**MICRO.AST.6.0 KIRBY BAUER DISK DIFFUSION TESTING**

**PRINCIPLE**

The standardized method recommended by the Clinical and Laboratory Standards Institute (CLSI formerly NCCLS) is based on that described by Bauer, Kirby, Sherris and Turck. This is a standardized disk diffusion test to determine the in vitro susceptibility of bacteria to various antimicrobial agents. Dried filter paper disks containing antimicrobics in various concentrations are applied to a Mueller Hinton plate which has already been inoculated with a standardized suspension of bacteria. The disks absorb water from the agar, dissolving the drug, which then migrates through the agar as the bacteria grow. Zones of inhibition are formed around the disks. The size of the zone is inversely proportional to the minimum inhibitory concentration (MIC) of the organisms. These zones are measured and can be used to predict the susceptibility of the bacteria to the antimicrobial agents tested. Current CLSI Antimicrobial Susceptibility Testing Standards are used to determine if an organism is susceptible, intermediate or resistant.

**DOCUMENT OWNER**

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**SPECIMEN**

1. Four or five isolated colonies of similar colony morphology grown overnight on a nonselective medium such as blood agar (if direct colony inoculation is used). Log phase testing can use colonies up to 48 hours old.
2. Isolates from frozen, lyophilized, or other stock conditions require two subcultures prior to testing.

**MEDIA AND REAGENTS**

1. Mueller Hinton Agar, 150 mm, stored at 2-8°C
2. Mueller Hinton Agar, 100 mm, stored at 2-8C
3. Mueller Hinton with Sheep Blood (5% ) agar, 150 mm, stored at 2-8C
4. Sterile saline, 0.9%, tubes, Vitek
5. Antimicrobic disks:
   * 1. Cartridges are supplied by Fisher Scientific or the manufacturer.
     2. Disks are stored at 2-8C unless otherwise specified by manufacturer on receipt until use. When a cartridge is in use, it is stored at 2-8C in an airtight container with a desiccant.
     3. After removing unopened containers of disks from freezer or refrigerator, allow them to equilibrate to room temperature at least one hour prior to opening to minimize condensation.
     4. Do not use disks beyond expiration date.
     5. Mueller Hinton Broth (BBL Ref: 296164) stored at 2-25C
6. McFarland standards, Remel (R20343), stored at 20-25C

**EQUIPMENT**

1. McFarland 0.5 standard, Remel (R20410)
2. Sterile cotton-tipped swabs
3. Forceps
4. Calipers
5. Light source
6. Vortex mixer
7. Multi-disk dispensing apparatus
8. Non CO2 incubator at 35C (for Staph)
9. Non CO2 incubator at 35-37C (for gnr)

**QUALITY CONTROL**

**NOTE**: QC testing of new lots or shipments of susceptibility supplies may be incorporated into the schedule of routine daily or weekly QC testing. The susceptibility supply must be tested and accepted before use on patient specimens. Weekly QC testing is performed. A 30 day study is performed prior to allowing a drug to be QC tested weekly. Refer to Disk Diffusion Antimicrobial Susceptibility Quality Control Plan.

1. Inoculate plates with 0.5 McFarland suspensions of the following organisms in Mueller Hinton broth or normal saline.
   * 1. Gram negative panel: performed weekly per IQCP  
        Use 0.9% normal saline with unsupplemented Mueller-Hinton agar

|  |  |  |
| --- | --- | --- |
| E. coli ATCC 25922  (acceptable range) | P. aeruginosa ATCC 27853  (acceptable range) | E. coli ATCC 35218  (acceptable range) |
| Ceftazidime (25-29mm) | Ceftazidime (25-33mm) | n/a |
| Ciprofloxacin (30-40mm) | Ciprofloxacin (25-33mm) | n/a |
| Gentamicin (19-26mm) | Gentamicin (16-21mm) | n/a |
| Piperacillin/tazobactam (24-30mm) | Piperacillin/taxobactam (25-33mm) | Piperacillin/tazobactam (24-30mm) |
| Trimethoprim/sulfamethoxazole (23-29mm) | n/a | n/a |
| Tobramycin (18-26mm) | Tobramycin (19-25mm) | n/a |
| Amikacin (19-26mm) | Amikacin (18-26mm) | n/a |
| Cefepime (31-37mm) | Cefepime (24-30mm) | n/a |
| Ampicillin/sulbactam (19-24mm) | n/a | Ampicillin/sulbactam (13-19mm) |
| Cefoxitin (23-29mm) | n/a | n/a |
| Meropenem (28-34mm) | Meropenem (27-33mm) | n/a |

* + 1. Gram negative supplemental panel: : performed weekly per IQCP

Use 0.9% normal saline with unsupplemented Mueller-Hinton agar

|  |
| --- |
| E. coli ATCC 25922 (acceptable range) |
| Ampicillin (16-22mm) |
| Nitrofurantoin (20-25mm) |
| Cefazolin (21-27mm) |
| Ceftriaxone (29-35mm) |

* + 1. Streptococcus panel: performed each day of patient testing  
       Use Mueller Hinton broth and Mueller-Hinton agar supplemented with 5% Sheep’s blood.

|  |
| --- |
| S. pneumoniae ATCC 49619 (acceptable range) |
| Levofloxacin (20-25mm) |
| Erythromycin (25-30mm) |
| Tetracycline (27-31mm) |
| Trimethoprim/sulfamethoxazole (20-25mm) |
| Clindamycin (19-25mm) |

* + 1. Gram positive panel: Performed each day of patient testing  
       Use 0.9% normal saline with unsupplemented Mueller-Hinton agar

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| --- |
| S. aureus ATCC 25923 (acceptable range) |
| Cefazolin (29-35mm) |
| Ciprofloxacin (22-30mm) |
| Clindamycin (24-30mm) |
| Erythromycin (22-30mm) |
| Cefoxitin (23-29mm) |
| Trimethoprim/sulfamethoxazole (24-32mm) |
| Penicillin (26-37mm) |
| Vancomycin (17-21mm) |
| Tetracycline (24-30mm) |
| Levofloxacin (25-30mm) |
| Nitrofurantoin (18-22mm) |

* + 1. Miscellaneous KB disks: Performed weekly per IQCP

|  |  |  |
| --- | --- | --- |
| Antibiotic | Media/incubation | QC organism(s) (acceptable range) |
| Cefoxitin | 0.9% normal saline with unsupplemented Mueller-Hinton agar, 35C ambient air, 16-18 hours | S. aureus ATCC 25923 (23-29mm) |
| Colistin | 0.9% normal saline with unsupplemented Mueller-Hinton agar, 35C ambient air, 16-18 hours | E. coli ATCC 25922 (11-17mm) P. aeruginosa ATCC 27853 (11-17mm) |
| Fosfomycin | 0.9% normal saline with unsupplemented Mueller-Hinton agar, 35C ambient air, 16-18 hours | E. coli ATCC 25922 (22-30mm) |
| Novobiocin | 0.9% normal saline with TSI w/ 5% sheep blood agar, 35C ambient air, 16-18 hours | S. saprophyticus ATCC 15305 (<=16mm) S. epidermidis ATCC 12228 (>=17mm) |
| Ertapenem | 0.9% normal saline with TSI w/ 5% sheep blood agar, 35C ambient air, 16-18 hours | E. coli ATCC 25922 (29-36mm)  P. aeruginosa ATCC 27853 (13-21mm) |
| Ceftazidime/avibactam | 0.9% normal saline with TSI w/ 5% sheep blood agar, 35C ambient air, 16-18 hours | P. aeruginosa ATCC 27853 (25-31mm)  K. pneumoniae ATCC 700603 (21-27mm) |

* + 1. Miscellaneous KB disks not listed above: Performed each day of patient testing as per CLSI M100-S22, do not report patient results unless QC is acceptable and documented in Access database. Notify Lead or manager of any not acceptable QC results.

1. Read and record results in Access database.
2. If the results are not in range due to disk failure notify manager and/or lead tech and a consecutive 5 day study is initiated. The drug is taken out of use until the problem is resolved. If the drug is out of control on one of the 5 days, daily testing must be performed until another 30 consecutive days of satisfactory performance is documented (see attached disk diffusion testing protocol).

**PROCEDURE**

1. Bring agar plates and disks to room temperature. Avoid prolonged exposure to elevated temperatures.
2. Using a sterile wooden swab or sterile loop, pick 3 to 5 isolated colonies (18-24 hour culture) with the same morphology by touching just the top of each colony, and inoculate into 0.9% saline or Mueller-Hinton broth.
3. Adjust turbidity to equal a McFarland 0.5 standard using the turbidity meter.
4. Within 15 minutes of adjusting the inoculum to a McFarland 0.5 standard, dip a sterile cotton swab into the suspension. Excess inoculum is removed by rotating the swab firmly against the wall of the tube above fluid level.
5. Inoculate the dried surface of a 150 Mueller Hinton agar plate by streaking evenly in three directions over the entire surface. Vary the angle of streak by 60° for each direction to ensure even distribution. A final sweep is made of the agar rim.

**NOTE**: If plates contain excess surface moisture, incubate at 35°C for 5-30 minutes, before inoculating.

1. Allow inoculated plates to dry a minimum of 3 minutes, but no longer than 15 minutes to allow for any excess moisture to be absorbed, before applying antibiotic-impregnated disks.
2. Using a disk dispenser, place appropriate disks on the agar surface.
   * 1. The dispenser is made so that each disk is pressed down to ensure contact with the agar surface. If necessary, press down gently on each disk with a sterile wooden applicator stick, forceps or loop, after dispensing.
     2. Disks must be distributed so as to be no closer than 24 mm from center to center. No more than 12 disks should be placed on one 150 mm plate. No more than 5 disks on a 100 mm MH plate. For *S. pneumoniae* no more than 9 disks on a 150mm plate or 4 disks on a 100mm plate (MH with 5% sheep blood).
     3. A disk should **not** be relocated after contact with the agar surface due to rapid diffusion of certain drugs. If necessary, place a new disk in another location on the plate.
3. Incubation:

Plates should be placed in the incubator within 15 minutes of disk application. Invert plates. Stack no more than 5 high. For all non- *S. pneumoniae* panels, incubate the plates for 16 to 18 hours at 35± 2°C in non-CO2. For *S. pneumoniae* panel incubate plate for 20 – 24 hours at 35± 2°C in CO2. For *Acinetobater* spp. and *Burkholderia cepacia* incubate plate for 20-24 hours.

**NOTE**: If oxacillin or vancomycin is being tested against *Staphylococcus* spp or vancomycin against *Enterococcus* spp, 24 hours of incubation are required before reporting as susceptible; other agents can be read and reported at 16 to 18 hours.

1. Reading and interpretation of zone sizes:
   * 1. Read plates after 16-18 hours of incubation, except for the organisms and situations described above. Read plates only if growth is confluent or nearly confluent.
     2. For Mueller Hinton, illuminate the back of the plate with light source at a 45°angle and with plate against a dark surface.
     3. The zone margin should be determined as the area showing no obvious visible growth. Minute colonies should be disregarded except for Staphylococci and Enterococci.
     4. Measure and record the zone sizes to the nearest whole millimeter by placed a ruler or caliper against the back of the plate.

**NOTE:** The following drug-bug combinations should be read using transmitted light (with the plate held up to the light): Staph / oxacillin / vancomycin and Enterococci / vancomycin. For these combinations any pinpoint growth or light film within the zone of inhibition of growth is indicative of resistance.

* + 1. For blood Mueller-Hinton agar, remove the plate cover and measure each zone on face of plate, illuminating agar surface at a 45° angle.
    2. Large colonies growing within a clear zone of inhibition may represent resistant variants or mixed inoculum. These require subculture, identification and retesting for susceptibility.
    3. The swarming of Proteus species should be disregarded and the margin of heavy growth is measured.
    4. Bacteria may go through several multiplications before inhibition occurs when testing sulfa antibiotics. Therefore, 80% inhibition (slight growth) is disregarded and the margin of heavy growth is measured.
    5. Zone sizes are interpreted according to the CLSI.

**REPORTING RESULTS**

1. Each zone size is recorded. The CLSI current guideline is consulted to determine how each zone size must be interpreted. Interpretations are susceptible (S), intermediate (I) and resistant (R).
2. Report oxacillin resistant staphylococci as resistant to all beta-lactam drugs, regardless of in vitro susceptibility results.
3. Results for nitrofurantoin or norfloxacin are reported for urinary tract isolates only.
4. For isolates of Salmonella and Shigella species, report only ampicillin, a quinolone and sulfa trimethoprim. Extraintestinal isolates of Salmonella should include a third generation cephalosporin (e.g. cefotaxime) and chloramphenicol.
5. Blood and CSF Enterococcal isolates should be reported with the comment “Combination therapy with ampillin, penicillin and vancomycin, plus an aminoglycoside is usually indicated for serious enterococcal infections. In Toplab, this comment is CTPA.
6. Antibiotics -- CSF:

The following antibiotics should NOT be reported on CSF isolates:

* + 1. Aminoglycosides, including gentamicin, tobramycin and amikacin
    2. First generation cephalosporins such as cefazolin and cephalothin
    3. Second generation cephalosporins such as cefamandole (with the exception of cefuroxime.
    4. Clindamycin
    5. Cefaoperazone

1. When reporting Kirby Bauer results in Toplab, interpretations must first be determined, then enter only S, I, or R in the appropriate field.

**PROCEDURE NOTES**

* 1. Strep pneumoniae is tested on Mueller Hinton with blood with a combination of E test and disks.
  2. Cefoxitin is used as the class drug to test Nafcillin, Methicillin and Oxacillin because it has the best stability and is a better predictor of mecA mediated resistance.
  3. Trimethoprim sulfa should not be tested on blood-containing media with one exception, S. pneumoniae.
  4. Oxacillin-resistant strains of Staphylococcus lugdunensis are not detected by disk diffusion using oxacillin but more accurately detected using the cefoxitin disk and S. aureus interpretive criteria.

**LIMITATIONS**

1. Slow growing organisms, obligate anaerobes and capnophiles should not be tested with this procedure. This procedure has been standardized for rapidly growing aerobes or facultative organisms. These include Enterobacteriaceae, Staphylococcus species, P. aeruginosa, Acinetobacter species, Enterococcus species, some Streptococcus species, and Listeria monocytogenes. Modifications have been made to standardize the testing of some fastidious organisms, such as Haemophilus species, Neisseria species (not N. meningitidis) and S. pneumoniae.

**NOTE**: If an organism not listed above is tested, report “Presumptive: disk diffusion testing not standardized for this organism”.

1. This method will not detect all Methicillin resistant Staphylococci nor will it detect all vancomycin resistant Enterococci.
2. Numerous factors can affect results: inoculum size, rate of growth, medium formulation and pH, incubation environment and length, disk content and drug diffusion rate, and measurement of endpoints. Therefore, strict adherence to protocol is required to ensure reliable results.

**REFERENCES**

* 1. NCCLS, Performance Standards for Antimicrobial Disk Susceptibility Tests - Sixth Edition, Approved Standard, NCCLS document M2-A6, 1997
  2. CLSI, Performance Standards for Antimicrobial Disk Susceptibility Tests - Ninth Edition, Approved Standard, CLSI document M2-A9, 2006
  3. NCCLS, Performance Standards for Antimicrobial Susceptibility Testing - Ninth Informational Supplement, NCCLS document M100-S9, 1999
  4. CLSI, Performance Standards for Antimicrobial Susceptibility Testing - Eighteenth Informational Supplement, CLSI document M100-S18, 2008
  5. Kirby-Bauer Antimicrobial disk Susceptibility Test procedure, SmithKline Beecham clinical Laboratories, August, 1997
  6. Isenberg, H.D., ed., Clinical Microbiology Procedures Handbook, American Society for Microbiology, 1992, 5.1
  7. Dipiro, Joseph, ed., Pharmaco Therapy (a Pathophysiologic Approach), 3rd edition, Appleton and Long, Stanford CT, 1997

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