

# **The Path Forward with Biofilms: Should CLSI Try to Develop Biofilm-Related Standard Methods? Relevance of Susceptibility Testing for Bacterial Biofilms**

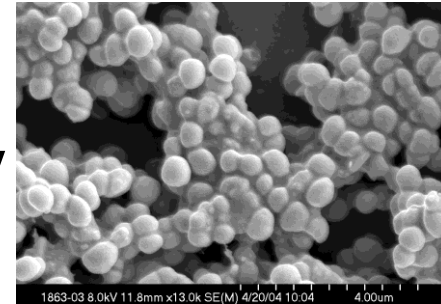
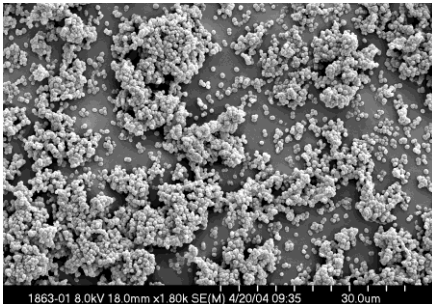
**January 21, 2012**

**Robin Patel, M.D.**

**Professor of Medicine and Microbiology**

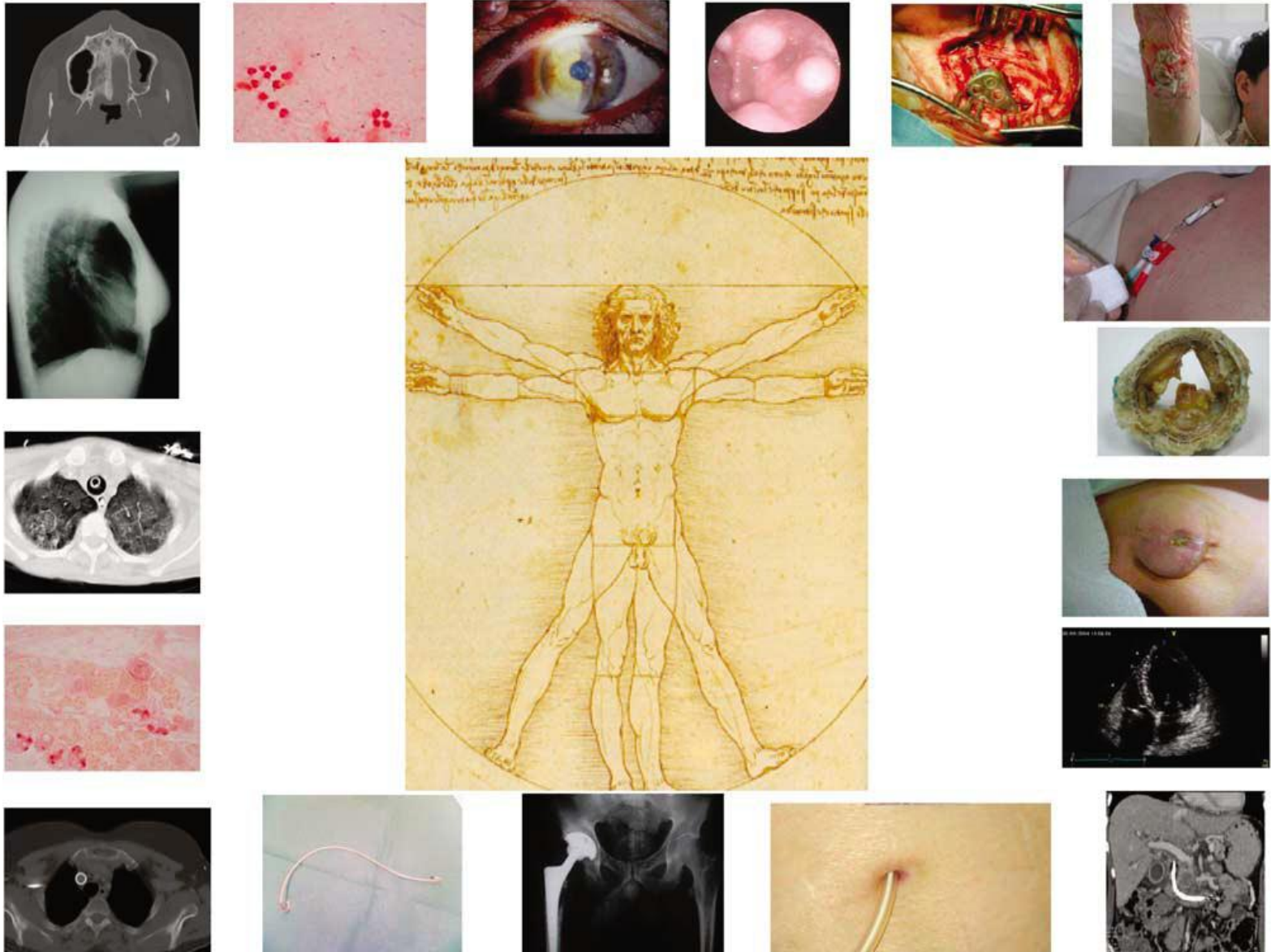
**Mayo Clinic, Rochester MN**

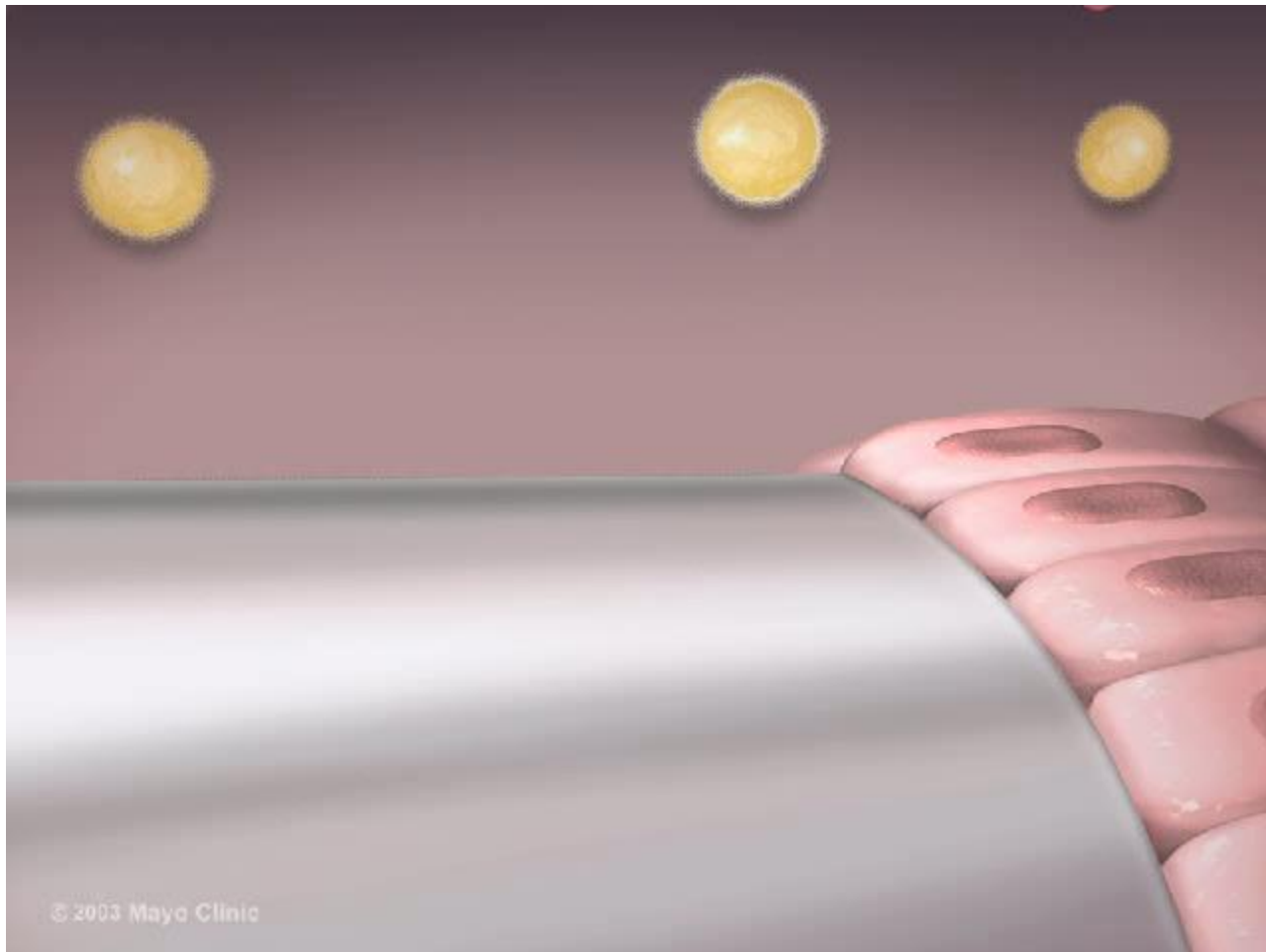
**[patel.rob@mayo.edu](mailto:patel.rob@mayo.edu)**



# Disclosures

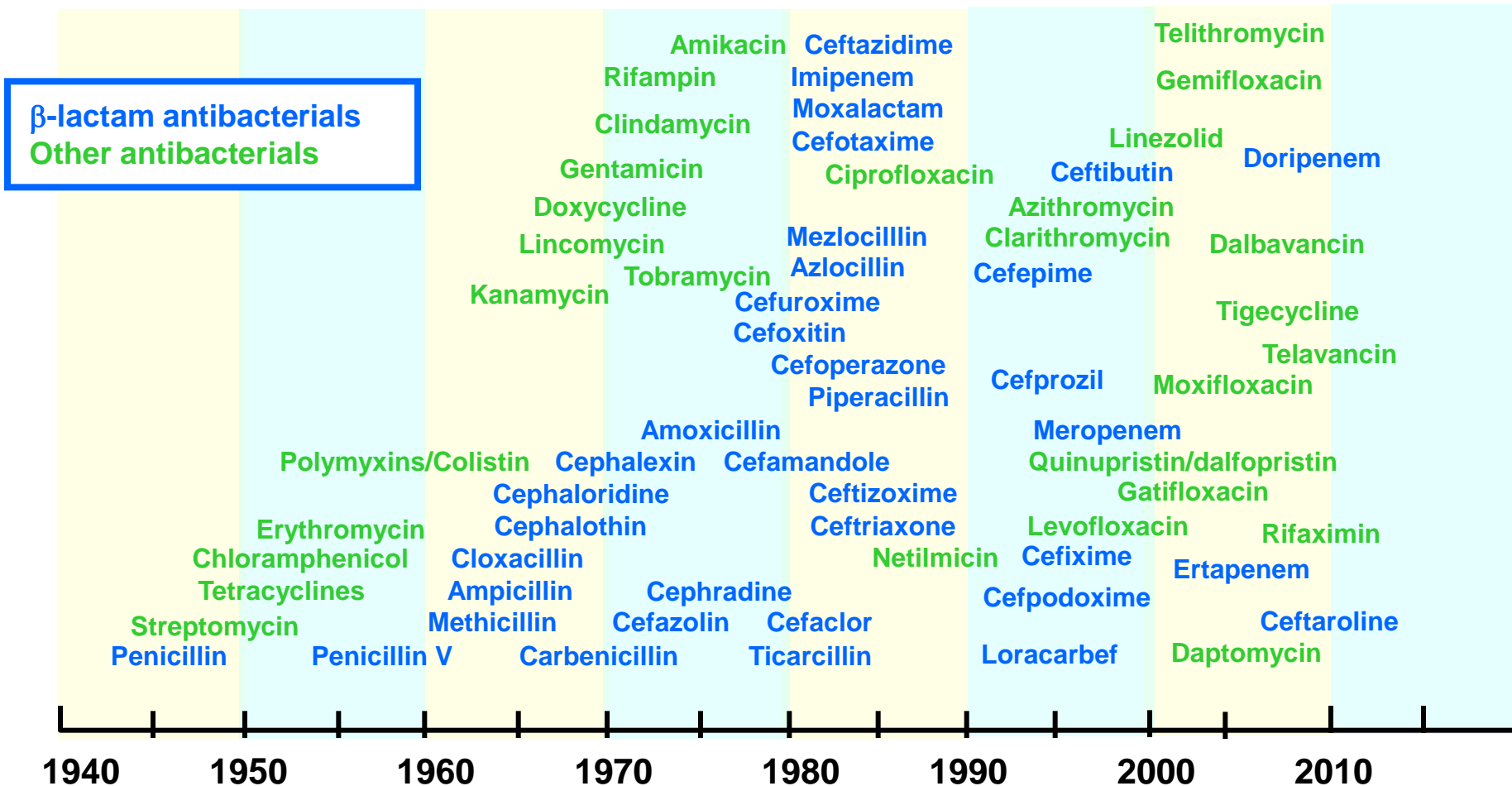
- **Grants**
  - **National Institutes of Health**
  - **Astellas**
  - **Tornier**
  - **Pfizer**
  - **Pradama**
  - **Pocared**
- **Patents**
  - ***B. pertussis/parapertussis* PCR**
  - **Method and apparatus for sonication (relinquished right to receive royalties)**
  - **Anti-biofilm substance (filed)**





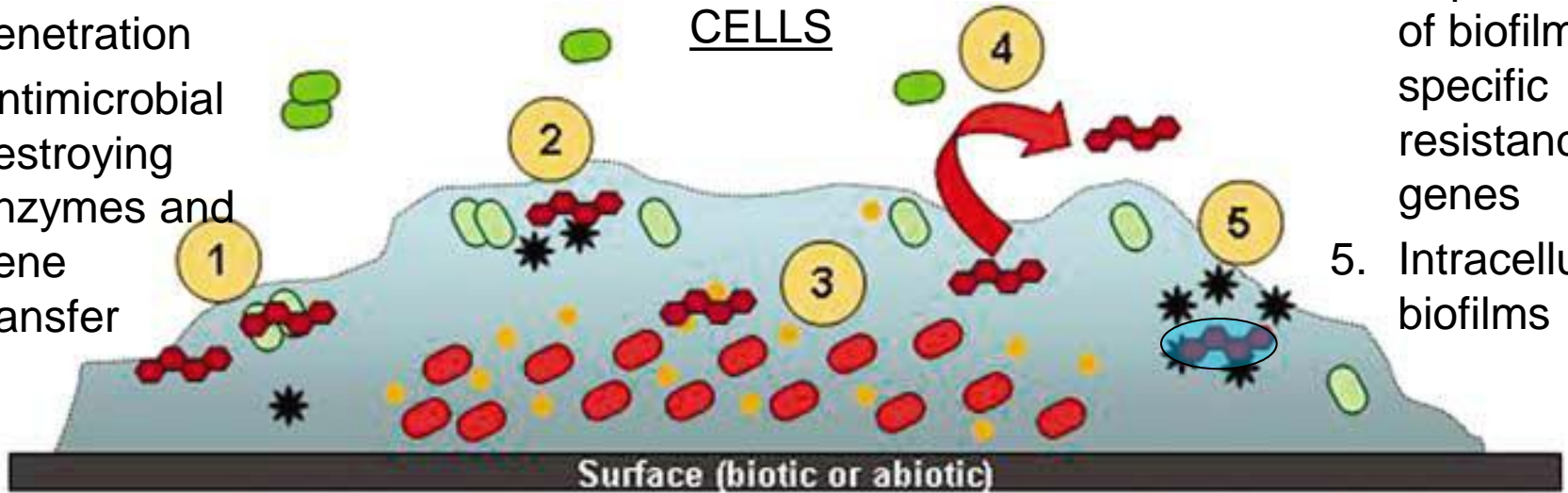


# Anti-Planktonic Bacterial Therapy



# Biofilm-Associated Antimicrobial Resistance

1. Restricted penetration
2. Antimicrobial destroying enzymes and gene transfer
3. PERSISTER CELLS
4. Expression of biofilm-specific resistance genes
5. Intracellular biofilms



Planktonic bacteria



Biofilm matrix (fully hydrated) with pH,  $pO_2$ , waste,... gradient



Biofilm active growing bacteria



Antibiotic chelator enzymes



Biofilm persister bacteria



Quorum sensors



Antimicrobial agent





# Biofilm-Associated Antimicrobial Resistance

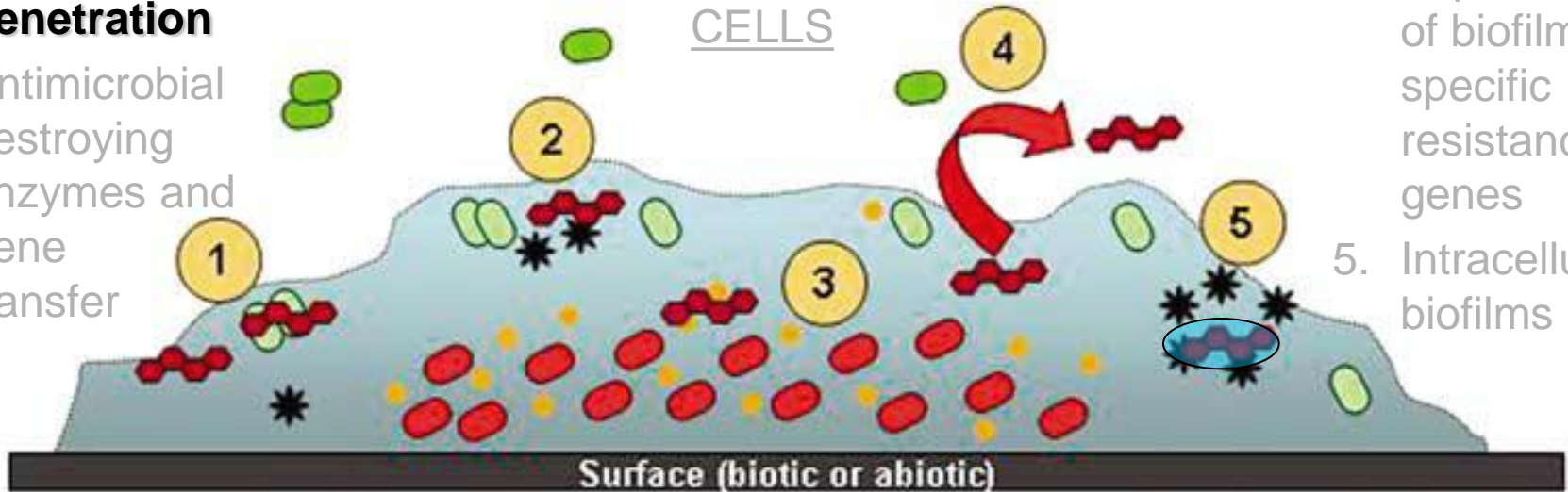
1. **Restricted penetration**

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3. PERSISTER CELLS

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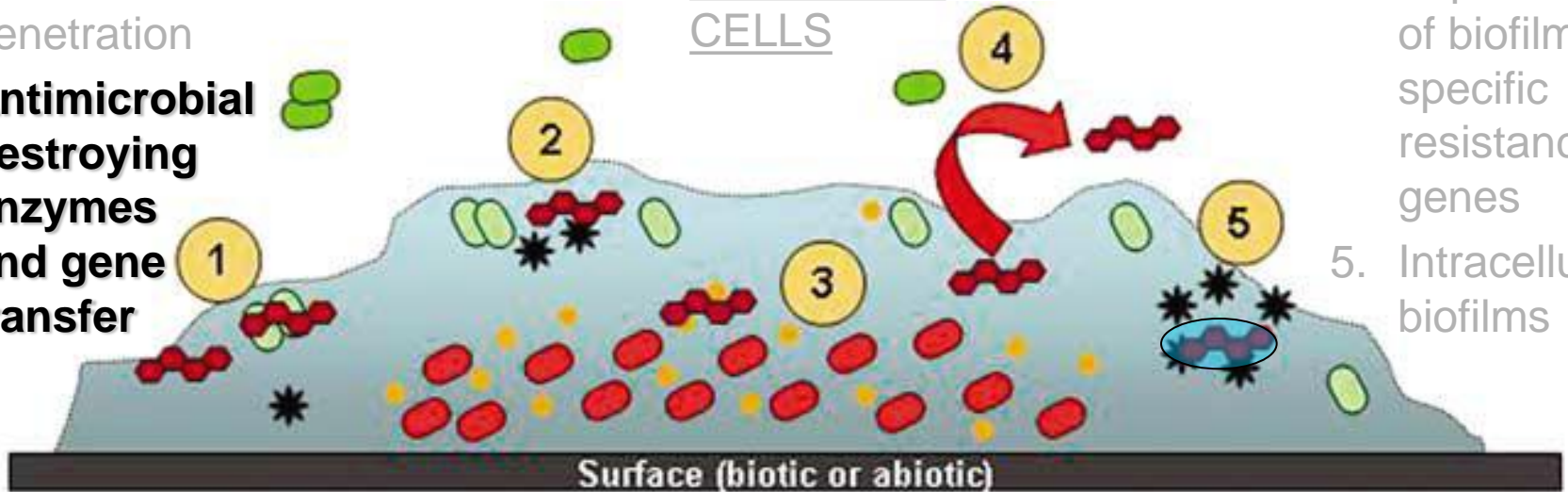
Quorum sensors



Antimicrobial agent

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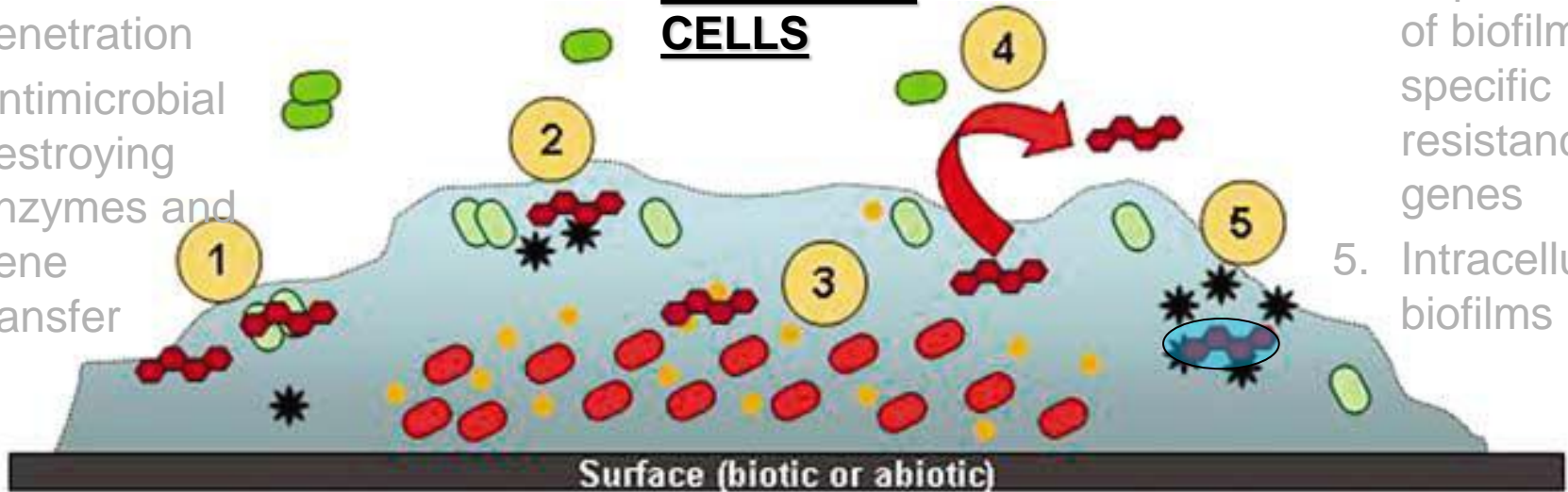
Antimicrobial agent





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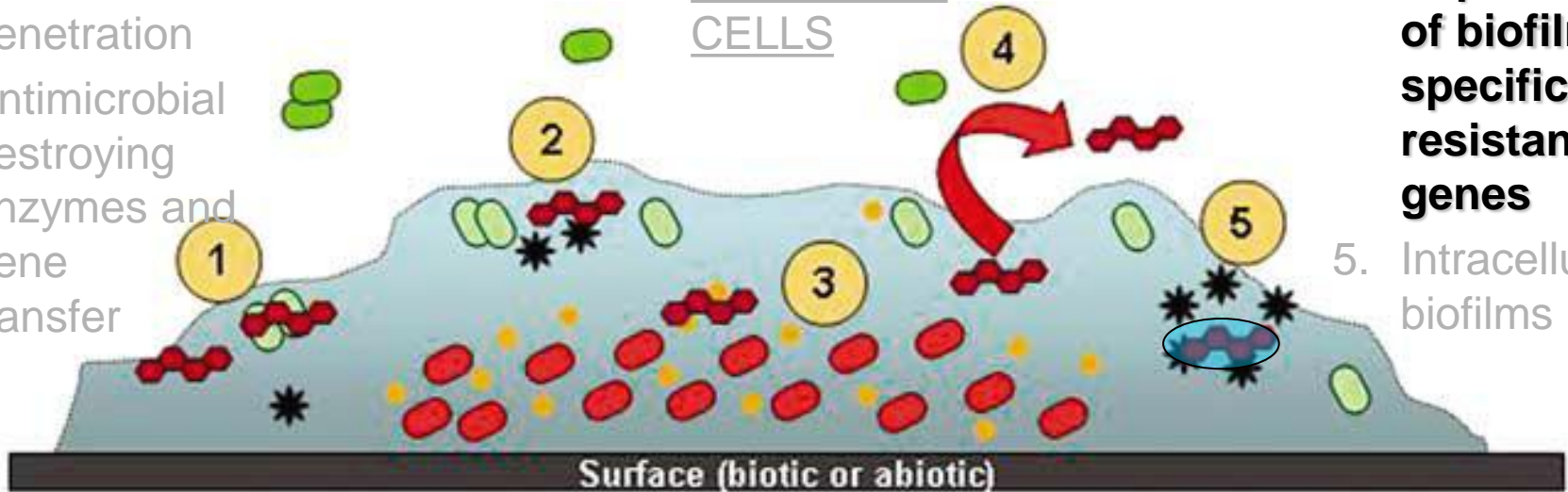


Antimicrobial agent



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Quorum sensors

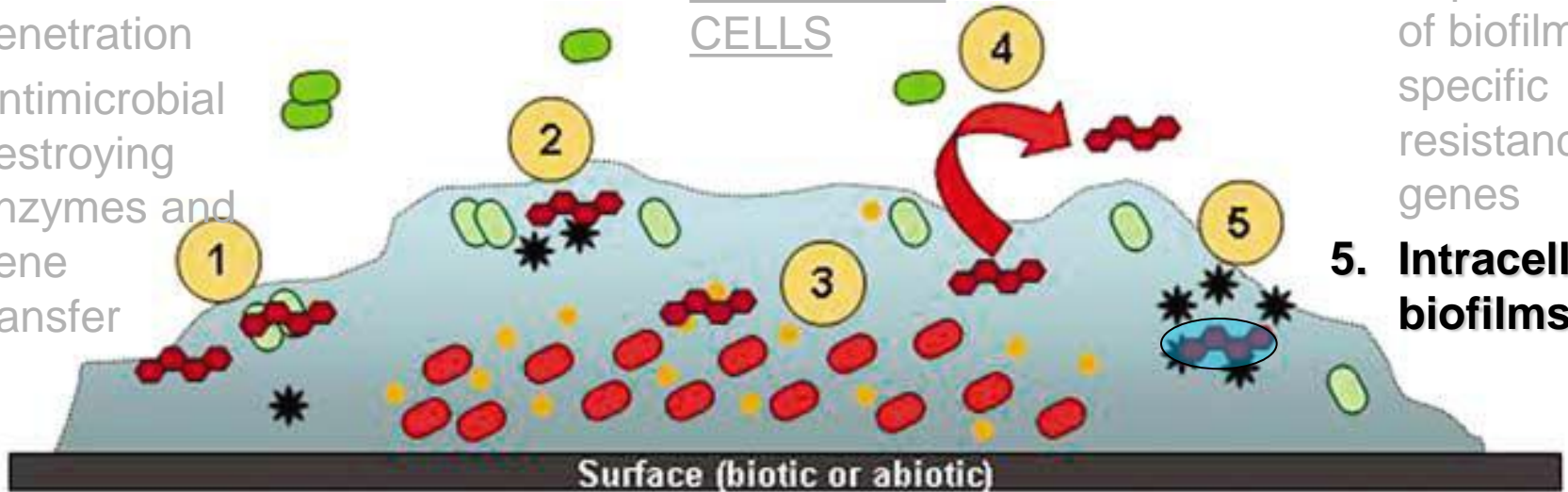


Antimicrobial agent



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Biofilm active growing bacteria



Antibiotic chelator enzymes



Biofilm persister bacteria



Quorum sensors



Antimicrobial agent

# Should CLSI Try to Develop Biofilm-Related Standard Methods?

- **Yes (my opinion)**
- **Rationale**
  - **Improve patient care**
  - **Prevent emergence of resistance**
  - **Guide development anti-biofilm agents**
    - **Pharma**
    - **Regulatory**

# **Criteria for Ideal Biofilm Antimicrobial Susceptibility Testing Methods**

- **Reproducible**
- **Cover range antibiotic concentrations**
- **Avoids contamination**
- **Easy to perform**
- **Applicable to numerous organism types**
- **Predictive of clinical outcome**
- **Inexpensive**
- **“Rapid” turnaround time**



# Systems for Experimental Investigation of Biofilms

- **Flow Systems:**

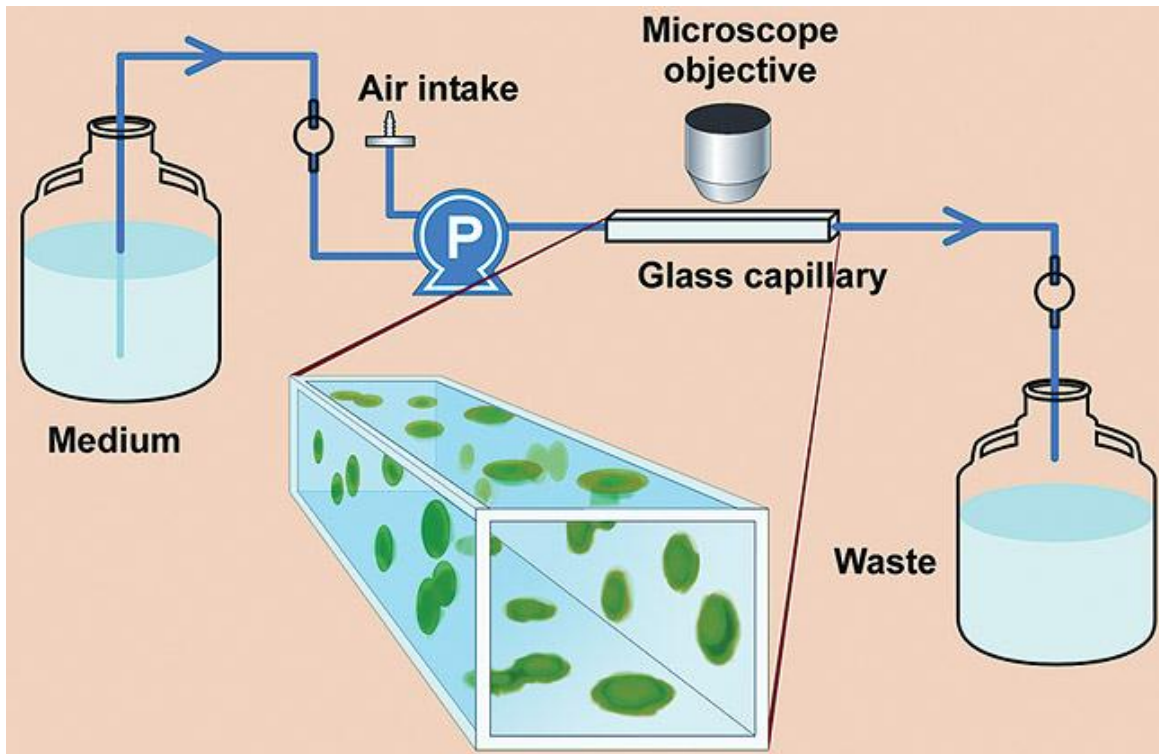
- Flow cell
- Drip flow reactor
- CDC biofilm reactor
- Rotating disk reactor
- Sorbarod filter

- **Static Systems:**

- Colony/membrane filter
- Agar-entrapment
- Microtiter plate
- Coupons/disks
- Peg lid microtiter plate



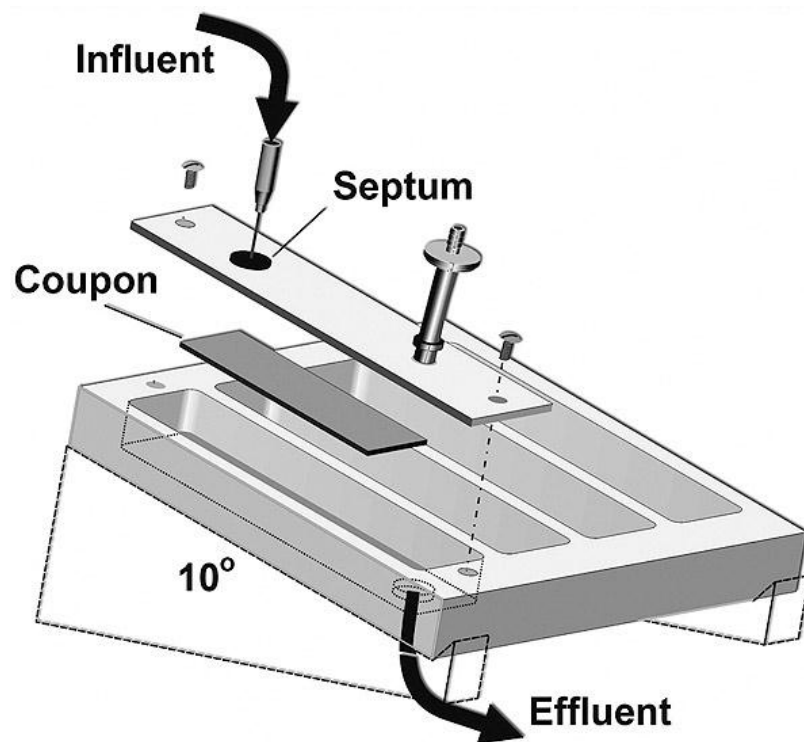
# Flow Cell Biofilm Assay



<http://cde.dentalaegis.com/courses/760-supragingival-and-subgingival-plaque-paradigm-biofilms>

Stovall Life Science, Inc.

# Drip Flow Reactor Biofilm Assay

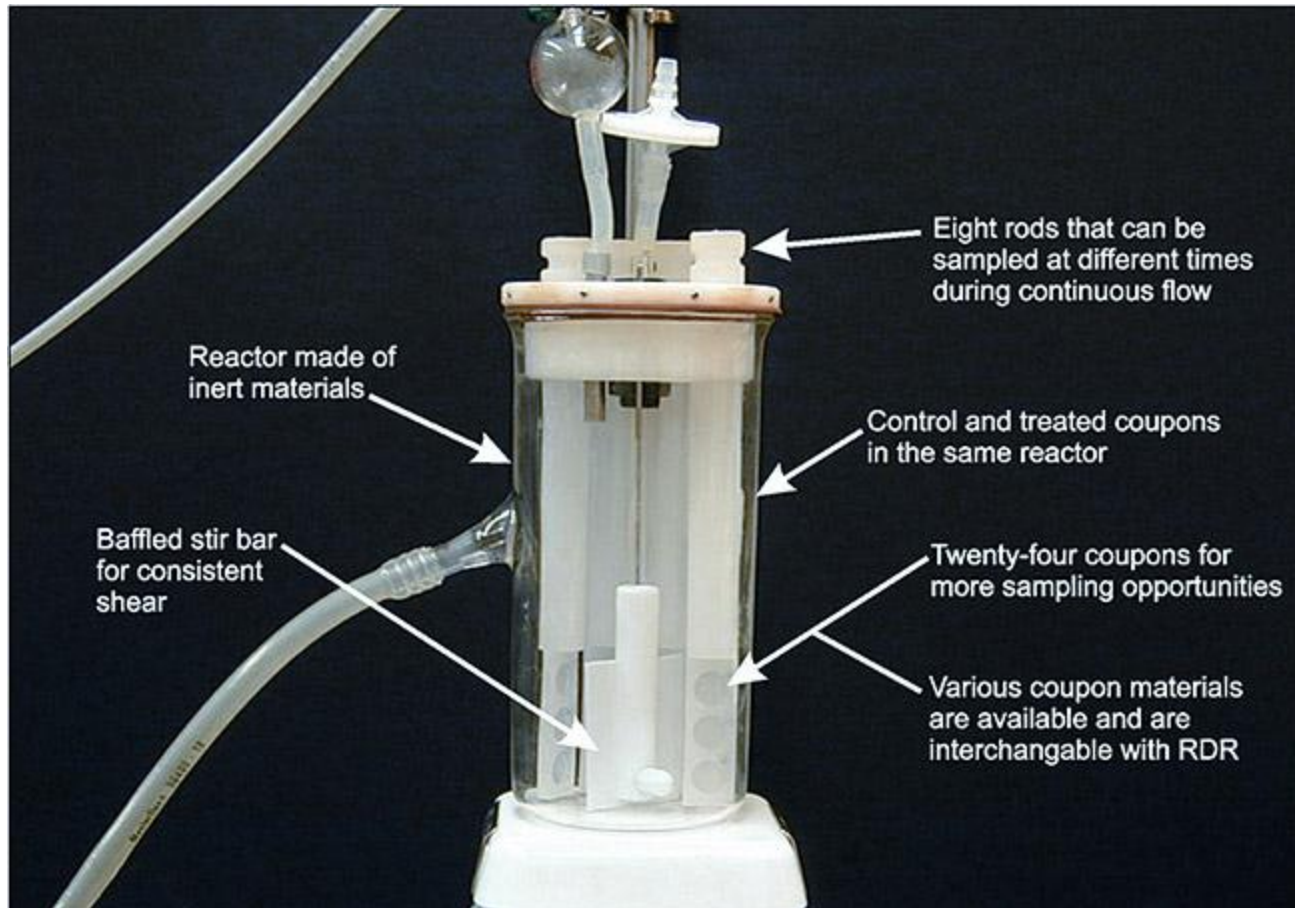


<http://cde.dentalaegis.com/courses/760-supragingival-and-subgingival-plaque-paradigm-biofilms>

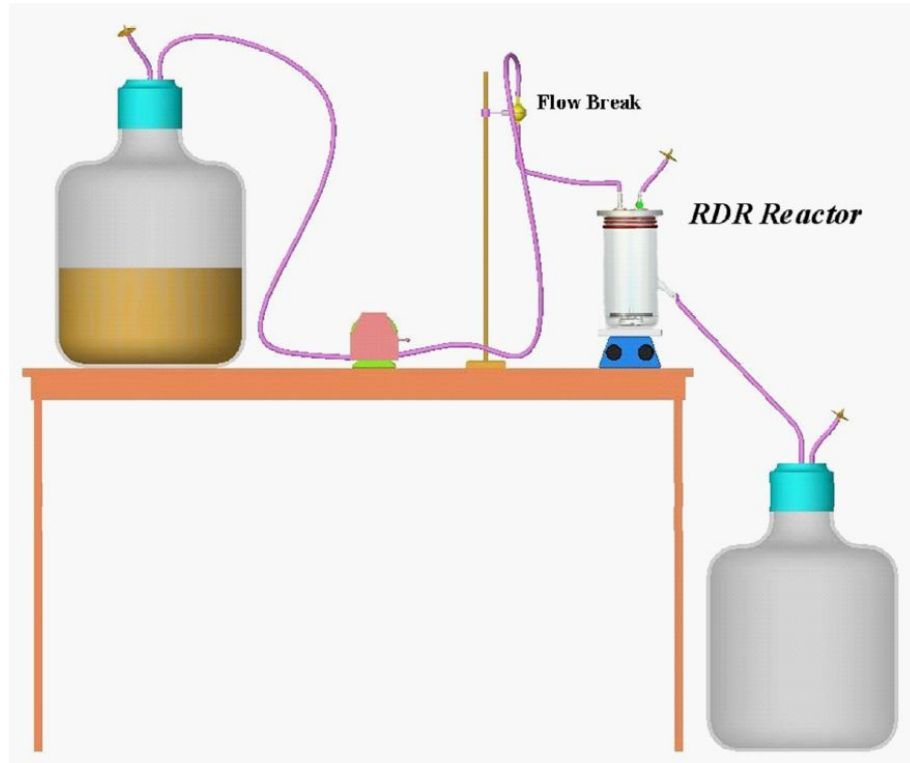


[http://www.astm.org/SNEWS/MJ\\_2008/biofilm\\_mj08.html](http://www.astm.org/SNEWS/MJ_2008/biofilm_mj08.html)

# CDC Biofilm Reactor



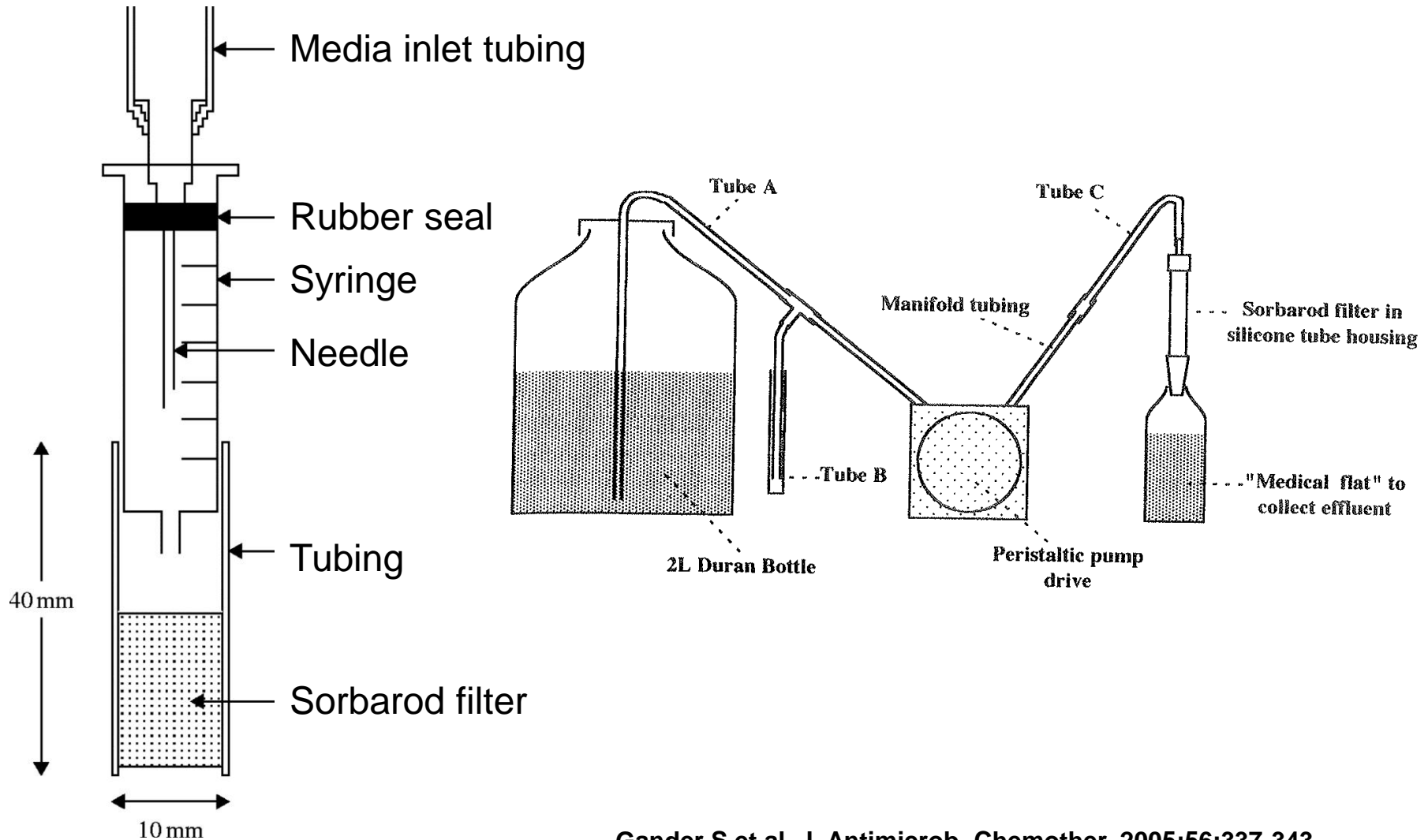
# Rotating Disk Reactor Biofilm Assay



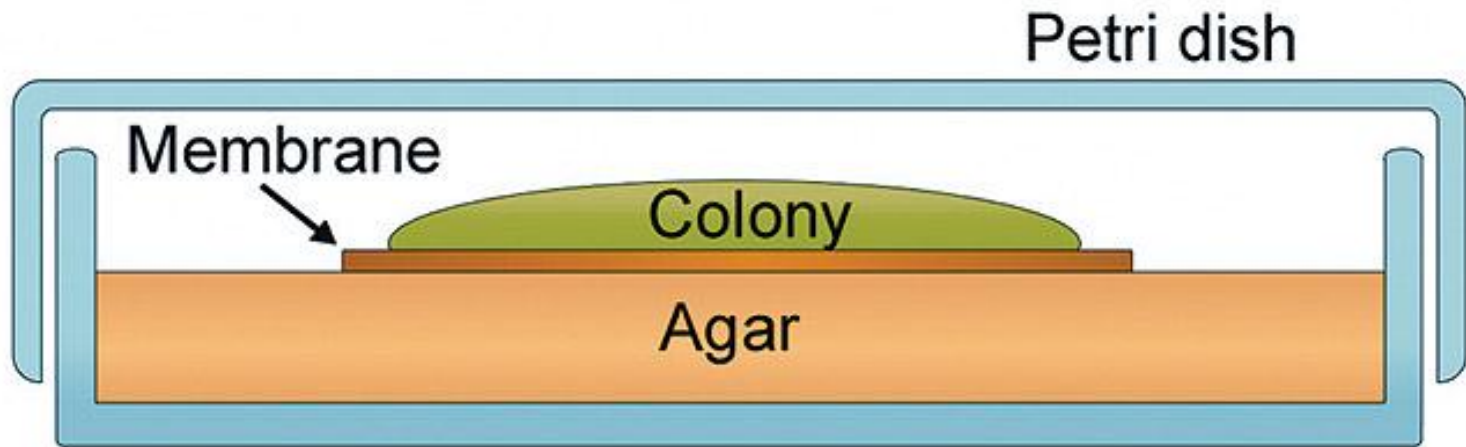
Garo et al. Antimicrob Ag Chemother 2007;51:1813



# Sorbarod Filter Plug Biofilm Assay

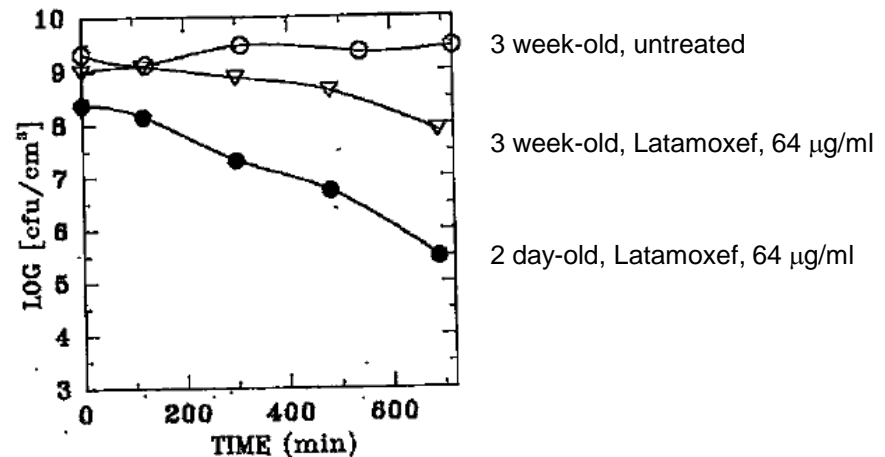
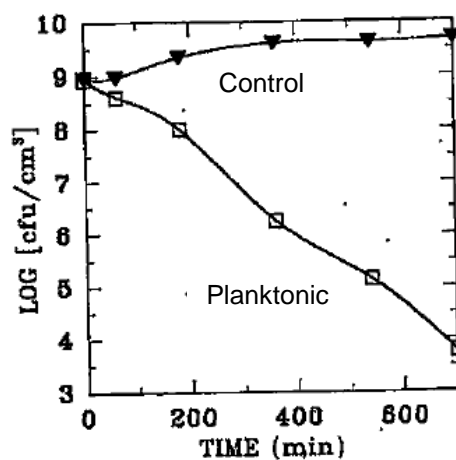
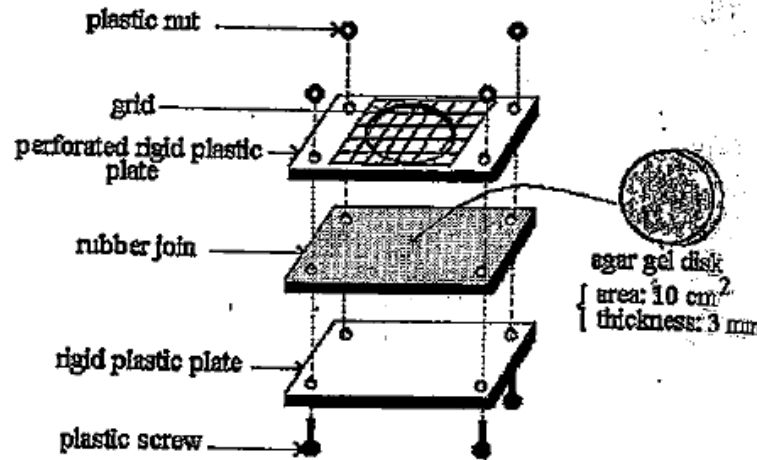


# Colony/Membrane Filter Biofilm Assay

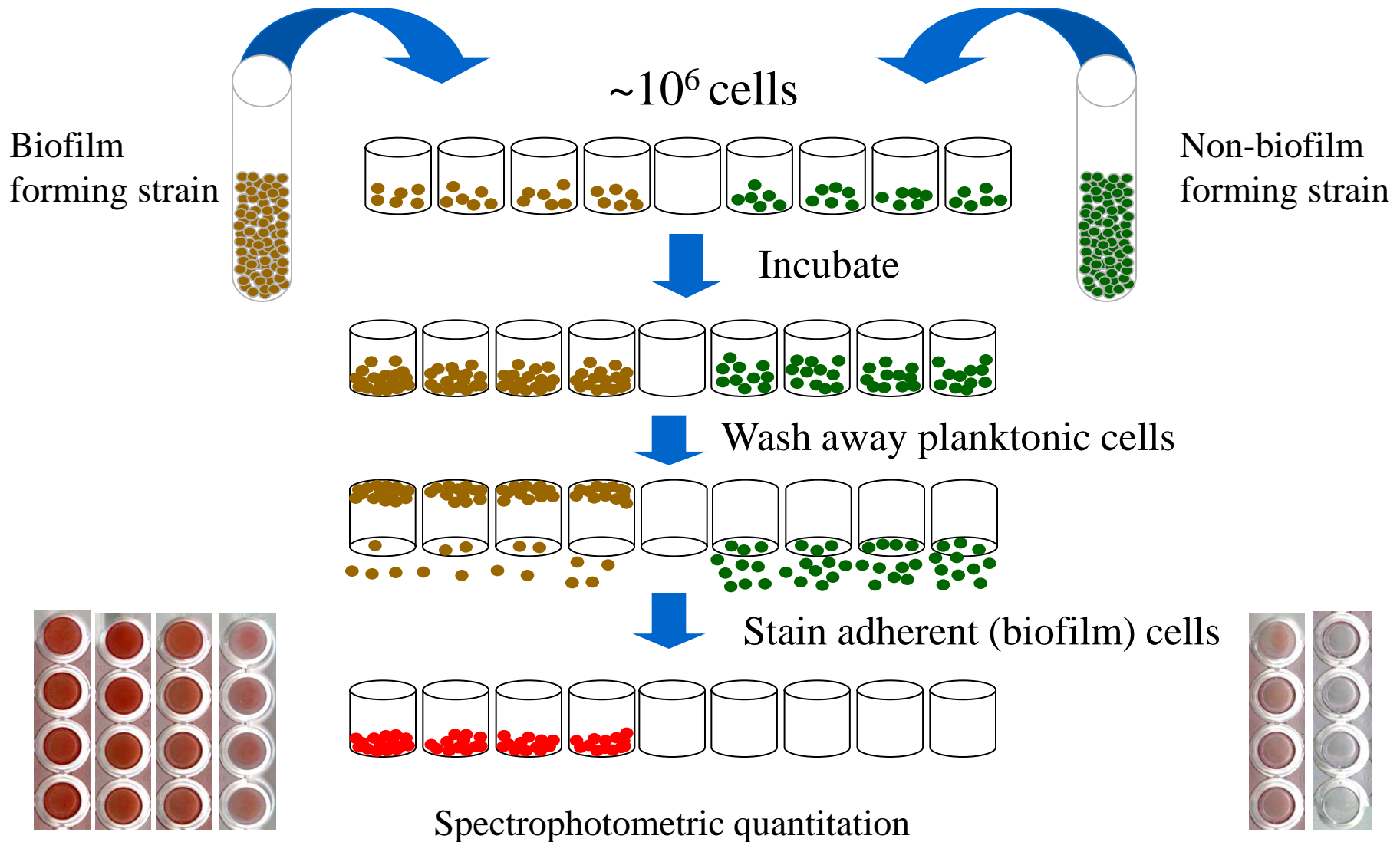


# Agar-Entrapped Biofilm Assay

## *Escherichia coli*



# Microtiter Plate Biofilm Assay



# Six-Well Plate Biofilm Assay

## MRSA-295



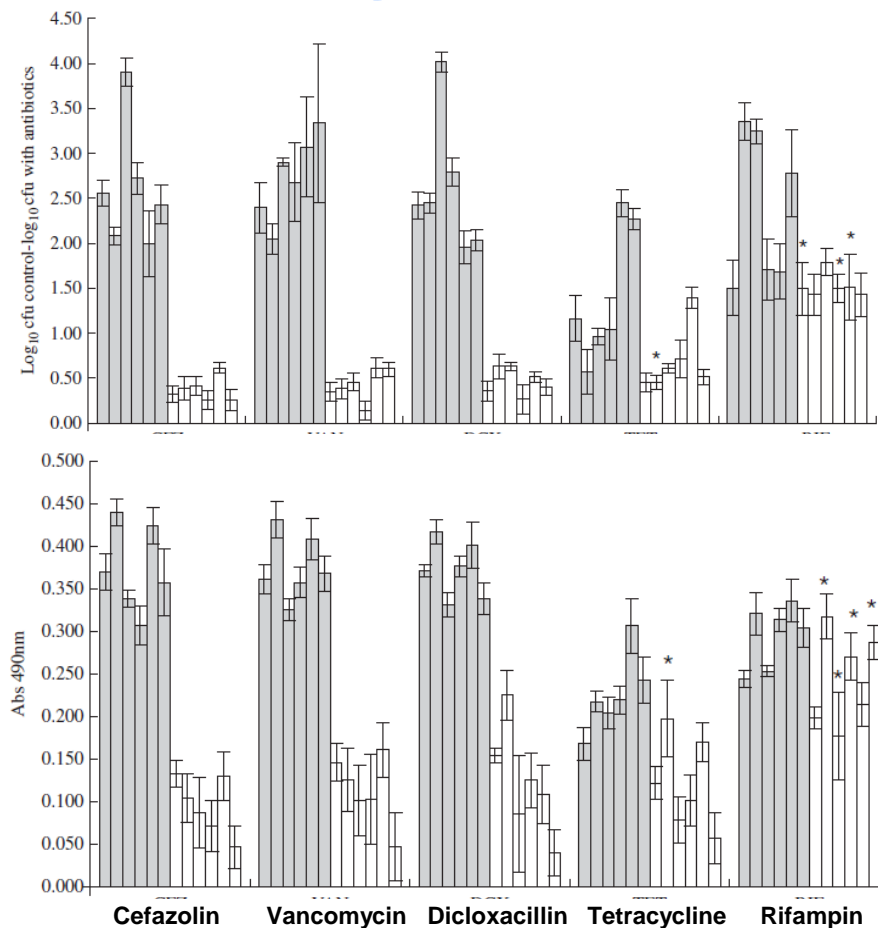
	MIC ( $\mu\text{g/ml}$ )	Concentration ( $\mu\text{g/ml}$ )	$\text{Log}_{10}$ CFU/biofilm (mean $\pm$ SD)
Inoculum			8.24 $\pm$ 0.16
Untreated			9.32 $\pm$ 0.07
Daptomycin	2	8	7.92 $\pm$ 0.16
		16	5.38 $\pm$ 0.28
Linezolid	2	>64	8.51 $\pm$ 0.07
Gentamicin	2	4	7.97 $\pm$ 0.14
		8	6.11 $\pm$ 0.21
TMP/SMX	1	>64	8.83 $\pm$ 0.01
Vancomycin	1	4	9.07 $\pm$ 0.09
		8	6.02 $\pm$ 0.30



# Microtiter Plate Biofilm Assay

## Quantitative Culture vs. XTT Colorimetry

### Coagulase Negative Staphylococci



# Microtiter Plate Biofilm Assay - Alamar Blue

	Planktonic MIC ( $\mu\text{g/ml}$ )	MBEC ( $\mu\text{g/ml}$ )
<b><i>Staphylococcus epidermidis</i></b>		
Gentamicin	16	>4,096
Bacitracin	32	>4,096
Vancomycin	2	>4,096
Ceftriaxone	32	>4,096
Oxacillin	8	>4,096
Rifampin	4	>1,024
<b><i>Staphylococcus aureus</i></b>		
Gentamicin	0.25	>2,048
Clindamycin	16	>2,048
Vancomycin	0.5	>2,048
Ciprofloxacin	0.25	>128
Ceftriaxone	2	>2,048
Penicillin	0.5	>2,048
Oxacillin	0.0625	>2,048
Rifampin	>0.0008	>2,048

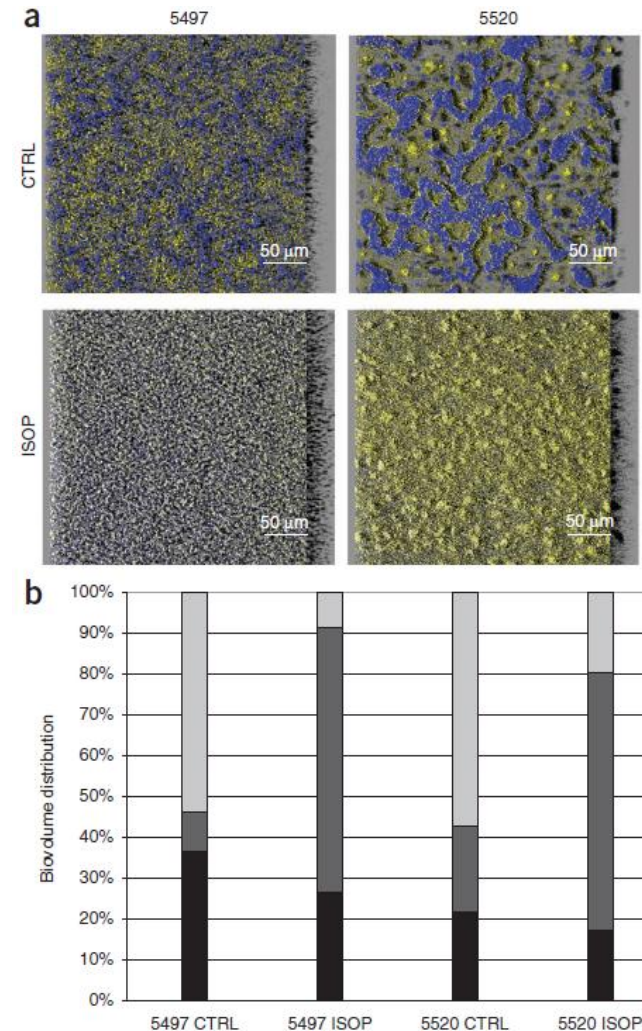
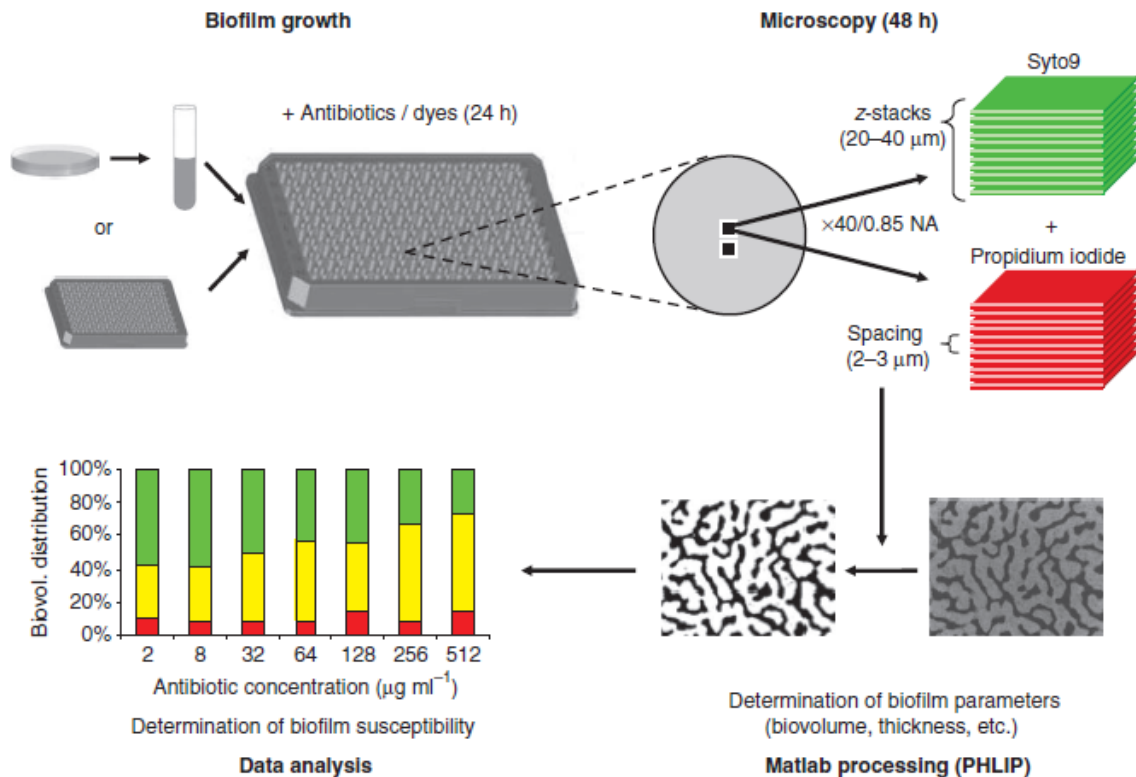
	Pearson's correlation coefficients with: Lysostaphin Alamar blue vs.	
<b><i>S. aureus</i></b>	<b>XTT</b>	<b>CFU/ml</b>
ATCC 2913	0.96	0.96
520009	0.99	0.98
520016	0.99	0.93
520020	0.95	0.97

Pettit et al. Ann Clin Microbiol Antimicrob 2009;8:28  
Pettit et al. Antimicrob Ag Chemother, 2005;49:2612  
Naves et al. J Chemother 2010;22:312



# Microtiter Plate Biofilm Assay

## Laser Confocal Microscopy Viability Staining

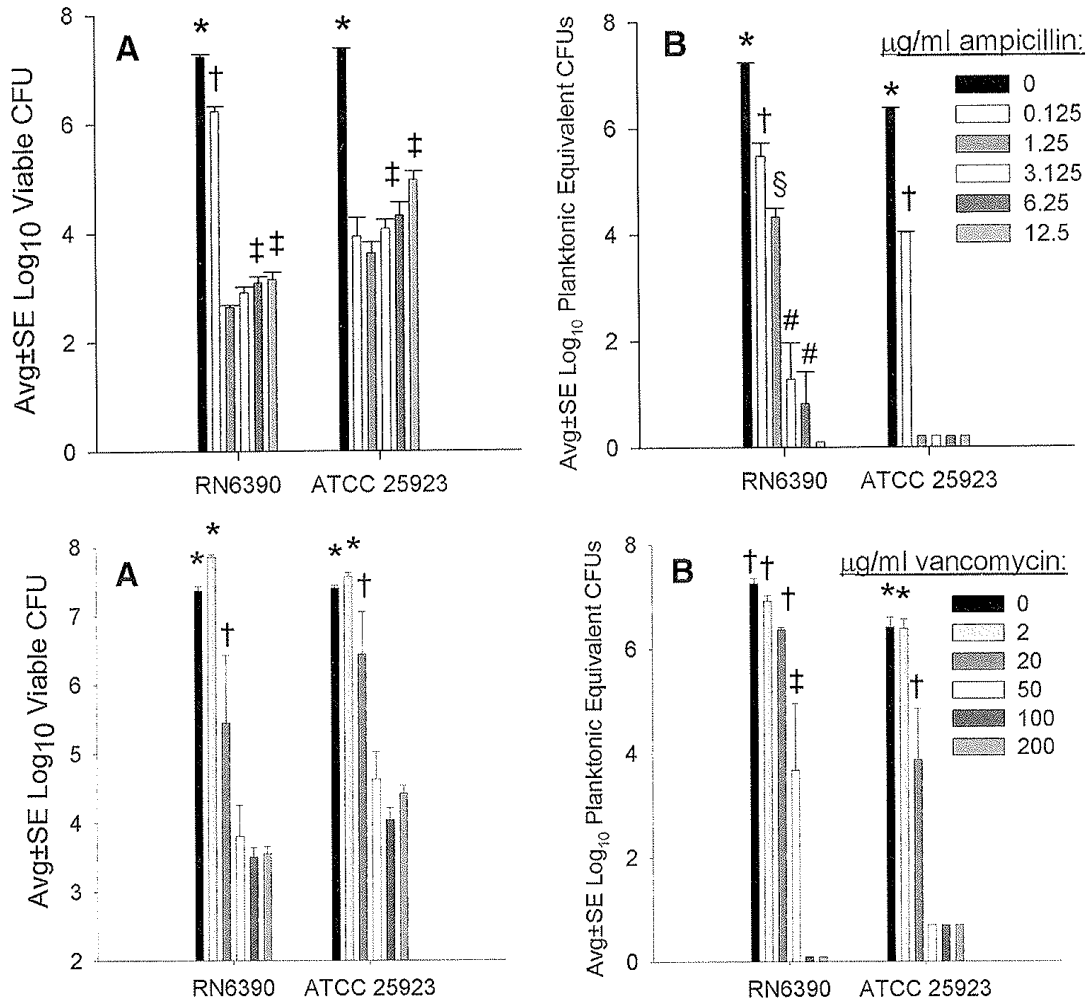


# Coupon/Disk Biofilm Assay

- **Silicone elastomer**
- **Cellulose nitrate membrane filter**
- **Polyvinyl chloride**
- **Stainless steel washers**
- **Contact lenses**
- **Hydroxyapatite**
- **Suture**
- **Polymethylmethacrylate**
- **Polyethylene**
- **etc.**

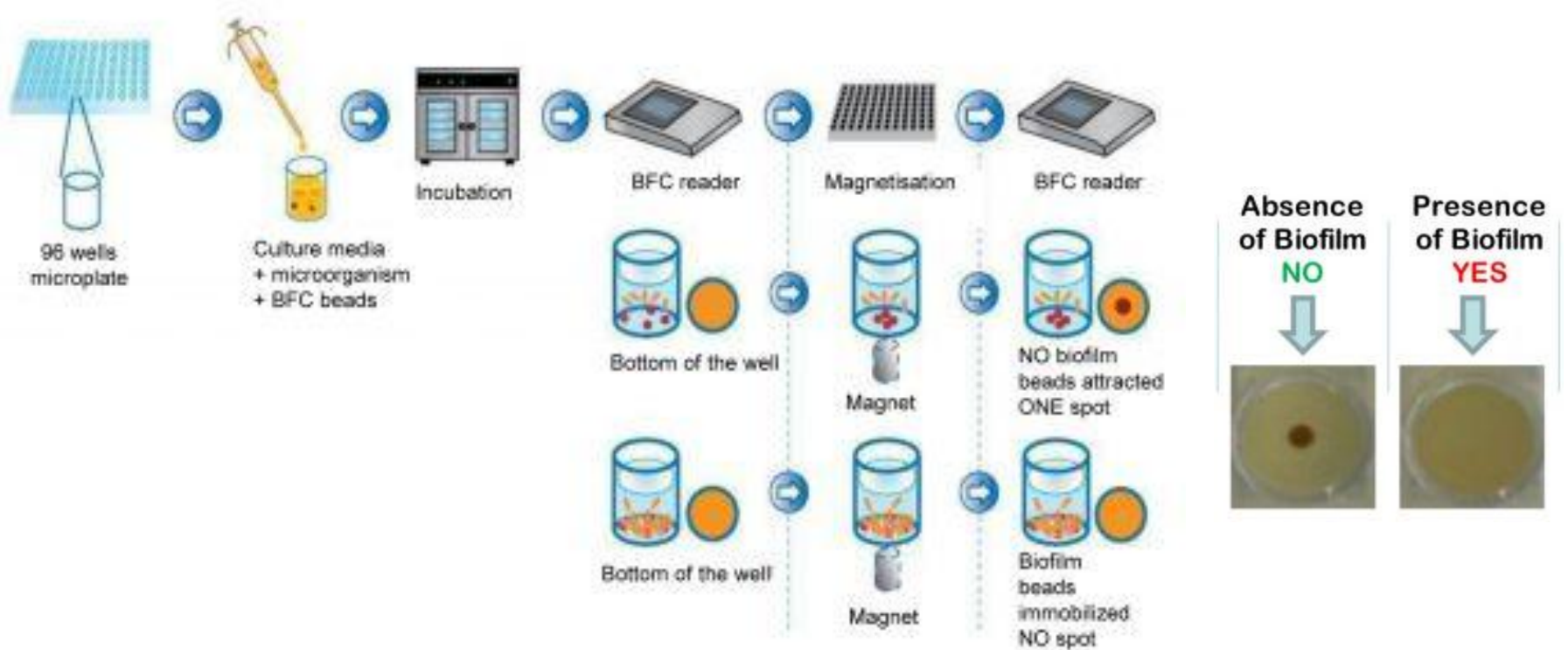


# Suture-Associated Biofilms, *S. aureus*

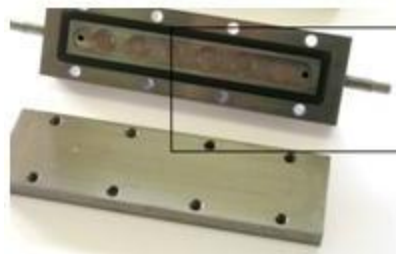
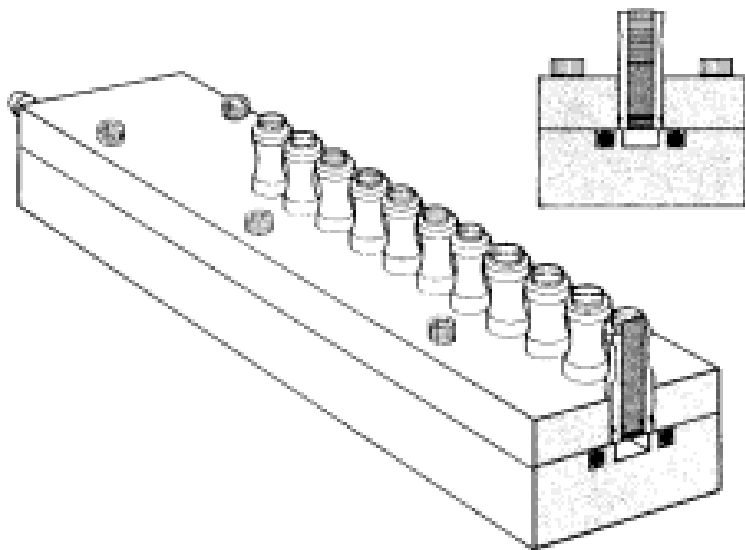




# Microtiter Plate Biofilm Assay BioFilm *Ring Test*®



# Modified Robbins Device



<http://www.advancesinpd.com/adv87/pt3in vitro87.html>

<http://www.ugent.be/fw/en/research/pharmaceutical-analysis/micro/equipment/mrd>

# Peg Lid Microtiter Plate Biofilm Assay

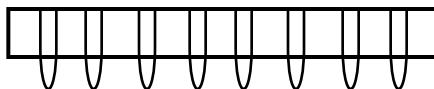


<http://cde.dentalaegis.com/courses/760-supragingival-and-subgingival-plaque-paradigm-biofilms>



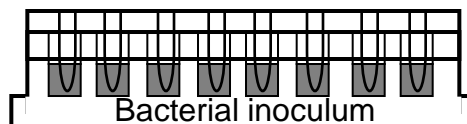
MAYO CLINIC

# Peg Lid Microtiter Plate Biofilm Assay



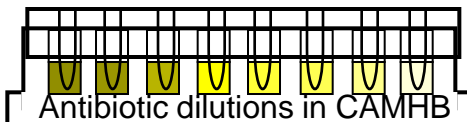
Pegged lid

22-24 h incubation, 37°C



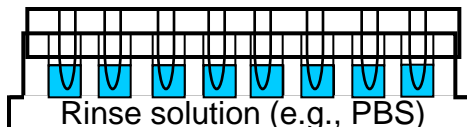
Bacterial inoculum

22-24 h incubation, 37°C



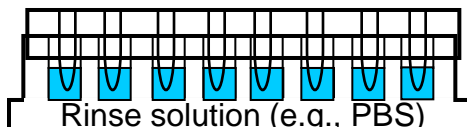
Antibiotic dilutions in CAMHB

Rinse lid



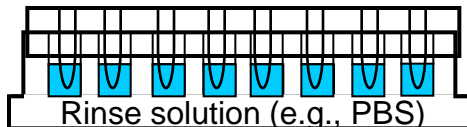
Rinse solution (e.g., PBS)

Rinse lid



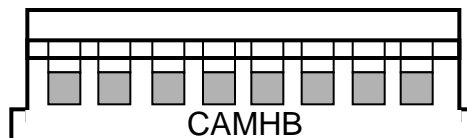
Rinse solution (e.g., PBS)

Rinse lid



Rinse solution (e.g., PBS)

Remove pegged lid  
Replace pegged lid with standard lid;  
Sonicate  
Measure OD<sub>600</sub>



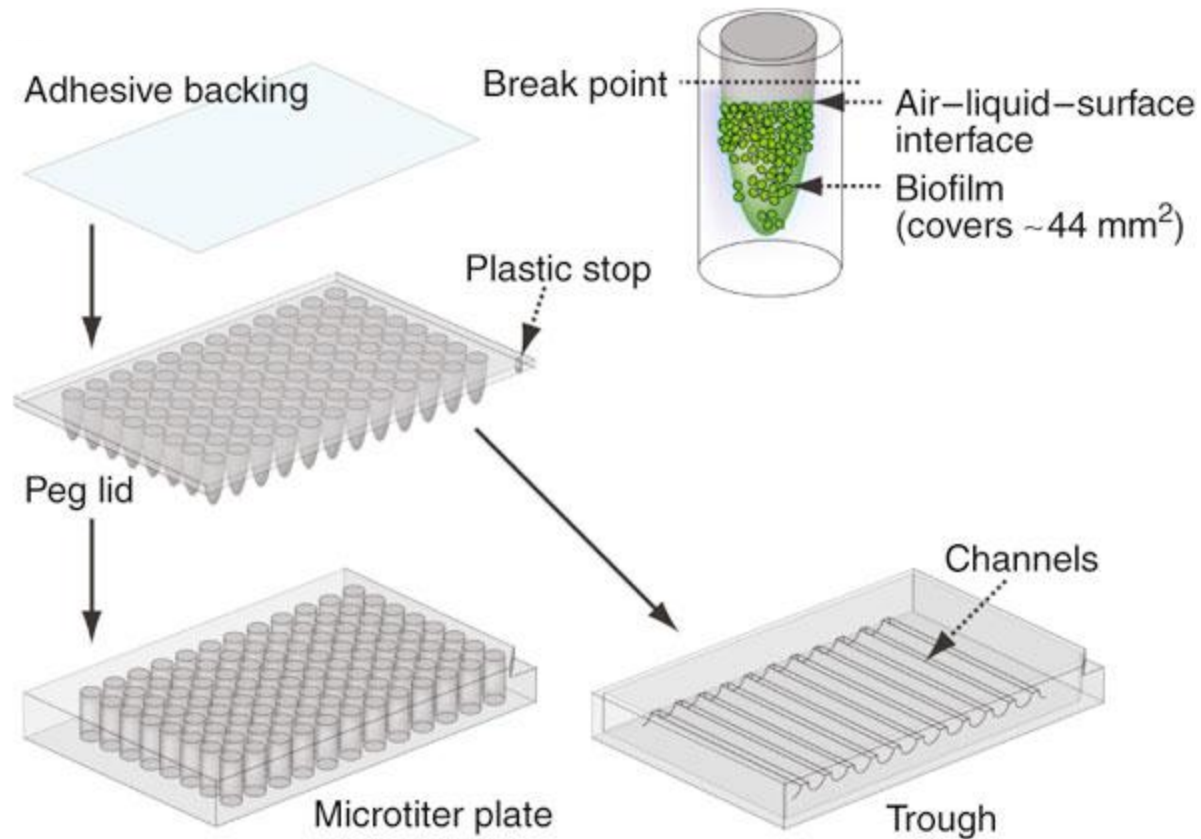
CAMHB

22-24 h incubation, 37°C;

Measure OD<sub>600</sub>



# Peg Lid Microtiter Plate Biofilm Assay



# Planktonic and Biofilm Susceptibility Peg Lid Microtiter Plate Biofilm Assay

	MIC (µg/ml)		MBEC (µg/ml)
	CLSI	Peg lid	
Staphylococcus aureus			
Cefazolin	0.5	0.5	>1,024
Ciprofloxacin	0.25	0.5	512
Clindamycin	0.12	0.25	128
Gentamicin	0.5	0.5	2
Oxacillin	0.12	0.25	>1,024
Vancomycin	1	1	>1,024
Pseudomonas aeruginosa			
Amikacin	2	4	16
Ceftazidime	1	2	>1,024
Ciprofloxacin	0.25	0.25	4
Gentamicin	2	4	128
Imipenem	1	4	>1,024
Piperacillin	2	16	>1,024
Tobramycin	0.5	1	2



# Planktonic and Biofilm Susceptibility Peg Lid Microtiter Plate Biofilm Assay *Staphylococcus lugdunensis* (n=15)

Antimicrobial Agent	MIC range ( $\mu\text{g/ml}$ )	MBEC range ( $\mu\text{g/ml}$ )
Cefazolin	$\leq 0.125 - 0.5$	1 - $>128$
Daptomycin	0.06 - 1	1 - $>128$
Linezolid	0.5 - 1	4 - $>128$
Moxifloxacin	0.125 - 0.25	$\leq 0.125 - 2$
Nafcillin	0.25 - 0.5	0.5 - $>128$
Quinupristin-dalfopristin	0.06 - 2	$\leq 0.125 - >128$
Rifampin	$\leq 0.03$	$\leq 0.125 - 64$
Tetracycline	0.06 - 1	1 - 128
Trimethoprim-sulfamethoxazole	0.125/2.4 - 16/304	0.125/2.4 - $>8/152$
Vancomycin	0.5 - 2	4 - $>128$

# Planktonic and Biofilm Susceptibility

## Peg Lid Microtiter Plate Biofilm Assay

### Cystic Fibrosis *Pseudomonas aeruginosa* (n=40)

	MIC (µg/ml)			MBEC (µg/ml)		
	MIC <sub>50</sub>	MIC <sub>90</sub>	Range	MBEC <sub>50</sub>	MBEC <sub>90</sub>	Range
Amikacin	0.5	4	0.25-8	32	512	2-1024
Cefazidime	2	8	0.25-64	64	1024	2-1024
Ciprofloxacin	0.125	1	0.06-2	16	128	0.5-1024
Gentamicin	0.5	1	0.125-4	32	128	0.5-1024
Tobramycin	0.25	0.5	0.06-1	64	128	2-512



# Planktonic and Biofilm Susceptibility

## Peg Lid Microtiter Plate Biofilm Assay

Chronic Periodontitis *Enterococcus faecalis* (n=23)

	MIC (µg/ml)			MBIC (µg/ml)			MBEC (µg/ml)		
	ATCC 29212	Range	MIC <sub>90</sub>	ATCC 29212	Range	MBIC <sub>90</sub>	ATCC 29212	Range	MBEC <sub>90</sub>
<b>Ampicillin</b>	0.5	0.12 to 0.5	0.5	2	1 to 2	2	>8,192	2,048 to >8,192	>8,192
<b>Erythromycin</b>	2	0.25 to 1,024	64	4	1 to 512	64	1,024	512 to 2,048	2,048
<b>Gentamicin</b>	8	4 to 1,024	32	16	8 to 8,192	32	32	16 to >8,192	256
<b>Tetracycline</b>	16	0.5 to 64	64	16	4 to 256	64	64	32 to 1,024	1,024
<b>Vancomycin</b>	2	1 to 4	2	4	2 to 8	4	>8,192	8,192 to >8,192	>8,192

# Peg Lid Microtiter Plate Biofilm Assay Batch Parameters for Growth on Peg Lids

Genus and species	Lid surface	Motion (rpm)	T (°C)	Time (h)	Inoculum (CFU/ml)	Growth Medium	Atmospheric gases	Mean cell count (log <sub>10</sub> CFU/peg)
<i>Acinetobacter baumannii</i>	M	O (110)	37	20	$1.0 \times 10^7$	TSB	ambient	$6.8 \pm 0.1$
<i>Alcaligenes xylosoxidans</i>	M	O (110)	37	20	$1.0 \times 10^7$	TSB	ambient	$6.9 \pm 0.4$
<i>Arcanobacterium pyogenes</i>	M	R (10)	37	24	$1.0 \times 10^7$	TSB + 2% FCS	air/CO <sub>2</sub> , 90:10	5.0
<i>Burkholderia cenocepacia</i>	M	O (125)	37	24	$1.0 \times 10^7$	CA-MHB	ambient	$\sim 4.3 \pm 0.4$
<i>Enterococcus faecalis</i>	M	R (NR)	35	72	$1.0 \times 10^7$	artificial test soil	ambient	$\sim 6.0 \pm 0.4$
<i>Escherichia coli</i>	M	O (110)	37	24	$1.0 \times 10^7$	TSB	ambient	$5.7 \pm 0.2$
<i>Haemophilus influenzae</i>	N	R (3.5)	35	16	$1.5 \times 10^8$	HTM	ambient	$\sim 6.0 \pm 0.6$
<i>Listeria monocytogenes</i>	M	R (3.5)	37	24	$1.0 \times 10^7$	BHI	ambient	$7.2 \pm 0.1$
<i>Moraxella catarrhalis</i>	M	O (110)	37	20	$1.0 \times 10^7$	TSB	ambient	$6.7 \pm 0.3$
<i>Pasteurella multocida</i>	M	R (10)	37	7	$1.0 \times 10^7$	TSB + 2% FCS	air/CO <sub>2</sub> , 90:10	5.0
<i>Proteus mirabilis</i>	M	O (125)	37	24	$1.0 \times 10^7$	artificial urine	ambient	$5.2 \pm 0.4 - 5.4 \pm 0.4$
<i>Pseudomonas aeruginosa</i>	M	O (125)	35	24	$1.0 \times 10^7$	LB	ambient	$6.8 \pm 0.6$
<i>Pseudomonas fluorescens</i>	M	O (100)	27	48	$1.0 \times 10^7$	LB	ambient	$6.0 \pm 0.8$
<i>Salmonella enterica</i>	N	static	16	48	$1.0 \times 10^7$	5% TSB	ambient	NR (CV)
<i>Staphylococcus aureus</i>	N	R (3.5)	35	24	$1.0 \times 10^7$	LB	ambient	$6.2 \pm 0.9$
<i>Staphylococcus epidermidis</i>	M	O (125)	37	24	$1.0 \times 10^7$	CA-MHB	ambient	$\sim 5.6 \pm 0.1$
<i>Staphylococcus lugdunensis</i>	N	static	37	24	$4.0 \times 10^6$	TSB + 1% glucose	ambient	NR (OD)
<i>Stenotrophomonas maltophilia</i>	M	O (110)	37	20	$1.0 \times 10^7$	NB	ambient	$6.4 \pm 0.2$
<i>Streptococcus agalactiae</i>	M	R (10)	37	7	$1.0 \times 10^7$	TSB	air/CO <sub>2</sub> , 90:10	5.2
<i>Streptococcus pneumoniae</i>	M	O (110)	37	20	$1.0 \times 10^7$	M260	ambient	$4.6 \pm 0.2$

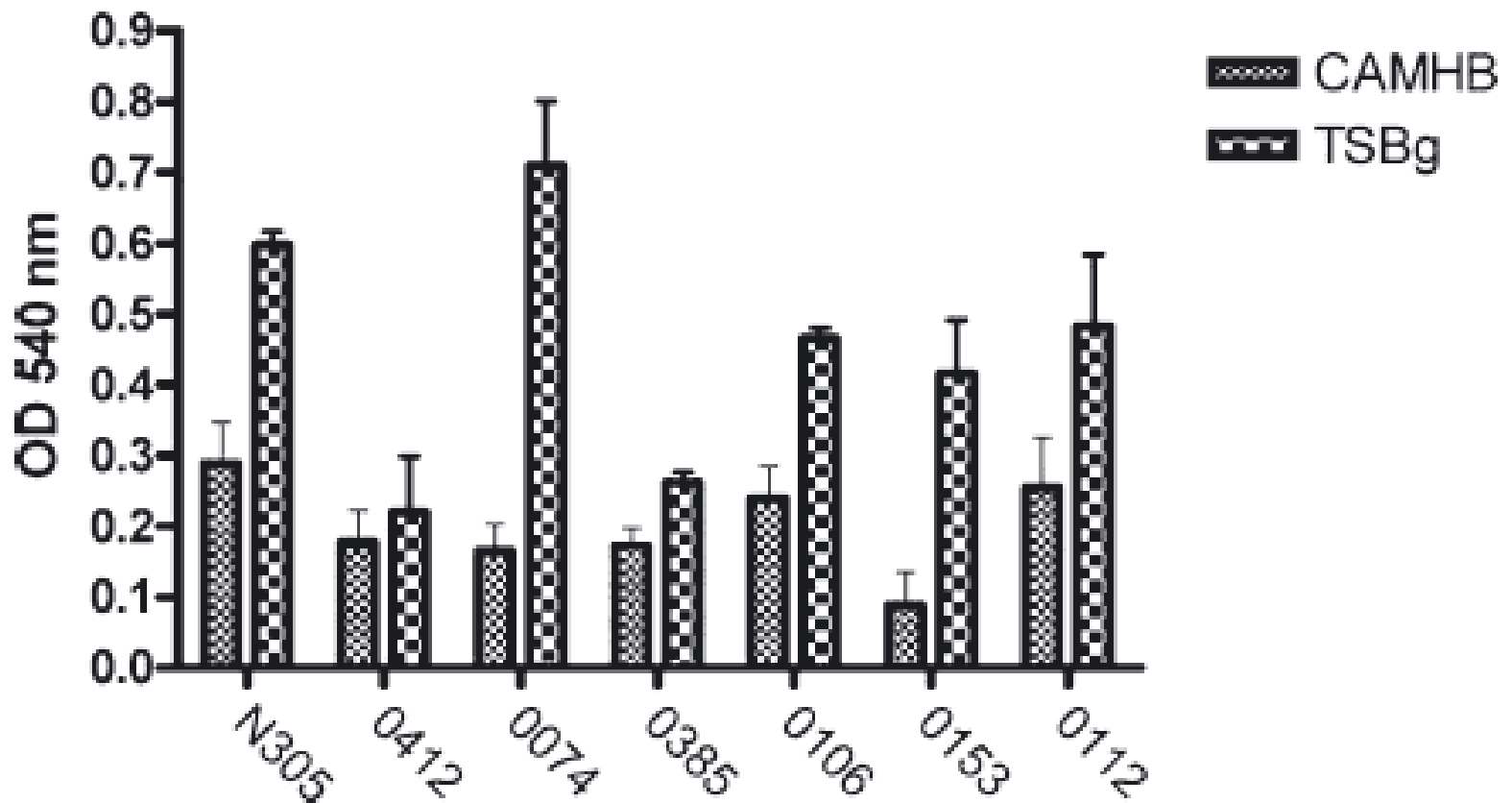


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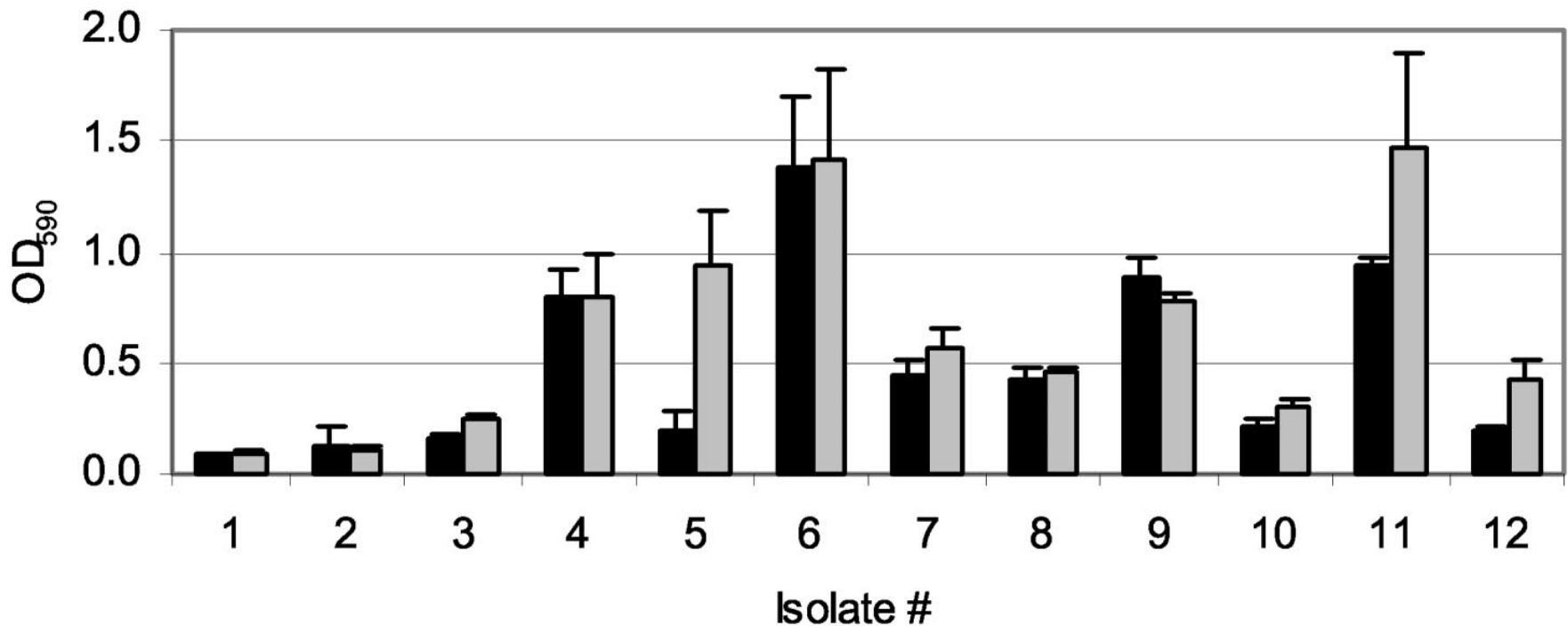
# Peg Lid Microtiter Plate Biofilm Assay

## *Staphylococcus aureus* Biofilm Formation

### Trypticase Soy Broth (with glucose) vs. CAMHB

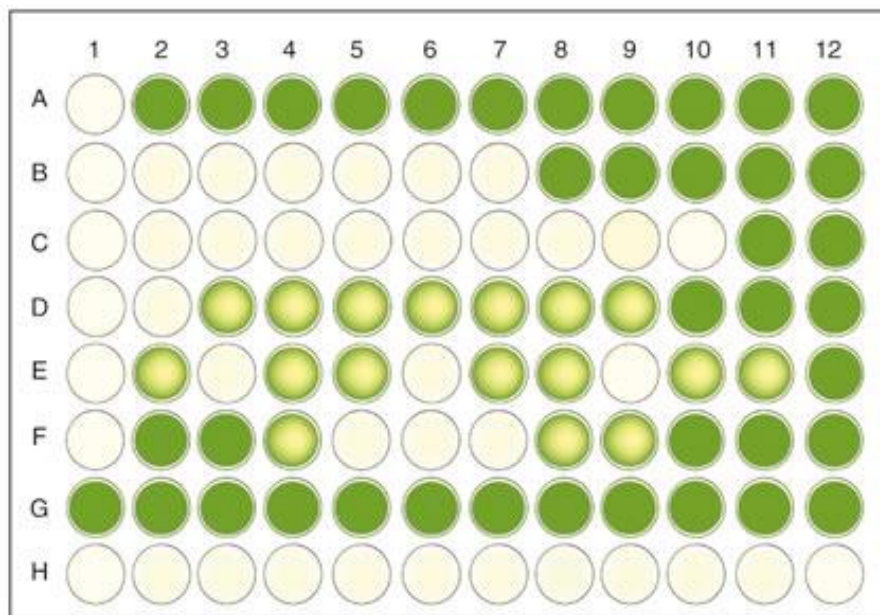


# Peg Lid Microtiter Plate Biofilm Assay *Pseudomonas aeruginosa* Biofilm Formation Sheer Stress versus Static Culture





# Peg Lid Microtiter Plate Biofilm Assay Interpretation



MBEC > x; if relevant, increase concentration range and repeat test

MBEC = x/32

MBEC ≤ x/512; if relevant, reduce concentration range and repeat test

MBEC = x; low numbers of biofilm survivors when concentration is >x/128

MBEC > x or insufficient biofilm cultivation time; repeat test

Asymmetrical biofilm formation; optimize growth conditions and repeat test, or Paradoxical killing of the microorganism by the antimicrobial agent

Recovery medium is likely contaminated; repeat test

Organism did not grow in recovery medium; repeat test

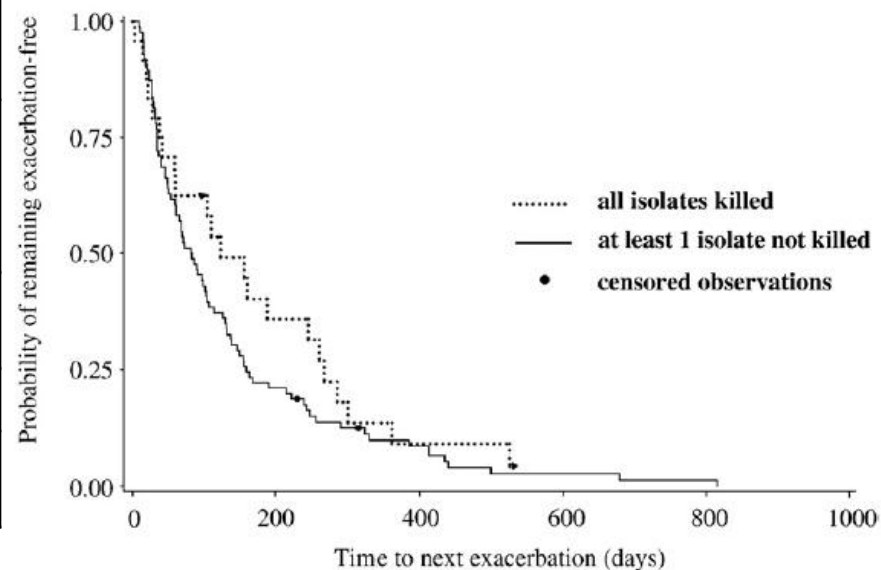
Sterility control ← Dilutions of antimicrobial agent → Growth control  
 x    x/2    x/4    x/8    x/16    x/32    x/64    x/128    x/256    x/512

# **Peg Lid Microtiter Plate Biofilm Assay Correlation with Rabbit *Escherichia coli* Catheter-Associated UTI**

	MIC	MBC	MBEC	Bladder mucosa	Urethra
	$\mu\text{g/ml}$			Mean $\log_{10}$ CFU/g tissue	
Fleroxacin	0.16	0.32	2	0	0
Ampicillin	2	4.0	>16	1.42	1.49
TMP-SMX	1	>128	>256	4.39	4.55
Gentamicin	0.5	1	>32	1.42	1.46
No treatment				4.21	3.89

# Peg Lid Microtiter Plate Biofilm Assay Correlation with Clinical Outcome

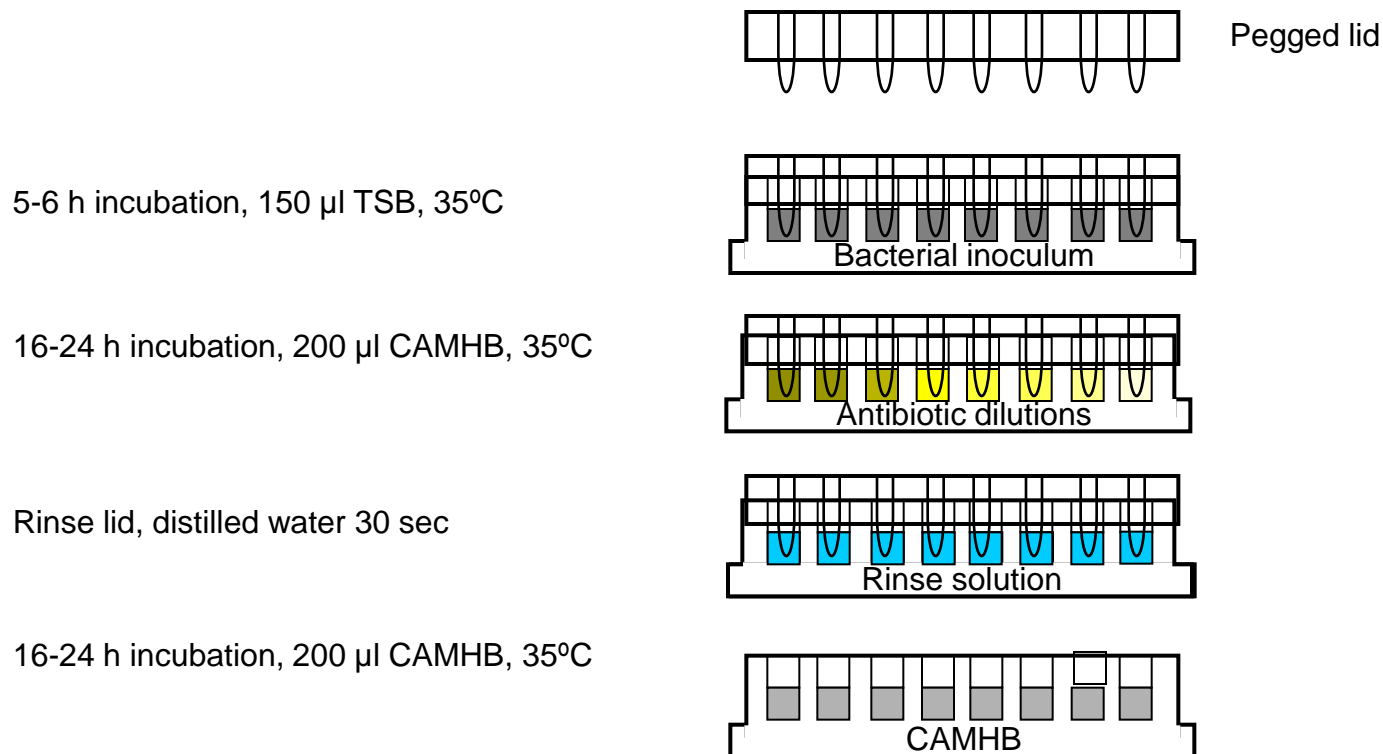
	At least one biofilm-grown isolate susceptible (n=61)	No biofilm-grown isolates susceptible (n=49)	P-value
Mean change in sputum bacterial density from day 0 to 14 in CFU/ml	-6.26 x 10 <sup>7</sup>	-1.50 x 10 <sup>7</sup>	0.02
Changes in FEV1 in L, day 0 to 14	0.26 (0.38)	0.23 (0.35)	0.73
Treatment failure (%)	2/61 (3.2)	6/49 (12.2)	0.14
Mean hospital length of stay in days	13.3 (6.9)	17.4 (11.3)	0.04



## Numbers at risk

All isolates killed	24	8	2	0	0	0
>1 isolate not killed	86	18	6	2	1	0

# Peg Lid Microtiter Plate Biofilm Assay bioFILM PA™ (innovotech, Edmonton, Canada)



## bioFILM PA™

# Reproducibility, Quality Control

- Guidance Document: Class II Special Controls Guidance Document: Antimicrobial Susceptibility Test (AST) Systems; Guidance for Industry and FDA
  - *Pseudomonas aeruginosa* ATCC 27853, 4 replicates, 3 separate days
    - Reproducibility MICs 99.5%
    - Reproducibility MBECs 92.3%
  - 10 *P. aeruginosa* (ATCC 27853 and 9 clinical isolates), triplicate, 3 separate days
    - Reproducibility MICs 99.9%
    - Reproducibility MBECs 95.0%
- Preliminary quality control limits established
- ASTM Standard method E2799-11
  - Testing disinfectant efficacy against *P. aeruginosa* biofilms

# Conclusions

- **Bacterial biofilms less susceptible to many antibiotics than planktonic counterparts**
  - **However, planktonic susceptibility is typically assessed based on inhibition and biofilm susceptibility based on killing**
- **Multiple methods used to assess MBEC (BBC)**
  - **Some may meet criteria for development of standards**
- **Limited data indicates that MBEC (BBC) correlates with clinical outcome**
  - **However, MBEC (BBC) values vary between methods**
  - **NEED standards!**



# Moving Forward...

- **Method**
  - **Starting inoculum**
  - **Biofilm age**
- **Target organism(s)**
- **Target antimicrobics**
- **QC strain(s)**
- **Interlaboratory multicenter study**



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