JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1994, p. 2564–2568
0095-1137/94/$04.00+0

Vol. 32, No. 10

NOTES

Testing Antimicrobial Susceptibilities of Adherent Bacteria by a Method That Incorporates Guidelines of the National Committee for Clinical Laboratory Standards

G. DOMINGUE,[1]* B. ELLIS,[2] M. DASGUPTA,[3] AND J. W. COSTERTON[4]

Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, British Columbia, V6T 2B5,[1] Department of Biological Sciences, University of Calgary, Calgary, Alberta, T2N 1N4,[2] and Department of Nephrology and Urology, University of Alberta Hospital, Edmonton, Alberta, T6G 2E5,[3] Canada, and Centre for Biofilm Engineering, Montana State University, Bozeman, Montana 59717[4]

Received 10 January 1994/Returned for modification 17 May 1994/Accepted 14 July 1994

Membrane filters (0.22-μm pore size) were colonized with 10^4 CFU of logarithmic-phase bacteria per filter under laminar flow conditions in a Modified Robbins Device. Colonized filters were then placed upon agar dilution plates for MIC determinations. Subsequently, filters were transferred to control plates and incubated to obtain MBCs.

The biofilm mode of bacterial growth, with its sessile (adherent) phenotype, is common in diseases, e.g., device-related infections. Here it poses a major problem because of a phenotypic resistance to antimicrobial agents (6, 10, 15). Despite its acknowledged importance, biofilm technology has not appeared yet in National Committee for Clinical Laboratory Standards (NCCLS) documentation.

As a result, susceptibility testing of surface-grown bacteria by nonclinical procedures has been described in the literature (5, 7, 11, 14, 16, 20). Apart from not conforming to clinical guidelines, these methods do not distinguish between loosely adherent and firmly adherent populations. This is because no method involves the formation of colonies under laminar flow conditions, i.e., shear-inducing circumstances, after the attachment phase. This is a prerequisite for true biofilm development and represents the forces found within medical catheters in situ or in hemodynamic systems (6, 9). The chemostat approach (3) does incorporate firmly adherent biofilms, but it is a valuable research tool rather than a method directly applicable to infectious diseases and clinical microbiology.

We describe an attempt to unite NCCLS guidelines and biofilm concepts with a view to promoting consideration of this philosophy in the clinical arena. The method incorporates both shear forces and testing by using NCCLS guidelines for MICs and MBCs (12, 13). This parallels the well-regulated quantitative testing of antimicrobial susceptibilities of bacteria using the planktonic (nonadherent, i.e., floating or freely swimming bacteria grown in flasks or test tubes) phenotype commonly examined in clinical laboratories (8, 12, 13, 17).

The control strains were *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853. The clinical strains were a continuous ambulatory peritoneal dialysis isolate of *S.*

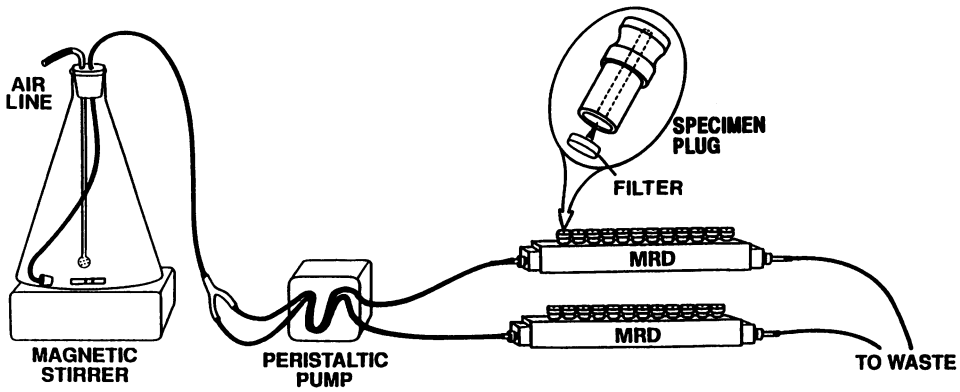

FIG. 1. Diagrammatic representation of MRDs and apparatus used to provide a continuous flow of bacteria and MH broth.

* Corresponding author. Mailing address: Department of Pathology and Laboratory Medicine, University of British Columbia, 2211 Wesbrook Mall, Vancouver, BC, V6T 2B5, Canada. Phone: (604) 822-7094. Fax: (604) 822-7635.

TABLE 1. Rates of colonization of membrane filters recovered from specimen plugs of MRDs

Time (h)	Colonization (10^4 CFU [±SD]/filter) with:	
	Staphylococci[a]	Pseudomonads[b]
0.5	1.79 ± 0.83	1.50 ± 0.75
1.0	4.84 ± 1.30	2.73 ± 0.83
1.5	5.65 ± 0.54	3.52 ± 0.57
2.0	6.00 ± 0.48	4.95 ± 0.23
2.5	6.20 ± 1.02	6.10 ± 0.98
3.0	6.59 ± 1.56	6.99 ± 1.22

[a] n = 10; values for both strains were similar and were therefore combined.
[b] n = 6; values for both strains were similar and were therefore combined.

epidermidis and a urinary tract infection isolate of *P. aeruginosa*. Both had been implicated as the causative agents in refractory infections despite MICs of a range of antibiotics in broth dilution for these strains, which would be indicative of susceptibility according to NCCLS guidelines (12); MBCs in broth dilution for these strains were also low (13). This pilot study incorporated cefuroxime (Glaxo, Vancouver, British Columbia, Canada) and vancomycin (Lilly, Toronto, Ontario, Canada) for the staphylococci and piperacillin (Lederle, Calgary, Alberta, Canada) and tobramycin (Lilly) for the pseudomonads. Each antibiotic was tested in a minimum of three experiments against each organism, and modal MICs and MBCs (5) were determined. When a modal value was not obtained, tests were repeated in triplicate.

Membrane filters (0.22-μm pore size, mixed cellulose esters; Nucleopore Corporation) were reduced to 6 mm in diameter with a cork borer and fitted into the specimen plugs of Modified Robbins Devices (MRDs). These are laminar-flow chambers made according to our original design (Fig. 1) (15) and now available commercially (Tyler Engineering, Edmonton, Alberta, Canada). The MRDs were then gas sterilized.

Paired MRDs were connected aseptically and in parallel to a flask containing a stir bar and 1 liter of Mueller-Hinton (MH) broth at 35°C. Previously, Ca^{2+} and Mg^{2+} had been adjusted to recommended levels for the pseudomonads (12). The whole assembly was maintained at 35°C. Flasks were inoculated with an overnight, shaken MH culture of *Staphylococcus* or *Pseudomonas* spp., and the inoculum target density of approximately 10^6 CFU ml^{-1} was confirmed by viable counts.

Fifteen liters of air h^{-1}, a previously determined saturating level for all planktonic (nonadherent) and sessile (adherent) populations, was delivered through a sparger into the flask. Stirring was started, and at between 1.5 and 2.0 h later, a flow of logarithmic-phase bacteria (approximately 2×10^6 CFU ml^{-1}) was initiated at 300 ml h^{-1} over the filters.

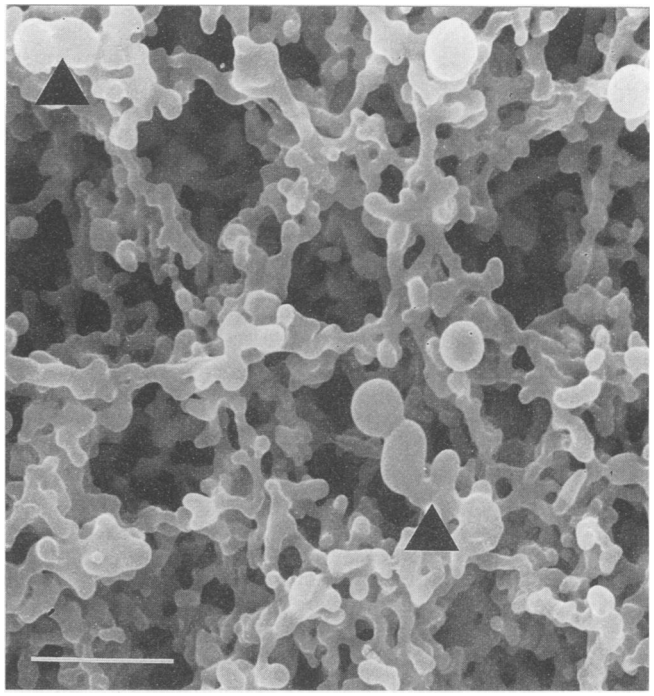

FIG. 2. Scanning electron micrograph of staphylococci colonizing a membrane filter recovered from a specimen plug of an MRD after 1 h of planktonic flow (arrowheads indicate dividing microcolonies) Bar, 5 μm.

In early experiments, the flow was stopped at selected time points and the level of filter colonization was determined by a modification of our disruption method (15). Specimen plugs were removed and washed with 5 ml of sterile phosphate-buffered saline (PBS), pH 7.4, to displace planktonic bacteria. Replicate filters were removed, and each was placed in a sterile, 35-by-10-mm-diameter petri dish. To reduce static buildup, 20 μl of PBS was placed on each filter, which was then scraped and macerated thoroughly with a sterile scalpel. Scalpel blades and the contents of each plate were transferred with 10-ml PBS rinses to conical centrifuge tubes. These were sonicated on low power (5 min, 1.0 A, 50 to 60 Hz),

TABLE 2. Staphylococcal 18-h broth dilution MICs and MBCs of vancomycin and cefuroxime[a]

Drug[b]	Value for the following strain from the indicated inoculum:											
	ATCC 29213						*S. epidermidis* (CAPD[c])					
	MICs (μg/ml)			MBCs (μg/ml)			MICs (μg/ml)			MBCs (μg/ml)		
	Tube[d]	Planks[e]	Dis sess[f]	Tube	Planks	Dis sess	Tube	Planks	Dis sess	Tube	Planks	Dis sess
VAN	1	1	0.5	1	1	2	2	1	2	2	2	2
CXM	1	0.5	1	1	1	1	1	1	2	2	1	1

[a] MICs and MBCs are as defined in references 12 and 13, respectively.
[b] VAN, vancomycin; CXM, cefuroxime.
[c] CAPD, continuous ambulatory peritoneal dialysis.
[d] Conventional inoculum (12).
[e] Inoculum taken from planktonic bacteria flowing over filters.
[f] Inoculum of disrupted sessile bacteria from homogenized filters.

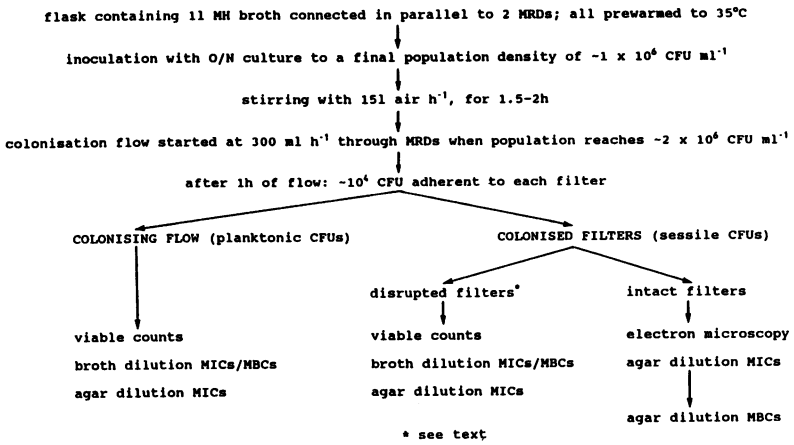

FIG. 3. Flow sheet of the experimental design in which the colonizing flow of planktonic (nonadherent) bacteria and the resultant sessile (adherent) population were tested for antibiotic susceptibilities.

vortexed vigorously for 1 min, and centrifuged (15 min, 10,000 × *g*, 4°C). The supernatants were discarded, and the deposits were processed for viable counts. Results indicated that 1 h of colonization flow routinely produced approximately 10^4 CFU adherent to each filter. These were present as actively dividing microcolonies, with the staphylococci generally reaching plateau first (Table 1; Fig. 2). This method therefore produced the equivalent of the approximately 10^4 CFU that is delivered to a 5- to 8-mm-diameter area by the pins of an inoculator when determining an agar dilution MIC (12).

Postcolonization events are depicted in Fig. 3. The planktonic population flowing over the specimen plugs was sampled for viable counts, broth dilution MICs and MBCs, and the agar dilution MICs described below. Only agar dilution MBCs of β-lactams are recognized for planktonic bacteria (13) and so would have been incomplete because of the inclusion of tobramycin and vancomycin.

Adherent growth was controlled by disrupting sessile bacteria by the method described above and then processing the deposits of disrupted bacteria for viable counts, broth dilution MICs and MBCs, and agar dilution MICs. For the reason stated above, which was extended to these disrupted sessile bacteria because they are in effect rendered planktonic, agar dilution MBCs were not determined.

The agar dilution MICs were determined by following NCCLS guidelines (12). Each plate in a series was positioned on a template. Intact, replicate filters were either placed, colonized surface up and without air bubbles, on the plates or processed for electron microscopy. Cells that originated from conventionally grown cultures, from the planktonic flow, and from disrupted filters of all strains were inoculated onto the plates; each plate ultimately bore inocula derived from each type of population. After absorption, these were incubated at 35°C. The endpoint was the lowest concentration that completely inhibited growth, i.e., the definition of MIC used in the agar dilution method (12), and this was assessed at 18 and 36 h by plate microscopy.

Sessile MBCs (it not being possible to determine planktonic MBCs for the previously stated reason) subsequently were determined by removing replicate intact filters from the MIC plates to control (drug-free) MH plates which were incubated at 35°C with observation at 18 and 36 h. The sessile endpoint was the lowest concentration that resulted in no growth, i.e., the definition of MBC employed in the broth microdilution method for MBCs determined with a multipoint inoculator (13); in effect, the colonized filters were being utilized as points.

All broth dilution MICs and MBCs for the conventionally grown, planktonic, and disrupted sessile populations denoted sensitivity (Tables 2 and 3). Eighteen-hour agar dilution MICs for the conventionally grown, planktonic, disrupted sessile, and intact sessile populations and agar dilution MBCs for sessile populations are presented in Tables 4 and 5 (there was no significant difference between values obtained at 18 and 36 h; data not shown). The elevated, sessile MBCs demonstrate the antibiotic tolerance of adherent bacteria, which accords with

TABLE 3. Pseudomonal 18-h broth dilution MICs and MBCs for tobramycin and piperacillin[a]

Drug[b]	Value for the following strain from the indicated inoculum:											
	ATCC 27853						*P. aeruginosa* (UTI[c])					
	MICs (μg/ml)			MBCs (μg/ml)			MICs (μg/ml)			MBCs (μg/ml)		
	Tube[d]	Planks[e]	Dis sess[f]	Tube	Planks	Dis sess	Tube	Planks	Dis sess	Tube	Planks	Dis sess
TOB	1	0.5	1	2	2	2	1	1	1	2	1	1
PIP	1	1	2	4	2	2	2	2	2	4	4	4

[a] MICs and MBCs are as defined in references 12 and 13, respectively.
[b] TOB, tobramycin; PIP, piperacillin.
[c] UTI, urinary tract infection.
[d] Conventional inoculum (12).
[e] Inoculum taken from planktonic bacteria flowing over filters.
[f] Inoculum of disrupted sessile bacteria from homogenized filters.

TABLE 4. Staphylococcal 18-h agar dilution MICs and MBCs for vancomycin and cefuroxime[a]

Drug[b]	Value for the following strain from the indicated inoculum:									
	ATCC 29213					*S. epidermidis* (CAPD[c])				
	MICs (μg/ml)				MBCs (μg/ml), int sess	MICs (μg/ml)				MBCs (μg/ml), int sess
	Plate[d]	Planks[e]	Dis sess[f]	Int sess[g]		Plate	Planks	Dis sess	Int sess	
VAN	1	1	1	1	>16	1	1	1	1	>16
CXM	1	0.5	0.5	0.5	>32	0.5	0.5	0.5	0.5	>32

[a] MICs are as defined in reference 12.
[b] VAN, vancomycin; CXM, cefuroxime.
[c] CAPD, continuous ambulatory peritoneal dialysis.
[d] Conventional inoculum (12).
[e] Inoculum taken from planktonic bacteria flowing over filters.
[f] Inoculum of disrupted sessile bacteria from homogenized filters.
[g] Inoculum of intact sessile bacteria adherent to intact filters; MBC method in text.

biofilm theory (6). This highlights the lack of biofilm-effective antimicrobial agents (3), even for the American Type Culture Collection strains, and in this sense, the method emphasizes testing for antibiotic resistance (18). Generally, MBC endpoints were not found with the agar dilution ranges employed in this pilot study, despite the misleadingly low MICs for the intact microcolonies. These low MICs reflected the nascent aspect of the biofilms, i.e., conforming to NCCLS guidelines, they were approximately 2 h old and not fully formed and consequently did not show elevated MICs (1, 15).

With practice, establishing the inoculum density and the filter colonization rate became routine. Dark filters became available after the conclusion of experiments, and in limited tests, they appeared to be a worthwhile visual aid.

The approach with MRDs described here will be useful for the screening of novel compounds with biofilm activity. The approach could also include synergy testing against colonized filters in agar dilution checkerboards or those checkerboards incorporating antimicrobial agents and, e.g., biofilm-active proteases (19). In a departure from clinical guidelines, extended colonization times could be used to produce "old" biofilms for determination of the biofilm eliminating concentration (1). Importantly, the MRD method is to date the only one that can be used to investigate the enhancement by direct-current electric fields of the efficacy of antimicrobial agents against biofilms (2, 4).

TABLE 5. Pseudomonal 18-h agar dilution MICs and MBCs for tobramycin and piperacillin[a]

Drug[b]	Value for the following strain from the indicated inoculum:									
	ATCC 27853					*P. aeruginosas* (UTI[c])				
	MICs (μg/ml)				MBCs (μg/ml), int sess	MICs (μg/ml)				MBCs (μg/ml), int sess
	Plate[d]	Planks[e]	Dis sess[f]	Int sess[g]		Plate	Planks	Dis sess	Int sess	
TOB	1	1	0.5	1	8	1	1	2	2	16
PIP	2	2	4	4	>32	2	2	2	2	>16

[a] MICs are as defined in reference 12.
[b] TOB, tobramycin; PIP, piperacillin.
[c] UTI, urinary tract infection.
[d] Conventional inoculum (12).
[e] Inoculum taken from planktonic bacteria flowing over filters.
[f] Inoculum of disrupted sessile bacteria from homogenized filters.
[g] Inoculum of intact sessile bacteria adherent to intact filters; MBC method in text.

Currently, agar dilution MBCs are recognized for β-lactams only (13). However, routine determinations of MBCs of other antibiotics against strictly planktonic bacteria are feasible by replacing multipoint inoculator transfer with membrane filter transfer (5, 11, 14). Calibrated bacterial suspensions could be dropped onto filters, and after incubation on antibiotic agar, filters and their potential colonies could be transferred to control plates for MBC determinations.

The MRD approach described above probably would not become a routine test but rather a subspeciality-driven service in large diagnostic laboratories or in infectious disease projects. However, it brings closer together the concepts of bacterial biofilms and susceptibility testing by using clinical microbiology standards.

G.D. acknowledges a Research Fellowship of the Heart and Stroke Foundation of Canada.

REFERENCES

1. **Anwar, H., and J. W. Costerton.** 1990. Enhanced activity of a combination of tobramycin and piperacillin for eradication of sessile biofilm cells of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **34**:1666–1671.
2. **Anwar, H., and J. W. Costerton.** 1992. Effective use of antibiotics in the treatment of biofilm-associated infections. ASM News **58**:665–668.
3. **Anwar, H., M. K. Dasgupta, and J. W. Costerton.** 1990. Testing the susceptibility of bacteria in biofilms to antibacterial agents. Antimicrob. Agents Chemother. **34**:2043–2046.
4. **Blenkinsop, S. A., A. E. Khoury, and J. W. Costerton.** 1992. Electrical enhancement of biocide efficacy against *Pseudomonas aeruginosa* biofilms. Appl. Environ. Microbiol. **58**:3770–3773.
5. **Caufield, P. W., D. N. Allen, and N. K. Childers.** 1987. In vitro susceptibilities of suspected periodontopathic anaerobes as determined by membrane transfer assay. Antimicrob. Agents Chemother. **31**:1989–1993.
6. **Costerton, J. W., K.-J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie.** 1987. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. **41**:435–464.
7. **Dix, B. A., P. S. Cohen, D. C. Laux, and R. Cleeland.** 1988. Radiochemical method for evaluating the effect of antibiotics on *Escherichia coli* biofilms. Antimicrob. Agents Chemother. **32**:770–772.
8. **Ericsson, H. M., and J. C. Sherris.** 1971. Antibiotic sensitivity testing. Report of an international collaborative study. Acta Pathol. Microb. Scand. Sect. B Suppl. 217.
9. **Gristina, A. G., C. D. Hobgood, L. X. Webb, and Q. N. Myrvik.** 1987. Adhesive colonization of biomaterials and antibiotic resistance. Biomaterials **8**:423–426.
10. **Gristina, A. G., R. A. Jennings, P. T. Naylor, Q. N. Myrvik, and L. X. Webb.** 1989. Comparative in vitro antibiotic resistance of surface-colonizing coagulase-negative staphylococci. Antimicrob. Agents Chemother. **33**:813–819.
11. **Millward, T. A., and M. Wilson.** 1989. The effect of chlorhexidine on *Streptococcus sanguis* biofilms. Microbios **58**:155–164.
12. **National Committee for Clinical Laboratory Standards.** 1990. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, vol. 2. Approved standard M7-A2. National Committee for Clinical Laboratory Standards, Villanova, Pa.
13. **National Committee for Clinical Laboratory Standards.** 1992. Methods for determining bactericidal activity of antimicrobial agents. Tentative standard M26-T. National Committee for Clinical Laboratory Standards, Villanova, Pa.
14. **Nichols, W. W., M. J. Evans, M. P. E. Slack, and H. L. Walmsley.** 1989. The penetration of antibiotics into aggregates of mucoid and non-mucoid *Pseudomonas aeruginosa*. J. Gen. Microbiol. **135**: 1291–1303.

15. **Nickel, J. C., I. Ruseska, J. B. Wright, and J. W. Costerton.** 1985. Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. Antimicrob. Agents Chemother. **27:**619–624.
16. **Prosser, D., D. Taylor, B. Dix, and R. Cleeland.** 1987. Method of evaluating effects of antibiotics on a bacterial biofilm. Antimicrob. Agents Chemother. **31:**1502–1506.
17. **Reeves, D. S., I. Phillips, J. D. Williams, and R. Wise (ed.).** 1978. Laboratory methods in antimicrobial chemotherapy. Churchill Livingstone, London.
18. **Sanders, C. C.** 1991. A problem with antimicrobial susceptibility tests. ASM News **57:**187–190.
19. **Selan, L., F. Berlutti, C. Passariello, M. R. Comodi-Ballanti, and M. C. Thaller.** 1993. Proteolytic enzymes: a new treatment strategy for prosthetic infections. Antimicrob. Agents Chemother. **37:** 2618–2621.
20. **Widmer, A. F., R. Frei, Z. Rajacic, and W. Zimmerli.** 1990. Correlation between in vivo and in vitro efficacy of antimicrobial agents against foreign body infections. J. Infect. Dis. **162:**96–102.