Antimicrobial Susceptibility Testing by the
Kirby-Bauer Disc Diffusion Method

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ABSTRACT

Antimicrobial susceptibility testing by the disc diffusion method is discussed with emphasis on the need for standardization of methodology. The Kirby-Bauer method is currently widely used by clinical laboratories and best incorporates many of the features recommended by the World Health Organization. It is relatively simple from a techniological standpoint and accurate and reproducible so long as the methodology is followed with care. Modifications should be accepted only after full documentation of the effects on inhibition zone sizes.

Introduction

The physician's decision to use an antimicrobial drug and to select the most appropriate agent involves consideration of a large number of factors. From the clinical standpoint, the nature and localization of the disease, concurrent medical conditions of the patient and possible patient allergic sensitivity to the drugs are of great importance. Similarly, an understanding of the pharmacologic action of the various chemotherapeutic agents must be considered. The actions may include possible toxicity, their methods of administration and excretion, the obtainable blood and tissue levels, their mode of action and whether or not bacteriocidal or bacteriostatic are of great importance. Lastly, the isolation and identification of the offending organism and the determination of its in vitro antimicrobial susceptibility must be accomplished. While all the above factors must be based upon sound clinical judgment when treating a patient, it is usually only the isolation and identification of the organism and the in vitro determination of its antimicrobial susceptibility which are routinely and readily submitted to objective laboratory analysis.

In vitro systems of testing antimicrobial sensitivity are accomplished in a milieu free of those influences which are often important in clinical infections. Factors such as host immunological competence and in vitro inactivation of antimicrobial agents by metabolic breakdown are not evaluated. However, attempts to demonstrate the influence of antimicrobial agents on bacterial growth under laboratory conditions have been found in most situations to correlate well with the clinical outcome of infected patients treated with those agents. Thus, if bacterial growth is inhibited by a particular agent in vitro, favorable clinical response to treatment of an infection with that agent can usually be anticipated. Similarly, failure of an antimicrobial
agent to inhibit growth of a bacterium in the laboratory usually accurately predicts that agent's inability to cure a clinical infection when it is used therapeutically. In addition to the above, quantitative factors are of great importance. First, the inhibition of bacterial growth by antimicrobial agents is not usually an all or nothing phenomenon, but rather it tends to be dose related. Thus, some bacteria may be inhibited by a low concentration of a particular agent and others may require a higher concentration for inhibition. Still others may actively proliferate in spite of very high concentrations of the antimicrobial agent, and lastly, some organisms may actually become dependent upon antimicrobial agents for growth. Secondly, knowledge of the physiology of each antimicrobial agent leads to appreciation of the obtainable blood or tissue levels resulting from administration of that agent in the recommended dose via a particular route. Therefore, in order for a bacterial species to be considered sensitive to a particular agent, critical blood or tissue concentrations of that agent must be obtainable when it is administered therapeutically. Furthermore, since the in vivo administration of these agents is a dynamic situation resulting from absorption, excretion, metabolic catabolism and inactivation by protein binding of the agents, tissue levels of two to four times the in vitro minimal inhibitory concentration are generally regarded as necessary for a clinical response. In order for in vitro antimicrobial testing to be most meaningful, therefore, it must correlate minimal inhibitory concentrations with the usually obtainable blood or tissue levels.

There are generally three basic approaches to performing antimicrobial susceptibility tests, namely, broth dilution, agar dilution and disc diffusion methods. The broth dilution method consists of preparing multiple concentrations of the antimicrobial agents in tubes of broth and subsequently inoculating them with the organisms to be tested. The resulting inhibition of bacterial growth can be clearly related to the concentration of antimicrobial agent. The lowest concentration resulting in inhibition of growth is referred to as the minimal inhibitory concentration (M.I.C.). Agar dilution methods are similar except that the dilutions are made in agar and the bacteria to be tested are inoculated onto the surface of multiple agar plates containing variable concentrations of the antimicrobial agents. Results of bacterial growth or inhibition are related to the concentration of the antimicrobial agents and reported in terms of M.I.C. Although both of these methods provide excellent correlation with clinical response, they have been adopted by clinical laboratories only to a limited extent because of their complexity and requirement of large amounts of technical time for completion.

Disc diffusion methods, on the other hand, have always enjoyed great popularity in busy clinical microbiology laboratories because of their relative simplicity and ability to easily test multiple antimicrobial agents on each bacterial isolate. The method involves the placing of antimicrobial impregnated paper discs onto the surface of agar which has previously been seeded with the bacteria to be tested. The antimicrobial agent subsequently diffuses into the agar where it may inhibit bacterial growth in a zone surrounding the disc. Early applications of the method sought only the demonstration of an inhibition zone with little regard for the nature of the agent or zone size. While extensively used, however, many serious workers soon became aware of the lack of standardization and inability to duplicate results and make consistent interlaboratory comparisons. It began to be appreciated, for example, that the amount of bacterial inoculum, rate of bacterial growth, pH of the media and the type and depth of media influenced the size
of inhibition zones. Variations in disc preparation, such as antimicrobial content, storage conditions and type of paper, were also found to be important. Many modifications appeared, each attempting to standardize the many variables in a better way. Recommendations were made for single high or low potency discs as well as two or more discs of variable potency. Many of these methods tended to approach inhibition zones from a qualitative sense; that is, the presence of an inhibition zone was regarded as evidence of sensitivity to a given antimicrobial agent rather than critically evaluating its size. In 1958, Hoette and Struyk described the dynamic sequences of antimicrobial agent diffusion from the impregnated discs into the agar. A diffusion gradient resulted in the highest concentrations of antimicrobial agent in the agar at the edge of the disc with gradually falling concentrations found peripherally as the distance from the disc increased. The gradient was most steep in the first few hours. By 24 hours, however, equilibrium was established resulting in antimicrobial agent concentration that was essentially the same at the edge of the disc and up to the 30 mm diameter mark. Hoette and Struyk proposed a method of altering the disc antimicrobial content so that the resulting concentration in the agar at the 10 mm mark, after approximately six hours diffusion, was similar to blood levels obtained therapeutically. They then defined organisms as resistant to the agent if the inhibition zone was smaller than 10 mm in diameter and sensitive if the zone was greater than 10 mm in diameter. The work of the World Health Organization Collaborative Study for the Standardization of Methods for Conducting Microbic Sensitivity Tests has served to emphasize the great need to develop a relatively standard approach to antimicrobial susceptibility testing. Similarly, a Supreme Court decision in 1969 has required the Food and Drug Administration to regulate and control antimicrobial disc content through certification and to make recommendations as to its proper use. The method described by Bauer, Kirby, Sherris and Turck correlates zone sizes with dilution techniques, incorporates many of the recommendations of the WHO group and has been widely accepted by many clinical laboratories. It is this method that is currently recommended and will be subsequently discussed.

**Principle**

The extent of diffusion of the antimicrobial agent into the agar is influenced by many physiochemical properties of both the agent and the agar including its solubility, the pH, temperature and the agar concentration and depth within the plate. This diffusibility differs greatly between individual antimicrobial agents and is unique to each agent. The antimicrobial agent concentrations in the agar are the result of dynamic events with multiple variables and, hence, bear no numerical relationship to the amount of agent impregnated into the disc. The established diffusion gradient is fairly steep for up to six hours, after which progression toward equilibrium results in gradual falling off of the concentration near the disc edge. A zone of inhibition of bacterial growth results when an antimicrobial concentration in the agar equal to or greater than the minimal inhibitory concentration acts upon a critical population of bacterial organisms which have been inoculated on the agar surface. Following inoculation of the organisms on the agar surface there is a variable lag time of two to four hours before active bacterial proliferation begins. It is during this period that the zone diameter is formed and its size determined by the effective concentration of an antimicrobial agent at some specific distance from the disc edge. Beyond this specific distance, the lower antimicrobial concentration allows the bacteria to
proliferate. Subsequent incubation produces minimal change in the zone radius until the gradient drops to the point at which decreasing inhibition, in the absence of bactericidal effects, allows multiplication of the population.

For a given antimicrobial agent, there is an approximate linear relationship between the logarithm of the minimal inhibitory concentration and the zone diameter for organisms with reasonably comparable growth rates. Using their own standardized technique, Bauer et al. and Sherris and Schoenhect have correlated zone diameters of multiple antimicrobial agents with M.I.C.s as determined by tube and agar dilution methods. From knowledge of the usually obtainable blood and/or urine concentrations resulting from standard therapeutic regimes, organisms have been described as sensitive, intermediate or resistant to given antimicrobial agents based on the diameter of inhibition zones.

Discussion

In 1972, six years after its introduction, the Kirby-Bauer method has been widely adopted by clinical laboratories. Multiple authors have attested to its great reproducibility and relative simplicity. All authors have emphasized the need to follow the technique as described and to accept modifications only after full documentation of the effects of any such changes on inhibition zone diameters.

The use of Mueller-Hinton agar was suggested by the WHO group because it was regarded as a relatively simple medium which could be prepared by a variety of commercial firms in a fairly uniform manner. Furthermore, its relative simplicity made it unlikely to inhibit or augment the action of multiple antibiotics and, specifically lacking sulfonamide inhibitors, it was useful for testing those agents. The pH of the agar medium has been found to be variable requiring continual surveillance and periodic alteration. Growth of fastidious organisms may require addition of blood. Zone sizes are not thus influenced except in the case of the highly protein bound antibiotic novobiocin and sulfonamides which may be inhibited by para-amino benzoic acid. Tetracycline zone sizes vary, according to the concentrations of di- and multivalent cations in the medium, which leads to varying degrees of chelation and partial inactivation of the antibiotic. Similarly, gentamycin testing of Pseudomonas aeruginosa has been shown to be influenced by divalent cations, particularly magnesium, in the agar medium with high concentrations resulting in smaller inhibition zones. Esser et al. have suggested the use of Oxoid medium for the Kirby-Bauer method citing clearer and more distinct inhibition zones, less swarming of proteus organisms and better growth of all organisms in general. Slight changes in zone diameters have been noted which should be further verified prior to adoption of this modification.

Perhaps one of the most difficult areas to control is the antimicrobial disc. Commercially available discs meet FDA certification standards, and the appropriate potency must be used if the Kirby-Bauer zone-size interpretative charts are to be used. Many antimicrobial agents are thermolabile, requiring refrigeration and protection from hydration for storage. Extra care is required of the penicillins and cephalosporins which are particularly labile and should be stored at freezing temperatures. The discs are frequently subjected to wide temperature fluctuations during shipment from distributors to laboratories, resulting in uncertain degrees of deterioration. Because of the instability of many antibiotics at incubator temperatures, inhibition zones should not be read later than 24 hours after the plates have been inoculated. Inhibition zones previously formed may subsequently be overgrown by bacteria which are no longer
inhibited by deteriorated antibiotics if this precaution is not observed. Lastly, periodic blank discs devoid of antimicrobial activity result either from manufacturing mishaps or subsequent deterioration. All of the aforementioned serve to emphasize the need for continual quality control efforts. Only by such tests can one hope to detect the sporadic occurrence of faulty technique or deteriorated reagents. Stable colonies of *Staphylococcus aureus* and *Escherichia coli* should be maintained and tested at regular intervals. By continual observation of inhibition zone diameters, gradual or abrupt changes can signal possible difficulties with the procedure.

The method of inoculating the plates with bacteria must be carefully controlled. The effect of small zones produced by large inoculations and large zones resulting from light inoculations has been repeatedly demonstrated. Particularly because of the inability to control the size of the inoculum, sensitivity tests performed on direct clinical materials are often misleading. If, however, gram stains of clinical materials suggest that only a single type of organism is present, preliminary sensitivity studies may be performed directly to serve as an early guide to therapy. These tests should be repeated, however, after isolation with a carefully controlled inoculum. Another method of inoculating the plates has been proposed by Barry et al. They have suggested incorporating the organisms in melted agar to form an overlay on the petri dish. Using Mueller-Hinton agar, it was indicated that the inhibition zones are clearer although of the same size diameter as those formed with the standard Kirby-Bauer method. Barry et al. believe that their method is more practical for testing rapidly growing pathogens, including *Enterobacteriaceae, S. aureus* and *P. aeruginosa*.

The disc diffusion method of antimicrobial susceptibility should be regarded, therefore, as a reproducible semiquantitative estimation of the minimal inhibitory concentration for fast growing organisms if standardized methodology is rigorously followed. The disc diffusion method should not be used for slow growing organisms or for sulfonamide testing of meningococci because of the clinical and epidemiological significance of low levels of resistance. It is also regarded as unsatisfactory for testing of *N. gonorrhoea*. Disc diffusion methods are sometimes difficult to interpret for agents, such as colistin and polymyxin B, which diffuse poorly. Broth or agar dilution methods may be required in those instances. All methods of susceptibility testing suffer from periodic contamination with other bacteria, but such contamination is easier to detect with the agar tests.

**Clinical Interpretation**

The designation of organisms as resistant, intermediate or sensitive relates to correlation of the usually obtainable blood or urine concentration of the antimicrobial agent when it is given in the usual therapeutic dose. While many factors must come into play in the selection of an appropriate antimicrobial agent, the results of *in vitro* testing have proved most helpful. In general, when organisms are demonstrated to be resistant to a particular antimicrobial agent, it is illogical to consider use of that drug. Only in those instances where the drug can be given in massive doses, should such be considered if resistance or intermediate status of the bacteria has been demonstrated. For example, massive doses of penicillin with streptomycin are often successfully employed in subacute bacterial endocarditis due to resistant or intermediate enterococci. Demonstration of susceptibility to a particular agent, on the other hand, represents only one facet in the decision to employ a given drug. If, however, the agent can be safely administered to an immunologically competent patient, and adequate levels of the agent can reach
the site of infection, clinical response usually can be anticipated.

References


“Men do not stumble over mountains, but over molehills.”

Confucius