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Detailed Methodology and Implementation of a Semiautomated Serial Dilution Microtechnique for Antimicrobial Susceptibility Testing

JAMES D. MACLOWRY, MARY J. JAQUA, AND SALLY T. SELEPAK

Clinical Pathology Department, Clinical Center, National Institutes of Health, Bethesda, Maryland 20014

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The detailed methodology and implementation of a semiautomatic microtechnique for performing serial dilution antimicrobial susceptibility studies are described. Quantitative susceptibility studies to a battery of antimicrobials are performed routinely on all significant clinical isolates. Results are reported as the minimal inhibitory concentration in micrograms per milliliter of broth. Guidelines relating standard doses of antimicrobials with expected blood and urine levels are presented to facilitate the use of the quantitative data. This microtechnique is used to measure serum and other body fluid levels of antimicrobial agents to document the level attained with a specific course of therapy. This technique is highly reproducible and has a high correlation with, and is at least 10 times faster than, standard glass tube techniques.

A microtechnique for routine serial dilution antimicrobial susceptibility testing has been developed and implemented in this laboratory. Widespread interest and continuing experience with this technique dictate a detailed description of the methodology including equipment used, methods of equipment calibration, sterilization procedures, media, sources and preparation of antimicrobial agents, operating details, quality control procedures, and the methods of determining antibacterial levels in body fluids. A brief discussion of the actual incorporation of this method into the clinical laboratory is included to assist others who may wish to introduce this quantitative technique into their routine.

EXPERIMENTAL

Equipment. The autodiluter (4) is a semiautomatic instrument designed by the Biomedical Engineering and Instrumentation Branch, Division of Research Services, National Institutes of Health, which performs serial microdilutions. A commercial version of this instrument is available (American Instrument Co., Silver Spring, Md.). This instrument holds 12 50-μliter microdiluters (Cooke Engineering Co., Alexandria, Va.) which are used to make serial twofold dilutions in Microtiter plates (Cooke Engineering Co.). A hand-held multidiluter handle frame is available and has accuracy comparable to the autodiluter but is slower and more cumbersome to use with large numbers of plates.

The 50-μliter microdiluters have been found to have a mean volume of 50.4 μliters and standard deviation (SD) of 0.65 with 0.9% NaCl. After 8 months of use, the mean volume was 51.6 with an SD of 0.64. When Trypticase soy broth or serum is used, the mean volumes are 49.4 and 49.9 μliters, and the SD is 0.36 and 0.71, respectively. Each morning before use, the microdiluters are flamed to incandescence, quenched in distilled water, touched to blotting paper to remove most of the water, and flamed briefly to dryness. After this procedure, they are ready for use.

Disposable flex vinyl "U"-bottom plates (Cooke Engineering Co.) with 96 wells, each with a capacity of 250 μliters, are used for the dilutions. The "U"-bottom plate is preferred to the "V"-bottom plate because the latter has a small button of plastic at the bottom of each well which interferes with reading of the end point. Rigid polystyrene "U"-bottom plates, both clear and frosted, are commercially available but are not used in this laboratory because the end points are more difficult to read. The flex vinyl "U"-bottom plates are sterilized with ethylene oxide and allowed to remain at room temperature for at least 2 days before use. Sterilization tape is placed on each lot to detect adequate sterilization. There has been no evidence of inhibition of

bacterial growth or alteration of antimicrobial potency with this type of plate.

Calibrated 50-μliter pipettes (Cooke Engineering Co.) are used to dispense diluent broth and the test organism. These pipettes are calibrated to deliver 50 μliters of 0.9% NaCl per drop. Fluids having surface tensions different from 0.9% NaCl cause the drop size to vary accordingly. The average drop size determined gravimetrically of 0.9% NaCl is 50.0 μliters; of human serum, 48.8 μliters; of Mueller-Hinton broth, 41.1 μliters; and of Trypticase soy broth, 40.8 μliters. The decreased size of the drop is quite consistent and depends on the type of solution used. Although a smaller drop alters the accuracy of twofold dilutions, no specific correction is made since the error is small and consistent throughout all dilutions. After the pipettes have been used, they are placed in a solution of Amphyl (National Laboratories, Lehn and Fink Industrial Products Division, Sterling Drug Co., Inc.) for a period of 2 to 6 hr, rinsed three to four times in distilled water, turned upside down, and allowed to dry overnight before resterilization. Each pipette is wrapped in a paper towel, closed with masking tape, gas sterilized with ethylene oxide in a manner similar to the plates, and allowed to remain at room temperature for 1 to 2 days before reuse.

Much of the initial work in this laboratory was performed by dispensing the 50 μliters of diluent broth with the calibrated pipettes. Recently an instrument (designed and built by Biomedical Engineering and Instrumentation Branch, Division of Research Services, National Institutes of Health) which dispenses 50 μliters of broth by positive displacement has been used. Since this is a prototype, it will not be described in detail. A commercially available hand or pneumatic-operated dispensing device (Astec Inc., Orange, Conn.) was tested briefly in this laboratory. This instrument gave results comparable to the calibrated pipette and was somewhat faster in its operation. There were difficulties in using this device because the broth media tended to clog some of the internal parts. Newer versions of this instrument may not have this problem.

The accuracy of the twofold dilutions in an aqueous medium was determined by diluting a standard sodium chloride solution and measuring in duplicate the chloride concentration in each of the wells by using the Aminco-Cotlove Titrator. The eighth dilution was ± 5% of the calculated value, and all of the intervening values were less than this deviation. The first few dilutions were within ± 1% of the calculated chloride concentration.

Media. Trypticase soy broth is used for most antimicrobial susceptibility studies. It is stored at 4 C in screw-top bottles and tubes to prevent interference from cotton fibers which are frequently found when cotton plugs are used. Other media are selected depending on the growth requirements of the organism to be tested. Trypticase soy broth with 5% Yeast Hemin Extract (BBL, Division of Bioquest, Cockeysville, Md.) is used for hemolytic and nonhemolytic *Haemophilus*. Todd-Hewitt broth is used for beta-hemolytic and occasionally for alpha-hemolytic streptococci. No protein or *p*H indicators are added to any of the media.

Antimicrobial agents. The most cumbersome aspect of the method is the procurement and preparation of antimicrobial standards. The different antimicrobial agents are provided generously by many pharmaceutical firms, usually as secondary reference standards. The methods of preparation of the antimicrobial standards are described in detail in the Code of Federal Regulations (2) and are summarized in Table 1 with the modifications used in this laboratory. None of these antimicrobial solutions is sterilized after preparation and there has been no problem with bacterial contamination. All of the buffers, weighing vials, and plastic containers for the standards are sterile.

After the antimicrobial standards have been prepared, portions of approximately 1 ml are put into sterile Lexan cups and frozen at −20 C. The Lexan cups are held in metal racks which are stored inside ultraviolet (UV)-sterilized rigid plastic boxes for ease of handling. The cups are not sealed while in the plastic boxes, so it is important that they not be stored in automatic defrosting refrigerators, since this will cause dehydration and loss of diluent from the standard. Antimicrobial solutions are prepared in sufficient volume for 4 weeks. The two exceptions to this are ampicillin and carbenicillin, which must be prepared every 14 days or stored at −120 C to maintain potency for at least 4 weeks.

The choice of antimicrobial agents to test is frequently related to local preferences and usage. The standards are stored in three groups: those used for gram-positive organisms, those for gram-negative organisms, and those for organisms isolated from the urine. The antibiotics included for the gram-positive organisms include penicillin G, ampicillin, oxacillin, erythromycin, cephalothin, tetracycline, kanamycin, carbenicillin, gentamicin, and lincomycin. Those which are used for gram-negative organisms are penicillin G, ampicillin, cephalothin, tetracycline, chloramphenicol, streptomycin, kanamycin, colistin,

TABLE 1. *Preparation of antimicrobial standard solutions, summary of Code of Federal Regulations*

Antimicrobial agent	Solvent	Diluent[a]	Drying requirements for antimicrobial powder[b]
Ampicillin, sodium		0.1 M Potassium phosphate buffer, *p*H 8	
Cephalothin		1% Potassium phosphate buffer, *p*H 6	60 C, 5 mm of Hg pressure, 3 hr
Chloramphenicol	Ethyl alcohol	1% Potassium phosphate buffer, *p*H 6	
Cloxacillin, sodium		1% Potassium phosphate buffer, *p*H 6	
Colistin sulfate	Distilled water	10% Potassium phosphate buffer, *p*H 6	60 C, 5 mm of Hg pressure, 3 hr
Erythromycin	Methyl alcohol	0.01 M Potassium phosphate buffer, *p*H 8	60 C, 5 mm of Hg pressure, 3 hr
Gentamicin sulfate		0.1 M Potassium phosphate buffer, *p*H 8	110 C, 5 mm of Hg pressure, 3 hr
Kanamycin sulfate		0.01 M Potassium phosphate buffer, *p*H 8	
Lincomycin		Distilled water	
Methicillin, sodium		1% Potassium phosphate buffer, *p*H 6	
Nalidixic acid	1 N NaOH	Distilled water	
Neomycin sulfate		0.1 M Potassium phosphate buffer, *p*H 8	60 C, 5 mm of Hg pressure, 3 hr
Nitrofurantoin		Distilled water	
Oxacillin, sodium		1% Potassium phosphate buffer, *p*H 6	
Penicillin G, sodium		1% Potassium phosphate buffer, *p*H 6	60 C, 5 mm of Hg pressure, 3 hr
Polymyxin B sulfate	Distilled water	10% Potassium phosphate buffer, *p*H 6	60 C, 5 mm of Hg pressure, 3 hr
Streptomycin		Distilled water	60 C, 5 mm of Hg pressure, 3 hr
Tetracycline	0.01 N HCl	1% Potassium phosphate buffer, *p*H 6	
Vancomycin		Distilled water	60 C, 5 mm of Hg pressure, 3 hr

[a] In this laboratory, 0.1 M potassium phosphate buffer is used whenever potassium phosphate buffer is designated.

[b] In this laboratory, all standards which require drying are dried at 60 C for 3 hr at reduced pressure of approximately 380-mm Hg.

carbenicillin, and gentamicin. If the organism to be tested is from the urinary tract, then penicillin G, ampicillin, oxacillin, cephalothin, streptomycin, kanamycin, tetracycline, gentamicin, colistin, nalidixic acid, nitrofurantoin, and carbenicillin are used. The sulfonamides are not tested by serial dilution broth technique since an adequate method is not available, but are tested by a disc technique on Mueller-Hinton agar.

Operating conditions. The autodiluter is under continuous UV irradiation in a hood, except during the time that dilutions are made. The UV light is turned on if the technologist leaves the instrument for more than a 15-min interval. The autodiluter has been operated up to 6 hr at a time without theUV light with no contamination from the hood environment. A few boxes of sterile plates are stored in the hood during the operation of the machine and are opened one at a time. A supply of wrapped sterile pipettes is kept in the hood and they are opened individually as needed. Each morning the plastic boxes with the racks of antimicrobial standards are removed from the freezer and placed in the hood to thaw. Once thawed, they are either used immediately or stored at 4 C until use later that day. Any antimicrobial solutions remaining at the end of the day are stored at 4 C overnight so that they are available for further evaluation if the quality control organisms reveal an unexpected problem. The residual solution is used the second day and discarded after that time.

The method of preparation of the plates has been described previously (4) in more detail. A 50-μliter amount of diluent broth is added to each well of a sterile plate. The plate is placed on the autodiluter, and the microdiluters are loaded with the antimicrobial agent by touching to the surface of the standards. The microdiluter chambers are checked visually to be sure that no bubbles are present. The dilution process is begun by lowering the microdiluters into the first row of wells and automatically rotating six times. As the dilutions are made one row after the next, the operator observes the height of diluent in each of the wells to be certain that it does not rise above the top of the microdiluter chamber. This is done at a glance after the operator has become familiar with the technique and serves as a safeguard in case the diluent has inadvertently been doubled or not added to a well. The height to which the diluent rises on the microdiluter chamber is constant and is seen as a ring of reflected light around the chamber. When the dilutions are completed, the microdiluters are rotated in sterile distilled water, blotted on filter paper, and flamed briefly to dryness. It is not necessary to reflame the diluters to incandescence at the end of each dilution. With the plate still on the plate holder, 50 μliters of an approximate 1:1,000 dilution of the organism suspension to be tested is added to each well with a calibrated pipette. The approximate 1:1,000 dilution is made from a moderately turbid suspension which has been prepared by mixing 6 to 10 isolated colonies in 2 ml of broth. A 1:10,000 dilution is used with *Proteus* species since the end points are much sharper and more reproducible with the smaller inoculum size. After the diluted suspension of organisms has been added to each well, the plate is immediately sealed with plastic sealing tape on a tape-sealing stand (Cooke Engineering Co.). This tape sealing stand has been modified to allow punching of holes in the tape. After the tape has been smoothed down with a roller, a specially designed hole puncher is used to make a small hole over each well. The hole puncher is flamed to sterilize its 96 needles. The tape covering the plate is cut from the roll. The plate is then removed and labeled with the appropriate accession number and with the name and source of the organism. The antimicrobial standards are identified across the plate as an aid in more rapidly reading the end points.

Usually 12 antimicrobial agents are used at one time and no positive or negative controls are included. When the technique was first used, positive and negative controls were included on all plates, but this was found unnecessary. With the wide variety of agents used, there will usually be growth at some concentration which serves as a positive internal control, and those having no growth serve as negative controls. The plates are incubated, usually at 37 C for approximately 18 hr, but the conditions of incubation are varied, depending on the organisms being tested. Increased carbon dioxide, anaerobiosis, or lower temperatures are used for appropriate cultures.

The plates are read by placing on a test reading stand (Cooke Engineering Co.) and overlaying a plastic sheet lined with thin black strips which produce a dark background. The use of the plastic sheet has greatly facilitated identification of the end points. The end point or minimal inhibitory concentration (MIC) is defined as that well which has the lowest concentration of antimicrobial agent in which there is no macroscopic bacterial growth. Bacterial growth in this system is usually manifested either by a button of bacteria which has settled to the bottom of the well, generalized turbidity of the medium, or a combination of the two. Some organisms which grow as very small granules or streaks in the well may be difficult to see. Frequently staphylococci with erythromycin and many different organisms with tetracycline and chloramphenicol are more difficult to interpret because there is a small amount of growth which occurs in many of the wells. An obvious difference between the usual growth pattern and the tiny button of organisms is easily seen. For this type of growth, the end point has been arbitrarily designated as the lowest concentration in which the button is less than 0.5 ml in diameter. By using this criterion, reproducible results are obtained by different observers. The MIC is recorded for each of the agents with the tested organism, and these results are reported in micrograms per milliliter of broth.

If the organism is a rapid grower, a preliminary reading can often be made after 4 to 6 hr of incubation. The requesting physician is advised that the final reading may be somewhat different, but usually the results do not differ by more than two dilutions.

Minimal bactericidal levels (MBC) are not routinely performed unless specifically requested, or if the clinical situation justifies such determinations. To perform an MBC for a given antimicrobial and organism, the tape over wells showing no growth is removed by touching with a heated loop. The contents of the well are aspirated with a sterile Pasteur pipette and inoculated onto a blood-agar plate. The MBC is considered to be the lowest concentration of antimicrobial in which there is complete or virtually complete inhibition of growth of the organism on blood-agar after 24 hr of incubation.

Quality control. Although this technique is simple in principle, there are many steps of

operation which must be monitored frequently. The items which require careful control are potency of the antimicrobials, calibration of the microdiluters and pipettes, inoculum concentration, and purity of the test organism. The calibration of the microdiluters and pipettes has been discussed previously. Recalibration of the microdiluters and pipettes is performed at 6-month intervals or at such time that the differences in results cannot be accounted for by alteration of potency of the antimicrobials. The procedures used for assaying the potency of the standards indirectly reflect the calibration of the equipment. Thus far, the instances in which the MIC for the quality control organism was outside the normal limits have been due to antimicrobial deterioration or changes in the indicator strain rather than changes in calibration of the pipettes or microdiluters.

Each work day an *Escherichia coli* and *Staphylococcus aureus* are used for quality control. The *E. coli* is tested with those agents used for gram-negative rods and the *S. aureus* with those used for gram-positive cocci. The MIC of some agents, especially with the *S. aureus*, is less than the minimum dilution which is determined with the standard working concentration. Once or twice a week, the standard concentration of these agents is diluted before use to a concentration so the MIC will fall within the range of dilutions. The quality control organisms are handled in a comparable way to the routine organisms in regard to inoculum size and evaluation of purity.

A summary of the variation of the MIC of these two indicator strains over a 6-month interval with 14 antimicrobial agents is listed in Table 2. The results are expressed as the percentage of determinations which are ± 1 and ± 2 dilutions of a modal MIC. Although some antimicrobial agents have a wider distribution of the MIC than others, most have 90% of the values within ± 1 dilution of the modal MIC.

General guidelines have been established for evaluating deterioration of the antimicrobials. No change is made if there is a one-tube variation from the modal MIC; an occasional two-tube variation is noted, and, if the two-tube variation persists for more than 2 days, the solution is discarded and a fresh supply is prepared. With the exception of ampicillin and carbenicillin, most antimicrobials retain full potency for at least 4 weeks.

Purity of the tested organism is critical to broth dilution studies. Because the growth characteristics of the organism in the Microtiter plates are unreliable indicators of culture purity, the following procedure is used. After the 1:1,000 dilution of the organism is made and the appropriate amount of broth is removed to inoculate the Microtiter plate, the remaining broth is flooded onto a Mueller-Hinton agar plate. The plate is tilted and excess broth is suctioned off. A number of antimicrobial discs are placed on the agar for the sole purpose of producing zones of inhibition in the bacterial lawn. If the organism is pure, these zones of inhibition are clear-cut and usually do not have colonies within. If the culture is mixed, colonies are frequently seen within the inhibitory zones of one or more discs due to the varying susceptibility of different organisms to these agents. We find the most useful discs for this purpose are a combination of ampicillin (10 μg), tetracycline (30 μg), dihydrostreptomycin (10 μg), colistin (10 μg), and oxacillin (1 μg). A

TABLE 2. *Variations of MIC of quality control organisms over a six-month interval*

Organism	Penicillin	Ampicillin	Carbenicillin	Oxacillin	Cephalothin	Erythromycin	Chloramphenicol	Streptomycin	Kanamycin	Gentamicin	Colistin	Lincomycin	Nitrofurantoin	Tetracycline
Escherichia coli														
No. of determinations	170	160	115		172		168	181	176	84	180		170	179
Determinations (%) ± 1 dilution of modal MIC	97.9	89.5	84.3		94.8		93.4	90.7	95.4	89.2	90.5		90.6	95.0
Determinations (%) ± 2 dilutions of modal MIC	99.5	95.6	100.0		99.4		100.0	100.0	100.0	97.6	100.0		100.0	100.0
Staphylococcus aureus														
No. of determinations		103	87	79	114	107	124		121	77		77	104	105
Determinations (%) ± 1 dilution of modal MIC		88.1	90	100	97.6	97.5	99.2		85.2	87.0		98.7	98.0	97.5
Determinations (%) ± 2 dilutions of modal MIC		100.0	100.0	100.0	100.0	100.0	100.0		98.4	97.4		100.0	100.0	100.0

TABLE 3. *Antibiotics regions and expected blood and urine levels*[a]

Antibiotic	Route	Dose	Peak blood levels	Residual blood vessels	Active agent in urine
Ampicillin	Oral	250 mg	2 μg/ml @ 2 hr		~25–35% @ 6 hr
	IM	250 mg	Slightly higher than oral with peak @ 1 hr		~50% @ 6 hr
Cephalothin	IM	500 mg	6–10 μg/ml @ 30 min		65–80% @ 6 hr
		1 g	14–23 μg/ml @ 30 min	5 μg/ml @ 2 hr	
Chloramphenicol	Oral	2 g*	20–40 μg/ml @ 2 hr		5–10% @ 24 hr
Colistin	IM	2.5 mg/kg (Child)	5 μg/ml @ 1 hr	~1.5 μg/ml @ 3 hr	40–80% of dose @ 24 hr
		75 mg (adult)	3 μg/ml @ 2 hr	1 μg/ml @ 6 hr	
Demethylchlortetracycline	Oral	0.6 g (single)*	~1.5 μg/ml	Half-life 40% longer than tetracycline	½ Tetracycline level
Erythromycin (base or stearate) (if buffered, stearate levels same as base)	Oral	200–300 mg	1–4 μg/ml @ 2 hr		2–5% oral dose @ 24 hr
		500 mg (single)	2–20 μg/ml @ 2 hr		
		500 mg (q6h)	Up to 50 μg/ml		
Erythromycin (estolate)	Oral	Equivalent dose	Peak levels 2 × as high		
Gentamicin	IM	0.4 mg/kg (q8h)	1–3 μg/ml @ 1 hr		50–100% @ 24 hr
		0.8 mg/kg (q8h)	4–6 μg/ml @ 1 hr		
		1.6 mg/kg (q8h)	7–11 μg/ml @ 1 hr		
Kanamycin	IM	1 g*	20–35 μg/ml @ 1 hr	5–15 μg/ml @ 6 hr 1 μg/ml @ 12 hr	50–80% @ 24 hr
		500 mg	20 μg/ml		
		250 mg	10–15 μg/ml		
Lincomycin	Oral	500 mg	2–5 μg/ml @ 4 hr		10% Variable but limited
	IM	300 mg (q12h)	9–11 μg/ml @ 30 min	8–12 μg/ml @ 1–2 hr 2 μg/ml @ 12 hr 1 μg/ml @ 24 hr	
		600 mg (q12h)	15–20 μg/ml @ 30 min		
Methicillin	IM	1 g	10 μg/ml @ ½–1 hr	1 μg/ml @ 4–6 hr	⅔ IM dose @ 4 hr
		2 g	>20 μg/ml	8 μg/ml @ 4 hr	
	IV	1 g	~40 μg/ml	1 μg/ml @ 2 hr <1 μg/ml @ 4 hr	
Nalidixic acid	Oral	Almost completely absorbed	Blood levels too low for antibacterial activity		80% @ 24 hr
Nitrofurantoin	Oral	Completely absorbed	Blood levels too low for antibacterial activity		40% dose, avg concn 20 mg/100 ml
Oxacillin	Oral	500 mg	7–13 μg/ml @ 30 min	<1 μg/ml @ 4 hr	~20% Oral dose @ 6 hr
		1 g	10–16 μg/ml @ 30 min		
	IM	500 mg	10–16 μg/ml		40% @ 6 hr
Penicillin G (1 unit = 0.6 μg)	Oral	1.2 million units	5 μg/ml @ 30–60 min		20% of dose
	IM	300,000 units	5 μg/ml @ 15–30 min	Fall to ½ peak value after 1 hr	60–90% of aqueous penicillin, most in first hour
Penicillin G, benzathine (1 unit = 0.6 μg)	IM	1.2 million units	0.3 μg/ml @ 30 min	0.03 μg/ml @ 4 hr 0.018 μg/ml @ 24 hr 0.012 μg/ml @ 14 days	Same as penicillin G
Penicillin, phenoxymethyl	Oral	Dose equivalent to penicillin G	Level 2–5 × higher than penicillin G		Same as penicillin G
Penicillin G, procaine	IM	300,000 units	0.45–0.9 μg/ml @ 2 hr	0.03–0.12 μg/ml @ 12 hr 0.018–0.03 μg/ml @ 24 hr	

[a] The antibacterial level in the body fluid must be two to four times the MIC to obtain good clinical response in most infections. Asterisk (*) designates doses higher than ordinarily recommended.

TABLE 3—*continued*

Antibiotic	Route	Dose	Peak blood levels	Residual blood levels	Active agent in urine
Polymyxin B (10 units = 1 μg)	IM	2–4 mg/kg* Repeated injections	1–8 μg/ml Higher levels		20–100 μg/ml 60% Total dose over several days
Streptomycin	IM	1 g	25–30 μg/ml @ 1–2 hr	Falls to ½ peak value after 5 hr	50–60% @ 24 hr
		500 mg (q6h)	7–15 μg/ml @ 1–2 hr		Most @ 12 hr
Sulfisoxazole	Oral				60% of dose @ 24 hr
Tetracycline	Oral	250 mg (q6h)	1–3 μg/ml @ 2–4 hr		20–55% @ 24 hr
		500 mg (q6h)	3–5 μg/ml @ 2–4 hr		
		1 g (q6h)*	> 5 μg/ml @ 2–4 hr		
	IV (single dose)	250 mg	5–10 μg/ml @ 5 min	1–2 μg/ml @ 12 hr	20–60% @ 24 hr
		500 mg	15–30 μg/ml @ 5 min		
	IM	100 mg (q6h)	4–5 μg/ml @ 1 hr		

sulfonamide disc also is included to determine susceptibility, since a serial dilution broth technique is not practical for sulfonamides. The antimicrobial susceptibility discs are stored at −20 C until needed. The dispenser in which they are used is refrigerated except during actual use. Zone sizes around the discs are not measured, since determinations of susceptibility are not made from the discs except for sulfonamides.

After incubation, these purity control plates are examined along with the Microtiter plate, and if there is any evidence that the culture is not pure the MIC is not reported until the purity is established. The density of the bacterial lawn on these agar plates also serves as a check on the uniformity of the inoculum size. This is a somewhat cumbersome procedure, but the amount of time involved in setting up these additional plates with a few antimicrobial discs is not great, and the quantitative data derived from the Microtiter plates make this additional control not only worthwhile but mandatory. In the early phases of this technique, the number of organisms, taken from isolation plates in the main diagnostic laboratory and sent for susceptibility testing, which were mixed approximated 20%. As the insistence upon pure cultures continued, the percentage of mixed cultures decreased markedly, but new personnel have to be impressed with the necessity of careful selection of colonies.

Determination of antimicrobial levels in body fluids. For determining the serum level of an antimicrobial agent, whole blood is collected in a sterile tube and centrifuged, and the serum is removed aseptically into a sterile plastic cup. The serum dilution is performed in broth containing 10% horse serum in the same manner as the antimicrobials. A diluted suspension of either the patient's organism or an appropriate indicator strain is added to all of the wells. In this instance the final serum dilution range is from 1:4 to 1:512. It is sometimes necessary to dilute the serum with broth containing 10% horse serum before doing the serial dilutions to have the end point fall within the range of dilutions. Since it is difficult to predict which specimens will require this additional dilution, it can be performed along with the initial serum determination or the serum should be stored at 4 C until it is decided if higher dilutions are necessary.

Concurrent with the serum dilutions, the MIC of the antimicrobial standard with the test organism is determined by using broth containing 10% horse serum. The serum level is calculated by multiplying the MIC of the indicator organism as determined from the aqueous standard by the serum inhibitory dilution. Serum levels are usually performed in duplicate or triplicate plus an uninoculated serum control. An attempt is made to use at least two different indicator strains with different sensitivity to the antimicrobial being tested. It is absolutely essential that the laboratory have accurate information on the antimicrobial therapy being used. If a combination of antimicrobials is present in the serum, it is frequently practical to express results only in terms of a serum inhibitory level to the patient's organism. Methods are being explored to separate the effects of different antimicrobials by using indicator strains with varying antimicrobial sensitivities.

Fluids, such as urine, spinal fluid, and pleural fluids, are usually filtered through a 0.45-nm pore size membrane filter before use. Filtration is necessary to be certain that only the test organism is present in the system. Because of high concentrations of antimicrobial agents in the urine, it is frequently necessary to dilute the urine with diluent broth to 1:10 or 1:100 before performing the serial dilutions so that the end point falls within the range of dilutions.

Implementation of the method into routine use. Having proven the microtechnique to be reproducible, rapid, and adequate to perform the volume of tests for the routine clinical laboratory, the problem of presentation of this quantitative data had to be faced. Most physicians would like to have quantitative data on antimicrobial susceptibility in selected instances, particularly in patients who are critically ill with a number of complicating processes. It was not known how useful these quantitative data would be on more routine cultures from patients in whom the choice of antimicrobial therapy was not superficially so critical. The concept of an organism being "sensitive" or "resistant" is so deeply entrenched that many physicians do not think in terms of blood, urine, or tissue levels of antimicrobials. This is understandable but represents a problem in interpreting quantitative results. It was necessary to establish guide lines for antimicrobial therapy in terms of expected blood or urine levels, or both, so that an estimation could be made whether an organism with a given MIC to a certain agent might be treatable under the clinical circumstances.

In compiling guidelines, it became obvious how little is known about the pharmacology of many antimicrobials in disease states. Most of the studies have been carried out in normal volunteers or in patients who were not seriously ill, or who have not suffered severe depression of their immune mechanisms. Many of the values have been derived from single-dose experiments rather than from patients who were on continuous therapy. With all of these reservations in mind, a list of antimicrobial agents with various dosage regimens and projected blood and urine levels is summarized in Table 3. This table could be much more elaborate, but in this form it has proven to be useful. One advantage of the microtechnique is that predictions from this type of data can be readily tested by determining the antimicrobial level rather than speculating about anticipated levels.

The quantitative data were reported to the physicians only on significant isolates from blood and urine between June and September 1968. Since October 1968, after this introductory period, the MIC values have been reported routinely for all organisms from all sources which require susceptibility studies.

DISCUSSION

It is known that quantitative evaluations of antimicrobial susceptibility correlate better with in vivo response than do qualitative disc techniques. The technical difficulties of serial dilution methods in the numbers required of the clinical laboratory have precluded extensive use in clinical care. For this reason the attempt was made to make quantitative studies feasible for the routine laboratory. A microtechnique seemed reasonable since much was known about the reproducibility of techniques and equipment used in micro methods, particularly in virological serology. The reproducibility and precision of this method in comparison with standard glass-tube methods have been demonstrated by several authors (1, 3–5).

At present, three technologists perform 8,000 to 10,000 serial dilution tests per month. This technique is not simpler nor faster than disc techniques but is in the order of 10 times faster than the standard manual methods for performing serial dilution tests.

The two areas of its greatest usefulness are in allowing the physician to choose antimicrobial therapy on the basis of quantitative in vitro results and easily document the levels of antimicrobials obtained in body fluids. The use of quantitative data has not only immediate therapeutic application but represents a powerful epidemiological tool as well. Time will be the final arbiter in deciding whether these quantitative data are, in fact, more useful to the practicing physician, but it is hoped that this information will allow the practicing physician to make more rational choices of antimicrobial therapy than are presently being made.

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