0.25	0.5	4	4	8	8
NYT	2	1	0.5	2	16	16	16	64
Chlor	8	8	8	8	32	8	16	64
TBB	32	32	4	1	128	128	*	128
FLC	1	0.25	8	1	>256	>256	>256	>256
VRC	0.5	8	0.125	0.03	>256	>256	128	256
Ravu	0.1	0.06	0.125	0.1	128	128	*	128
Lip-AMB	0.5	0.06	0.06	0.5	0.25	0.25	1	*
Lip-NYT	0.5	0.06	0.5	0.5	8	16	32	*
ABLC	0.25	0.06	0.06	0.25	0.25	0.25	0.25	*
Casp	0.125	0.125	1	1	0.25	0.5	0.125	4
Mica	0.001	0.001	0.25	0.5	0.25	0.5	0.125	2

[a] Results are representative of at least two separate experiments. Lip-AMB and Lip-NYT are the lipid complex formulations of AMB and NYT. For details of methods used, see text. *, unable to determine MIC.

Kuhn et al. (2002) Antimicrob. Agents Chemother. 46:1773-1780

Evaluation of Caspofungin and Amphotericin B Deoxycholate against *Candida albicans* Biofilms in an Experimental Intravascular Catheter Infection Model

Jennifer A. Shuford[1], Mark S. Rouse[1], Kerryl E. Piper[1], James M. Steckelberg[1] and Robin Patel[1,2]

J Infect Dis. (2006) 194 (5): 710-713.

J Infect Chemother (2011) 17:634–639
DOI 10.1007/s10156-011-0224-3

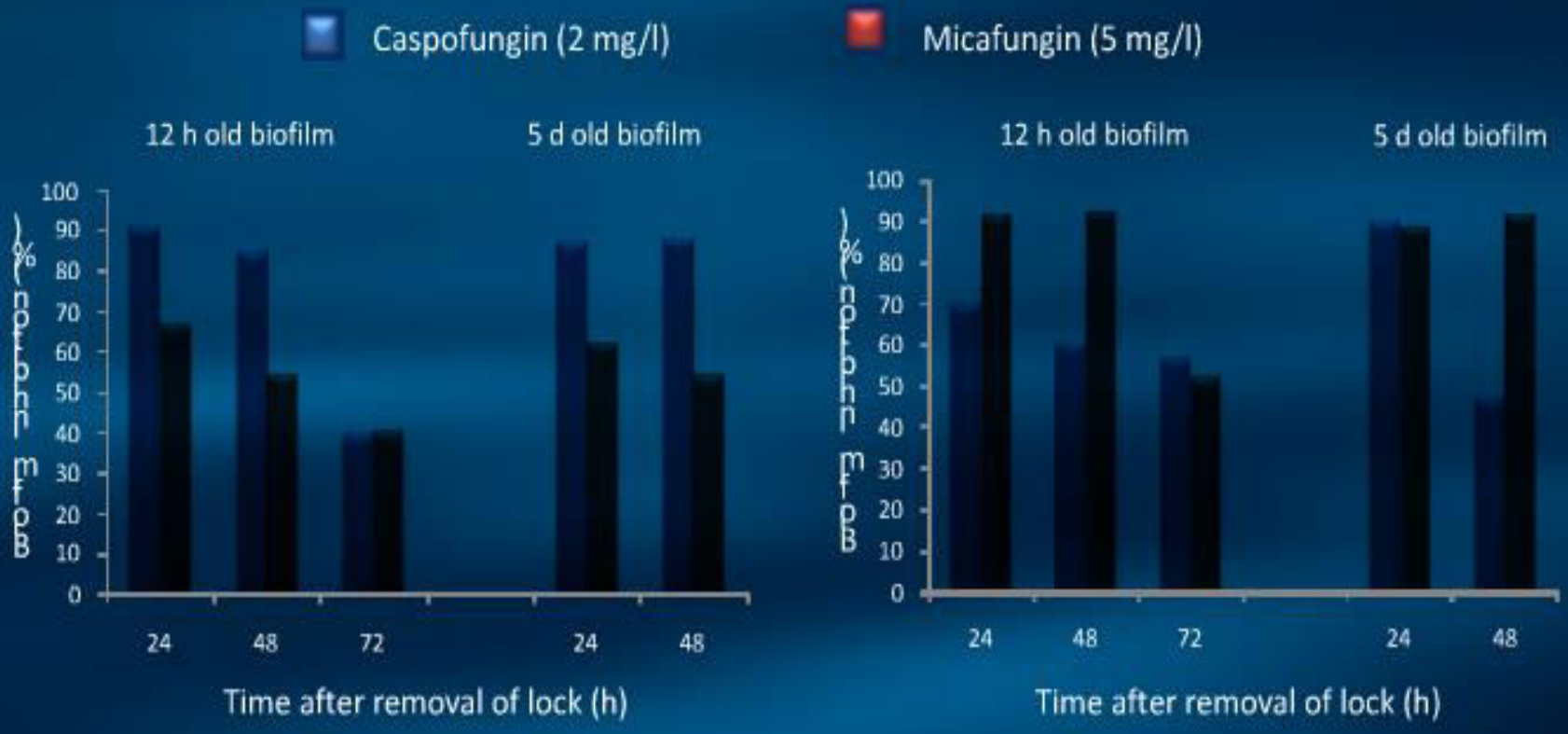
ORIGINAL ARTICLE

In vitro effectiveness of antifungal lock solutions on catheters infected with *Candida* species

Serkan Öncü

Activity of echinocandins against biofilms

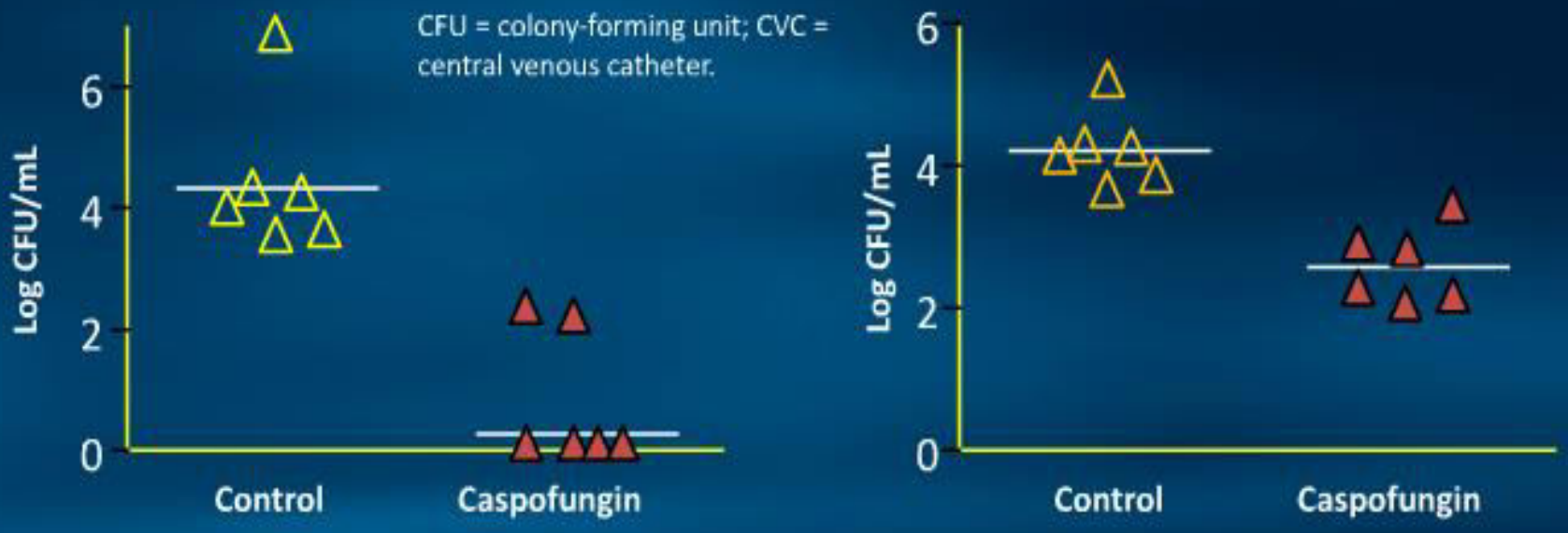
- Micafungin and caspofungin activity against intermediate and mature-phase biofilms of two separate *Candida albicans* strains
  - ATCC 3153 (left) and ATCC 66396 (right)



Cateau E, et al. *J Antimicrob Chemother* 2008;62:153–5

In vivo efficacy of echinocandins

Prevention and treatment of *C albicans* biofilms formed in mice CVC-associated candidiasis



Lazzell A et al. *J Antimicrob Chemother*. 2009:64;567–570.

Treatment of *Candida* biofilm

Antifungal Agent	Amph-B	Voricon-	Caspo-	Anidula-
Sessile MIC90 (µg/ml)	2	>256	2	≤0.03

In vitro sessile **antifungal** susceptibility of 30 clinical *C. albicans* isolates

Shuford JA et al Diagn Microbiol Infect Dis 2007; 57:277-281
Jacobson MJ et al AAC 2008;52:2242-43Choi HW et al AAC 2007;51:1520-1523,
Katragkou A et al AAC 2008;52: 357-360

Caspo-, Ampho-B, Posa-, in biofilm reduction regardless of the tested development phase

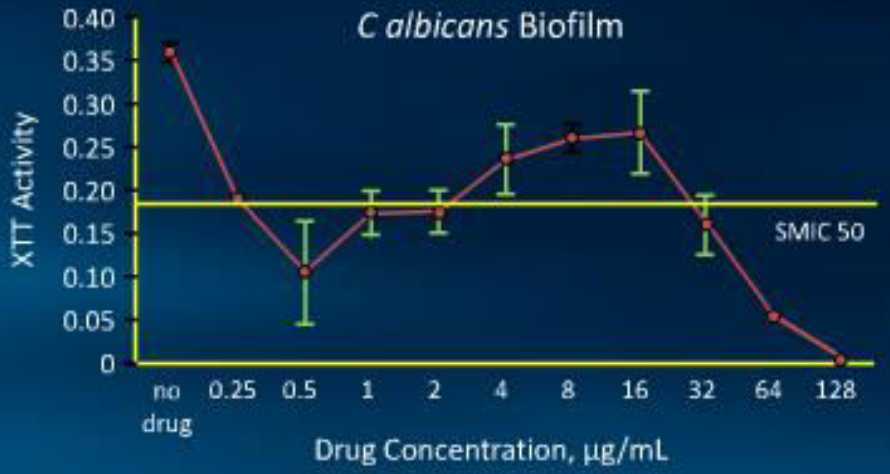
Caspofungin achieved a ≥50% reduction of 24- and 72-hour-old biofilms even at the low concentration of 1 x MIC.

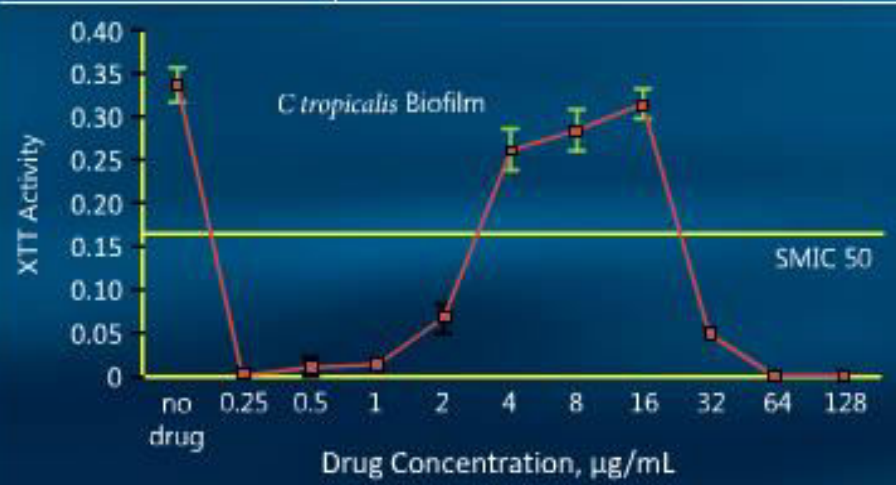
AmB = amphotericin B; CAS = caspofungin; MIC = minimum inhibitory concentration; POS = posaconazole.

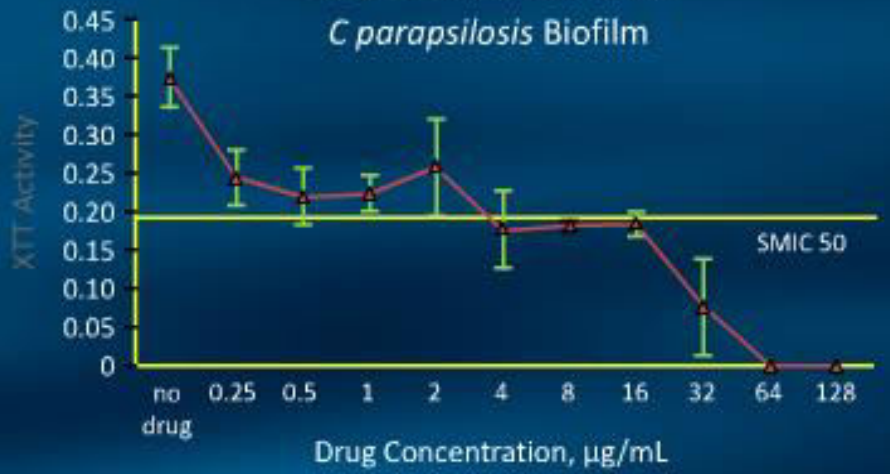
Tobudic S et al. *Mycoses*. 2009;53:208–214.

Inhibition of metabolic activity of *Candida* biofilms *in vitro*

Isolate	MIC (µg/mL) of CAS for: Planktonic cells[a]	Biofilms at 48 h (SMIC 50)[b]
C albicans (CA4)	0.0625	0.5
C parapsilosis (CP1)	0.25	4
C tropicalis (CT8)	0.0625	0.25



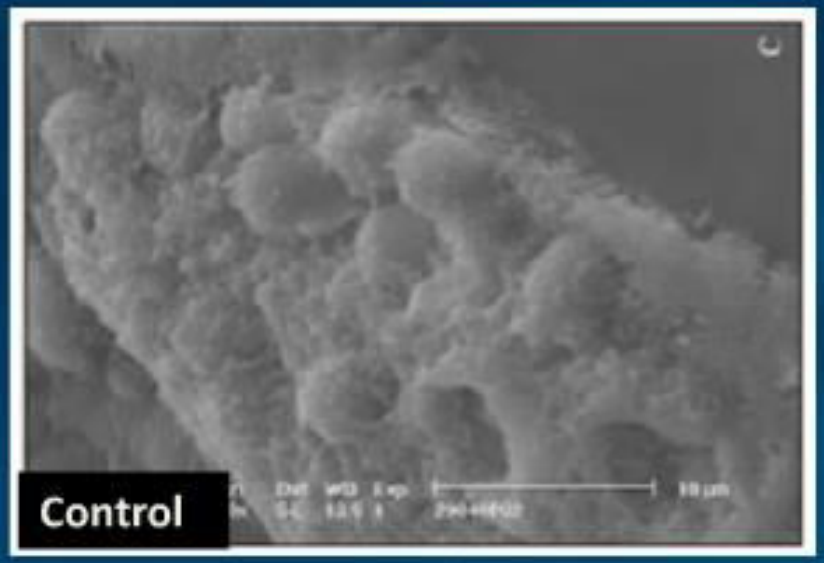


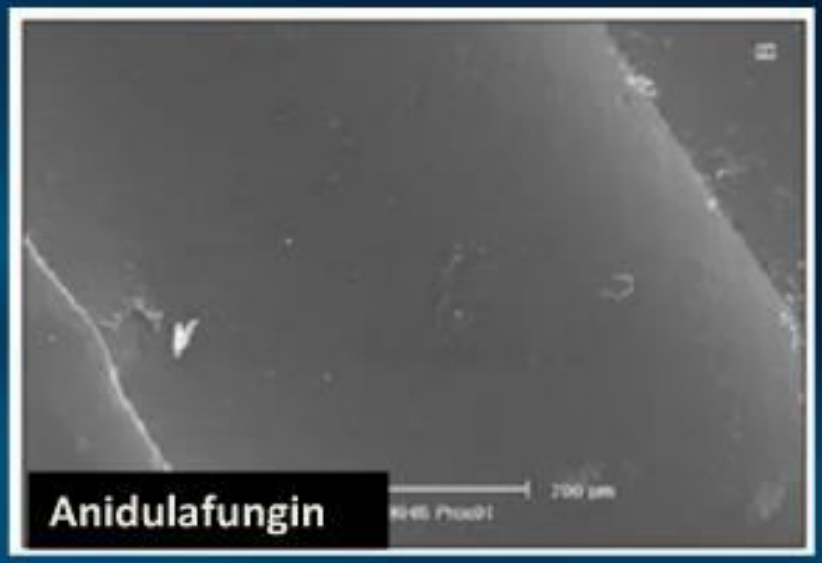


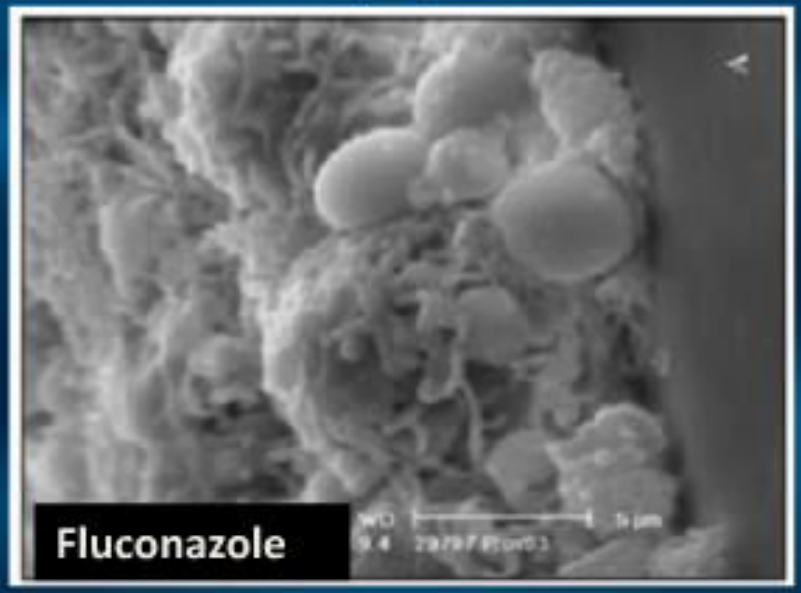
[a]The MIC end point for planktonic cells is based on visual determination of the lowest drug concentration that produced a prominent decrease in growth relative to the growth in the drug-free control well, [b]The MIC end point for biofilms is based on the lowest drug concentration producing a 50% RMA relative to the metabolic activity of the untreated growth control, as measured by the XTT reduction assay. CAS = caspofungin; MIC = minimum inhibitory concentration; RMA = reduction in the metabolic activity; SMIC = sessile minimum inhibitory concentration.

Ferreira J et al. *Antimicrob Agents and Chemother.* 2009;53:4377–4384.

Scanning electron micrograph of catheters removed from rabbits







MIC_{50} and MIC_{90} : planktonic form

	Anid	Mica	Caspo	Fluc	Itra	Fluco	Posa	Vori	AB
C. albicans									
MIC50	0,12	0,2	0,2	4	0,25	4	0,5	0,12	0,06
MIC90	0,5	0,5	1	16	1	32	4	0,5	0,1
C. tropicalis									
MIC50	0,06	0,06	0,06	0,25	0,25	2	0,5	0,12	0,1
MIC90	0,2	0,2	0,1	4	0,9	14	0,5	0,12	0,4
C. glabrata									
MIC50	0,03	0,04	1	0,18	0,5	48	0,5	0,25	0,2
MIC90	0,12	0,06	4	0,5	1,7	64	2,2	8	0,8
C. parapsilosis									
MIC50	0,12	0,09	0,375	0,12	0,375	3	0,25	0,09	0,045
MIC90	0,275	0,25	0,5	0,5	2	35,2	0,5	0,12	0,12
C. dubliniensis									
MIC50	0,265	0,265	1,125	0,56	0,53	0,185	1,005	0,265	0,28
MIC90	0,453	0,453	1,825	0,912	0,906	0,237	1,801	0,453	0,456
C. guiliermondii									
MIC50	1,25	1,125	8,5	0,185	2,5	2,125	0,31	0,28	0,375
MIC90	1,85	1,825	14,5	0,237	3,7	3,625	0,462	0,456	0,475
C. lusitaniae									
MIC50	0,265	0,14	0,185	32,06	0,185	0,31	0,13	0,28	0,375
MIC90	0,453	0,228	0,237	57,612	0,237	0,462	0,226	0,456	0,475

Velegraki A and Dimopoulos G , AAC (in press)

MIC_{50} and MIC_{90} : sessile form

	Anid	Mica	Caspo	Fluoc	Itra	Fluco	Posa	Vori	AB
C. albicans									
MIC50	1	2	2	16	8	16	4	8	4
MIC90	4	4	4	32	16	128	8	32	4
C. tropicalis									
MIC50	2	2	8	16	4	48	4	2	2
MIC90	4	4	32	64	16	64	28,8	7,2	4
C. glabrata									
MIC50	0,5	0,5	2	2	3	96	1,5	1	1
MIC90	1	1	4	4,4	8,8	256	4,4	2	2,2
C. parapsilosis									
MIC50	1,5	2	4	12	8	32	12	1,5	2
MIC90	2,2	4	35,2	83,2	32	83,2	64	4,4	4,4
C. dubliniensis									
MIC50	2,5	2,5	3	18	36	4,5	10	6	1,5
MIC90	3,7	3,7	3,8	29,2	58,4	7,3	14,8	7,6	1,9
C. guiliermondii									
MIC50	5	3	5	6	12	17	8	1	3
MIC90	7,4	3,8	7,4	7,6	15,2	29	8	1	3,8
C. lusitaniae									
MIC50	2	1	3	34	2	2,5	8	1,5	1
MIC90	2	1	3,8	58	2	3,7	8	1,9	1

Velegraki A and Dimopoulos G , AAC (in press)

Mechanism of Resistance in a biofilm

- Penetration of drugs is diminished
- Inhibited growth of the organism
- Upregulated Gene expression of efflux pumps

Mechanisms of Antifungal Resistance

- **Multifactorial**
  - Efflux pumps
  - Variation in membrane sterol patterns
- Phase-dependent

Mukherjee *et al.*, (2003). *Infect. Immunity* 71(8):4333-4340

Antimicrobial Agents with Reduced Efficacy Against Microorganisms in Biofilms

Biocides

Amphoteric surfactants	Hydrogen peroxide
2,2-Diromo-3-nitrilopropionamide	Iodine
2-Bromo-2-nitro-1,3-propanediol	Isothiazolone
Benzalkonium chloride	Monochloramine
Cetylpyridinium chloride	Ozone
Chlorhexidine	Peracetic acid
Chlorine	Polyhexamethylene biguanide
Chlorine dioxide	Potassium monopersulfate
Chlorosulfamate	Povidone iodine
Formaldehyde	Triclosan
Glutaraldehyde	

Antibiotics

Amikacin	Fosfomycin
Amphotericin B	Gentamicin
Ampicillin	Metronidazole
Aztreonam	Novobiocin
Cefazolin	Ofloxacin
Ceftazidime	Piperacillin
Cefuroxime	Rifampin
Ciprofloxacin	Tetracycline
Clindamycin	Tobramycin
Erythromycin	Trimethoprim-sulfa-methoxazole
Fluconazole	Vancomycin

Prevention of Biofilm formation

Silver zeolite-impregnated CVC and CVC related colonisation in the ICU

Type of catheter	Outcome		
	No growth (%)	Colonisation (%)	Tota
Silver zeolite-impregnated	51 (41.8)	71 (58.2)*	122
Control	33 (26.6)	91 (73.4)*	124

*P-value (<0.025).

The overall incidence of catheter tip colonisation

Episodes of CR-BSI	Isolates from silver zeolite-impregnated CVC	Isolates fro control CV
Definitive episodes		
Coagulase negative staphylococci	3	0
Staphylococcus aureus	1	1
Enterococcus species	0	1
Candida albicans	0	2
Suspected episodes unrelated to catheter		
Streptococcus pneumoniae	1	0
Coagulase negative staphylococci	2	1
No growth	5	4
Total	12	9

Microbiological analysis of clinically suspected episodes of CR-BSI

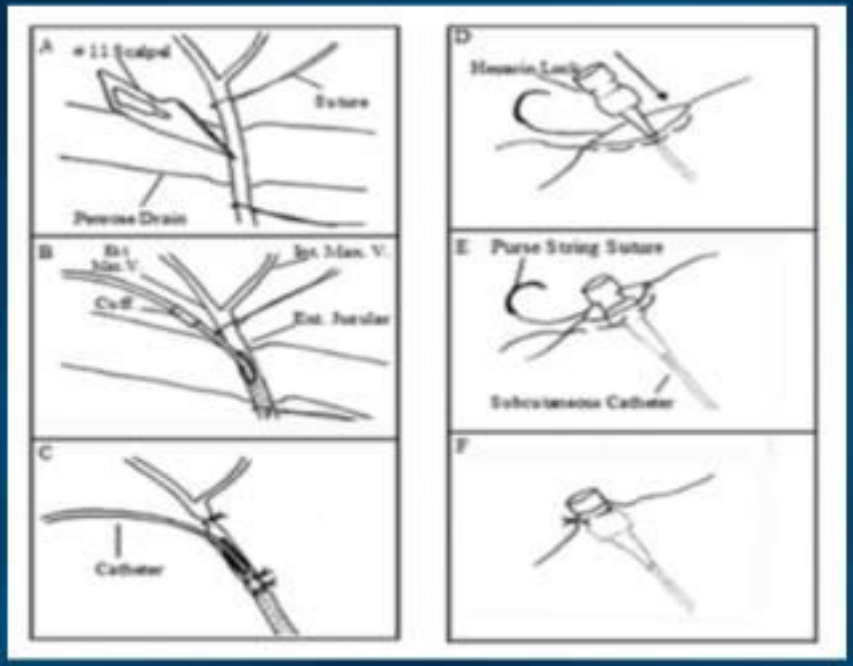
Khare et al, J Infection (2007) 54, 146e150

Strategy to Study Biofilms

- **Optimization of a Biofilm Model**
  - Determination of optimal conditions for the growth of **reproducible** *C. albicans* biofilms on bio-prosthetic surfaces
  - Quantitation of biofilm growth
- **Investigation of Biofilm Development and Architecture**
  - Visualization of biofilm: scanning electron, fluorescence and confocal microscopic analyses.

Courtesy to M. Ghanum

Development of an *in vivo* *C. albicans* Catheter-Associated Biofilm Model



Catheter insertion and attachment of the heparin lock device

Postoperative venogram of catheter placement

Schinabeck et al. (2004) Antimicrobial Agents and Chemotherapy 48, 1727-1732.

Targetting Micobes

- Blast them
- Fool them
- Do not irritate them