1988

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Citation/Publisher Attribution
Dix, B. A., Cohen, P. S., Laux, D. C., & Cleeland, R. (1988). Radiochemical method for evaluating the effect of antibiotics on *Escherichia coli* biofilms. *Antimicrob. Agents Chemother., 32*(5), 770-772. doi: 10.1128/AAC.32.5.770
Available at: http://dx.doi.org/10.1128/AAC.32.5.770

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1988, p. 770–772
0066-4804/88/050770-03$02.00/0

Vol. 32, No. 5

Radiochemical Method for Evaluating the Effect of Antibiotics on *Escherichia coli* Biofilms

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Received 8 September 1987/Accepted 5 February 1988

A simple radiochemical method for evaluating the action of antibiotics on *Escherichia coli* cells in biofilms is reported. After growth, biofilms of *E. coli* ATCC 25922 on disks of urinary catheter material were suspended in fresh medium containing or lacking an antibiotic, incubated for 4 h at 37°C, and pulse-labeled with [^{3}H]leucine for 5 min. Radioactivity in trichloroacetic acid-precipitable material in the biofilm and in the surrounding medium (planktonic *E. coli*) was then measured. Antibiotic-induced inhibition of incorporation of [^{3}H]leucine into the cells in the biofilm was far less pronounced than incorporation into planktonic cells and, furthermore, correlated well with loss in viable counts. The method is simple, inexpensive, and extremely timesaving.

A significant percentage of hospital patients receive urinary tract catheters during their stay. Of these patients, a large percentage develop urinary tract infections because of the catheters. That is, normal commensal bacteria adhere to the surfaces of urinary catheter material (3, 5). Once bound, these bacteria replicate, forming extensive biofilms (3). Biofilms then serve as a constant source of infection of the bladder or kidneys as individual cells break away into the surrounding medium (3).

Therapeutically, biofilms pose a major problem. That is, the organisms (sessile bacteria) in the biofilms are more resistant to the action of phagocytic cells and to antibiotics than are their free-floating (planktonic bacteria) counterparts (1–3, 5), presumably because of extensive bacterial synthesis of an exopolysaccharide barrier during biofilm formation (2–4). Clearly then, it is important to be able to screen new antibiotics as they become available in order to identify those effective against *E. coli* in biofilms formed on urinary tract catheters.

In our laboratory, biofilms on catheter disks are routinely treated with an antibiotic at a number of different concentrations for prescribed periods of time, after which the disks are scraped and viable counts are determined (6). This process is both time-consuming and expensive. We have therefore developed a simple, inexpensive radiochemical assay which monitors the incorporation of [^{3}H]leucine into trichloroacetic acid (TCA)-insoluble material in *E. coli* present in biofilms and which reflects results obtained by the viable count method.

E. coli ATCC 25922 was used in this study and grown in Minimal Broth Davis (Difco Laboratories, Detroit, Mich.) supplemented with 0.1% glucose (MBDG) in a 37°C water bath until the A_{650} was 0.5. Sterile disks of urinary catheter material (diameter, 0.5 cm^2) placed in wells of a 24-well Nunclon tissue culture plate (Sci-lab Co.) were inoculated with 1 ml of the *E. coli* suspension and incubated for 1 h at 37°C (adhesion period). Each disk was then washed twice with MBDG, and 1 ml of MBDG was added. Disks were then incubated for 20 to 24 h at 37°C (biofilm formation). Disks were then washed twice with MBDG, and 1 ml of MBDG or MBDG containing an antibiotic was added to each well. Plates were incubated for 4 h at 37°C. After incubation with or without an antibiotic, [^{3}H]leucine (1 μCi/ml, 16.7 mCi/mmol; New England Nuclear Corp., Boston, Mass.) was added to each well and the cells were pulse-labeled for 5 min. The MBDG in each well, i.e., the supernatant containing planktonic cells, was transferred to tubes containing 1 ml of 10% TCA supplemented with 1% Casamino Acids (Difco). *E. coli* cells were removed from the disks by incubating the disks in wells containing 0.5 ml of 5% sodium dodecyl sulfate for 3 h at 37°C. After incubation of the disks, the sodium dodecyl sulfate was transferred to tubes containing 0.5 ml of 10% TCA supplemented with 1% Casamino Acids. Each TCA precipitate was filtered through a prewetted Gelman Metricel membrane filter (pore size, 0.45 μm; Fisher Scientific Co., Pittsburgh, Pa.). Membranes were washed three times with 5 ml of 5% TCA containing 0.1% Casamino acids and dried under an infrared lamp, and radioactivity was counted in 10 ml of Filtron-X scintillation fluid (National Diagnostics). Control experiments showed that the rate of incorporation of [^{3}H]leucine into sessile and planktonic cells was linear for at least 10 min.

The effect of antibiotics on the incorporation of [^{3}H]leucine into planktonic and sessile cells is expressed as a percentage of the incorporation into control cells, i.e., cells incubated in the absence of an antibiotic. All assays were performed in duplicate.

In some experiments, viable bacterial counts of the biofilms were determined as described previously (6) by scraping the cells from the surfaces of the disks into 5 ml of phosphate-buffered saline. The disks and the scrapings were placed in sterile vials, vortexed for 1 min, and gently sonicated at 60/50 cycles in an Ultramet III sonic cleaner for 5 min. Viable counts were determined by using a Spiral plater and a laser bacterial colony counter (Spiral Systems Instruments, Inc., Bethesda, Md.).

The effect of several concentrations of three cephalosporins on the incorporation of [^{3}H]leucine into sessile and planktonic *E. coli* ATCC 25922 cells was measured; the cephalosporins were cefamandole (Eli Lilly & Co., Indianapolis, Ind.), cephaloridine (Sigma Chemical Co., St. Louis, Mo.), and ceftriaxone (Roche Research, Div. Hoffmann-La

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Roche Inc., Nutley, N.J.). Cefamandole and ceftriaxone were more effective against planktonic cells than against sessile cells, whereas cephaloridine was equally effective against sessile and planktonic cells (Fig. 1 and 2). Furthermore, as shown in Fig. 2, the radiochemical assay is very reproducible, the ceftriaxone experiments having been done on separate days.

A comparison between the effects of the cephalosporins on viability of bacteria in the biofilm and on incorporation of [^{3}H]leucine into bacteria in the biofilm was also made by comparing the concentration of antibiotic required to drop the viable count 2 orders of magnitude ($VC_{1\%}$; see Table 1) to the concentration of antibiotic required for a 50% inhibition of [^{3}H]leucine incorporation (II_{50}; see Table 1). As illustrated in Table 1, this comparison results in a good reproducible correlation between the two methods. Furthermore, it is important to note that the effectiveness of the antibiotics on biofilm cells did not correlate well with their MICs (Table 1).

Presently, we do not understand why the antibiotics induce levels of incorporation of [^{3}H]leucine into sessile cells greater than that of the control at low concentrations (Fig. 1 and 2), nor is it clear why when 99% of the sessile cells do not form colonies they still synthesize protein at 50% of the rate of the control (Table 1). Clearly, much is yet to be learned about the effects of antibiotics on the growth and metabolism of sessile bacteria on catheter material.

In summary, the [^{3}H]leucine method reported here is both simple and inexpensive and is less laborious than the viable count method. Furthermore, far more data can be obtained in less time. For example, routinely, the effects of six concentrations of five antibiotics on sessile cells can be tested in one working day as compared with a maximum of only three concentrations of three antibiotics by the viable count method in the same time. Moreover, viable count determination requires immediate processing, whereas after TCA precipitation samples need not be processed immediately. As an extra advantage, the [^{3}H]leucine assay allows determination of the effect of the antibiotics on planktonic cells with very little additional effort. In contrast, to make the same determination by the viable count method is a major undertaking.

TABLE 1. Comparison of antibiotic concentrations which cause a 50% inhibition of incorporation (II_{50}) and a 99% reduction in viable counts ($VC_{1\%}$) in biofilms

Antibiotic	$VC_{1\%}$ (μg/ml)[a]	II_{50} (μg/ml)[a]	MIC (μg/ml)[b]
Cefamandole	8.6 (9.4)	7.5 (9.7)	0.20
Ceftriaxone	0.4 (0.8)	0.4 (1.0)	0.01
Cephaloridine	4.5 (4.4)	3.4 (5.0)	5.0

[a] Viable counts and II_{50} values were determined on the same day. $VC_{1\%}$ values were extrapolated from semi-log plots of the data, whereas II_{50} values were extrapolated from data plotted as shown in the figures. In the absence of antibiotic, viable counts were routinely about 10^7 CFU per disk. Values in parentheses represent data from a second independent experiment.

[b] MICs were determined by the microtiter broth dilution method in MBDG inoculated with 10^5 CFU/ml.

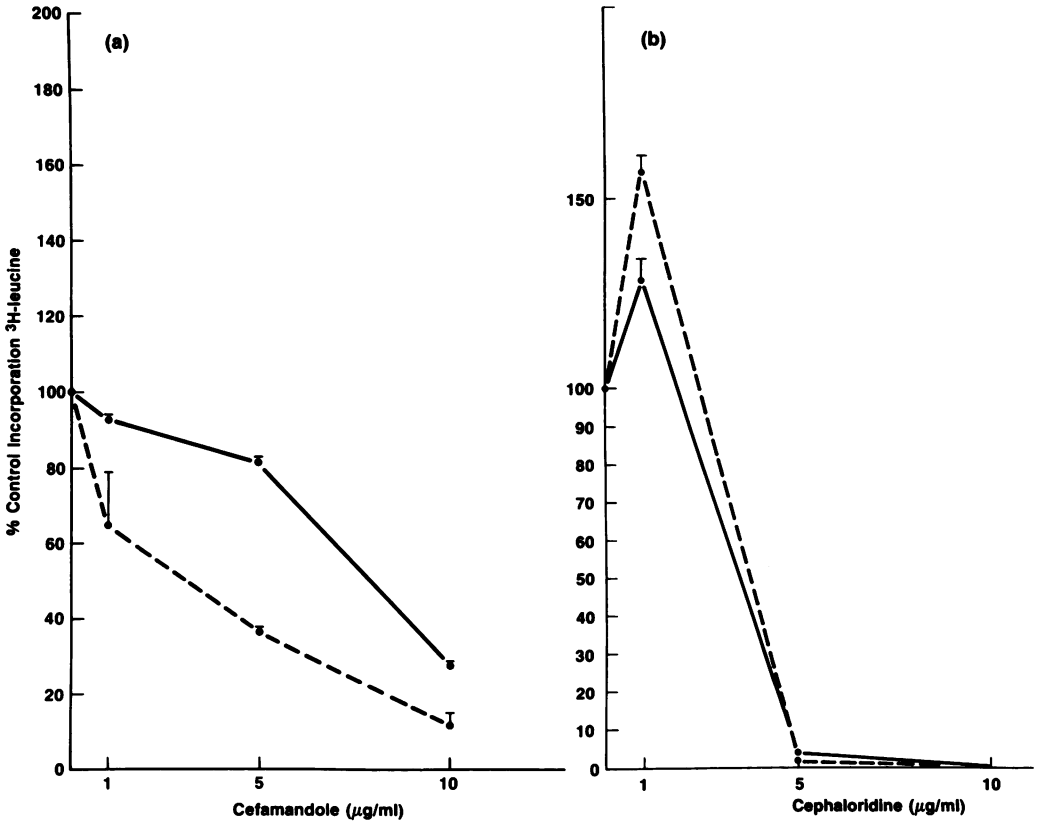

FIG. 1. Effect of cefamandole and cephaloridine on incorporation of [^{3}H]leucine into sessile and planktonic *E. coli* ATCC 25922. ——, Sessile cells; - - - - -, planktonic cells. (a) Cefamandole. Incorporation into sessile and planktonic cells in the absence of antibiotic was 8,975 cpm ± 636 and 16,121 ± 268 cpm, respectively. (b) Cephaloridine. Incorporation into sessile and planktonic cells in the absence of antibiotic was 6,508 ± 723 and 54,676 ± 2763 cpm, respectively. Each bar represents the standard error of the mean of duplicate samples.

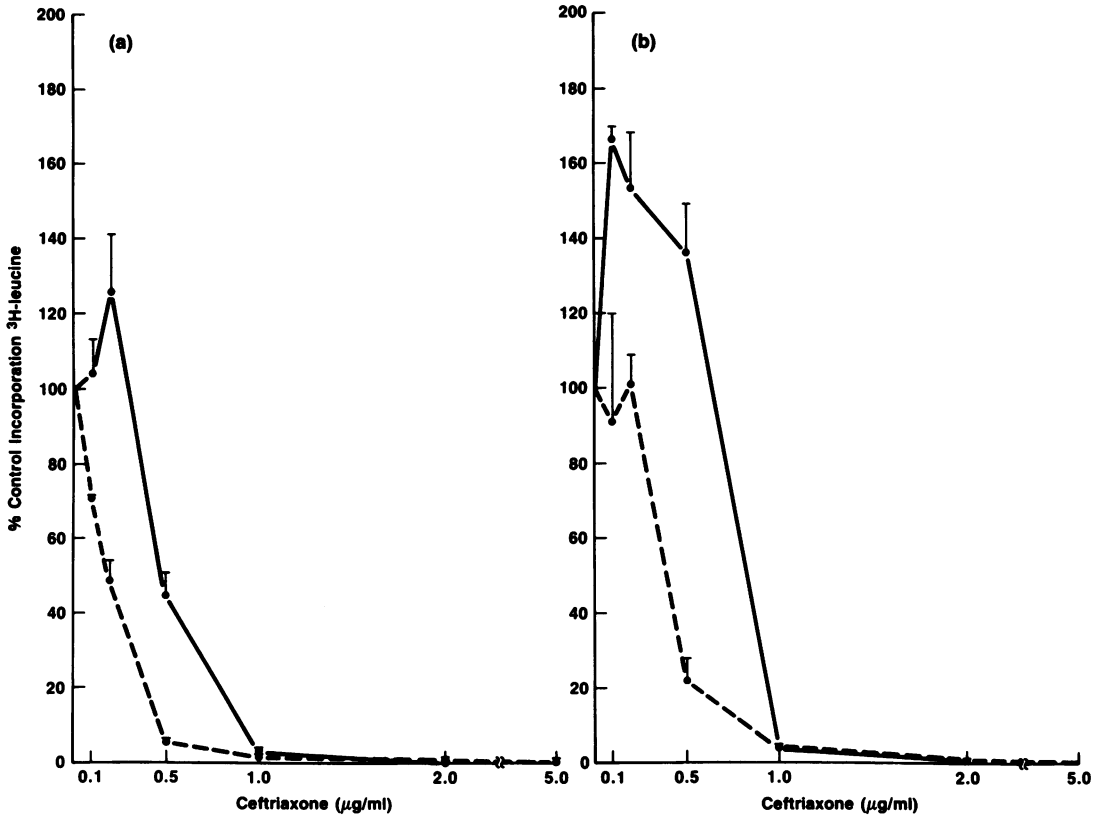

FIG. 2. Effect of ceftriaxone on incorporation of [^{3}H]leucine into sessile and planktonic *E. coli* ATCC 25922 in two separate experiments. ——, Sessile cells; - - - -, planktonic cells. The results shown in panels a and b were from experiments performed on separate days. Incorporation into sessile and planktonic cells in the absence of ceftriaxone was 6,508 ± 723 cpm and 54,676 ± 2763 cpm, respectively, in panel a and 6,137 ± 468 and 31,789 ± 1,061 cpm, respectively, in panel b. Each bar represents the standard error of the mean of duplicate samples.

Certainly, before the [^{3}H]leucine incorporation method can be accepted for widespread application, its efficacy must be confirmed by testing more antibiotics and other bacteria. Furthermore, it should be emphasized that we do not mean to imply that the [^{3}H]leucine incorporation method should completely replace the viable count method. We suggest, however, that the [^{3}H]leucine incorporation method can be used as a primary screen when there are many antibiotics to be tested. The effectiveness of those antibiotics which cause a very low II_{50} should then be confirmed by the viable count method.

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