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Method of Evaluating Effects of Antibiotics on Bacterial Biofilm

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Antibiotics are generally not effective against organisms in exopolysaccharide biofilms. A simple method of studying the effect of antibiotics on bacteria in established biofilms is reported. *Escherichia coli* ATCC 25922 cells grown overnight at 37°C on Mueller-Hinton agar were suspended in buffer and dispensed on 0.5-cm^2 catheter disks. The disks were incubated for 1 h at 37°C, washed, transferred to petri dishes containing 20 ml of broth, and incubated at 37°C for 20 to 22 h, at which time thick biofilms were established. Disks were washed, placed in broth or broth containing antibiotic, and incubated at 37°C for 4 h. The disks were removed, and viable counts were determined. This process was repeated at other selected time intervals (e.g., 8 and 24 h). Viable bacterial counts decreased from 10^3 to 10^4 CFU/cm^2 in 24 h with 400 μg of amdinocillin or cefamandole per ml. A combination containing 400 μg of each antibiotic per ml decreased the viable counts to an undetectable level (<100 CFU/cm^2) in 24 h. Other antibiotics and organisms were also examined in this system.

Bacterial populations form a biofilm by adhering to surfaces using pili and exopolysaccharides. The cells become enveloped in a matrix of hydrated exopolysaccharides termed a glycocalyx (1). The glycocalyx matrix modifies the environment of the adherent cells by concentrating nutrients and protecting the cells from surfactants, biocides, and phagocytic cells (2). Antibiotics are generally not very effective against organisms embedded in biofilms (sessile bacteria) in comparison with free-floating organisms (planktonic bacteria) (9, 13). A simple method of studying the effect of antibiotics on established biofilms has therefore been developed.

MATERIALS AND METHODS

Antibiotics. The following antibiotics were used in this study: amdinocillin (Hoffmann-La Roche Inc., Nutley, N.J.), cefamandole (Eli Lilly & Co., Indianapolis, Ind.), ceftriaxone (Roche), cephalexin (Lilly), cephalothin (Lilly), ceftazidime (Glaxo Pharmaceuticals, Ltd., Greenford, United Kingdom), gentamicin (Schering Corp., Bloomfield, N.J.), sulfamethoxazole (Roche), and trimethoprim (Roche).

Bacteria. The following strains of bacteria were used in this study: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* SDH-14 and EL 2522-27, *Enterobacter cloacae* 948-1, and *Serratia marcescens* #1-JM. All strains were obtained from the Chemotherapy Department culture collection at Hoffmann-La Roche Inc.

Methods. *E. coli* ATCC 25922 was grown overnight on Mueller-Hinton (MH) agar at 37°C. The cells were scraped from the agar surface with an inoculating loop and suspended in Hanks-HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (H-H) buffer, pH 7.4, to an optical density of 0.8 at 540 nm. The cell suspension was dispensed in 80-μl amounts on ethylene oxide-sterilized 0.5-cm^2 disks of silicon latex catheter material obtained from various manufacturers. The disks were incubated for 1 h at 37°C, washed once with H-H buffer, pH 7.4, transferred to petri dishes containing 20 ml of National Institutes of Health (NIH) broth (11a), and incubated at 37°C for 20 to 22 h. Disks were then washed in H-H buffer, placed in broth alone or broth containing antibiotic, incubated at 37°C for 4 h, washed in H-H buffer, and placed in fresh broth or broth containing antibiotic. This procedure was repeated at 8 and 24 h. At each time interval (0, 4, 8, and 24 h), disks were removed and the surface film was scraped with a sterile scalpel into 5 ml of phosphate-buffered saline. This procedure was modified in later experiments to include washing, sampling, and replacing with fresh broth or broth containing antibiotic at 0, 6, and 24 h (rather than 0, 4, 8, and 24 h), which yielded similar results. Each disk and the scrapings were placed in a vial, vortexed for 1 min, and gently sonicated for 5 min in an Ultramet III sonic cleaner (Buehler Ltd., Evanston, Ill.). By using a Spiral plater (model D) and a model 500A laser bacterial colony counter (Spiral System Instruments, Inc., Bethesda, Md.) (7), viable counts were determined. Disks were also removed and stained with 0.4% methylene blue for examination by light microscopy. Additional disks were processed for scanning electron micros-

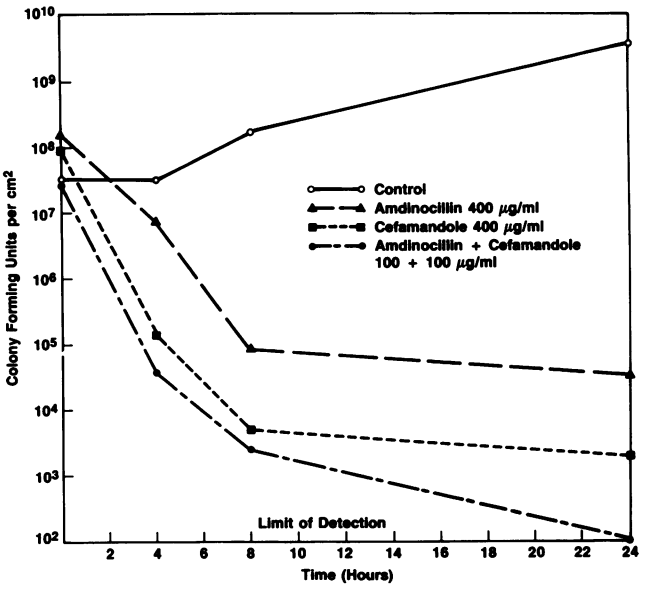

FIG. 1. Effect of amdinocillin and cefamandole alone and in combination on sessile populations of a 24-h *E. coli* ATCC 25922 biofilm.

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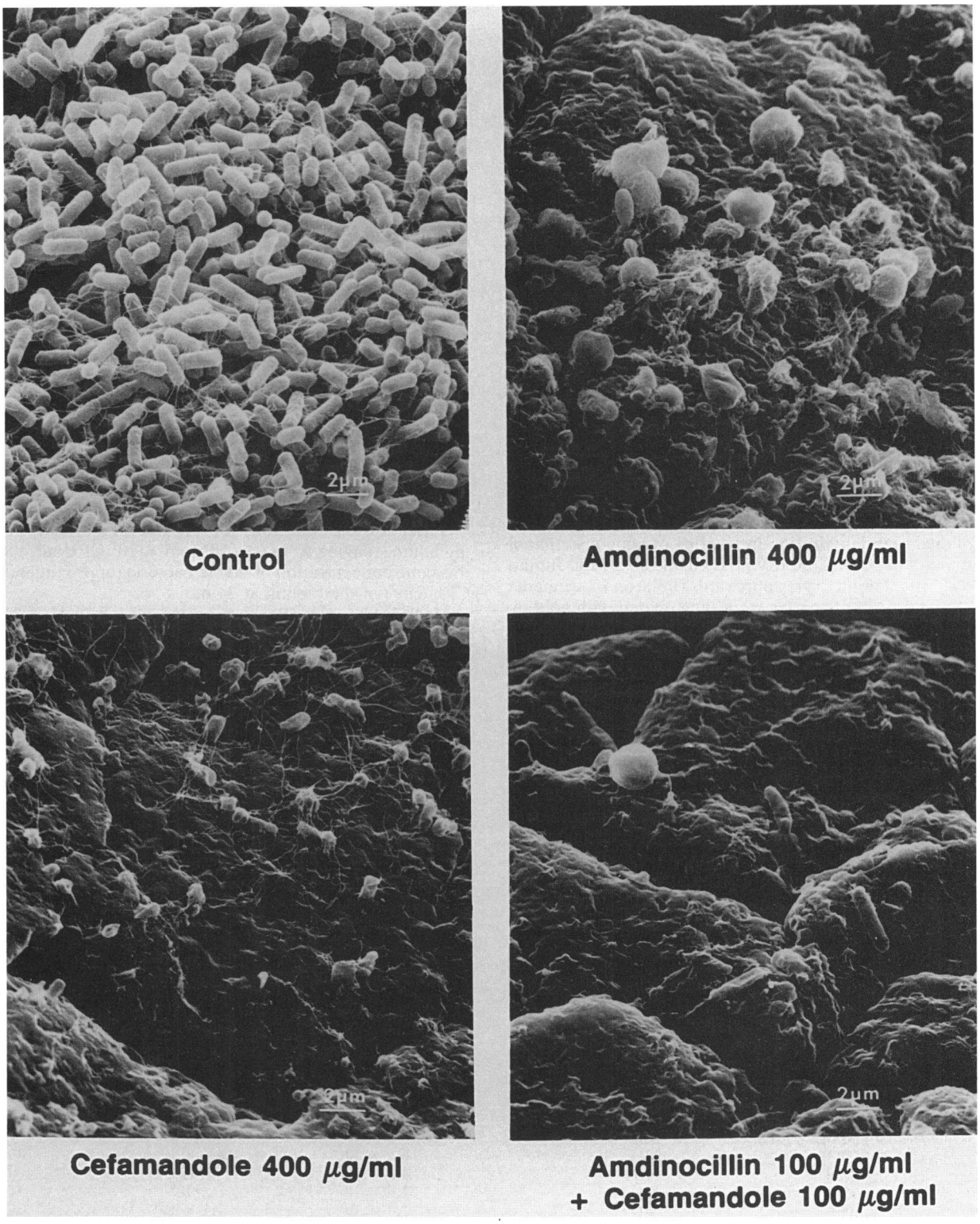

FIG. 2. In vitro effect of amdinocillin and cefamandole as single agents and in combination against an *E. coli* ATCC 25922 biofilm on latex catheter material. Incubation, 24 h.

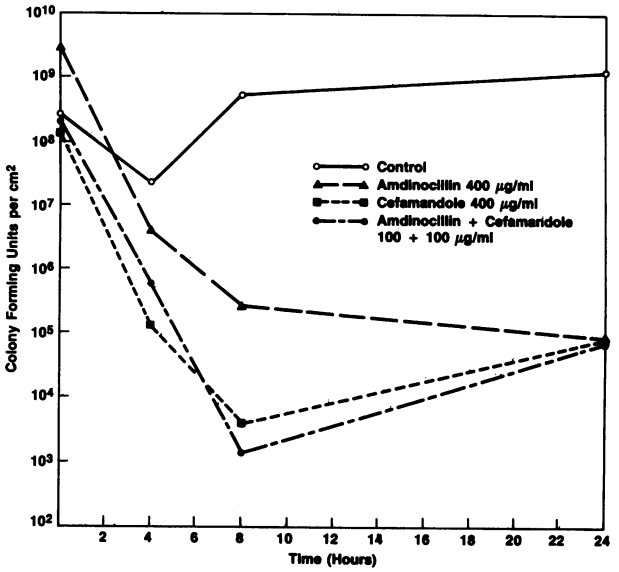

FIG. 3. Effect of amdinocillin and cefamandole alone and in combination on sessile populations of a 48-h *E. coli* ATCC 25922 biofilm.

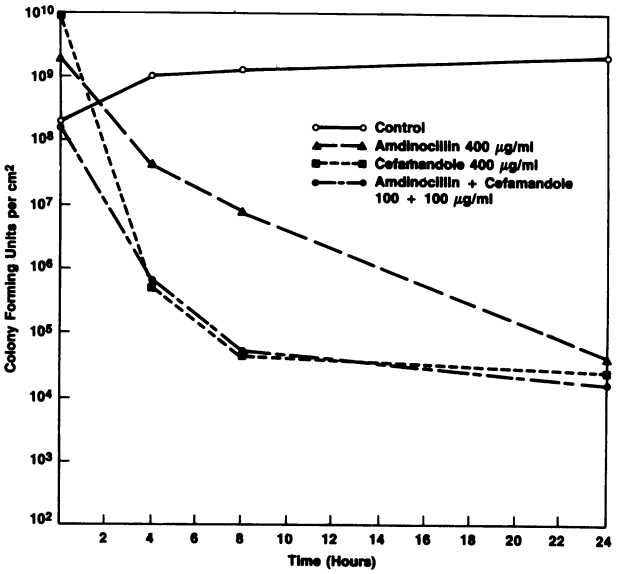

FIG. 4. Effect of amdinocillin and cefamandole alone and in combination on sessile populations of a 72-h *E. coli* ATCC 25922 biofilm.

copy by fixation overnight at 4°C in 2.5% glutaraldehyde in 0.1 M cacodylate buffer plus 0.2 M sucrose (pH 7.4). This was followed by dehydration in a series of aqueous ethanol solutions (30 to 100%) and drying by using an E3100 Jumbo Polaron Critical Point drying apparatus (Polaron Instruments Inc., Hatfield, Pa.) . Samples were then coated with gold by using a Polaron Instruments Inc. scanning electron microscope coating unit E5000 and examined by using a JEOL JSM-35 scanning electron microscope (JEOL USA, Inc., Peabody, Mass.) (8).

RESULTS

Growth. After inoculation of the disks with 10^9 CFU of *E. coli* ATCC 25922 per ml, approximately 10^6 CFU/cm^2 adhered to the catheter disk. After 24 h, 10^7 to 10^8 CFU/cm^2 were obtained.

Inoculum. Adhesion to the catheter material occurred equally well with an inoculum from an overnight MH agar or NIH broth culture as well as with a log-phase culture in NIH broth.

Serial 10-fold dilutions of an overnight agar culture inoculum, ranging from 6×10^9 to 6×10^1 CFU/ml, yielded the same concentration of viable bacteria (approximately 10^7 CFU/cm^2) in the biofilm at 24 h.

Media. After the adhesion period of 1 h in H-H buffer, incubation for 24 h at 37°C of the inoculated catheter disks in various broths, such as MH, NIH, trypticase soy broth, 50% artificial urine (10) plus 50% MH, and 50% artificial urine plus 50% trypticase soy broth, resulted in biofilms containing approximately 10^7 to 10^8 CFU/cm^2. However, minimal medium Davis was not as effective (i.e., biofilms contained 10^5 to 10^7 CFU/cm^2).

Effect of antibiotics. Concentrations of 400 μg of amdinocillin and cefamandole per ml were chosen for testing on the basis of the urinary levels of these agents still attainable approximately 2 h after standard doses (5, 6). Concentrations of 100 μg of each drug per ml were used in combination on the basis of the previously observed synergistic effect of amdinocillin and cefamandole (12). The single agents in NIH

TABLE 1. Effect of antibiotics on *E. coli* ATCC 25922 in a 24-h biofilm after treatment for 48 h

Antibiotic (manufacturer) [concn, μg/ml]	Broth	CFU/cm^2 at:		
		0 h	24 h	48 h
Control	MH	2.4×10^7	1.0×10^8	1.5×10^8
Ceftazidime (Glaxo) [100]	MH	6.5×10^7	4.1×10^2	<100[a]
Ceftriaxone (Roche) [5]	NIH	2.5×10^8	3.5×10^3	ND[b]
Ceftriaxone (Roche) [100]	MH	6.6×10^7	4.7×10^2	<100[a]
Ceftriaxone (Roche) [400]	MH	6.5×10^7	4.7×10^2	<100[a]
Ceftriaxone (Roche) [4,000]	NIH	4.3×10^7	<100[a]	ND
Cephalexin (Lilly) [400]	MH	1.6×10^7	$>10^6$	<100[a]
Cephalothin (Lilly) [400]	MH	2.3×10^7	1.1×10^4	<100[a]
Gentamicin (Schering) [90]	MH	1.9×10^7	4.0×10^2	<100[a]
Sulfamethoxazole (Roche) [400] + trimethoprim (Roche) [20]	MH + thy P-ase[c]	9.6×10^7	7.1×10^7	ND

[a] Minimum detectable level.
[b] ND, Not done
[c] Thy P-ase, 0.1% thymidine phosphorylase.

TABLE 2. Effect of amdinocillin and cefamandole alone and in combination at 6 and 24 h on sessile populations of various organisms in a 24-h biofilm

Organism	Antibiotic (concn, μg/ml)	CFU/cm² at:		
		0 h	6 h	24 h
K. pneumoniae SDH-14	Control	3.7×10^8	1.4×10^8	6.8×10^8
	Amdinocillin (400)		1.1×10^7	3.2×10^8
	Cefamandole (400)		1.0×10^5	6.2×10^4
	Amdinocillin (100) + cefamandole (100)		1.1×10^5	3.3×10^4
K. pneumoniae EL2522-27	Control	8.7×10^8	6.0×10^7	6.5×10^8
	Amdinocillin (400)		1.6×10^8	4.1×10^8
	Cefamandole (400)		6.6×10^5	5.3×10^4
	Amdinocillin (100) + cefamandole (100)		1.1×10^6	6.0×10^3
E. cloacae 948-1	Control	5.1×10^8	7.9×10^7	2.0×10^8
	Amdinocillin (400)		2.4×10^6	1.3×10^6
	Cefamandole (400)		3.6×10^7	8.4×10^7
	Amdinocillin (100) + cefamandole (100)		2.1×10^5	3.7×10^5
S. marcescens #1JM	Control	3.1×10^7	2.3×10^8	1.6×10^8
	Amdinocillin (400)		7.2×10^7	3.8×10^7
	Cefamandole (400)		2.7×10^7	8.5×10^7
	Amdinocillin (100) + cefamandole (100)		2.4×10^6	2.6×10^7

broth decreased the sessile bacterial concentration by 10^3 to 10^4 CFU/cm² in 24 h. The combination at 100 + 100 μg/ml in NIH broth decreased the sessile bacterial concentration by 10^5 CFU/cm² (Fig. 1). Raising the concentration of agents in the combination to 400 + 400 μg/ml reduced the count to an undetectable level (<100 CFU/cm²) in 24 h (data not shown). Scanning electron micrographs (Fig. 2) demonstrate the effect of the antibiotics on the bacteria in the biofilm after 24 h.

Effect of biofilm age. Biofilms established for 48 and 72 h were treated with the same concentrations of amdinocillin and cefamandole, and the viable counts of the sessile bacteria were determined (Fig. 3 and 4). Concentrations of the 48- and 72-h sessile bacteria were reduced by both the single agents and the combination to approximately the same endpoint (i.e., 10^4 to 10^5 CFU/cm²) after 24 h of exposure to the antibiotics.

Other antibiotics. We have also examined the effect of other antibiotics in MH broth (unless otherwise stated) on *E. coli* ATCC 25922 in a 24-h biofilm (Table 1). With the exception of 400 μg of cephalexin per ml and 400 μg of sulfamethoxazole per ml plus 20 μg of trimethoprim per ml, the antibiotics tested reduced the concentration of *E. coli* in the biofilm by 10^3 to 10^5 CFU/cm² in 24 h and, when determined, further reduced the bacterial concentration to an undetectable level (<100 CFU/cm²) in 48 h.

Ceftriaxone, at 5 μg/ml, in NIH broth decreased the sessile population by 10^4 to 10^5 CFU/cm² in 24 h. At 100 and 400 μg/ml in MH broth, it reduced the count to an undetectable level (<100 CFU/cm²) in 48 h. With 4,000 μg/ml in NIH broth, the organisms were eliminated in 24 h.

Cephalexin, at 400 μg/ml, had no effect on reducing viable counts at 24 h but did eliminate the organisms in 48 h.

Sulfamethoxazole and trimethoprim, at 400 + 20 μg/ml of MH broth plus 0.1% thymidine phosphorylase, were ineffective in eliminating organisms from the biofilm at 24 h.

MICs. A subculture was made from viable organisms isolated from the sessile population of *E. coli* ATCC 25922 after treatment with ceftriaxone at 400 μg/ml in MH broth for 24 h. By using National Committee for Clinical Laboratory Standards methods (11), broth macrodilution MICs were determined for both the subcultures and the control. The MICs for both were the same (i.e., 0.125 μg/ml), indicating that there was no difference in sensitivity in the remaining sessile population.

Other organisms. Other gram-negative organisms (*K. pneumoniae*, *Enterobacter cloacae*, and *S. marcescens*) have been shown to establish biofilms on the catheter disks similar to those observed with the *E. coli* strain. We have also tested a 24-h biofilm of *K. pneumoniae* SDH-14 and EL 2522-27, *Enterobacter cloacae* 948-1, and *S. marcescens* #1JM versus amdinocillin, cefamandole, and a 1:1 combination (Table 2). These organisms were chosen because they exhibited, on the basis of MICs, a wide range of susceptibilities to the individual agents from highly susceptible (*K. pneumoniae* SDH-14) to highly resistant (*S. marcescens* #1JM) and showed in two cases (i.e., *K. pneumoniae* EL 2522-27 and *Enterobacter cloacae* 948-1) significant synergy with the 1:1 combination (fractional inhibitory concentration, ≤0.25).

The combination of amdinocillin and cefamandole at 100 + 100 μg/ml decreased the concentration of cells in the biofilm of *K. pneumoniae* SDH-14 by 10^4 CFU/cm² and EL2522-27 by 10^5 CFU/cm² (but still left 10^3 to 10^4 CFU/cm² on the catheter).

Amdinocillin alone, at 400 μg/ml, was ineffective on either *Klebsiella* strain. Cefamandole, at 400 μg/ml, reduced the count of both organisms by 10^3 to 10^4 CFU/cm² in 24 h.

With *Enterobacter cloacae* 948-1, the combination reduced the count by 10^3 CFU/cm² and amdinocillin reduced it by 10^2 CFU/cm², whereas cefamandole was essentially ineffective.

The cell count in a biofilm of *S. marcescens* #1JM was not reduced by the single agents or the combination.

Reduction of viable counts generally correlated with susceptibility to the single agent or the combination.

DISCUSSION

Infection of prosthetic devices is a serious problem for many patients. Despite the large number of antibacterial agents available, none eradicate organisms from biofilms (9,

16). The usual method of treating this problem is to remove the infected material and replace it with a new device (3, 14).

Factors such as numbers of organisms (4), their metabolic state (15), and their protection by the exopolysaccaride glycocalyx (13) can affect the ability of an antibiotic to penetrate and kill organisms in a biofilm. The fact that the subculture made from the viable organisms isolated from the remaining sessile population of *E. coli* ATCC 25922 after treatment with 400 μg of ceftriaxone per ml for 24 h had the same MIC of drug as the control indicates that these organisms were not a population of resistant variants.

Earlier workers have used the modified Robbins device to determine the efficacy of antibiotics in penetrating and killing bacteria in a biofilm (13). However, this method is cumbersome, and a significant amount of time and effort is required to perform an assay. With the modified Robbins device, one is unable to control the large numbers of planktonic bacteria which can inactivate the antibiotic.

This study describes a simple, reproducible method for establishing a thick biofilm of gram-negative aerobic bacteria on catheter material. The model system is designed to allow determination of the activity of an antibacterial agent against the sessile bacterial population embedded in the biofilm without the problems of dealing with inactivation of the agent by heavy concentrations of planktonic bacteria.

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