Antibiotics in Laboratory Medicine

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Preface

Antibiotics* are among the most valuable and the most utilized therapeutic agents in medical practice. They account for over 30% of the budgets of hospital pharmacies. *In vitro* tests are the main guidelines for monitoring antibacterial therapy.

The growth of the field of antibiotic research and therapy, as related to the clinical laboratory, has resulted in an extensive literature. It is the purpose of *Antibiotics in Laboratory Medicine* to present in one reference volume the fundamental *in vitro* aspects of antibiotics, from the screening for new agents to their assay in various body fluids.

I have compiled this volume together with experts who have pioneered and developed by their own investigations the various areas of research and practice with antibiotics. Our efforts culminated in this comprehensive reference book. It contains an unprecedented amount of theoretical and practical information as to what can be done in the laboratory including methodology and the significance of results.

Each chapter contains an academic section which covers the field at the highest level of current expertise, as well as a practical part which describes in detail not only sophisticated technology, but also routine methods in such a way that scientists, physicians, and technologists should be able to reproduce the procedures described without the need for additional reading. Consistent with this approach, some duplication was not only accepted but also required. The reader can find all the specific information in the chapter consulted without the need of reference to other parts of the book. Each chapter is documented by an extensive bibliography.

It is my hope that this volume will help those in research as well as those whose daily task is concerned with the many laboratory aspects required for the care of patients receiving antibiotics.

It is an honor to introduce this book containing contributions from such eminent professionals who have donated so much of their valuable time and expertise toward its preparation. Their cooperation made the task of editing a real pleasure. I am confident the results of their efforts will prove to be invaluable to all those concerned in this field.

Special recognition must go to Drs. Max Finland, L. D. Sabath, and John Sherris who enthusiastically helped me to undertake the publication of this volume. My gratitude also goes to my Chief and Friend, Dr. Leopold Reiner, and to the administration of The Bronx Lebanon Hospital Center for creating conditions that allowed me to concentrate on the many endeavors required for the editing of this volume. Last but not least my sincere thanks to Mrs. Urbis C. Westray, my secretary, whose devoted help made this complex activity possible.

I would also like to thank all my friends at Williams & Wilkins whose expertise and kind cooperation made the process of publishing an enjoyable experience.

VICTOR LORIAN *New York, 1980*

* In this volume the word **antibiotic** will be used to cover all antimicrobial agents.

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chapter 1

Procedure for Testing Antibiotics in Agar Media: Theoretical Considerations

ARTHUR L. BARRY, Ph.D.

INTRODUCTION

Laboratory procedures involving antimicrobial agents are commonly performed in agar media. To determine microbial susceptibility to an antimicrobial agent, measured amounts of drug may be incorporated into an agar medium which is then inoculated with the microorganism to be tested. After appropriate incubation, one can determine whether the microorganism is capable of growing in the presence of that concentration of antimicrobic. When a series of petri plates are prepared with varying amounts of antimicrobic, one can determine the minimal concentration required to inhibit growth of the microorganism (the minimal inhibitory concentration or MIC). Such drug dilution tests carried out in agar media are commonly referred to as agar dilution procedures, as opposed to broth dilution procedures which are carried out in a broth medium.

Rather than preparing dilutions of each drug, antimicrobic susceptibility tests may be performed by agar diffusion methods. A single concentration of antimicrobic is applied to a reservoir on a seeded agar medium and the drug is allowed to diffuse into the surrounding medium. This exposes the test organism to a continuous gradient of drug concentrations, with diminishing concentrations at increasing distances from the reservoir. Agar diffusion techniques have also been adapted for measuring the concentration of antimicrobic in body fluids or other material (bioassay procedures). Although performed for entirely different purposes, bioassay and susceptibility tests are both agar diffusion procedures which are governed by the same theoretical principles.

The purpose of this chapter is to review theoretical considerations governing *in vitro* tests of antimicrobial agents in agar media. Both dilution and diffusion techniques will be reviewed in principle. Specific details of agar diffusion susceptibility and bioassay procedures are covered in Chapters 2 and 9. Antimicrobic tests performed in broth media are discussed in Chapter 4.

THE NATURE OF AGAR

Almost any nutrient broth can be solidified by adding 1.5–2.0% agar—a complex polysaccharide substance prepared from one of several species of red seaweed, the *Rhodophyceae*. The physical characteristic of agar which make it useful for microbiological work is its unique gelling capacity. It is liquid when heated to boiling temperatures and remains liquid when cooled to 45–50°C. At 30–45°C, it forms a firm gel and once gelled it does not liquefy until heated to near boiling temperatures again. Agar itself is essentially unaffected by microbial growth and thus it is ideally suited for solidifying many nutrient media.

Agar is a complex substance which is yet to be completely characterized and chemically synthesized. Because agar is a natural product, one would expect some lot-to-lot variability in the actual composition of different agars. The quality of a batch of agar will depend upon the method of extraction from the seaweed, the quality of the seaweed itself and the environment in which the seaweed is grown. Agar contains at least two types of polysaccharides; agarose and agaropectin. Agarose is essentially neutral, with few ionic charges. Most agars carry an overall negative charge, largely due to acid and sulfate groups on the polysaccharides. Normally a variety of metalic cations are bound to the negatively charged radicals on the polysaccharide. The concentration of such cations can be reduced by dialysis or by washing the agar with large volumes of water (22). Traces of calcium are necessary in order to maintain the ability of the agar to gel. In addition to cations, agars often contain other trace elements which may be either stimulatory or inhibitory to microbial growth. Complete removal of all contaminating substances might actually have a deleterious effect on the growth of some microorganisms and might stimulate the growth of other microorganisms. In the absence of a completely defined synthetic gelling agent, microbiological work in agar media is not likely to be extremely well standardized or completely reproducible.

Alternative Gelling Agents

Because of the many problems inherent in manufacturing consistently uniform agar media, alternative gelling agents deserve consideration. A chemically defined, synthetic product would be ideal if it is not affected by microbial growth and does not affect the activity of antimicrobial agents.

Separan NP 10 is one such substance which may reduce the amount of agar needed to produce a satisfactory gel. It is a high molecular weight polymer produced by polymerization of acrylamide. It is essentially nonionic in solution but has a preponderance of amide groups, a small proportion of which are hydrolyzed to anionic carboxyl groupings. The viscous polymer can be added to 0.5% agar to obtain a gel comparable to that obtained with 1% agar alone (30).

Another potentially useful substitute for agar is a polyoxyethylene polymer which was developed by Union Carbide (Tuxedo, New York) and has been given the trade name Neutra-Gel. It is a synthetic polymer which is converted to the polymer in standard plastic petri plates by a propriatory cross-linking process which produces carbon-carbon covalent bonds. The chemical structure is $(CH_2—CH_2—O)_n$. It is an inert, optically clear, stable, irreversible solid which is not affected by extremes in temperature.

Neutra-Gel is supplied as a presterilized gel which is tightly bonded to a plastic petri plate. It contains only distilled water, traces of ionic materials and the polymer. The amount of water and ionized materials can be adjusted at the time of manufacture, depending upon the needs of the user. For use, a 2–3 times concentrated nutrient broth is asceptically added to the hydrogel and then allowed to absorb into the hydrogel by standing several hours, preferably overnight. The appropriate concentration of nutrients in the broth will be provided when the concentrated broth is diluted by the water in the hydrogel layer. Only solubilized materials will be incorporated into the polymer, particulate substances will remain on the surface. The starch in some batches of Mueller-Hinton broth is not completely soluble and will tend to form a cloudy film over the surface of the hydrogel. Consequently, susceptibility testing on a Mueller-Hinton medium solidified with hydrogel is not entirely satisfactory.

By incorporating the synthetic amino acid medium (SAAM) (23) into the synthetic hydrogel, a completely defined medium can be prepared. The possibility of using such a defined medium for antimicrobic susceptibility testing has been investigated (16a, 27).

Table 1.1 includes the results of diffusion tests on Mueller-Hinton agars from three different manufacturers and on SAAM solidified with 0.9% Ionagar and with the synthetic hydrogel. The affect on zone sizes varied with the organism and antimicrobial agent being tested. Variability in the performance of Mueller-Hinton agars from different manufacturers has been shown to be statistically significant (6). On SAAM agar, the zones were generally larger than on Mueller-Hinton agar and the differences were especially marked when *Staphylococcus aureus* was being tested. The polymyxins were the major exceptions, they both gave zones which were essentially the same size on either agar medium. On the neutral polymer, the polymyxins gave much larger zones than on the agar media. Presumably, diffusion of the cationic polymyxin molecules is inhibited by agar but not by

Table 1.1
Mean Zone Sizes on Mueller-Hinton Agars from Three Commercial Sources and on a Synthetic Amino Acid Medium (SAAM) Solidified with 0.9% Ionagar and with a Synthetic Hydrogel (Neutra-Gel), All Inoculated by a Flooding Technique on Six Separate Drugs

Test Strain	Medium	Mean Zone Diameters (mm) Seven Replicates Each Drug									
		Polymyxin B	Colistin	Gentamicin	Streptomycin	Nitrofurantoin	Tetracycline	Cephalothin	Ampicillin	Carbenicillin	Chloramphenicol
Staphylococcus aureus	Mueller-Hinton agar:										
	Difco	9.4	6[b]	23.6	17.9	20.6	25.6	31.7	30.4	32.4	23.9
	Pfizer	10.0	6	23.7	19.7	20.5	26.6	32.1	30.4	33.1	23.6
	BBL	9.7	6	24.1	18.9	21.3	25.3	31.7	30.0	33.0	23.3
	SAAM										
	Ionagar	9.3	6	29.7	20.0	30.8	31.8	38.5	46.3	47.4	28.5
	Hydrogel	12.1	6	30.9	19.0	22.8	27.7	41.7	36.9	43.6	22.4
Escherichia coli	Mueller-Hinton agar										
	Difco	16.6	16.0	21.6	16.1	22.4	21.0	19.3	16.3	24.4	23.4
	Pfizer	13.9	13.3	21.4	16.7	23.1	22.9	23.1	18.1	25.9	24.5
	BBL	13.7	13.0	19.1	14.9	22.1	21.7	19.9	17.3	24.0	22.9
	SAAM										
	Ionagar	13.8	13.4	25.0	19.3	24.6	22.4	20.7	14.7	24.6	24.4
	Hydrogel	18.5	16.6	25.3	17.0	19.8	18.6	18.0	14.0	21.1	21.0
Pseudomonas aeruginosa	Mueller-Hinton agar:										
	Difco	16.9	14.9	23.3	14.9	6	9.4	6	6	29.1	14.9
	Pfizer	13.3	11.7	21.6	15.9	6	8.7	6	6	29.1	15.3
	BBL	14.1	13.0	23.6	16.7	6	10.3	6	6	31.7	16.0
	SAAM										
	Ionagar	15.1	13.9	23.7	9.7	6	11.7	6	6	30.0	15.4
	Hydrogel	20.6	17.6	25.4	10.3	6	8.7	6	6	25.1	14.0

[a] Adapted from Barry and Effinger (6, 6a)
[b] 6 = no zone of inhibition around the 6-mm disc

the inert hydrogel. Diffusion of the other antimicrobics through the hydrogel appears to be a little slower since the zones tend to be slightly smaller.

Since the SAAM-hydrogel medium is a completely defined synthetic medium, there should be a minimal amount of variation in the performance of different batches. Precision of diffusion tests in the SAAM-hydrogel medium is compared to that on a single lot of Mueller-Hinton agar (Difco, Control No. 579660) by the control data summarized in Table 1.2. Fifteen different determinations were made for each antimicrobic during a 4-month study period. The precision of tests on the SAAM-hydrogel medium was definitely inferior to the precision of tests on Mueller-Hinton agar, especially when the *S. aureus* control strain was being tested. In part, the variability in results of tests on SAAM-hydrogel plates reflects the relatively poor growth of *S. aureus* on SAAM. *Escherichia coli* provided satisfactory growth on both media and the precision of diffusion tests on the two media was more nearly comparable.

In summary, the synthetic polymer can be used to solidify a synthetic amino acid medium and the solid medium can be used for diffusion tests. The results of diffusion tests on such a chemically defined medium will differ from that on Mueller-Hinton agar and the differences will vary with the antimicrobic and type of microorganism being tested. At this point, it is only possible to document differences in results; it is not possible to decide which result is the "correct" one. Since the synthetic hydrogel is not yet available commercially, it appears that the use of agar media will continue for some time. Consequently, we should develop an appreciation for the ways in which agar affects antimicrobial agents.

Interaction between Antimicrobial Agents and Agar

Antimicrobics with cationic molecular structures may be electrostatically bound to acid or sulfate groups on bacteriologic agar and consequently the rate of diffusion through the agar gel is diminished. The polymyxins are examples of fairly large, strongly cationic molecules which diffuse at a slow rate and produce relatively small zones of inhibition with agar

Table 1.2
Precision of Diffusion Tests on Mueller-Hinton Agar (MHA) and on a Synthetic Amino Acid Medium (SAAM) Solidified with a Synthetic Polymer (Neutra-Gel), Expressed as Standard Deviations Calculated from 16 Replicate Tests[a]

Antimicrobic	Standard Deviation in Zone Diameters (mm)					
	Staphylococcus aureus		*Escherichia coli*		*Pseudomonas aeruginosa*	
	SAAM	MHA	SAAM	MHA	SAAM	MHA
Gentamicin	2.82[b]	0.89[b]	1.17	0.97	1.54	2.02
Streptomycin	0.84[b]	0.61[b]	1.06	0.78	1.65	1.98
Polymyxin B	2.03	0.77	0.86	0.41	1.60	0.49
Colistin	NZ[c]	NZ	0.62	0.36	1.68	0.52
Chloramphenicol	1.88	1.51	0.93	1.06	1.92	2.33
Tetracycline	2.40	1.53	1.17	1.19	2.02	1.35
Doxycycline	1.91	1.35	1.59	1.81	1.12	1.35
Nitrofurantoin	2.17	0.93	1.05	0.62	NZ	NZ
Carbenicillin	1.41[b]	1.98[b]	0.66	0.96	2.43	3.13
Ampicillin	2.41[b]	1.72[b]	1.25	1.62	NZ	NZ
Cephalothin	4.62	1.59	1.13	1.12	NZ	NZ

[a] Adapted from A. L. Barry and L. J. Effinger (6a).
[b] Standard deviations based on 8 replicates, rather than 16 tests.
[c] NZ = no zone of inhibition.

diffusion tests. Much larger zones on inhibition can be obtained with the polymyxins, if the agar is treated with protamine to block the acidic sulfate groups (26). Other drugs, such as the aminoglycosides might have a similar tendency for electrostatic binding to active groups on the agar molecule. The polymyxins and aminoglycosides tend to give larger zones of inhibition when tested in agarose (26, 33). The polymyxins produced much larger zones on a neutral polymer than on agar media (Table 1.1).

Calcium, magnesium, zinc and other cations are often found associated with bacteriologic agars. There is a great deal of variability in the cation content of different lots of Mueller-Hinton agars (Table 1.3). The concentration of calcium, magnesium and other divalent cations profoundly affects the activity of the aminoglycosides against *Pseudomonas* spp. For example, one strain of *Pseudomonas aeruginosa*, tested on the 14 lots of Mueller-Hinton agar described in Table 1.3, gave gentamicin MICs ranging from 0.5 to 6.0 μg/ml, tobramycin MICs ranging from 0.2 to 2.0 μg/ml and amikacin MICs varied from 0.8 to 11 μg/ml (42). The activity of the aminoglycosides against bacteria other than *Pseudomonas* sp. is not affected as dramatically by the calcium and magnesium ions in the agar medium. The concentration of cations in the culture medium also affects the activity of polymyxin B and tetracycline against *Pseudomonas* spp. (17). Mueller-Hinton agar is rich in phosphates. Magnesium and calcium ions tend to form stable polyphosphate complexes, especially when the agar medium is autoclaved. Antimicrobics are affected more by the cations which are free to ionize than by those that are bound in polyphosphates or other complexes. For that reason the data in Table 1.3 might be a little misleading since the measurements reflect both free and bound cation.

Table 1.3
Divalent Cation Content of 14 Different Lots of Mueller-Hinton Agar from Three Manufacturers (4–5 Lots from Each)[a]

Cation	Cation Concentration (μg/ml)[b]		
	Minimum	Maximum	Mean
Calcium	6.58	85.01	50.51
Magnesium	2.89	46.42	19.83
Copper	0.068	0.665	0.141
Zinc	0.285	1.220	0.662
Iron	0.551	4.030	1.587

[a] Data adapted from Washington et al. (42).
[b] Constituted medium (μg/ml) as determined by atomic absorption spectrophotometry after wet-ashing with nitric and perchloric acid.

The mechanism by which metalic cations influence the activity of antimicrobial agents is not entirely understood. The most likely explanation lies in the observation that divalent cations may be essential for maintaining structural integrity of the cell wall. The lipopolysaccharides in the cell wall of *P. aeruginosa* are probably stabilized by cross-linking with divalent cations. Removal of the cell wall cations may increase the permeability to a variety of unrelated molecules. When cells of *P. aeruginosa* are grown in media with abundant amounts of calcium and magnesium, the cations should be incorporated into the cell wall thus making it less permiable to aminoglycoside drugs and to other compounds. Consequently, the microorganism would require somewhat greater concentrations of drug for inhibition. Alternatively, the cations may compete with the drug for binding sites on or in the