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TENTATIVE STANDARD

Performance Standards for Antimicrobial Disc Susceptibility Tests, as used in Clinical Laboratories

(November 13, 1972)

Attached is the tentative standard on the above subject for your review and study. This standard will remain tentative for a period of one year while comments are being received from members or others who may have an interest in this standard. At the end of the year, the comments will be studied by the Board and the respective area committees and a determination made as to whether the standard whould be submitted for adoption as an NCCLS Standard.

You are urged to give this serious consideration so that the final standard will represent a real consensus.

PERFORMANCE STANDARDS FOR ANTIMICROBIAL DISC SUSCEPTIBILITY TESTS, AS USED IN CLINICAL LABORATORIES

1. INTRODUCTION

A variety of laboratory techniques can be used to measure the *in vitro* susceptibility of bacteria to antimicrobial agents. Recent recommendations of the International Collaborative Study and regulations proposed by the Food and Drug Administration represent important steps in the development of standardized methodology. The NCCLS subcommittee on antimicrobial susceptibility testing has reviewed and coordinated its efforts with those of these other groups. The subcommittee's recommendations are consistent with the efforts of these other groups.

The following document is concerned only with disc diffusion methods that can be used for routinely testing the common rapidly growing bacterial pathogens in clinical laboratories. The procedures recommended in this report are outlined in order to establish a minimum standard of performance which we consider to represent the most practical but yet accurate and precise methods now available. It is anticipated that improvements in the current methodology may be developed but alternative methods can not be accepted until they have been adequately tested and shown to be at least as precise and accurate as the currently recommended technique. The type of information which would be required to properly evaluate other techniques is included in this report to serve as a guide to those who wish to propose other methods. Although the following recommendations are limited to a description of disc diffusion methods to be used routinely in clinical diagnostic laboratories there is no intent to suggest that other approaches such as broth or agar dilution techniques can not also be used for this purpose.

2. INDICATIONS FOR PERFORMING SUSCEPTIBILITY TESTS

2.1 Susceptibility testing is indicated for any organism contributing to an infectious process warranting chemotherapy, if its susceptibility cannot be predicted from knowledge of its identity. Susceptibility tests therefore are most often indicated when the causative organism has been identified as a species known to be capable of exhibiting resistance to commonly used antimicrobic agents, e.g. Staphylococcus species and the Enterobacteriaceae. Some organisms have predictable susceptibility to antimicrobial agents; susceptibility tests are unnecessary when the infection is due to a microorganism that is invariably susceptible to a highly effective drug, for example the apparently universal susceptibility to penicillin of Streptococcus pyogenes, S. pneumoniae and N. meningitidis in the U.S.

2.2 Isolated colonies of organisms which may be playing a pathogenic role should be identified on the primary agar plates and then tested under controlled conditions. Mixtures of different types of microorganisms should not be tested on the same susceptibility test plates. Therefore, the practice of conducting susceptibility tests directly with clinical material should be avoided except in clinical emergencies when the direct gram-stain suggests a single pathogen. In instances when testing has been carried out directly with clinical material, the susceptibility test should always be repeated using the standardized methodology. When the nature of the infection is not clear and the specimen contains mixed growth or normal flora in which the organisms probably bear little relationship to the infectious process being treated, susceptibility tests are often wasteful and may be grossly misleading.

3. SELECTION OF ANTIMICROBICS TO BE TESTED ROUTINELY

3.1 In order to minimize further confusion, all antimicrobic agents should be referred to by generic names only and laboratory reports should not refer to specific trade names.

3.2.1 In order to simplify the routine susceptibility test it is necessary to limit sharply the number of drugs to be tested routinely. In general, routine tests should include only one representative of each group of antimicrobics with closely related *in vitro* activity. Tests should be limited to those drugs which represent currently useful agents which are commonly utilized within the institution where the tests are being performed and which are appropriate for use in treatment of the specific pathogen under test. Clearly, the agents routinely tested against gram positive cocci should differ from those tested against gram negative bacilli. In addition, certain antimicrobics are limited in use to the treatment of urinary tract infections, i.e. nitrofurantoin and nalidixic acid. These agents should not be used to test microorganisms recovered from material other than urine.

3.2.2 *In vitro* tests with methenamine manadelate should not be performed because the activity of this drug *in vivo* depends on the attainment of a urinary pH of 5.0 or less and *in vitro* test conditions may bear no relationship to the situation in the urine, the only possible site of antibacterial activity in the patient.

3.2.3 The following guidelines can be used for selecting agents to be tested routinely.

PENICILLINS - Generally, it is advisable to test all staphylococci against benzyl penicillin (penicillinase labile) and against one penicillinase stable drug such as methicillin, oxacillin or nafcillin. Cloxacillin or dicloxacillin should not be used for this purpose because they may fail to detect certain heteroresistant strains. In addition, ampicillin should be tested with gram negative bacilli. There is no reason to include ampicillin in tests with staphylococci because of its lesser activity and similar susceptibility to penicillinase as compared to penicillin G. Carbenicillin may need to be tested against the gram negative bacilli, especially Pseudomonas sp.

CEPHALOSPORINS - Of the available cephalosporanic acid derivatives only cephalothin need be tested routinely. However, testing with other cephalosporins be indicated for testing the occasional gram negative bacillus which appears to be resistant to cephalothin and alternative drugs are unsatisfactory. Because of its increased susceptibility to penicillinase, cephaloridine should not be tested against staphylococci.

TETRACYCLINES - Drugs within this group are closely related and only tetracycline should be tested routinely.

POLYMYXINS - Polymyxin B and polymyxin E (colistin) are closely related and only one needs to be tested routinely. The polymyxins diffuse poorly in agar and the accuracy of the diffusion method is therefore less than with other antibiotics. Resistance is always significant, but when treatment of systemic infections due to apparently susceptible strains is being considered, it is wise to confirm the results of a diffusion test with a dilution method.

AMINOGLYCOSIDES - This group of chemically related drugs includes kanamycin, neomycin, streptomycin and gentamicin. Their relationship is not complete enough to assume that if an organism is resistant or susceptible to

one it will be resistant or susceptible to another.

MACROLIDES - This group of drugs includes erythromycin and oleandomycin which have similar activity but incomplete cross-resistance. For susceptibility testing the two drugs cannot be used interchangeably Erythromycin is usually the preferred therapeutic agent and is therefore used most frequently for susceptibility testing.

THE LINCOMYCIN GROUP - These drugs have an *in vitro* and clinical spectrum of activity similar to that of erythromycin. However, cross resistance is not complete and the lincomycin group (lincomycin and clindamycin) cannot be used to replace erythromycin in susceptibility testing.

3.2.4 The list of drugs in the following table represents a compromise based on various considerations including microbiologic factors and clinical-pharmacologic considerations. It fulfills the basic requirements for routine use in most clinical laboratories. Additional drugs should be available for use with special problems of the individual patient. Drugs other than those appropriate for use in therapy may be tested to provide epidemiologic information and taxonomic data; however, routine reports to physicians should include only those appropriate for therapeutic use in order to avoid misleading information. For example, susceptibility to nitrofurantoin or nalidixic acid should not be reported with organisms isolated from sites other than the urinary tract.

Staphylococcus aureus [a]	Enterobacteriaceae	Pseudomonas sp.
1. Penicillin G	1. Ampicillin	1. Polymyxin B.
2. Methicillin or Oxacillin or Nafcillin	2. Cephalothin	2. Gentamicin
3. Cephalothin	3. Kanamycin	3. Carbenicillin
4. Erythromycin	4. Polymyxin B	4. Kanamycin [d]
5. Clindamycin	5. Gentamicin	5. Chloramphenicol [d]
6. Chloramphenicol [b]	6. Tetracycline	6. Tetracycline [d]
7. Gentamicin [b]	7. Chloramphenicol	7. Sulfonamide [c,d]
8. Tetracycline [b]	8. Nitrofurantoin [c]	
9. Kanamycin [b]	9. Nalidixic Acid [c]	
10. Vancomycin [b]	10. Sulfonamides [c]	

[a] These drugs may be used for testing enterococci but ampicillin should be added and kanamycin, gentamicin and clindamycin should be omitted, methicillin may be tested for taxonomic purposes only.

[b] May be tested with Staphylococcus aureus, but as secondary drugs only.

[c] Only with microorganisms recovered from urinary tract infections.

[d] May be indicated for testing some Pseudomonas species, other than P. aeruginosa.

4. CURRENTLY RECOMMENDED DISC DIFFUSION TECHNIQUES

4.1 For susceptibility testing disc diffusion methods that employ interpretive standards relating the presence of any zone of inhibition to susceptibility of the organism to the drug under test are not acceptable. More reliable results can be obtained with disc diffusion tests which utilize the principle of zone diameter measurement correlated with minimal inhibitory concentrations and the behavior of strains among known clinically sensitive and resistant species. However, the technical details of such procedures must be carefully standardized and controlled in order to obtain optimal results. The procedure described below (4.3) is one technique for which sufficient data are now available to evaluate its accuracy and precision.

4.2 The disc diffusion test currently recommended by the NCCLS subcommittee on antimicrobial susceptibility testing, is a slight modification of that described by Bauer, Kirby, Sherris and Turck (Am. J. Clin. Pathol. 45; 493, 1966) Of all the diffusion tests proposed in this country, this represents the most completely described method for which interpretive standards have been developed and supported by a great deal of clinical and laboratory evidence. The only alternative method which has been adequately studied and shown to give comparable zone sizes, similar precision and satisfactory correlation with minimal inhibitory concentrations, is the agar overlay modification of Barry, Garcia and Thrupp (Am. J. Clin. Pathol. 53; 149, 1970). This involves an acceptable alternative method for standardizing the inoculum for testing the commonly isolated rapidly growing bacterial pathogens such as S. aureus, members of the Enterobacteriacae and Pseudomonas aeruginosa, but it is not applicable to tests with other microorganisms such as the streptococci or Hemophilus sp.

Plates inoculated by either of these methods can be interpreted with the same zone size standards.

4.3 Agar Medium

4.3.1 Of the many media now available, the subcommittee considers Mueller-Hinton agar to be the best compromise for routine susceptibility tests, since it shows rather good batch-to-batch reproducibility for susceptibility testing and is low in sulfonamide and tetracycline inhibitors and gives good growth of rapidly growing pathogens. In addition, a large amount of data has been collected with tests performed with this medium and if another agar medium were to be selected most of the previous studies would have to be repeated and new zone size interpretive standards established. With the addition of 5% defibrinated sheep, horse or other animal blood, Mueller-Hinton agar can be used for testing certain fastidious microorganisms which cannot grow on Mueller-Hinton agar without the added supplement. The blood must be free of antimicrobial activity.

Although an ideal medium is not yet available it would have the following characteristics:

1. The contents should be defined at least to the point of specific production details for crude components such as "peptone" and agar.
2. Susceptibility test results should be reproducible on different batches of the medium prepared by different manufacturers.
3. Without enrichment, the medium should support growth of the majority of pathogens for which susceptibility tests are required.
4. The medium should be free of components known to be antagonistic to the common agents for which susceptibility tests are made.
5. The medium should not be subject to marked pH shifts especially to the acid side during growth of common pathogenic organisms.

6. The agar and broth version of the medium should have the same formulation, except for the presence or absence of the solidifying agent.
7. The medium should be approximately isotonic and the agar medium appropriate for addition of blood when required for growth of fastidious organisms.

The subcommittee recognizes the need for a medium which better meets the above requirements. If economically feasible, this defined medium might be used routinely in diagnositc laboratories. If not economical for such use, a defined medium could serve as a standard to which the performance of more complex media could be compared.

4.3.2 Mueller-Hinton agar is prepared according to the manufacturer's recommendation and immediately after autoclaving it should be allowed to cool in a 45° - 50° C waterbath. If needed, the defibrinated blood is added to the cooled medium to give a final concentration of 5% (v/v). When necessary, the blood-containing medium may be "chocolatized" in order to support growth of Hemophilus sp. The freshly prepared and cooled medium is poured into petri dishes on a level, horizontal surface so as to give a uniform depth of approximately 4 mm. This corresponds to approximately 60 ml of medium for plates of 14 cm internal diameter and approximately 25 ml for plates of 9 cm internal diameter. Either glass or plastic petri dishes are suitable but the bottom half of the plates must be flat. The agar medium should be allowed to cool to room temperature and unless used the same day should be stored in a refrigerator (approximately 2° - 8°C). These plates should be used within seven days after preparation unless adequate precautions, such as wrapping in plastic, have been taken to minimize evaporation and they have

been shown to perform correctly with the control organism as specified in Section 4.9. Samples of each batch of blood containing plates should be tested for sterility, by incubating at 35° to 37°C for 24 hours.

Just before use, the plates should be placed in an incubator (35° to 37°C) with lids ajar until excess surface moisture is lost by evaporation (usually 10 minutes). There should be no droplets of moisture on the surface of the medium or on the petri dish covers when the plates are inoculated. If the agar overlay method of inoculation is used (4.5.2), the plates must be warmed to room temperature but the surface should not be dried before inoculation.

4.3.3 The pH of each batch of Mueller-Hinton agar should be checked at the time the medium is prepared. The exact method by which this is done will depend largely upon the type of equipment available in each laboratory, but the agar medium should have a pH of 7.2 to 7.4 at room temperature and must therefore be checked after gelling. This can be achieved by macerating a small amount of agar in a small volume of distilled water, or by allowing a small amount of agar to solidify around a pH electrode in a small beaker or by using a properly calibrated surface electrode.

4.4 Storage of antimicrobic discs. Cartridges containing filter paper discs specifically certified for susceptibility testing are generally supplied in separate jars, each containing desiccant (preferably an indicating desiccant). The jars should be kept refrigerated (approximately 4°C) or frozen (-14°C or less) until needed. Discs containing those drugs belonging to the penicillin family and cephlosporins should always be kept frozen to maintain their potency except for a small working supply which can be held at 4°C for up to one week. The unopened jars should be removed from the refrigerator or freezer one or two hours before the discs are to be used and allowed to equilibrate to room temperature

before being opened. This is done in order to minimize the amount of condensation that would occur when warm room air reaches the cold jars. If a disc dispensing apparatus is used it should be tightly sealed and supplied with an adequate indicating desiccant, also it should be allowed to warm to room temperature before being opened. When not in use the dispensing apparatus containing discs should always be kept refrigerated. Only those discs that have not reached the manufacturers stated expiration date on the label should be used. Discs must be discarded on the stated expiration date.

4.5 Inoculation of test plates

4.5.1 The currently recommended method is performed as follows:

A. Select at least four to five well isolated colonies of the same morphologic type from an agar plate culture. Touch the top of each colony with a wire loop and transfer the growth to a tube containing 4 to 5 ml of a suitable broth medium such as soy bean casein digest broth.

B. Allow the broth culture to incubate at 35° to 37°C until it achieves or exceeds the turbidity of the standard described below (usually 2 to 5 hours).

C. Adjust the turbidity of the actively growing broth culture with sterile saline or broth so as to obtain a turbidity visually comparable to that of the turbidity standard described below. In order to perform this step properly there must be an adequate source of light, and reading should be made against a white background with a contrasting black line, to aid in the visual comparison.

D. 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 0.36N H_2SO_4 (1% v/v). Four to six ml are then distributed into screw cap tubes of the same size as used in growing the broth culture inoculum. These tubes are tightly sealed and stored in the dark at room temperature. This turbidity standard must be vigorously agitated on a mechanical vortex shaker just before use. A fresh standard must be prepared at least once every six months. There is some evidence that deterioration may occur and close scrutiny is indicated.

The subcommittee recognizes the potential source of error associated with the use of such a $BaCl_2$ turbidity standard and efforts are now under way to define a more reliable permanent turbidity standard or a more acceptable method for adjusting the turbidity of the inoculum.

E. Within 15 minutes after adjusting the density of the inoculum suspension, a sterile cotton swab on a wooden applicator (plastic applicator swabs should not be used) is then dipped in the standardized suspension and the excess inoculum is removed from the swab by rotating it several times with a firm pressure on the inside wall of the test tube above the fluid level.

F. Inoculate the dried surface of a Mueller-Hinton agar plate by streaking the swab over the entire sterile agar surface. Repeat this streaking procedure two more times rotating the plate approximately 60° each time so as to insure an even distribution of inoculum. If the plate is satisfactorily streaked, the zones of inhibition will be uniformly circular and there will be a uniform

confluent or almost completely confluent lawn of growth. Replace the plate top and allow the inoculum to dry for three to five minutes but no longer than 15 minutes before applying the drug impregnated discs.

4.5.2 An alternative method for inoculating disc diffusion test plates which is acceptable for routine use is the agar overlay method. This method is applicable only to tests with commonly isolated rapidly growing bacterial pathogens such as S. aureus, Enterobacteriacae and Pseudomonas aeruginosa.

A. Select 4 to 5 isolated colonies of the same morphologic type from an agar plate culture and prepare a visibly turbid suspension in 0.5 ml of brain heart infusion broth in a 13 x 100 mm tube. To avoid changes due to evaporation during storage of this small volume of broth, it is transferred asceptically into sterile tubes on the day that it is to be used.

B. Incubate the small volume broth cultures in a 35°-37°C waterbath or heating block for at least four hours but no longer than eight hours

C. Transfer a 0.001 ml calibrated loopful of the well mixed broth culture to 9.0 ml of a 1.5% aqueous solution of agar which has been melted and held one to eight hours in a 45°-50°C heating block (in 16 x 125 mm screw cap tubes). The caps are tightened after the agar is melted and unused tubes are discarded at the end of the day to avoid changes in agar concentration due to evaporation.

D. Quickly mix this seeded agar by gentle inversion and then spread over the surface of a 150 x 15 mm plastic petri plate containing Mueller-Hinton agar (4mm in depth). To facilitate this procedure the plates are brought to room temperature before attempting to spread the thin layer of seeded agar.

E. Allow the inoculated plates to stand three to five minutes undisturbed on a flat and level surface and then apply susceptibility discs as described below.

4.6 Test Procedure

A. Place the appropriate drug impregnated discs on the surface of the agar plate inoculated by one of the two methods described above. With sterile forceps or needle tip gently press down each disc to insure complete contact with the agar surface. The discs may be placed individually or with a dispensing apparatus, but they must be distributed evenly so that they are no closer than 15 mm from the edge of the plate and no two discs are closer than 24 mm from center to center. Since some diffusion of drug is almost instantaneous, a disc should not be moved once it has come in contact with the agar surface.

B. The plates are then inverted and placed in a 35°-37°C incubator within 15 minutes after the discs are applied. The plates should not be incubated under an increased concentration of CO_2 because the interpretative standards were developed using aerobic incubation and CO_2 will significantly alter the size of the inhibitory zones with some agents.

C. After 16 to 18 hours of incubation examine each plate and measure the diameters of the zones of complete inhibition (as judged by the unaided eye) including the diameter of the disc, to nearest whole millimeter using sliding calipers, a ruler or a template prepared for this purpose, held on the back of the petri plate illuminated with reflected light. Systems using transmitted light may be used if comparable zone sizes are obtainted by the quality control procedure in 4.9. If

blood is added to the agar base the zones should be measured from the surface illuminated with reflected light with cover removed. The end point should be taken as the area showing no obvious, visible growth that can be detected with the unaided eye. Colonies which can be detected only by varying the angle of illumination are ignored. Strains of Proteus mirabilis and P. vulgaris may swarm into areas of inhibited growth around certain antimicrobics. The zones of inhibition are usually clearly outlined and this veil of swarming growth is ignored. With the sulfonamides, organisms may grow through several generations before inhibition takes effect. Slight growth (80% or more inhibition) with sulfonamides is therefore disregarded and the margin of heavy growth is measured to determine the zone diameter.

4.7 Interpretation of zone diameters.

The sizes of the zones of inhibition are then interpreted by referring to Table 4.7 - I, which represents the subcommittees present recommendations. A categorization of "susceptible" implies that an infection due to the strain tested may be expected to respond to the recommended dosage of antimicrobic for that type of infection and infecting species. Resistant strains, on the other hand, are not completely inhibited by concentrations within the therapeutic range. The intermediate category includes strains which may respond to concentrations attainable by unusually high dosage or in areas, such as portions of the urinary tract, where the antimicrobic is concentrated. The intermediate category also comprises a "buffer zone" which should prevent major interpretative discrepancies from small uncontrolled technical factors.

The categories listed in Table 4.7 - I were developed by comparing zone

Table 4.7 - I

Zone-Size Interpretive Standards and Approximate M I C Breakpoints for the Disc Diffusion Technique[a]

Antimicrobial Agent	Disc Potency	Inhibitory Zone Diameter (to nearest mm)			Approx M I C Breakpoints	
		Resistant	Intermediate	Susceptible	Resistant	Susceptible
Penicillin G and Ampicillin	10 U 10 µg					
Staphylococci		20 or less	21 - 28	29 or more	Pen'ase[b]	≤0.1 µg/ml
Enterobacteriaceae & enterococci		11 or less	12 - 13	14 or more	≥32 µg/ml	≤5-15µg/ml[c]
Other organisms		11 or less	12 - 21	22 or more	≥32 µg	≤1.5 µg/ml
Methicillin	5 µg	9 or less	10 - 13	14 or more		≤2.5 µg/ml
Nafcillin or Oxacillin	1 µg	10 or less	11 - 12	13 or more		≤0.6 µg/ml
Vancomycin	30 µg	9 or less	10 - 11	12 or more		≤5 µg/ml
Cephalothin	30 µg	14 or less	15 - 17	18 or more	≥32 µg/ml	≤10 µg/ml
Cephaloridine	30 µg	11 or less	12 - 15	16 or more	≥40 µg/ml	≤10 µg/ml
Carbenicillin	50 µg					
Pseudomonas sp		12 or less	13 - 14	15 or more	≥250 µg/ml	≤125 µg/ml
Proteus & E. coli		17 or less	18 - 22	23 or more	≥32 µg/ml	≤16 µg/ml
Polymyxin B[d]	300 U	10 or less	11 - 14	15 or more	mean	≤12.5 U/ml
Chloramphenicol	30 µg	12 or less	13 - 17	18 or more	≥25 µg/ml	≤12.5µg/ml
Tetracycline	30 µg	14 or less	15 - 18	19 or more	≥12.5µg/ml	≤4 µg/ml
Erythromycin	15 µg	13 or less	14 - 17	18 or more	≥8 µg/ml	≤2 µg/ml
Lincomycin	2 µg	9 or less	10 - 14	15 or more	≥8 µg/ml	≤2 µg/ml
Clindamycin	2 µg	11 or less	12 - 15	16 or more	≥8 µg/ml	≤2 µg/ml
Kanamycin	30 µg	13 or less	14 - 17	18 or more	≥25 µg/ml	≤6 µg/ml
Neomycin	30 µg	12 or less	13 - 16	17 or more		≤10 µg/ml
Streptomycin	10 µg	11 or less	12 - 14	15 or more	≥15 µg/ml	≤6 µg/ml
Gentamicin	10 µg	12 or less	13 - 14	15 or more	>12.5µg/ml	≤6 µg/ml
Sulfonamides[e,f]	300 µg	12 or less	13 - 16	17 or more	>35 mg%[d]	≤10 mg%[d]
Nitrofurantoin[f]	300 µg	14 or less	15 - 18	19 or more	>100 µg/ml	≤25 µg/ml
Nalidixic Acid[f]	30 µg	13 or less	14 - 18	19 or more	>12.5µg/ml	≤12.5µg/ml

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

b - Penicillinase - producing staphylococci

c - M I C dependent upon dilution method used.

d - Polymyxin B diffuse poorly in agar and the accuracy of the diffusion method is thus less than with other antibiotics. Resistance is always significant, but 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.

e - 300 µg or 250 µg sulfonamide discs can be used with the same standards of zone interpretation (M I C values are for sulfamethizole).

f - Urinary tract infections only.

sizes with minimal inhibitory concentrations (MIC's) in broth and agar dilution tests. These were related to blood levels usually obtained with frequently used dose schedules or in the case of nitrofurantoin or naladixic acid, with urine levels. These breakpoints were tested against the distribution of zone sizes and MIC's among a variety of species of known clinical responsiveness or lack of responsiveness in order to check their appropriateness.

4.8 Limitations of the Method.

The disc methods described above have been standardized for testing rapidly growing pathogens, especially the Enterobacteriacae, S. aureus and Pseudomonas sp. In addition, limited experience has been gathered suggesting that the interpretative standards are applicable to tests with Hemophilus sp. and streptococci, provided that the agar medium is supplemented with blood. The alternate agar overlay method cannot be used when blood must be added to the agar medium. Although S. pyogenes and S. pneumoniae may be tested by the recommended method, routine testing of these organisms is generally unnecessary because they remain susceptible to penicillin G; isolates obtained from patients allergic to penicillin might be tested against erythromycin and tetracycline in order to detect the strains resistant to these alternative drugs. Sufficient studies have not yet been undertaken to develop reproducible definitive standards for interpretation of disc tests with microorganisms which are fastidious in their nutritional requirements, require an anaerobic atmosphere or increased concentration of CO_2 for growth, demonstrate an unusually slow growth rate or manifest marked strain-to-strain variation in the rate of growth. Such organisms should not now be tested by disc diffusion methods since the results can not be properly interpreted. Some studies have indicated that a slight modification of the recommended method

may be applicable to tests of N. meningitidis for the purpose of detecting sulfonamide resistant strains (Bennett, Kamp and Eickhoff, Appl. Microbiol. 16, 1056, 1968). The standard method probably is not reliable for testing N. gonorrheae which should be tested by dilution methods if tests are to be performed. This disc diffusion method appears to be satisfactory for detecting most strains of S. aureus which are resistant to methicillin and other penicillinase-stable penicillins, in spite of the marked heterogeneity of such cultures. The alternative agar overlay method has not proven to be as satisfactory for detecting methicillin-resistant staphylococci. In this regard the sensitivity of disc diffusion tests may be more efficient if the plate is incubated at 30° C. Methicillin-resistant staphylococci generally appear sensitive to cephalosporins by the diffusion technique but there is evidence that they are more resistant to these antibiotics than methicillin susceptible strains. However, the clinical significance of this observation remains uncertain. For this reason, cephalosporin susceptibility of methicillin-resistant strains should be considered equivocal.

4.9 Quality Control Procedures

4.9.1 In order to control the precision and accuracy of the test procedure stock cultures of Staphylococcus aureus (ATCC 25923) and Escherichia coli (ATCC 25922) should be obtained from a reliable source and tested daily (or each time a batch of tests is performed) and inhibitory zone diameters should be recorded. Solid and liquid media used for propagation and storage of these cultures should be soy bean casein digest agar (USP) and soy bean casein digest broth (USP). For prolonged storage, laboratories with the required equipment may store these stock cultures at -20°C in a suitable stablizer such as 50% fetal calf serum in broth, in a lypholized state or in

liquid nitrogen, without significant risk of alteration in antibiotic susceptibility. Alternatively, cultures may be grown on soy bean casein digest agar slants and stored under refrigeration (4°-8°C). Such cultures stored in the refrigerator should be subcultured to prepare fresh slants every two weeks. For testing, the cultures should be inoculated into the broth, incubated for four to eighteen hours and then streaked onto the agar plates to obtain single colonies. Colonies are then picked for testing according to the recommended susceptibility testing procedure. Such cultures may be used to monitor precision and accuracy as long as there is no significant change in the mean zone diameter which can not be attributed to methodology. Such a change indicates contamination or change in susceptibility of the organism. Fresh cultures should be obtained from a reliable source of such changes occur.

4.9.2 Adequate precision and accuracy in this procedure can be determined by standard statistical methods. Laboratory workers experienced in these computations may establish acceptable precision by calculating the standard deviation for each drug tested with the control cultures. Table 4.9 - I lists acceptable maximum standard deviation which if not exceeded will assure with 99% or greater confidence that serious interpretive errors will not be made due to inherent test variability. These values are based on the differences between the minimum zone size for susceptible strains and maximum zone size for resistant strains as listed in Table 4.7 - I.

Accuracy may be established by comparing the observed mean zone diameter with the accepted range around the currently accepted true mean value. The acceptable range can be calculated according to the following formula: $\bar{X}' \pm \frac{3S}{\sqrt{N}}$

Where $\bar{X}'$ is the accepted true mean listed in Table 4.9 - I, S is the maximum permissible standard deviation listed in Table 4.9 - I and N = the number of

observations used in determining the observed mean. An observed mean which lies outside this range indicates a significant difference from the accepted true mean ($p<.01$).

4.9.3 Alternatively, a worker may establish acceptable precision and accuracy by the following procedure which requires no statistical computations. Each laboratory should record the results of consecutive separate analyses with the recommended control cultures for each antimicrobic to be controlled. These observations are taken sequentially in groups of five observed values each. Precision (reproducibility) may be evaluated by determining the range (R) of values in each group of 5 observations (Maximum value minus Minimum value). This range should not exceed the maximum limit listed in Table 4.9 - II and in a series of ranges obtained from consecutive groups of 5 tests each, the average range should approximate the listed value.

Accuracy of results can be evaluated by comparing the mean value obtained with each group of 5 observations, with the expected range of mean values for each antimicrobic-organism combination being tested. Table 4.9 - II lists the acceptable range of mean values about the true mean.

4.9.4 If either of the control limits established above are exceeded, there is a significant probability that technical variation exists which can lead to clinically significant misinterpretation of test results. These variations must be investigated and corrected in order to assure valid results.

The control limits described above are based on data gathered by the Food and Drug Administration in its phase II collaborative study, and represents a degree of precision and accuracy which is practically attainable in clinical microbiology laboratories. The experience of several subcommittee members suggests that the test can be performed with even more precision than that permitted by the described standards.

Table 4.9 - I Maximum Acceptable Standard Deviations and Mean Zone Diameters that should be expected with the E. coli (ATCC 25922) and S. aureus (ATCC 25923)

Antimicrobic	Disc Content	Maximum Acceptable Stand. Dev.	Currently accepted true mean zone diameter (mm) E. coli	S. aureus
Penicillin	10 units	2.9	*	31.5
Ampicillin	10 mcg			
Staphylococci		2.9	*	29.5
Enteric bacilli & enterococci		1.3	17.5	*
Methicillin	5 mcg	1.6	*	19.5
Nafcillin & Oxacillin	1 mcg	1.3	*	----
Cephalothin	30 mcg	1.3	20.5	31.0
Cephaloridine	30 mcg	1.6	--	*
Carbenicillin	50 mcg			
Pseudomonas sp		1.3	—	*
Proteus & E. coli		1.6	--	*
Chloramphenicol	30 mcg	1.9	24.0	22.5
Tetracycline	30 mcg	1.6	21.5	23.5
Erythromycin	15 mcg	1.6	11.0	26.0
Lincomycin	2 mcg	1.9	*	—
Clindamycin	2 mcg	1.6	*	—
Kanamycin	30 mcg	1.6	21.0	22.5
Neomycin	30 mcg	1.6	20.0	22.0
Streptomycin	10 mcg	1.3	16.0	18.0
Gentamicin	10 mcg	1.3	22.5	23.0
Sulfonamides	300 mcg	1.6	—	*
Nitrofurantoin	300 mcg	1.6	—	*
Nalidixic Acid	30 mcg	1.9	—	*
Polymyxin B	300 units	1.6	14.0	*
Vancomycin	30 mcg	1.3	*	17.0

* data not relevant

— data not yet established

Table 4.9 - II Control Limits for Evaluation of Precision and Accuracy of Inhibitory Zone Diameters (mm) obtained in groups of 5 separate observations.

Antimicrobic Agent[a]	Disc content	*E. coli* (ATCC 25922) Accuracy Control Zone Diam. (mm) MEAN of 5 values	*E. coli* (ATCC 25922) Precision Control Range[b] of 5 values Maximum	*E. coli* (ATCC 25922) Precision Control Range[b] of 5 values Average[c]	*S. aureus* (ATCC 25923) Accuracy Control Zone Diam. (mm) MEAN of 5 values	*S. aureus* (ATCC 25923) Precision Control Range[b] of 5 values Maximum	*S. aureus* (ATCC 25923) Precision Control Range[b] of 5 values Average[c]
Ampicillin	10 mcg	15.8 - 19.2	5	2.9	25.8 - 33.2	11	6.4
Penicillin	10 units	---- ----	-	---	27.8 - 35.2	11	6.4
Methicillin	5 mcg	---- ----	-	---	17.8 - 21.2	5	2.9
Cephalothin	30 mcg	18.8 - 22.2	5	2.9	27.0 - 35.0	12	7.0
Chloramphenicol	30 mcg	22.0 - 26.0	6	3.5	20.2 - 24.8	7	4.1
Tetracycline	30 mcg	19.2 - 23.0	7	4.1	20.5 - 26.5	9	5.2
Erythromycin	15 mcg	9.0 - 13.0	6	3.5	23.3 - 28.7	8	4.7
Polymyxin B	300 units	12.7 - 15.3	4	2.3	---- ----	-	---
Kanamycin	30 mcg	18.3 - 23.7	8	4.7	20.2 - 24.8	7	4.1
Neomycin	30 mcg	18.0 - 22.0	6	3.5	19.3 - 24.7	8	4.7
Streptomycin	10 mcg	13.3 - 18.7	8	4.7	15.3 - 20.7	8	4.7
Gentamicin	10 mcg	20.2 - 24.8	7	4.1	20.3 - 25.7	8	4.7
Vancomycin	30 mcg	---- ----	-	---	15.7 - 18.3	4	2.3

[a] Data not yet available for the control of tests with nafcillin, oxacillin, cephaloridine, carbenicillin, clindamycin, lincomycin, sulfonamides, nalidixic acid or nitrofurantoin.

[b] The maximum value minus the minimum value obtained in a series of 5 consecutive tests should not exceed the listed maximum limits and the MEAN should fall within the range listed under "accuracy control".

[c] In a series of ranges from consecutive groups of 5 tests each, the average range should approximate the listed value.

5. RECOMMENDATIONS FOR EVALUATING ALTERNATIVE DISC DIFFUSION TECHNIQUES

5.1 There is little question that the currently recommended methods can be improved upon in a number of ways but the subcommittee is concerned with the fact that modifications are often introduced without first adequately evaluating the effect of altering the test system. For those investigators who wish to propose any modification the following guide lines are established to define the type of data that will be needed in order to determine whether the "new" method is acceptable. Without such data any deviation from the described techniques must be considered unsatisfactory.

5.2 A series of recent clinical isolates must be tested by the proposed method in parallel with the currently recommended technique, recording the observed zone diameters around all relevant antimicrobic discs. For each agent the diameters observed on the two types of test plates can then be plotted against one another and a regression analysis used to determine the degree of correlation. If a significant difference exists, the regression line may be used to suggest new interpretive standards for the proposed method. The number of tests necessary for statistically significant results is difficult to determine but approximately 50 to 100 selected isolates would be needed, providing zone diameters are evenly distributed and that there are no points along the line where excessive numbers of data points are accumulated.

5.3 The accuracy of the disc diffusion method must be evaluated by a correlation between inhibitory zone diameters and minimal inhibitory concentrations (broth or agar dilution tests). Selected strains obtained during the comparative study described above (5.2) should be further tested by a broth or agar dilution technique in parallel with a repeated disc diffusion test. Strains

selected for such a study should include all strains with which the interpretation of the two disc diffusion techniques disagreed and all strains which gave intermediate zone sizes. In addition, a few strains which were susceptible by both methods and a few strains which were resistant by both disc methods should be tested by antibiotic dilution techniques. For each drug at least 50 organisms should be tested in this manner.

5.4 The precision of the proposed diffusion methods must also be documented. On at least 50 different occasions the two control organisms described in 4.9 should be tested by the currently recommended technique and at the same time by the proposed method. The resulting data then can be used to calculate and compare the mean and standard deviation for zone diameters around each of the antimicrobic agents tested. To be acceptable the proposed technique must be capable of producing a standard deviation no greater than that of the recommended procedure and both should be well within the tolerance limits described item 4.9.

6. RECOMMENDATIONS FOR A NATIONAL REFERENCE CENTER

It has become increasingly obvious that standardization can be obtained only when there is some sort of reference laboratory which will continually evaluate various reference materials such as media, stock cultures, antimicrobial agents, etc. In addition, a central laboratory could provide an invaluable service by collecting, storing and analyzing relevant data which have been collected in regional reference centers and by other accomplished investigators. Finally, a national reference laboratory could undertake specific investigational studies to provide the information that is needed for the further development of standard methods and to study problems which arise when new antimicrobial agents are developed. One central reference laboratory and four or five regional reference centers should be designated in order to obtain these goals. Such a national reference center must maintain close communication with other agencies and professional organizations concerned with the general problem.

This laboratory should function under the continuing guidance of a group of individuals designated through the cooperation of the NCCLS subcommittee on antimicrobial susceptibility testing, the FDA and the CDC. This group of selected individuals should meet with the central laboratory staff at regular intervals in order to develop the programs to be undertaken by the central reference laboratories and to evaluate relevance and priority of the problems to be assessed. This group should also systematically review additional data collected by the central reference laboratory and its regional reference laboratories. On the basis of these continuing studies this panel would prepare future technical and interpretative recommendations to the NCCLS subcommittee and to the FDA. In this way it would be possible to systematically prepare

definitive standards when new antibiotics are introduced or other changes are indicated.

The Food and Drug Administration has the legal responsibility for certification of antibiotic impregnated discs. A national reference center which can consider questions that fall outside the responsibility of the FDA is essential, since FDA has no direct affiliations with clinical or public health laboratories. Obviously, such a center must work closely and collaboratively with FDA and other agencies and professional organizations to derive the maximum benefit and to avoid unnecessary duplication.

The NCCLS subcommittee on antimicrobial susceptibility tests formally recommend that a national reference laboratory be established at the Center for Disease Control, Atlanta, Georgia working in collaboration with regional reference centers located in academically oriented medical centers. The regional reference centers should be headed by recognized authorities in the area of in vitro susceptibility testing. Representatives from the Food and Drug Administration National Center for Antibiotic Analysis and from other agencies and professional organizations concerned with susceptibility testing should be included in decision making. Certain members of the NCCLS subcommittee on antimicrobic susceptibility testing shall serve in an advisory capacity to the national reference laboratory and in this way shall be instrumental in conveying to the reference center the concerns of the subcommittee and bring recommendations to the NCCLS membership. Without such centralized directed effort it will be difficult or impossible to maintain current updated recommendations within this complicated multifaceted field.