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Bacterial resistance and susceptibility to chemotherapeutic agents

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PREFACE

The outcome of treatment of bacterial infections with antibiotics is dependent upon many factors. Our capability to predict and to carry out successful therapy is modified by our inability to assess some of these factors particularly by conventional laboratory susceptibility testing. Many of the factors are related to conditions within the patient but many others are influenced by the structure, genetics and growth characteristics of the bacteria under test. My years as a physician, microbiologist and investigator have taught me that to understand the effective use of antibiotics and to improve on some of the important deficiencies of these agents, one should have an integrated overview of antibiotic activity. This view must take into account drug distribution and elimination in the patient, host tissue antagonism of antibiotic activity, toxicity, mechanisms of action and bacterial targets, drug penetration into bacteria, acquired and natural bacterial resistance mechanisms, the epidemiology of resistance, a clear view of where therapeutic problems exist among various clinically important bacteria and the weaknesses of our susceptibility testing systems.

Such an integrated view of antibiotics is not only useful to use drugs properly but it is useful to place emphasis in our diagnostic laboratories on overcoming such problems as slow answers to physicians on bacterial susceptibility, misleading test results, and uninterpretable and uninterpreted results. In investigational laboratories it points out where we should 'spend our money' to solve problems of therapeutic failure of antibiotics.

This book is intended to provide an overview of the many facets involved in antibiotic activity. Why is a bacterium susceptible or not susceptible to an antibiotic under a specific set of conditions? Why do bacteria change in susceptibility to antibiotics? An attempt has been made to review mechanisms of antibiotic action

and resistance in the conventional manner isolated from bacteria but also in place in the whole bacterial cell. Around this approach I have tried to outline the principles influencing antibiotic activity in the patient and in the laboratory test circumstance and to point out those bacteria and those antibiotics that pose some of our major problems of antibiotic therapy. Finally I have considered some of the things we can do to overcome loss of susceptibility.

It is probably necessary that a single author write a book such as this which attempts an integrated overview of antibiotics. It is a book intended to be read rather than to be encyclopedic. At the same time, I hope it has been written with enough detailed information to provide a satisfying substance to the reader. Needless to say, as a single author, it is difficult to cover many aspects of antibiotics and to give due credit to all individuals who have made worthwhile contributions. It is also difficult to keep such a broad field up to date. Only time will tell how successfully I have met my aims and overcome the hazards of a single-author book.

I am grateful to my colleagues especially Harvey Rabin and Allan Godfrey at the University of Calgary for their part in the development of an environment in which to write this book. My thanks to those individuals who sent information before publication, in particular Dennis Kopecko, Tim Foster, Naomi Datta, Bob Hancock and Thalia Nicas. I am indebted to Joan Godfrey for typing the manuscript. Finally, I express my gratitude to my family for their interest, encouragement and especially their patience in waiting for all those late meals.

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Antibiotic susceptibility and resistance – definition and detection

Criteria for the definition of susceptibility and resistance

The objective of laboratory testing of bacterial susceptibility to antibiotics is to identify microorganisms producing infections which either will or will not respond to conventional courses of antibiotic therapy. Such information can be used to direct initial therapy, evaluate therapy in progress or to develop susceptibility profiles of bacteria within a defined population (e.g. a hospital) as an aid to initial therapy. Response to therapy depends on both the course of antibiotics and on host factors including defense mechanisms and drug distribution. Laboratory testing methods, in general, do not fully take into account many of the factors which affect the susceptibility of bacteria within host tissues. These are discussed in later sections. The inability to assess 'host factors' means that laboratory susceptibility testing cannot give an absolute indication of *in vivo* antibiotic susceptibility. In order to provide a reasonably reliable prediction of the efficacy of an antibiotic for treating infections due to specific microorganisms, several types of criteria have been used to set susceptibility or resistance standards.

Clinical efficacy

Susceptibility of a bacterial isolate to an antibiotic is most reliably defined in relation to the record of clinical efficacy of that antibiotic. The initial assessment of efficacy (and toxicity) comes from experimental animal infections with a variety of selected bacteria. Prior to the introduction of an antibiotic, limited trials of human use are performed which provide further information on antibiotic effectiveness. These studies along with microbiological investigations provide data to establish toxic and therapeutic doses of antibiotics. However, to assess the relationship between

susceptibility testing and efficacy accurately, many years of use of the agent in humans are required.

Due to a wide variation in clinical circumstances and manner of antibiotic use, it may be difficult to make absolute correlations between efficacy and the patterns of drug use. Thus, it is important to continue assessment of therapeutic response to antimicrobial agents and to re-evaluate susceptibility criteria over several years.

A practical approach to define bacterial susceptibility based on clinical response to antibiotics is to compare the susceptibility of an organism of known susceptibility status (i.e. a treatment response to a standard antibiotic dose) with that of the organism isolated from an infection. This approach requires the determination of susceptibilities of both organisms by identical diffusion or dilution methods. If the clinical isolate is inhibited by antibiotic concentrations equal to or less than that required to inhibit the control organism, it may be regarded as susceptible. If it is distinctly less sensitive the organism is regarded as resistant. Between these categories organisms may be regarded as moderately resistant or intermediate.

Control organisms are best selected by their susceptibility to systemic or urinary antibiotic concentrations rather than being the same species as the test organism. However, it is important on certain occasions to test control species of the same type as the test isolate. *Pseudomonas aeruginosa* susceptibility to aminoglycoside antibiotics is highly dependent on the concentration of magnesium and calcium in the growth medium. Thus, a clinical isolate should be compared to a *P. aeruginosa* control similarly affected by divalent cations. This organism is also relatively resistant to many of the antibiotics with which it is normally treated. It is difficult to detect small increases in resistance which may be of marked clinical significance unless the same species is used as a control. If a diffusion method is used and fastidious organisms are being treated, it is preferable to use an identical control species or an organism with similar growth characteristics.

This procedure can be sophisticated by the use of a variety of organisms susceptible to a range of antimicrobial concentrations obtained in different tissue compartments. Control organisms should cover urinary and vascular antibiotic concentrations,

but on occasion controls for central nervous system, intraocular, bronchopulmonary and prostatic tissue concentrations are advisable. For ideal use of this approach numerous control strains are required which are susceptible to various concentrations of different antibiotics in tissues and which provide for the circumstances where a species of the same type is required.

Serum antibiotic levels

A second general method of defining susceptibility is based on a comparison between susceptibility concentrations and obtainable serum antibiotic levels. Inhibitory concentrations of the antibiotic in question are determined by one or more standard methods and related to serum levels produced by commonly used doses and routes of administration of the antibiotic. It has been common practice to utilize peak serum or mean (midpoint between highest and lowest serum concentrations) serum levels for comparison purposes. There are a host of variables affecting serum levels including dose, route of administration, rapid or slow infusion, renal function, body surface to mass ratio, etc. Due to the difficulty in precise definition of serum levels a safety factor has been added. Thus, it is often suggested the MIC (minimal inhibitory concentration) should be one-quarter or less of the mean serum level.

In the case of organisms for which the MIC is very much lower than serum concentrations, this method works well. Under these conditions tissue concentrations, which are often much lower than serum levels, usually are adequate to inhibit growth of the bacterial species. This relationship is illustrated by comparing commonly obtained MICs for *Streptococcus pyogenes* with benzylpenicillin blood levels obtained after oral or parenteral dosage with all but long acting penicillins. MIC values are usually in the range of 0.01 μg/ml and are well below serum levels. The predictive value of this approach is shown by a successful history of clinical efficacy of benzylpenicillin for *S. pyogenes* infection. However, in the case of many other antibiotics, MICs are much closer to peak or mean serum levels. An example is the susceptibility of *Pseudomonas aeruginosa* to carbenicillin. Frequently, MICs of carbenicillin for *P. aeruginosa* are only slightly below or equal to

obtainable peak or mean serum levels. In recent years in some institutions there has been a tendency to suggest that bacteria are susceptible to antibiotics if the MIC does not exceed peak or mean serum levels. However, the evidence is that, in the case of *P. aeruginosa* and carbenicillin, this relationship does not hold as carbenicillin is frequently ineffectual as a single agent for systemic infections with this bacterium.

The problems in directly relating achievable serum concentrations and MICs fall mainly into two areas. Different methods used to determine MICs yield different results particularly for certain antibiotics. The relationship of a particular susceptibility method to circumstances within the host is usually not clear. A striking illustration of the problem is seen with determinations of MICs of aminoglycosides for *P. aeruginosa*. Alteration of divalent cation content of the medium can markedly change MIC values. A second concern is that infections are frequently partially or completely sequestrated to tissue compartments in the host. The level of antibiotic appearing in that tissue compartment and particularly at the site of infection is usually much below peak or mean serum levels.

The use of trough blood levels (blood levels taken immediately prior to the next dose) may represent a more direct relationship between MICs and serum concentrations for many antibiotics. Trough serum levels which are detectable and stable after several doses may represent an equilibrium established between the vascular compartment, the various tissue compartments and the excretion of the drug. Unfortunately no simple relationship exists between MICs and serum levels. Tissues may hold antibiotics by extensive protein binding and concentrations may occasionally be above trough levels. This is particularly likely to be the case if levels are based on single doses or widely spaced doses. It has been shown, for example, that penicillin may persist in tissues after its disappearance from blood. However, in these circumstances the tissue levels were less than 5% of peak serum levels.

In general if serum antibiotic level and MIC relationships are to be established it seems unwise to use peak levels. Mean or particularly trough levels are more likely to represent tissue concentration.

Concentrations of an antibiotic in the urine may be much above serum levels if the antibiotic is concentrated during renal excretion. For lower urinary tract infections, urinary concentrations determine the efficacy of many antibiotics. To establish susceptibility criteria for urine isolates, MICs should be related to urinary and not serum concentrations.

Population distribution of bacterial susceptibility

The third method of defining susceptibility criteria is based on comparison of the susceptibility level of a bacterial isolate to that of a large population group of the same species or similar species. Should members of that population appear which are less susceptible, they may be designated as relatively or absolutely resistant. An example of this type of situation has been observed with *Neisseria gonorrhoeae* and response to penicillin therapy. This organism responded successfully to penicillin over a span of many years. In this interval population members with reduced susceptibility to penicillin appeared. These organisms had susceptibility levels several times those of the most sensitive members of the group and were associated with an increased incidence of treatment failures. However; it was possible to successfully treat urethral infections with such gonococci using an increased dose of penicillin. Eventually a third population group appeared which had resistance levels several times those of the moderately resistant group. This group had a very high proportion of therapeutic failures and could be regarded as fully resistant to penicillin.

Resistance mechanisms

In the case of certain antibiotics it is possible to define resistance mechanisms. Bacterial isolates can be examined for the possession of such resistance mechanisms and, if found to possess them, can be regarded as resistant. This situation is best developed for penicillins. The demonstration of a β-lactamase active on penicillin G in *Staphylococcus aureus* or active on ampicillin in strains of *Hemophilus influenzae* is a clear indication of antibiotic resistance.

Unfortunately, although much information is available on other

resistance mechanisms, many of these are not readily detected. In addition, resistance may be associated with a combination or variety of resistance mechanisms. However, in spite of these reservations, the possession of, for example, β-lactamase activity under defined circumstances is a clear indication of resistance. The problem is that a lack of such a resistance mechanism may not always be associated with susceptibility.

In practise the definition of susceptibility to antimicrobial agents is frequently the result of testing and studies in each of the four areas noted above. Bacterial isolates which are highly susceptible or highly resistant to antimicrobial agents do not normally pose significant problems. Most bacterial isolates which are presented to clinical laboratories tend to fall into either of these two categories. Bacterial isolates that cause difficulties in defining susceptibility and which point out the limitations of our currently used methods are those of borderline antibiotic susceptibility. These bacteria are important causes of nosocomial infections. Organisms like *Streptococcus fecalis*, *Pseudomonas aeruginosa*, several species of nonfermentative bacteria, and many gram-negative enteric aerobic and anaerobic bacteria may fall into this group on occasion. For example, while a particular isolate of *E. coli* may be clearly susceptible to ampicillin in the urinary tract, susceptibility of the same strain causing a serious pneumonia in a patient with compromised host defenses will be very much less clear. This is often rationalized on the basis that the host has impaired defenses. However, these circumstances only illustrate that our methods of determining susceptibilities depend heavily on host defenses. In such conditions the response of a microorganism to an antimicrobial agent depends mainly on the amount of active antibiotic delivered to the infection site and the susceptibility of the organism under those conditions. It is these circumstances which are most challenging to susceptibility testing and which are the least well-explored set of variables.

Susceptibility testing methods

Methods for testing bacterial susceptibility to antibiotics fall into three general groups: diffusion and dilution assays and detection of resistance mechanisms.

Diffusion methods

Disc diffusion. In these procedures antibiotic from a central diffusion source, most commonly a paper disc, is allowed to diffuse into an agar medium during the growth of a microorganism. A continuous concentration gradient between the disc and the most peripheral antibiotic results. After a period of growth, examination is made for a zone of inhibition of bacterial growth.

The significance of the zone can be assessed by several types of criteria. It can be compared to zones produced by control 'susceptible' organisms tested under identical conditions. This is the basis of the comparative and Stokes methods of disc susceptibility testing. Zones of inhibition may, alternatively, be related to minimal inhibitory concentrations (MICs) of antibiotics. This procedure requires that a relationship be established prior to the use of the method. The procedure must be very highly standardized so that a reproducible relationship of zone and MIC can be obtained. In general a large number of strains have their MICs and zones of inhibition for individual antibiotics determined and related by regression lines obtained from plots of zone diameter versus $\log_2$ MIC. Zone criteria for susceptibility are usually based on antibiotic concentrations in blood or urine.

It is obvious that many potential problems exist for the zone:MIC method. These include variation of both MIC (a 'discontinuous' method) and zone diameter (a 'continuous' method) measurements, requirement for strictly standardized methodology, a poor appreciation of which antibiotic blood level (e.g. peak, trough or mean) to use as well as other problems.

The formation of a zone of inhibition of bacterial growth requires that a critical concentration of antibiotic be obtained in the medium which is adequate to keep growth below a critical observable cell density. When the antibiotic concentration in the medium is less than the critical concentration, growth occurs and a zone is established. A close relationship exists between the critical concentration and the MIC of a strain when determined under similar conditions.

The size of the zone depends at what distance from the central antibiotic source the critical concentration is formed. This distance depends on the rates of diffusion of the drug and of growth of the

bacteria. Diffusion rates depend mainly on size, polarity, lipid solubility and chelating capabilities of the drug; temperature of incubation; concentration gradients; and thickness, pH, ionic strength and other components of the growth medium. Growth rates depend on the organism, temperature of incubation, growth atmosphere, pH, initial inoculum, nature of growth medium, the presence of antibiotic antagonists (e.g. thymidine with trimethoprim) etc.

In practise the following items require careful control particularly for the Kirby–Bauer and Ericcson diffusion methods.

Inoculum. The size of inoculum influences the time required before a critical cell density is obtained. Inoculum size is particularly important among bacteria producing β-lactamases especially those where the mechanism of resistance is principally a population phenomenon (see Chapter 5). Inoculum size is also critical in determining sulfonamide susceptibility due to the presence of adequate preformed *p*-aminobenzoic acid to allow enough growth to obscure a zone. Inocula may be applied by flooding (Ericcson), swab inoculation (Kirby–Bauer, comparative, Stokes methods) or agar overlay (Barry modification of Kirby–Bauer). In most cases semi-confluent to barely confluent growth are widely used as inocula.

Composition of the medium. The nature of the medium may markedly alter rates of antibiotic diffusion and bacterial growth. Additionally some medium constituents may antagonize or enhance antibiotic activity. Antagonists include: intermediates and end products of folate metabolism (particularly thymidine) inhibit trimethoprim and sulfonamides, preformed *p*-aminobenzoic acid antagonizes sulfonamide activity; divalent cations antagonize aminoglycoside cell entry and cell binding of polymyxins; divalent cations may chelate with tetracyclines to reduce tetracycline activity; increased phosphate concentration may reduce aminoglycoside activity; high salt concentration reduces aminoglycoside but increases bacitracin and fusidic acid activities; alkaline pH increases activity of aminoglycosides, macrolides, lincomycins and less frequently some other agents; acid pH increases activity of

tetracyclines, fusidic acid, novobiocin and occasionally other agents; agar mainly through sulfonate and sulfonic acid residues bind positively charged drugs (aminoglycosides, polymyxins).

In general the addition of 5–10% blood or serum to medium tends to produce relatively small effects but could conceivably reduce zones for highly protein-bound drugs. For some highly protein-bound drugs this may more closely resemble *in vivo* conditions, although higher serum concentrations are needed to show much effect. The type of growth medium may alter zones in other undefined ways. Unfortunately batch to batch variation of the composition of growth media remains a problem. In the Kirby–Bauer method, the medium is specified as Mueller–Hinton (MH) and in the Ericcson method as MH or PDM antibiotic sensitivity medium. Those procedures using comparative zones can be performed with a variety of testing media as the control is grown under the same conditions.

Temperature of incubation. Temperature changes alter rates of antibiotic diffusion and bacterial growth. *Staphylococcus aureus* may not produce detectable methicillin resistance at temperatures greater than 35 °C.

Depth of medium. A reduction in thickness of agar depth changes rates of antibiotic diffusion. This is most pronounced with very thin agar layers where zones may markedly increase in size. A depth of 4 mm is recommended.

Conditions of incubation. Carbon dioxide (5–10%) in the atmosphere may decrease pH and reduce the activity of erythromycin, lincomycin and aminoglycosides. Anaerobic conditions markedly antagonize aminoglycoside activity. Obviously anaerobic growth conditions are required to test susceptibility of aero-sensitive bacteria.

Disc application and storage. Prediffusion of antibiotic prior to inoculation increases zone sizes. Pre-incubation of inoculated plates prior to addition of antibiotic decreases zone sizes. It is very important to dry plates before adding antibiotic discs to prevent antibiotic leaching from the disc into moisture on plates.

Although cups or wells may be used to store the antibiotic, paper dics are most widely used. Discs should be stored preferably at −20°C except for those in current use which can be stored at 4 °C. This is particularly important for various penicillins, cephalosporins and tetracyclines. It is essential that discs be kept dry until used by storage with a desiccant.

Disc content refers to the total amount of drug per disc. Contents of discs are standardized for each of the methods although more rigidly for the Kirby–Bauer and Ericcson methods. Control strains are used in all methods and usually involve strains of *S. aureus, E. coli* and *P. aeruginosa.* Specific strain numbers have been recommended in certain cases.

Zones of inhibition, if measured, should be read repeatedly in the same manner. Considerable variation in the size of the same zones recorded by different individuals can be readily observed. Comparison of zone sizes to those of susceptibility categories is best achieved through the use of templates or calipers. Lighting and definition of the zone margin are variables that require careful standardization. Rigorous quality control standards have been developed for the Kirby–Bauer method.

Other diffusion procedures. Other reservoirs of antibiotic may replace paper discs. These include paper strips, cups or cylinders, agar wells, antibiotic tablets and others. Although of value when carefully performed, these methods are in limited use.

Dilution methods

Dilution methods are used to determine MICs and may be used to determine minimal bactericidal concentrations (MBCs) of antibiotics. These procedures may be carried out with broth or in agar medium. The former is more readily adapted for determining MBCs.

Dilution methods are most widely performed using doubling or halving concentrations in broth or agar medium based on the unit of 1 (e.g. 0.25, 0.5, 1, 2, 4 etc.). As such, the method involves a discontinuous concentration gradient which partly accounts for the widely held view that the method is more precise than diffusion testing which involves a continuous antibiotic concentration