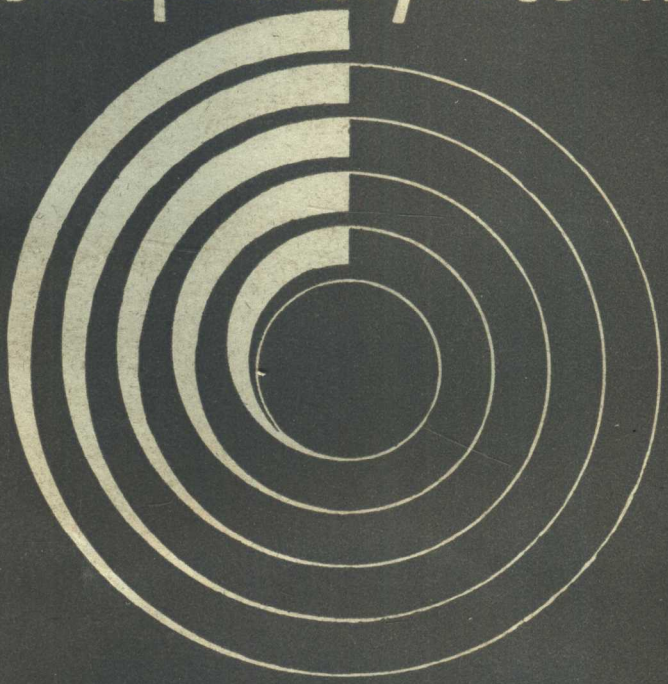
Current Techniques for Antibiotic Susceptibility Testing



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FOREWORD

THE GENESIS OF THIS SERIES, *The American Lecture Series in Clinical Microbiology,* stems from the concerted efforts of the Editor and the Publisher to provide a forum from which well qualified and distinguished authors may present, either as a book or monograph, their views on any aspect of clinical microbiology. Our definition of clinical microbiology is conceived to encompass the broadest aspects of medical microbiology not only as it is applied to the clinical laboratory but equally to the research laboratory and to theoretical considerations. In the clinical microbiology laboratory we are concerned with differences in morphology, biochemical behavior and antigenic patterns as a means of microbial identification. In the research laboratory or when we employ microorganisms as a model in theoretical biology, our interest is often focused not so much on the above differences but rather on the similarities between microorganisms. However, it must be appreciated that even though there are many similarities between cells, there are important differences between major types of cells which set very definite limits on the cellular behavior. Unless this is understood it is impossible to discern common denominators.

We are also concerned with the relationships between microorganism and disease—any microorganism and any disease. Implicit in these relations is the role of the host which forms the third arm of the triangle: microorganism, disease and host. In this series we plan to explore each of these; singly, where possible, for factual information and in combination for an understanding of the myriad of interrelationships that exist. This necessitates the application of basic principles of biology and may, at times, require the emergence of new theoretical concepts which will create new principles or modify existing ones. Above all, our aim is to present well-documented books which will be informative, instructive and useful, creating a sense of satisfaction to both the reader and the author.

Closely intertwined with the above *raison d'etre* is our desire to produce a series which will be read not only for the pleasure of knowledge but which will also enhance the reader's professional skill and extend his technical ability. *The American Lecture Series in Clinical Microbiology* is dedicated to biologists—be they physicians, scientists or teachers—in the hope that this

series will foster better appreciation of mutual problems and help close the gap between theoretical and applied microbiology.

In the spring of 1972 a Seminar on Current Techniques for Antibiotic Susceptibility Testing was sponsored by Canalco, Incorporated in a deliberate attempt to review and discuss the state of the art. The meeting was well attended and, by all measurable parameters, was a success. The presentations were thorough and timely; the open discussions were frank and comprehensive. The prevailing opinion was that while many problems still persist, a great deal of useful and informative data were presented and the movement toward the establishment of standardized procedures for performing *in vitro* antimicrobic susceptibility tests was well under way. It was felt that the proceedings of this symposium would serve as a welcome source of authoritative information to clinical microbiology laboratories. Accordingly, the oral presentations were converted into manuscripts and assembled in this volume of *The American Lecture Series in Clinical Microbiology*. Taken either as individual papers or collectively, I believe this addition will serve the purpose for which it was intended—to make available the current thinking and practices that prevail today in the performance of this most important laboratory test. As more clinical laboratories recognize the need for and adopt standardized susceptibility test procedures —be they disc diffusion, agar or broth dilution, or the forthcoming automated instruments—we will be able to provide clinicians with the information they need to initiate and monitor therapy of patients with infectious diseases.

ALBERT BALOWS
EDITOR

PREFACE

I WISH TO EXPRESS a sense of deep satisfaction in having had the opportunity to sponsor this seminar. The large number of participants, and their enthusiasm for the subject, gave evidence of the need for the gathering. Having heard what all the speakers had to say, listened to the questions from participants, and taken part in informal "corridor conversations," I was struck not only with the need for this seminar, but also with its timeliness. There seemed to be an almost audible sigh of relief that at last all the main points of view were being brought together in one place to be heard, to be challenged, and to be clarified.

The protagonists responded with a dignified restraint of partisan expression that betokened a sincere desire to meld the best of all available evidence. They showed unequivocally their dedication to the common goal of advancing the art as rapidly as possible for improvement of the practice of medicine and for the good of humanity. This spirit, which dominated the entire event, contributed as much to the success of the symposium as did its solid content.

It is a tribute to your professional stature and wide circle of friends in microbiology that such a stellar platform of speakers was assembled, and to them that such a large turn out responded. As a corporation, it was our pleasure to serve as the administrative sponsor of this event, and as individual participants I and my staff came away far better prepared to develop our area of responsibility in the provision of instrumentation responsive to the needs of those concerned.

RALEIGH HANSL, JR.
President, Canalco, Inc.

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CURRENT TECHNIQUES FOR ANTIBIOTIC SUSCEPTIBILITY TESTING

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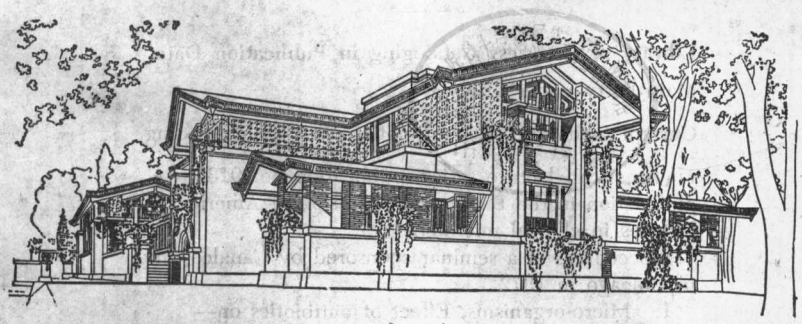

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CHAPTER I

INTRODUCTION TO "IN VITRO" SUSCEPTIBILITY TESTING

ALBERT BALOWS

ONE OF THE FIRST recorded observations of *in vitro* antibiosis was made by van Leeuwenhoek in 1676. You may recall he related that his living animalcules disappeared after he placed them in some "pepper water." In fact, in his presentation to the Royal Society of London, he commented on the action of drugs based on these observations. From that time until now, reports on the *in vitro* demonstration of growth inhibited by an antibiotic constitute one of the largest segments of microbiologic literature. Of major importance has been the shift in emphasis, so that nowadays growth inhibition by antibiotics is more than a research tool. The determination of antibiotic susceptibility has a most practical application, and it has profoundly altered the course of many infectious diseases—for the better in most cases. Clinical microbiology has experienced a growth over the past thirty years that remarkably and understandably parallels the discovery and subsequent development of modern day antimicrobial agents. In the previously held traditional view, infection encompassed the microbe and the host; we now more correctly refer to the *triad of infection,* which indicates the vast change brought about by the introduction of antibiotics in the therapy of bacterial diseases. This triad is represented by a triangle in virtually all textbooks dealing with infectious diseases. It is very important to keep this triad in mind—the host, the bacterium, and the antimicrobic agent —each symbolized by the side of a triangle touching the other two sides.

This interdependence has placed considerably more responsibility on the clinical microbiology laboratory than ever before, and, among other things, demands that microbiologists be prepared to provide what is obviously expected of them. In order for the clinician to carry out antibacterial therapy, on a rational basis, he rightfully expects the laboratory to provide him with (1) the identity of the infecting organism(s) and (2) accurate and reliable guidance as to which antibiotics can be used and which cannot; that is, which antibiotics will be effective *in vivo.* Extensive investigations were initially set up with animal models to assess the activity of a given antibiotic in treating a specific bacterial disease. This approach was not only time consuming and costly, but, more to the point, it failed notoriously

to provide in any degree of reliability the kind of therapeutic guidelines sought. Attention was then focused on the development of *in vitro* susceptibility tests which would, with proper interpretation, provide the needed information. It is not my intention to recap the historical development of *in vitro* susceptibility tests, but, in a way, one might liken it to the California Gold Rush. Practically everyone came up with an *in vitro* method, and in no time at all a dozen or more methods were being used in the United States alone. When you add to this the modifications that any given laboratory is likely to make in adapting a given procedure, then we can visualize twelve squared, or perhaps cubed, as indicating the number of different procedures used. The reagents were initially homemade but in a number of instances were produced commercially by increasing numbers of industrial companies. Little, if anything, existed in the way of controls in production or procedures. As a result, it became increasingly evident that clinical microbiology laboratories were not only failing to meet their obligations but, in some instances, were providing incorrect results or misinterpretations of their results or both. Many concerned individuals viewed the situation as indeed chaotic and called for corrective measures. The combined efforts of individuals from industry, academia, medical institutions, and federal agencies paved the way for these corrective measures.

This resulted first with the Food and Drug Administration establishing, in 1959, regulations controlling the manufacture of antibiotic-impregnated discs that are either produced in, or to be used within, the United States. Simultaneously, concerted efforts were made to develop procedures that would provide meaningful data. By meaningful data, I mean a way of determining whether a given organism is susceptible or sensitive to a given antibiotic in a stated concentration. In 1961 a group of knowledgeable World Health Organization consultants provided a working definition of a susceptible organism. A bacterium is considered to be susceptible if the concentration attainable *in vivo* exceeds the concentration required to inhibit the growth of the bacterium *in vitro*. Admittedly, this definition fails to take into consideration these three factors: (1) the host defense mechanisms may act either in an additive or an antagonistic way to the antibiotic (2) maximum or average dosage of a given drug will result in different blood levels in different individuals, and the crest or nadir in blood levels following administration will differ and (3) the blood level may have no relationship at all to the actual concentration of the drug at the site of infection. Despite these shortcomings, this definition of antibiotic susceptibility is still in use, and it has been the pivotal point around which good testing methods have survived. Those methods that are not good have faded away, albeit with some difficulty in several instances. The various methods and variations within a given method, along with the arbitrary choice of end

points, led to such divergent results that serious doubts were raised regarding the validity and usefulness of antimicrobial susceptibility tests.

The past ten years have been most productive in a positive and corrective fashion. We have identified and attempted to eliminate those practices that were impractical, nonreproducible, or ill defined. We have retained those methods that can be controlled and which give reproducible results that lend themselves to interpretation along the lines of the definition mentioned earlier.

I do not wish to imply that we now have all the answers. On the contrary, we still have problems, but solutions are on the horizon. This seminar should bring you up to date on the current status of *in vitro* antibiotic susceptibility testing. Through the remainder of the sessions today and tomorrow, you will hear presentations on susceptibility testing and the different techniques that are held to be reliable and reproducible. A glance at the program indicates that we will cover four major aspects of susceptibility testing: first, dilution techniques, which basically consist of exposing the test culture to increasing concentrations of an antibiotic in either broth or agar medium usually by serial twofold dilutions which yield what we have termed the Minimal Inhibitory Concentration, or MIC; and second, diffusion methods. Initially, there were many such methods, but these have now more or less settled down to the use of impregnated discs of filter paper in a prescribed manner.

Parenthetically, for those of you who are history buffs, it may be of interest to note that in 1947 Bondi and his coworkers, who were among the early investigators describing a disc diffusion technique for susceptibility testing, actually recognized the zone size around the disc to be a function of both the concentration and diffusibility of the antibiotic *and* the relative susceptibility of the test organism. In other words, these workers described what we now refer to as interpretive tables drawn from regression curve analyses. Because this concept was poorly understood, many modifications of Bondi's method resulted, and this, in turn, led to the confusion and, to some extent, the frank expressions of doubt regarding the reliability of this method.

Third, and next will be presented an introduction to some interesting research which represents a solid beginning to answering the question of how meaningful susceptibility tests for anaerobic bacteria can be performed. Fourth, the last aspect of susceptibility testing that we will discuss is the current status of mechanizing or automating susceptibility testing. I want to emphasize that this is an appraisal of *current status* because I am firmly convinced that within the next few years, we will witness a new era in susceptibility testing in both manual and automated methods.

CHAPTER II

THE AGAR DIFFUSION ANTIMICROBIAL SUSCEPTIBILITY TEST

CLYDE THORNSBERRY

INTRODUCTION

FOR ALMOST THREE DECADES, microbiologists have used paper discs to determine whether or not a culture was susceptible to an antimicrobial agent.[1,2] Either single or multiple discs containing varying concentrations of the antimicrobic have been used with these techniques. Methods of interpretation have varied from susceptibility based on zone-no-zone readings to susceptibility based on the measurement of the zone diameter.

One of the most significant contributions in this area has been made by Doctors Bauer, Kirby, Sherris and their colleagues at the University of Washington, Seattle. They were able to develop a single disc technique that could be interpreted on a quantitative basis. Furthermore, they were able to promote their concept to such an extent that it has been adopted in most clinical bacteriology laboratories in this country. This procedure has been commonly referred to as the Kirby-Bauer antimicrobial susceptibility test.

The basic concept of the Kirby-Bauer procedure is that the size of the zone of inhibition can be correlated with the clinical susceptibility of an organism to an antimicrobic. This concept obviously demanded that the procedure be standardized before a set of interpretive zone diameters could be developed. The standard procedure provided for the use of Mueller-Hinton agar, a standard inoculum applied in a standard manner, and a single disc of a predescribed potency for each antimicrobic tested. For determining interpretive zone sizes, both agar diffusion and dilution tests were performed on a number of a variety of the appropriate species of bacteria isolated from recent infections. The zone diameters and minimal inhibitory concentrations were compared to ascertain the degree of correlation between the two sets of values. On the basis of this correlation, and within the limits of the readily achievable concentrations of antimicrobic in the serum, the zone sizes representing an interpretation of susceptible, resistant, and intermediate (or equivocal) could be delineated. These interpretations

were confirmed by clinical efficacy studies. Interpretive values have been determined for most of the commonly used antimicrobics, and tables of these values have been distributed to most of the bacteriology laboratories in this country. However, it should be emphasized that these interpretive standards can only be used with the rapidly growing bacteria, and should not be used for the more fastidious, slower growing organisms and seldom isolated organisms which have not been studied by this procedure.

Although the Kirby-Bauer procedure is often referred to as a qualitative test, it is, in reality, a quantitative test because the interpretations are based on the diameters of the zones of inhibition and are directly related to minimal inhibitory concentrations. When performed in the standardized manner, with proper control of variables and with a quality control program to assure accuracy and precision, this test generates reliable susceptibility data and can be readily used in most bacteriology laboratories.

Unfortunately, the test is often misused. Many laboratories do not follow the standard procedure, yet they use the Kirby-Bauer standards for interpretations. Other laboratories use this technique or a similar one, but base their interpretations on the presence or absence of a zone of inhibition. It is likely that most of the errors made in disc susceptibility testing are due to failure to control the variables in the procedure.

Recently, two organizations have recommended that either the Kirby-Bauer standardized method [3] or an agar overlay method [4] be used for routine susceptibility testing in clinical laboratories. These organizations are the Food and Drug Administration (FDA),[5] and the National Committee for Clinical Laboratory Standards Subcommittee on Antimicrobial Susceptibility Testing.[6] The recommendations made by these organizations will be fully discussed in other parts of the symposium.

The purpose of this part of the symposium is to review the Kirby-Bauer agar diffusion procedure, point out areas where problems may occur, and make some recommendations concerning proper performance of the test and interpretation of the results.

THE KIRBY-BAUER PROCEDURE

This procedure should be used only for the commonly isolated, rapidly growing bacterial pathogens such as *Staphylococcus aureus*, the *Enterobacteriaceae*, and *Pseudomonas aeruginosa*. Results obtained for these bacteria with this standardized procedure can be interpreted with the zone size standards shown in Table II–I.

Mueller-Hinton agar should be used for the performance of this test. Defibrinated blood may be added to the cooled medium in a concentration of 5 percent; the blood-containing medium may also be chocolatized. Approximately 60 ml of medium should be poured into 14 cm petri plates or 25 ml into 9 cm petri plates. The medium should have a pH of 7.2 to 7.4 at

TABLE II-I

ZONE-SIZE INTERPRETIVE STANDARDS FOR THE DISC DIFFUSION TECHNIQUE[a]

Antimicrobial Agent	*Disc Potency*	*Inhibitory Zone Diameter (to nearest mm)*		
		Resistant	*Intermediate*	*Susceptible*
Penicillin G and	10 U			
Ampicillin	10 ug			
Staphylococci		20 or less[b]	21–28	29 or more
Enterobacteriaceae and enterococci		11 or less	12–13	14 or more
Other organisms		11 or less	12–21	22 or more
Methicillin	5 ug	9 or less	10–13	14 or more
Nafcillin or Oxacillin	1 ug	10 or less	11–12	13 or more
Vancomycin	30 ug	9 or less	10–11	12 or more
Cephalothin	30 ug	14 or less	15–17	18 or more
Cephaloridine	30 ug	11 or less	12–15	16 or more
Carbenicillin	50 ug			
Pseudomonas sp.		12 or less	13–14	15 or more
Proteus & E. coli		17 or less	18–22	23 or more
Polymyxin E (colistin)[c]	10 ug	8 or less	9–10	11 or more
Polymyxin B[c]	300 U	8 or less	9–11	12 or more
Chloramphenicol	30 ug	12 or less	13–17	18 or more
Tetracycline	30 ug	14 or less	15–18	19 or more
Erythromycin	15 ug	13 or less	14–17	18 or more
Lincomycin	2 ug	9 or less	10–14	15 or more
Clindamycin	2 ug	11 or less	12–15	16 or more
Kanamycin	30 ug	13 or less	14–17	18 or more
Neomycin	30 ug	12 or less	13–16	17 or more
Streptomycin	10 ug	11 or less	12–14	15 or more
Gentamicin	10 ug	12 or less	13–14	15 or more
Sulfonamides[d,e]	300 ug	12 or less	13–16	17 or more
Nitrofurantoin[e]	300 ug	14 or less	15–18	19 or more
Nalidixic Acid[e]	30 ug	13 or less	14–18	19 or more

[a] As modified from Bauer et al. (1968). Prepared by NCCLS Subcommittee on Anti-microbial Susceptibility Testing (June 1971).

[b] Penicillinase-producing staphylococci.

[c] Polymyxins diffuse poorly in agar, and the accuracy of the diffusion method is thus less than with other antibiotics. Resistance is always significant, but some relatively resistant strains of *Enterobacter* or *Klebsiella* may give zones in the lower end of the sensitive range (up to 15 mm). When treatment of systemic infections due to susceptible strains is considered, it is wise to confirm the results of a diffusion test with a dilution method.

[d] 300 ug or 250 ug sulfonamide discs can be used with the same standard of zone interpretation (MIC values are for sulfamethizole).

[e] Urinary tract infections only.

room temperature after gelling. The plates can be stored at 2 to 8C, but should be used within seven days, unless they are wrapped in plastic to prevent evaporation. Just before use, the plates should be placed in an incubator with the lid ajar to permit evaporation of the excess surface moisture.

The discs used in the test should contain the concentration of antimicrobic shown in Table II-I. Except for a small working supply, cartridges containing these discs should be stored with a desiccant at −14C or less until needed. The working supply can be safely stored at 2 to 8C for a week if they are kept dry. To minimize condensation, the discs should be allowed to come to room temperature before the container or dispensing apparatus is

opened. When not in use the container(s) should be returned to the refrigerator. Discs should not be used after the stated expiration date.

Four to five well isolated colonies of the same morphological type should be selected from an agar plate culture for use in preparing the inoculum. The top of each should be touched with a wire loop and the growth transferred to a tube containing 4 to 5 ml of broth (such as soy bean casein digest broth). Cultures should be incubated at 35C until growth equals or exceeds the standard described below. The turbidity of the culture should be adjusted to equal that of the standard as judged by visual comparison of the two with the aid of adequate lighting and a white background with a contrasting black line. The turbidity standard is prepared by adding 0.5 ml of 0.048 M $BaCl_2$ (1.175% w/v $BaCl_2 \cdot 2H_2O$) to 99.5 ml of 0.36 N H_2SO_4 (1% w/v). After the standard is mixed, 4 to 5 ml are distributed into tubes of the same size as those used for the broth cultures. The tubes should be sealed to prevent loss of fluid. The standard should be observed for evidence of deterioration and should be vigorously vortexed just prior to use.

Within fifteen minutes after the density of the inoculum is adjusted, a sterile cotton swab should be dipped into the culture and rotated against the inner wall of the tube to remove excess inoculum. Then a dried Mueller-Hinton plate is inoculated by streaking the swab over the entire surface of the agar. This procedure is repeated twice, and the plate is rotated approximately 60° each time. The lid is replaced and the excess moisture is allowed to absorb for not more than fifteen minutes. The appropriate discs are placed on the inoculated plate and gently pressed onto the agar surface with sterile forceps. The plates are inverted and incubated aerobically at 35C for eighteen to twenty-four hours. The incubator should not contain an increased concentration of CO_2. After incubation a confluent or nearly confluent lawn of growth should be observed. The diameter of each zone of inhibition is measured to the nearest mm with either calipers, a ruler, or a properly prepared template held on the back of the petri plate and illuminated with reflected light. If the medium contains blood, measurements should be made on the surface with the cover removed. The end point should be regarded as the area showing no obvious visible growth that can be detected with the unaided eye. Interpretations of the zone sizes are made as sensitive, intermediate, or resistant by referring to the standards shown in Table II–I.

FACTORS AFFECTING THE KIRBY-BAUER TEST

There are several variables in the test that can cause discrepancies in results if they are not properly controlled. A discussion of these variables follows. Unless otherwise indicated, one brand of Mueller-Hinton at pH 7.2 to 7.4, one brand of discs, and a temperature of 35C were used in these studies.

Medium

Although it is recognized that Mueller-Hinton agar is not the ideal medium for this purpose, it is the medium that must be used with this test. There is a need to develop a defined medium that can be used not only in agar diffusion tests but also in other kinds of susceptibility tests. In our laboratory, Mueller-Hinton agars from three commercial sources have been compared in a limited study. An example of the zone diameters obtained with the three is shown in Table II–II. The variation in the zone diameters obtained with the three media are within the limits generally accepted for the Kirby-Bauer procedure. Medium No. 3 is no longer commercially available.

The pH of the test agar can substantially affect the diameter of the

TABLE II–II

DIAMETERS OF ZONES OF INHIBITION OBTAINED WITH THREE DIFFERENT BRANDS OF MUELLER-HINTON AGAR

	Zone Diameter (mm)					
	S. aureus			*E. coli*		
	Medium			*Medium*		
Antimicrobic	*1*	*2*	*3*	*1*	*2*	*3*
Ampicillin	27	26	27	15	15	14
Cephalothin	28	26	29	19	20	19
Chloramphenicol	19	20	19	21	23	21
Gentamicin	20	21	20	20	18	19
Kanamycin	20	20	19	18	17	17
Streptomycin	16	16	16	14	13	14
Tetracycline	22	20	19	19	19	20

TABLE II–III

DIAMETERS OF ZONES OF INHIBITION OBTAINED WITH *S. AUREUS* AT DIFFERENT pH LEVELS

	pH of Mueller-Hinton Agar						
Antimicrobic	*6.0*	*6.5*	*7.0*	*7.2*	*7.4*	*8.0*	*8.5*
Penicillin	29	28	26	25	26	26	28
Ampicillin	24	25	26	25	25	26	29
Oxacillin	20	18	17	17	17	16	16
Cephalothin	28	26	25	25	25	28	29
Kanamycin	16	18	18	19	20	18	17
Neomycin	14	17	18	19	20	21	21
Streptomycin	11	13	15	15	16	18	18
Tetracycline	24	20	18	17	16	12	12
Chloramphenicol	19	18	18	18	19	20	20
Bacitracin	16	17	17	18	18	18	18
Erythromycin	15	20	20	21	21	26	27
Lincomycin	9	13	16	18	19	19	20