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Automatic Radiometric Measurement of Antibiotic Effect on Bacterial Growth

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A new method for rapid, automatic radiometric measurement of antibiotic effects on bacterial growth was developed and compared with a conventional broth dilution technique. The radiometric method measures the amount of radioactive CO_2 generated by the bacterial metabolism of ^{14}C-glucose in the presence of antibiotics. Antibiotic effect on bacterial growth was standardized by measuring the evolution of $^{14}CO_2$ 3 hr after inoculation. This measurement was found to be quantitatively related to increasing concentration of antibiotic provided the organism was susceptible to the antibiotic tested. In 50 of 179 experiments (28%), each testing one organism against serial concentrations of an antibiotic, the concentration of antibiotic producing a 50% reduction of $^{14}CO_2$ within 3 hr after inoculation in comparison with a control culture was the same as the minimal inhibitory concentration (MIC) determined by the broth dilution technique. In 129 experiments (72%), the antibiotic concentrations inhibiting $^{14}CO_2$ release to 50% of the control level were less than the MIC values. Results of the radiometric method were related to those of the broth dilution method by constant factors characteristic of the organism and antibiotic tested. Our results indicate that the radiometric method provides a reproducible, quantitative, rapid, and sensitive measurement of the inhibitory effects of antibiotics on bacterial growth. The constant relationship between the results of the radiometric and conventional technique should facilitate the adaptation of the automated method to clinical testing of antibiotic susceptibility.

Currently accepted methods for the determination of antibiotic susceptibility are based upon the visual estimation of bacterial growth in the presence of antibiotics. Techniques employed include the use of antimicrobial discs, agar well diffusion, agar dilution, microtitration, and serial broth dilution (1, 5, 6). The serial broth dilution methods, which are generally considered the reference to which other methods are compared (4), require 24 to 48 hr to complete in most laboratories.

We have previously described an automated radiometric method for the detection of bacterial growth and its clinical application in blood cultures (2, 3). The present report describes the use of this principle for rapid automated quantitative measurement of antibiotic effect on bacterial growth.

MATERIALS AND METHODS

The antibiotics tested, with the bacterial species they were tested against given in parentheses, were as follows: ampicillin (*Escherichia coli, Staphylococcus aureus, Pseudomonas*), carbenicillin (*Enterobacter*), cephalothin (*E. coli, S. aureus*), chloramphenicol (*S. aureus*), cloxacillin (*S. aureus*), colistin (*E. coli, Enterobacter, S. aureus, Pseudomonas*), erythromycin (*E. coli, S. aureus*), gentamicin (*E. coli, Enterobacter, S. aureus, Pseudomonas*), kanamycin (*E. coli, Enterobacter*), lincomycin (*S. aureus*), methicillin (*S. aureus*), nafcillin (*S. aureus*), oxacillin (*S. aureus*), penicillin (*S. aureus*), streptomycin (*E. coli, S. aureus, Pseudomonas*), tetracycline (*E. coli, Enterobacter. S. aureus*), vancomycin (*S. aureus*), and novobiocin (*S. aureus*). Commercially available stock standards of the antibiotics were procured in a desiccated form and were stored at 4 C. Weighed quantities of each antibiotic were dissolved in sterile distilled water at a concentration of 1,000 μg/ml. The stock solutions were stored in 1.4-ml portions at −20 C and were used within 2 weeks of preparation. On the day of each experiment, samples were thawed and diluted in nutrient broth.

Two of the organisms chosen for testing, an *E. coli* strain and an *S. aureus* strain, are widely used standards for susceptibility testing. Three organisms, *S. aureus* 213JHML, *Enterobacter* 2JHML, and *P. aeruginosa* 2JHML, are used routinely in the Johns Hopkins Microbiology Laboratory as added standards for the same purpose. Members of the other two

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genera studied, *Enterobacter* (8 strains) and *Klebsiella* (3 strains), comprise 11 pathogenic strains isolated from patients at Johns Hopkins Hospital.

Overnight cultures of each organism were diluted to a turbidity equal to that of a no. 2 McFarland barium sulfate standard, equivalent to 1.5×10^8 organisms per ml. Cultures were further diluted to achieve approximate concentrations of 10^6 organisms per ml (gram-negative species) and 10^7 organisms per ml (gram-positive species). Cell numbers were confirmed by pour plate colony counts. Samples from the final dilutions were used as inocula for susceptibility testing. Gram-negative species were tested as inocula of 10^5 organisms/ml and gram-positive species as inocula of 10^6 organisms/ml. Measurement of antibiotic effect on bacterial growth of one or more organisms in nutrient broth was determined for each antibiotic by a standard broth dilution method and by the automatic radiometric technique, run in parallel.

Broth dilution method. In the broth dilution method, appropriate antibiotic solutions in selected serial concentrations were prepared daily by serially diluting the stocks in sterile water. Samples (0.1 ml) from each concentration of antibiotic to be tested were then added to a series of tubes containing 0.8 ml of nutrient broth; 0.1 ml of bacterial inoculum was then added to each tube. The final dilutions produced serial 50% reductions in antibiotic concentration corresponding to serial broth dilutions. One tube without antibiotic was used as a growth control. The tubes were incubated at 37 C for 48 hr. The lowest concentration of antibiotic in a tube without visible bacterial growth at 48 hr after inoculation was considered the minimal inhibitory concentration (MIC).

Radiometric method. The radiometric method measures the amount of radioactive CO_2 generated by the bacterial metabolism of ^{14}C-glucose. Serial measurements at hourly intervals are taken by an instrument (Bactec Bacterial Growth Detector, Johnston Laboratories, Cockeysville, Md.) that permits handling of 25 samples automatically. Sealed glass vials containing specimens to be tested are inserted into a circular holding tray within an enclosed incubation chamber kept at 37 C. Release of $^{14}CO_2$ into the air within the vial is facilitated by the constant stirring action of a magnetic rod. The tray containing 25 samples revolves, so that each vial is brought into position underneath two sampling needles, which descend through the rubber stopper into the air above the culture broth. While the needles are in the vial, 200 ml of filtered air is flushed through the system, forcing the air within the culture vial into an ionization chamber. The $^{14}CO_2$ formed by bacterial metabolism is measured by the ionization chamber, which produces an electrical potential that is proportional to the amount of radioactivity detected. The electrical potential is converted to a digital index between 0 and 99, which is printed on paper tape for a permanent record. An index of 99 is equivalent to 0.025 μCi of $^{14}CO_2$ or 2.5% of the total amount of radioactive glucose (1.0 μCi) within each culture vial. An index of 20, our established threshold for detection of bacterial growth (5), is equivalent of 0.005 μCi, or 0.5% of the substrate used. The measurement cycle for each sampling required 72 sec, or 30 min for 25 vials.

The vials used for susceptibility testing each contained 10 ml of nutrient broth, 10 μCi of ^{14}C-glucose, and a magnetic stirring rod. Samples from appropriate antibiotic solutions prepared from the stock were added to the vials to produce the desired antibiotic concentrations. A 1-ml amount of bacterial inoculum was then added to each vial, producing a series of cultures with concentrations of antibiotic and bacteria identical to those present in the broth dilutions. In each experiment, one culture without antibiotic was used as a control to measure the $^{14}CO_2$ released by bacterial growth of each organism in the absence of antibiotics.

Immediately after inoculation, the vials were inserted into the incubation chamber of the instrument and sampled hourly for the evolution of radioactive CO_2. Table 1 shows data as recorded in a typical study. The numbers in the central four columns are indexes of bacterial growth proportional to the amount of radioactive CO_2 released from the cultures at 1, 2, 3, and 4 hr after inoculation. Indexes below 20, lower than our threshold for a positive reading, are represented as dashes. The columns at the right indicate the presence (+) or absence (0) of growth in the corresponding broth dilutions 24 and 48 hr after inoculation.

To standardize the technique, we selected from our results the minimal concentration of antibiotic in each experiment that produced at least a 50% reduction of the release of radioactive CO_2 at 3 hr after inoculation in comparison with a control. We compared that concentration with the minimal concentration in the broth dilutions that produced no visual evidence of bacterial growth at 48 hr (the MIC). In the experiment illustrated in Table 1, there was at least a 50% reduction of release of $^{14}CO_2$ 3 hr after inoculation from the culture vial containing 1.56 μg of methicillin per ml. The MIC determined by the broth dilution method at 48 hr

TABLE 1. *Comparison of serial radiometric growth indexes with broth dilution results in a typical experiment*[a]

Methicillin concn (μg/ml)	Serial growth index				Broth dilution result	
	1 hr[b]	2 hr	3 hr	4 hr	24 hr[b]	48 hr
6.25	—	—	—	—	0	0
3.12	—	—	—	—	0	0
1.56	23	24	22	19	+	+
0.78	29	43	56	58	+	+
0.39	31	57	89	98	+	+
0.00[c]	30	48	69	95	+	+

[a] The results shown were obtained with *Staphylococcus aureus* strain 213.
[b] Hours after inoculation.
[c] Control.

was 3.12 μg/ml, and was within one serial twofold dilution of the radiometric result.

RESULTS

In 179 experiments, each testing a single organism against one of the antibiotics listed in Materials and Methods, there was a quantitative relationship between the amounts of radioactive CO_2 released from the cultures and serial concentrations of the antibiotic tested, provided the organism was susceptible to the antibiotic. In these experiments, antibiotic effect on the release of CO_2 was always detectable within 3 hr after the beginning of testing by comparison of the $^{14}CO_2$ indexes from those cultures containing antibiotics with the indexes from the control cultures. In 50 experiments (28%), the antibiotic concentrations producing a 50% reduction of $^{14}CO_2$ released at 3 hr after inoculation were the same as the MIC determined by the serial broth dilution method at 48 hr. In 129 experiments (72%), the antibiotic concentrations inhibiting $^{14}CO_2$ release to 50% of the control level were less than the MIC. These data are illustrated in Fig. 1.

To determine the reproducibility of the radiometric method and compare it with that of the broth dilution technique, we repeatedly tested 11 different combinations of organisms and antibiotics by both methods in parallel. Each combination was tested 10 times on 10 different days. As can be seen in Table 2, the reproducibility of the radiometric technique was at least as good as that of the broth dilution method. The repeated results were reproducible to within one serial twofold dilution in every instance.

Analysis of the data in Table 2 discloses an apparently constant relationship between the concentration of antibiotic producing 50% reduction of $^{14}CO_2$ 3 hr after inoculation and the MIC determined by the broth dilution method. In our study, this relationship was characteristic of the organism and antibiotic tested. From these data, the relationship can be expressed as the number of serial dilutions that separate the results of the radiometric method from those of the broth dilution technique. These numbers can be used as conversion factors relating the results of the two techniques. Some examples, derived from the data in Table 2, are illustrated in Table 3.

Results from experiments in which the organ-

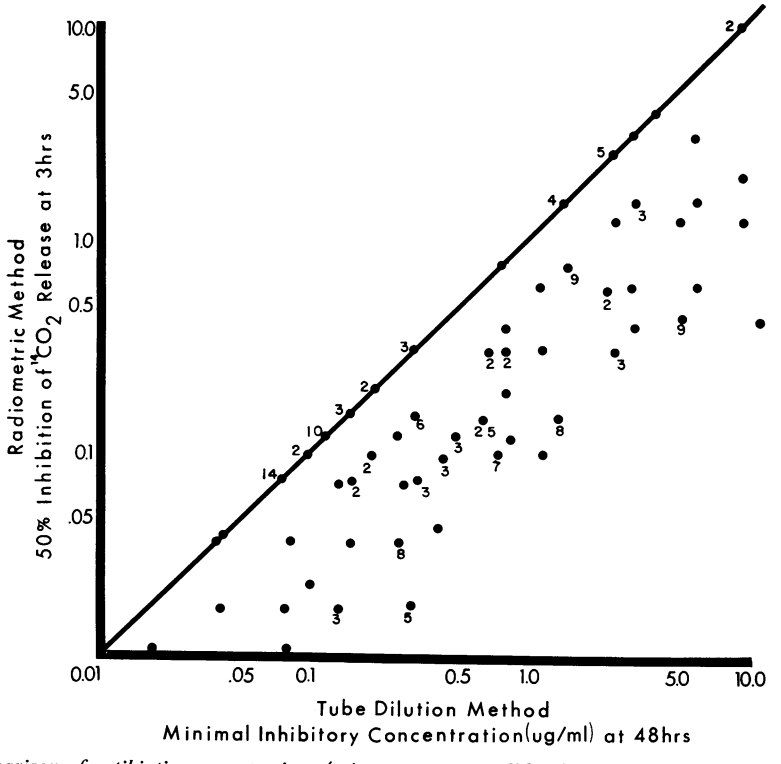

FIG. 1. *Comparison of antibiotic concentrations (micrograms per milliliter) causing 50% reduction of $^{14}CO_2$ release measured by the radiometric method at 3 hr with the minimal inhibitory concentrations determined by the broth dilution method at 48 hr.*

TABLE 2. *Results from serially repeated tests comparing the reproducibility of the radiometric and broth dilution methods*

Combination te ted	Radiometric method		Broth dilution method	
	Drug concn[a] (μg/ml)	No. of tests	MIC (μg/ml)	No. of tests
Erythromycin vs. *Staphylococcus*	0.039	8	0.156	1
	0.078	2	0.312	9
Tetracycline vs. *Staphylococcus*	0.156	10	0.625	6
			1.25	4
Lincomycin vs. *Staphylococcus*	0.156	10	0.625	9
			1.25	1
Methicillin vs. *Staphylococcus*	1.25	6	2.5	10
	2.5	4		
Cephalothin vs. *Staphylococcus*	0.1	5	0.2	4
	0.2	5	0.4	8
Ampicillin vs. *Staphylococcus*	0.16	8	0.16	7
	0.08	2	1.08	3
Chloramphenicol vs. *E. coli*	0.625	10	5	9
			10	1
Kanamycin vs. *E. coli*	0.156	10	0.625	7
			1.25	3
Streptomycin vs. *E. coli*	0.2	8	0.8	9
	0.4	2	1.6	1
Gentamicin vs. *Enterobacter*	0.2	8	0.16	6
	0.4	2	0.32	4

[a] Concentration causing 50% inhibition of $^{14}CO_2$ release.

TABLE 3. *Dilution factors relating radiometric to broth dilution results*

Combination tested	No. of serial dilutions
Erythromycin vs. *Staphylococcus*	+3
Tetracycline vs. *Staphylococcus*	+2
Lincomycin vs. *Staphylococcus*	+2
Methicillin vs. *Staphylococcus*	+1
Cephalothin vs. *Staphylococcus*	+1
Ampicillin vs. *Staphylococcus*	0
Chloramphenicol vs. *E. coli*	+3
Kanamycin vs. *E. coli*	+2
Streptomycin vs. *E. coli*	+2
Gentamicin vs. *Enterobacter*	+3

isms tested were resistant to the antibiotics used usually demonstrated no inhibition of CO_2 release within 3 hr after inoculation; these results corresponded to bacterial growth in the broth dilutions at 24 and 48 hr. One exception was a group of experiments in which penicillin was tested against pencillinase-producing strains of *S. aureus*. In 12 strains of this species, penicillin caused prolonged delay of $^{14}CO_2$ release, in concentrations as low as 0.625 units of penicillin/ml, though there was bacterial growth, as evidenced by turbidity, in concentrations as high as 10 μg/ml in the broth dilutions at 48 hr. In these experiments, there was invariably release of $^{14}CO_2$ at a later time from those cultures containing higher concentrations of antibiotics corresponding with the concentrations permitting bacterial growth in the broth dilutions. Data for these strains are summarized in Table 4. The concentrations of antibiotic diminishing $^{14}CO_2$ release 3 hr after inoculation to 50% of the control level are compared with later radiometric readings and with the broth dilution results.

The effects of antibiotics against organisms found in blood cultures from four patients in Johns Hopkins Hospital were tested radiometrically, each against a range of three concentrations of different antibiotics. The tests were performed on the morning the cultures were noted to be positive. Table 5 lists the organisms and antibiotics tested and the concentrations of antibiotics which produced 50% reduction of the evolution of radioactive CO_2 in comparison with the control 3 hr after the beginning of testing.

Serial dilutions of serum from two patients receiving antibiotic therapy for septicemia were similarly tested against the respective organisms isolated from the patients. Inhibitory effects were noted radiometrically within 3 hr after inoculation up to a 1:80 dilution of one patient's serum against a *Klebsiella* strain and up to a dilution of 1:100 of the other patient's serum against

TABLE 4. *Comparison of 3-hr and later radiometric readings with broth dilution results in experiments testing the effect of penicillin concentrations ranging from 0.625 to 10 μg/ml on penicillinase-producing Staphylococcus aureus strains*

Strain[a]	3-hr readings[b]	Later readings		Broth dilution result[d]	
		Antibiotic concn[c] (μg/ml)	Time of delayed reading (hr)	24 hr	48 hr
213	2.5	10.0	10	10.0	10.0
O.S. −49	1.25	5.0	24	10.0	10.0
		10.0	48		
72-01 (0.064)	1.25	5.0	19	10.0	10.0
		10.0	48		
71-12 (0.043)	1.25	2.5	22	2.5	10.0
		10.0	48		
72-12 (0.035)	1.25	5.0	20	5.0	10.0
		10.0	48		
71-11 (0.002)	2.5	5.0	6	10.0	10.0
		10.0	8		
71-09 (0.002)	0.625	10	48	2.5	10.0
72.01 (0.66)	0.625	2.5	48	2.5	10.0
		10	48		
7	0.625	2.5	48	1.25	5.0
F-8739	0.625	2.5	22	1.25	10.0
		10.0	48		
O.S. −37	1.25	10	17	10	10
71-11 (0.001)[e]	0.625	1.25	23	0.625	5.0
		5.0	24		

[a] Strain designations are Johns Hopkins Hospital identification numbers.
[b] Results expressed as the antibiotic concentration (micrograms per milliliter) causing a 50% reduction of $^{14}CO_2$ release.
[c] Concentration causing $^{14}CO_2$ release equal to the control reading at 3 hr.
[d] Expressed as the maximal antibiotic concentration (micrograms per milliliter) showing bacterial growth.
[e] Low penicillinase.

a *S. aureus* strain. The broth dilution method showed no growth at 24 hr up to the 1:60 and 1:100 dilutions, respectively.

DISCUSSION

The results of our experiments suggest that the radiometric method may provide a faster and more sensitive measure of the inhibitory effects of antibiotics on bacterial growth than techniques in current use. The method can automatically provide data relevant to the quantitative determination of antibiotic susceptibility within 3 hr of the detection of bacterial growth, a considerable saving of time in comparison with current serial broth dilution techniques, whether they are interpreted at 24 or 48 hr.

Our results showed rapid inhibition of $^{14}CO_2$ release by the antibiotics to which organisms were susceptible by standard techniques. We chose 50% reduction of $^{14}CO_2$ release at 3 hr after inoculation to standardize our technique, to serve as a basis of comparison with the serial broth dilutions, and to facilitate use of the rapid results provided by the radiometric method. The finding that our results can be related to those of standard serial dilution techniques by constant factors should facilitate the adaption of the radiometric method to routine testing of bacterial susceptibility to antibiotics.

The radiometric technique depends upon measuring the inhibitory effects of antibiotics on glucose metabolism evidenced by the release of $^{14}CO_2$ as a by-product. In all bacterial strains tested, except penicillinase-producing *S. aureus* strains, we found an excellent correlation between rapid suppression of glucose metabolism and absence of visual evidence of bacterial growth at 48 hr after inoculation. The effect of penicillin against penicillinase-producing *S. aureus* strains was different. In these experiments, though penicillin caused rapid suppression of glucose metabolism, there was invariably release of $^{14}CO_2$ in delayed readings up to 48 hr, which correlated with turbidity in the broth

TABLE 5. *Radiometric measurements of antibiotic effects on the growth of organisms found in blood cultures from four patients at Johns Hopkins Hospital*

Organism	Antibiotic	Concn (μg/ml) producing 50% inhibition of CO_2 production at 3 hr
Escherichia coli (E-924)	Kanamycin	0.625
	Colistin	0.625
	Tetracycline	0.3125
	Chloramphenicol	1.25
	Cephalothin	1.25
	Ampicillin	1.0
	Streptomycin	0.625
	Erythromycin	>2.5
Klebsiella (C-524)	Kanamycin	0.625
	Colistin	0.625
	Tetracycline	>20.0
	Chloramphenicol	30.0
	Cephalothin	20.0
	Ampicillin	2.0
	Streptomycin	5.0
	Penicillin	>10.0
Staphylococcus aureus (C-951)	Lincomycin	1.25
	Erythromycin	0.625
	Penicillin	1.25
	Methicillin	5.0
	Cephalothin	1.25
	Chloramphenicol	5.0
	Streptomycin	0.625
	Tetracycline	0.312
S. epidermidis (C-593)	Methicillin	2.5
	Cephalothin	0.156
	Lincomycin	1.25
	Erythromycin	0.156
	Penicillin	2.5
	Streptomycin	0.625
	Clindamycin	0·156

dilutions. Therefore, in testing strains of *S. aureus* against penicillin, early suppression of $^{14}CO_2$ released could not be used as evidence of sufficient antibiotic effect for clinical purposes. The evidence of delayed glucose metabolism in these experiments apparently correlated well with the resistance of the bacteria to penicillin demonstrated by the broth dilution technique.

We have attributed this effect to the action of the penicillinase frequently associated with *S. aureus*. However, its mechanism remains unknown to us. We have also tested strains of other penicillinase-producing organisms (*Klebsiella* and *E. coli*) against penicillin. Results from these experiments show no inhibition of $^{14}CO_2$ release.

Most clinically significant bacteria metabolize ^{14}C-glucose in quantities sufficient to produce enough $^{14}CO_2$ for detection by the radiometric method. In our experience with 2,967 blood cultures processed by the radiometric method (2), the accuracy of the radiometric method employing ^{14}C-glucose as the only substrate compared favorably with routine bacteriological techniques. We are currently making use of a combined substrate containing other ^{14}C-labeled substrates in addition to ^{14}C-glucose for the detection of unusual organisms that do not metabolize glucose. Results to date are very encouraging, and suggest that the use of this combined substrate will also permit application of radiometric susceptibility testing to these organisms.

Since MIC values determined by serial dilution techniques are frequently used as guidelines to regulate antibiotic therapy, the rapidity of the method of quantitative susceptibility testing that we have described may lead to improved correlation between treatment regimens and in vitro testing. For example, in our application of the radiometric method to 2,967 blood cultures (2), 65% of the positive cultures were detected on the day of inoculation. In those patients with symptoms and signs suggesting a high probability of septicemia, e.g., a history of rheumatic fever, recent dental extraction, chills, fever, and a heart murmur, it may be possible to obtain quantitative information much more rapidly by inoculating media containing antibiotics at the same time as the initial radiometric cultures. Comparison of the results from cultures containing antibiotics with those from cultures without antibiotics might be capable of providing quantitative antibiotic susceptibility data within 1 day.

Vials containing precise quantities of antibiotics in dried stable form can be manufactured. Testing a number of antibiotics at different concentrations would be accomplished by simply adding to the vials a specified volume of liquid medium containing radioactive glucose and the bacterial inoculum. The amount of radioactivity required is extremely small, and proper handling of solutions containing ^{14}C, a beta emitter, is nonhazardous to the laboratory worker. The technique offers the potential to introduce automation to quantitative antibiotic susceptibility testing. The reduction in the amount of personnel time required and the ability to perform large numbers of tests simultaneously and automatically would make quantitative susceptibility testing more readily available.

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