Disk Diffusion Susceptibility Testing (Kirby-Bauer Method)

1. Isolated colonies of each organism that might be playing a pathogenic role should be selected from primary agar plates and tested for susceptibility. Identification procedures may be performed at the same time. Mixtures of different types of microorganisms should not be tested on the same plate. The practice of conducting susceptibility tests directly with clinical material should be avoided. 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 infection being treated, susceptibility tests are often not necessary and the results can be grossly misleading.

2. Of the many media available, NCCLS recommends Mueller-Hinton agar due to: it results in good batch-to-batch reproducibility; it is low in sulfonamide,trimethoprim, and tetracycline inhibitors; it results in satisfactory growth of most bacterial pathogens; and a large amount of data has been collected concerning susceptibility tests performed with this medium. If batches of media do not support adequate growth of organism, the zone size will be larger and provide false result. Thus, only media from manufacturers following the NCCLS standards are to be used.

3. The agar medium should have pH 7.2 to 7.4 at room temperature. The surface should be moist but without droplet of moisture. The antibiotic disks should be maintained at 8°C or lower or freeze at -14°C or below until needed, according to the manufacturer's recommendations. Allow the disks to warm to room temperature before use. Don't use expired disks.

4. To standardize the inoculum density, a BaS04 turbidity standard is used (0.5 McFarland standard,approx. 10^8 organism per mL).

5. The steps of the standard method are as follows:

- a. Select at least 4 to 5 well-isolated colonies of the same morphological type from an agar plate. 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 tryptic-soy broth. Allow the broth culture to incubate at 35°C until it achieves or exceeds the turbidity of 0.5 McFarland standard. For routine susceptibility tests, however, the inoculum can also be prepared by making a direct saline or broth suspension of colonies that are selected from an 18 to 24-hour agar plate (a nutrient, non-selective agar such as blood agar plate must be used).

- Adjust the turbidity with sterile saline or broth. Use adequate light, and, to aid in the visual comparison, read the tube against a white background with contrasting black lines.

- Within 15 minutes after adjusting the turbidity of the inoculum suspension, dip a sterile non-toxic swab on an applicator into the adjusted suspension. Rotate the swab several times, pressing firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab.

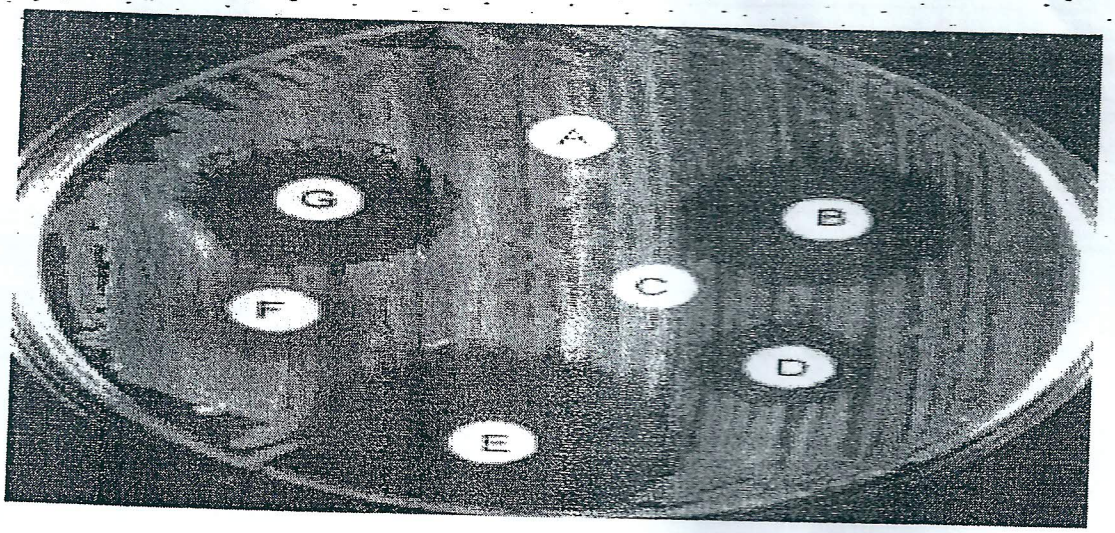
- Inoculate the dried surface of a Muller-Hintonagar plate by streaking the swab over the entire sterile agar surface. Repeat this procedure two more times, and rotate the plate 60° each time to ensure an even distribution of inoculum. Replace the plate top and allow 3 to 5 minutes, but no longer than 15 minutes, for any excess surface moisture to be absorbed before applying the antibiotic disks. There should be an almost confluent lawn of growth when done properly. If only isolated colonies grow, the inoculum was too light and the test should be repeated. To avoid extremes in inoculum density, never use undiluted overnight broth cultures for streaking plates.

- Place the appropriate disks evenly (no closer than 24 mm from center to center) on the surface of the agar plate either by using a sterile forceps or the dispensing apparatus. No more than 12 disks should be placed on one 150 mm plate or more than 5 disks on a 100 mm plate. A disk is not to be moved once it has come in contact with the agar surface since some of the compound diffuses almost instantaneously.

- Invert the plate and place them in an incubator at 35°C within 15 minutes after disks are applied. The plates should be incubated aerobically (no C02). After 16-18 hrs.of incubation, examine each plate and measure the diameters of the zones of complete inhibition, including the diameter of the disk. Measure the zones to the nearest millimeter using a ruler. Large colonies growing within a clear zone of inhibition should be subcultured. reidentified and retested./li>

- Interpret the zone sizes by referring to the manufacturer provided standard table and report the organism to be either susceptible, intermediate, or resistant. Never compare the zone sizes of two different antibiotics and judge their effectiveness accordingly.

- Quality control organisms such as E. coli ATCC 25922, and S.aureusATCC 25923 should be tested periodically to validate the accuracy of your procedures.



ABLE 2. *Zone Diameter Interpretive Standards and Approximate Minimum Inhibitory Concentration (MIC) Correlates*

ntimicrobial Agent	Disk Content	Zone Diameter, nearest whole mm: Resistant	Intermediate[b]	Moderately Susceptible[b]	Susceptible	Approximate MIC Correlates[a]: Resistant	Susceptible
nikacin[e]	30µg	≤ 14	15–16	—	≥ 17	≥ 32µg/mL	≤ 16µg/mL
mpicillin[d] when testing gram-negative enteric organisms	10µg	≤ 11	12–13	—	≥ 14	≥ 32µg/mL	≤ 8µg/mL
when testing staphylococci[e]	10µg	≤ 28	—	—	≥ 29	β-lactamase[e]	≤ 0.25µg/mL
when testing *Haemophilus* species[f]	10µg	≤ 19	—	—	≥ 20	≥ 4µg/mL	≤ 2µg/mL
when testing enterococci[g,h]	10µg	≤ 16	—	≥ 17[h]	—	≥ 16µg/mL	—
when testing non-enterococcal streptococci and *Listeria monocytogenes*[g,h]	10µg	≤ 21	—	22–29	≥ 30	≥ 4µg/mL	≤ 0.12µg/mL
gmentin when testing *Haemophilus* & staphylococci[e,f]	20/10µg	≤ 19	—	—	≥ 20	—	≤ 4/2µg/mL
when testing other organisms	20/10µg	≤ 13	14–17	—	≥ 18	≥ 32/16µg/mL	≤ 8/4µg/mL
locillin when testing *Pseudomonas*[e]	75µg	≤ 14	15–17	—	≥ 18	≥ 256µg/mL	≤ 64µg/mL
ireonam	30µg	≤ 15	—	16–21	≥ 22	≥ 32µg/mL	≤ 8µg/mL
rbenicillin when testing the Enterobacteriaceae[e]	100µg	≤ 17	18–22	—	≥ 23	≥ 32µg/mL	≤ 16µg/mL
when testing *Pseudomonas*	100µg	≤ 13	14–16	—	≥ 17	≥ 512µg/mL	≤ 128µg/mL
lamandole[j]	30µg	≤ 14	15–17	—	≥ 18	≥ 32µg/mL	≤ 8µg/mL
fazolin[j]	30µg	≤ 14	15–17	—	≥ 18	≥ 32µg/mL	≤ 8µg/mL
lonicid[j]	30µg	≤ 14	15–17	—	≥ 18	≥ 32µg/mL	≤ 8µg/mL
foperazone[j]	75µg	≤ 15	—	16–20	≥ 21	≥ 64µg/mL	≤ 16µg/mL
fotaxime[j]	30µg	≤ 14	—	15–22	≥ 23	≥ 64µg/mL	≤ 8µg/mL
foxitin[j]	30µg	≤ 14	15–17	—	≥ 18	≥ 32µg/mL	≤ 8µg/mL
ft[illegible]e[j]	30µg	≤ 14	15–17	—	≥ 18	≥ 32µg/mL	≤ 8µg/mL
ftizoxime when testing, urinary isolates of *P. aeruginosa*	30µg	≤ 10	—	≥ 11	—	≥ 64µg/mL	—
when testing other organisms	30µg	≤ 14	—	15–19	≥ 20	≥ 32µg/mL	≤ 8µg/mL
ftriaxone	30µg	≤ 13	—	14–20	≥ 21	≥ 64µg/mL	≤ 8µg/mL
furoxime[j]	30µg	≤ 14	15–17	—	≥ 18	≥ 32µg/mL	≤ 8µg/mL
phalothin[j]	30µg	≤ 14	15–17	—	≥ 18	≥ 32µg/mL	≤ 8µg/mL
loramphenicol	30µg	≤ 12	13–17	—	≥ 18	≥ 25µg/mL	≤ 12.5µg/mL
[illegible]xacin[k]	100µg	≤ 14	15–18	—	≥ 19	≥ 64µg/mL	≤ 16µg/mL
ndamycin[l]	2µg	≤ 14	15–16	—	≥ 17	≥ 2µg/mL	≤ 1µg/mL
xycycline[m]	30µg	≤ 12	13–15	—	≥ 16	≥ 16µg/mL	≤ 4µg/mL
thromycin	15µg	≤ 13	14–17	—	≥ 18	≥ 8µg/mL	≤ 2µg/mL
ntamicin[e]	10µg	≤ 12	13–14	—	≥ 15	≥ 8µg/mL	≤ 4µg/mL
penem	10µg	≤ 13	14–15	—	≥ 16	≥ 16µg/mL	≤ 4µg/mL
amycin	30µg	≤ 13	14–17	—	≥ 18	≥ 25µg/mL	≤ 6µg/mL
hicillin when testing staphylococci[n]	5µg	≤ 9	10–13	—	≥ 14	—	≤ 3µg/mL
locillin[e]	75µg	≤ 12	13–15	—	≥ 16	≥ 256µg/mL	≤ 64µg/mL
cycline[m]	30µg	≤ 14	15–18	—	≥ 19	≥ 16µg/mL	≤ 4µg/mL
dlactam[j]	30µg	≤ 14	—	15–22	≥ 23	≥ 64µg/mL	≤ 8µg/mL
illin when testing staphylococci[n]	1µg	≤ 10	11–12	—	≥ 13	—	≤ 1µg/mL
dixic Acid[k]	30µg	≤ 13	14–18	—	≥ 19	≥ 32µg/mL	≤ 8µg/mL
imi[illegible]c	30µg	≤ 12	13–14	—	≥ 15	≥ 32µg/mL	≤ 12µg/mL
[illegible]in[k]	300µg	≤ 14	15–16	—	≥ 17	≥ 100µg/mL	≤ 25µg/mL
loxacin	10µg	≤ 12	13–16	—	≥ 17	≥ 16µg/mL	≤ 4µg/mL
cillin when testing staphylococci[n]	1µg	≤ 10	11–12	—	≥ 13	—	≤ 1µg/mL
when testing pneumococci for penicillin G susceptibility	1µg	≤ 19	—	—	≥ 20	—	≤ 0.06µg/mL
cillin G when testing staphylococci[e]	10 units	≤ 28	—	—	≥ 29	β-lactamase[e]	≤ 0.1µg/mL
when testing *N. gonorrhoeae*	10 units	≤ 19	—	—	≥ 20	β-lactamase	≤ 0.1µg/mL
when testing enterococci[g,h]	10 units	≤ 14	—	≥ 15[h]	—	≥ 16µg/mL	—

when testing non-enterococcal streptococci and *L. monocytogenes*[g,h]	10 units	≤19	—	20–27	≥20	≥ 4μg/mL	≤ 0.12μg/mL
'acillin[e]	100μg	≤14	15–17	—	≥18	≥ 256μg/mL	≤ 64μg/mL
ɔlomycin	10μg	≤11	12–14	—	≥15	—	—
namides[k,o]	250 or 300 μg	≤12	13–16	—	≥17	≥ 350μg/mL	≤ 100μg/mL
cycline[m]	30μg	≤14	15–18	—	≥19	≥ 16μg/mL	≤ 4μg/mL
cillin[e]	75μg	≤11	12–14	—	≥15	≥ 128μg/mL	≤ 64μg/mL
ɔmycin[e]	10μg	≤12	13–14	—	≥16	≥ 8μg/mL	≤ 4μg/mL
ɔthoprim[k,o]	5μg	≤10	11–15	—	≥16	≥ 16μg/mL	≤ 4μg/mL
ɔthoprim-sulfamethoxazole[o]	1.25/23.75μg	≤10	11–15	—	≥16	≥8/152μg/mL	≤2/38μg/mL
omycin	30μg	≤ 9	10–11	—	≥12	—	≤ 8μg/mL

hese correlates are not meant for use as breakpoints for susceptibility categorization with ilution MIC tests as described in NCCLS M7-T. Use M7-T for categorization with dilution ests.

he category "Intermediate" should be reported. It generally indicates that the test result is quivocal (see 2.3.2.3). When designated in this table, a "moderately susceptible" result should e reported to indicate susceptibility under certain conditions (see 2.3.2.2). Other β-lactams re currently being considered for definition of a moderately susceptible category.

he zone sizes obtained with aminoglycosides, particularly when testing *P. aeruginosa*, are en[illegible]um dependent because of variations in divalent cation content. These interpretive lar[illegible] are to be used only with Mueller-Hinton medium that has yielded zone diameters ithin the correct range shown in Table 3 when performance tests were done with *P. aeruginosa* TCC® 27853. Organisms in the intermediate category may be either susceptible or resistant hen tested by dilution methods and should therefore more properly be classified as "indo-rminant" in their susceptibility.

ass disk for ampicillin, amoxicillin, bacampicillin, cyclacillin, and hetacillin.

esistant strains of *S. aureus* produce β-lactamase and the testing of the 10 unit penicillin sk is preferred. Penicillin G should be used to test the susceptibility of all penicillinase-producing enicillins, such as ampicillin, amoxicillin, azlocillin, bacampicillin, hetacillin, carbenicillin, ezlocillin, piperacillin, and ticarcillin/Results may also be applied to phenoxymethyl penicillin phenethicillin.

x testing *Haemophilus* use Mueller-Hinton agar supplemented with 1% hemoglobin (or 5% orse blood) and 1% IsoVitaleX (BBL), Supplement XV (Difco) or an equivalent synthetic upplement. Adjust pH to 7.2. Prepare the inoculum by suspending growth from a 24-hour ocolate agar plate in Mueller-Hinton broth to the density of a turbidity standard (see 4.1.3). e vast majority of ampicillin-resistant strains of *Haemophilus* produce detectable β-lacta-ase.

r enterococci, other *Streptococcus* spp. and non-penicillinase-producing penicillin-sensitive ganisms, and *Listeria monocytogenes*, the former intermediate interpretation should be ported as "Moderately Susceptible." Results in this category include *enterococci* and *steria* which for blood or serious invasive tissue infections require high dosage of penicillin ampicillin, often combined with an aminoglycoside (gentamicin) for improved therapeutic sponse and bactericidal action.

r streptococci, staphylococci, and other penicillin-sensitive organisms, "*Susceptible*" sults should be regarded as "*Very Susceptible*." Enterococci strains (*S. faecalis*, *S. oclum*, and *S. durans*) producing zones ≥30mm for ampicillin or ≥28mm for penicillin e quite unusual and the streptococcal speciation procedures should be reexamined.

j. Cefazolin, cefamandole, cefonicid, cefuroxime, cefoxitin, cefotaxime, moxalactam, ceftizoxime, ceftazidime, ceftriaxone, cefoperazone, and aztreonam are recently studied beta-lactams having a separate diagnostic disk and a wider spectrum of antimicrobial activity, especially against gram-negative bacilli than do other previously approved cephalosporins. Therefore, the 30 μg cephalothin disk cannot be used as the class representative for these drugs even though cefazolin is considered a "first generation" drug. The cephalothin disk should only be used for testing susceptibility to cephalothin, cefaclor, cefadroxil, cephalexin, cephapirin, and cephradine. Cefazolin, cefamandole (or cefonicid or cefuroxime or ceforanide), cefoxitin, cefoperazone, cefotaxime (or aztreonam or ceftazidime or ceftizoxime or ceftriaxone or moxalactam) should be tested separately against the enteric bacilli (see Table 1). *S. aureus* exhibiting resistance to one of the penicillinase-resistant penicillins (MRSA) *must* be reported as resistant to cephalosporin-like antimicrobial agents, regardless of *in vitro* disk test results, because in most cases of documented MRSA infection have responded poorly to cephalosporin chemotherapy. Methicillin-resistant, coagulase-negative *Staphylococcus* spp. also appear not to respond well to cephalosporin treatment.

k. Susceptibility data for cinoxacin, nalidixic acid, nitrofurantoin, norfloxacin, sulfonamides, and trimethoprim apply only to organisms isolated from urinary tract infections.

l. The clindamycin disk is used for testing susceptibility to both clindamycin and lincomycin.

m. Tetracycline is the class disk for all tetracyclines, and the results can be applied to chlortetracycline, demeclocycline, doxycycline, methacycline, minocycline, and oxytetracycline. However, certain organisms may be more susceptible to doxycycline and minocycline than to tetracycline. (See footnote i, Table 1.)

n. Of the antistaphylococcal, β-lactamase resistant penicillins, either oxacillin, nafcillin, or methicillin may be tested, and results can be applied to the other two of these drugs and to cloxacillin and dicloxacillin. Oxacillin is preferred due to more resistance to degradation in storage and its application to pneumococcal testing (methicillin also usable) and because it is more likely to detect heteroresistant strains more easily. Do not use nafcillin on blood-containing media. Cloxacillin disks *should not* be used because they may not detect methicillin-resistant *S. aureus*. When an intermediate result is obtained with staphylococci, the strains should be further investigated to determine if they are heteroresistant.

o. The sulfisoxazole disk can be used for any of the commercially available sulfonamides. Blood-containing media, except media containing lysed horse blood, are *not* satisfactory for testing sulfonamides. The Mueller-Hinton agar should be as thymidine-free as possible for sulfonamide and/or trimethoprim testing. (See footnote a, Table 3.)

From M2-A3, *Performance Standards for Antimicrobial Disk Susceptibility Tests, Third Edition; Approved Standard*
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