TITLE: ANTIMICROBIAL SENSITIVITY TESTING BY DISK DIFFUSION

**MOP MICROBIOLOGY STANDARD OPERATING PROCEDURE**

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**1. Aim**

To measure the susceptibilities of pathogenic bacteria to appropriate antimicrobials by a standardised disk diffusion method. For Etest measurement see MOPSOP\_4b\_Etest Antimicrobial Senstivity Testing.

**2. Principle**

Standard amounts of antibiotics impregnated onto absorbent paper are placed onto a specified agar plate seeded with a known concentration of bacteria. These plates are then incubated overnight in defined conditions (e.g. temperature and atmosphere). The antibiotic diffuses through the medium from the disk and if the bacteria are sensitive to the antibiotic then growth will be inhibited leaving a zone of clearing. The diameter of this zone is measured and compared with CLSI guidelines for zone sizes to determine whether the bacteria should be classified as sensitive, intermediately sensitive or resistant to the antibiotic.

Various factors have been identified as influencing disk diffusion susceptibility tests. These include the type of medium, excess surface moisture on the medium, agar depth, disk potency, inoculum concentration, pH, cation content, incubation atmosphere, temperature and duration, and β-lactamase production by test organisms. It is therefore of the utmost importance to follow the CLSI guidelines on testing methods precisely in order for the CLSI interpretation tables to be valid. Regular quality control is also essential.

CLSI methods are regularly reviewed and updated and so it is important that the most recent versions of CLSI standard documents are used.

In some circumstances, accurate results cannot be achieved by simple disk diffusion testing and Etest MICs are required. This is described in a separate SOP.

**3. Method**

***3.1 Reagents and Equipment***

Agar plates (3-4 mm deep) Antibiotic impregnated disks Sterile saline (2-5ml)

Sterile cotton tipped swabs

Automatic disk dispenser or template with 5 or 6 disk spacing pattern

Forceps

Incubator with correct atmosphere at appropriate temperature

Calipers/ruler

***3.2 Inoculation***

1. Subculture the organism to be tested onto a non-selective agar plate and incubate for 18-

24 hours to obtain a pure growth.

2. Remove the antibiotic disks from the fridge so they reach room temperature before the container is open (to avoid condensation and subsequent deterioration). Containers must contain active desiccant. Replace the disks and container in the refrigerator as soon as you have finished using them. Do not use disks past their expiry date.

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3. Using a straight wire or loop, touch at least six individual colonies from the pure culture and transfer them into sterile saline solution.

4. Emulsify the colonies in sterile saline to give an equivalent turbidity of 0.5 McFarland Standard (equivalent to a growth of 1-2 X 108 CFU/mL for *E. coli* ATCC 25922). Check the turbidity either using the Oxoid turbidometer or by comparing with the standard by eye.

5. Within 15 minutes after adjusting the turbidity of the inoculum, immerse a sterile cotton swab into the emulsion. Press the swab against the inner side of the tube, above the fluid level, to remove excess fluid.

6. Use the appropriate plate and incubation conditions according to Table 1. If the organism in question does not appear in Table 2 or 3, consult CLSI documents M02-A11, M100- S23 or M45-A2 (or the latest versions of these documents) for further guidance.

**Table 1.**

**Incubation media, atmospheres and times according to CLSI M100-S23**

|  |  |  |
| --- | --- | --- |
| **Organism** | **Media** | **Incubation conditions** |
| *Enterobacteriacae*  *Pseudomonas aeruginosa*  *Aeromonas* spp.  *Vibrio* spp. | Mueller Hinton | 35-37°C in air for 16-18h |
| *Acinetobacter* spp.  *Burkholderia cepacia Stenotrophomonas maltophilia Burkholderia pseudomalleia* | Mueller Hinton | 35-37°C in air for 20-24h |
| *Staphylococcus aureus* | Mueller Hinton | 35-37°C in air for 16-18h  (**35°C for 24 hours for oxacillin and cefoxitin**) |
| Coagulase negative staphylococci | Mueller Hinton | 35-37°C in air for 24 hours |
| Enterococci | Mueller Hinton | 35-37°C in in air for 16-18h  (24h for vancomycin) |
| *Haemophilus* species | Haemophilus Test  Medium | 35-37°C in 5% CO2 for 16-18h. |
| *Neisseria gonorrhoeae* | GC agar base and 1%  defined growth supplement | 35-37°C in 5% CO for 20-24h  2 |
| *Moraxella catarrhalis* | Mueller Hinton | 35-37°C in 5% CO2 for 20-24h |
| *Neisseria meningitidisb and* | Mueller Hinton with 5%  blood | 35-37°C in 5% CO2 for 20-24h |
| *Streptococcus pneumoniae*, Beta- haemolytic streptococci and other  streptococci | Mueller Hinton with 5%  blood | 35-37°C in 5% CO2 for 20-24h |

Notes:

a. There are no published CLSI disk diffusion interpretative criteria for *B. pseudomallei*.

MIC testing is recommended in document M45. However, local experience suggests that disk diffusion testing can be used for all antibiotics except co-trimoxazole, for

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which Etest MICs should be performed if the organism appears resistant by disk testing.

b. The inoculum for *N. meningitidis, N. gonorrhoeae* and *Haemophilus* spp. should be prepared from a chocolate agar plate incubated in 5% CO2 for 20-24 hours. 0.9% PBS (pH 7.0) as opposed to ordinary saline is recommended for making the suspension of *N. gonorrhoeae*. All antimicrobial susceptibility testing of *N. meningitidis* should be done in a Biosafety cabinet as manipulating heavy suspensions of this organism species outside a BSC has been associated with meningococcal disease in laboratory workers.

c. If the organism you want to test is not specifically listed in this SOP there may still be CLSI interpretative criteria for disk testing or other methods may be recommended – consult CLSI documents.

7. Inoculate the entire agar surface of the plate, either using a rotary plater or by spreading the plate 3 times, rotating the plate 60° between the streaks and then swabbing the rim of the agar surface.

 The plate to be inoculated should be moist, but no droplets of moisture should be apparent on the surface of the medium or on the Petri dish covers. If so, the plate and its lid should be left between 10-30 minutes in the Biosafety cabinet to dry.

 Care should be taken not to mark the agar by too much pressure when streaking and that there is evenness of spread, particularly at the edge.

 The plate may be left to dry for 3-5 minutes (no more than 15 minutes) after streaking to allow for any excess surface moisture to be absorbed.

***3.3 Application of disks***

8. Place disks of the appropriate antibiotics for the species in question (Table 2 or 3) on the plate using the automatic disk dispenser or 5 disk spacing template. Single disks may be handled using forceps.

 AVOID PLACING PENICILLIN AND CEPHALOSPORIN DISKS NEXT TO EACH OTHER.

 Disks need to be applied evenly on the agar surface; press gently on the disk after application.

 Because some antibiotics diffuse almost instantaneously, a disk should not be relocated once it has come into contact with the agar surface. Instead, place a new disk in another location on the agar.

 Disks should be applied no later than 15 minutes after the plates have been inoculated. Similarly, once the disks are applied, they should be put in the incubator within a 15 minutes interval to prevent pre-diffusion of the antimicrobial at room temperature.

 Disk diffusion testing is not reliable for some organism/antibiotic combinations (e.g. *B. pseudomallei* and co-trimoxazole). In these circumstances an Etest MIC may need to be undertaken.

9. Invert the plates and incubate in the correct atmosphere for the appropriate time as indicated in Table 1.

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 Agar plates should not be placed in stacks of more than 10 because the middle plates will take longer to reach the incubator temperature. This delay could cause overlarge zones.

***3.4 Reading and interpreting results***

10. After the incubation is complete, remove the plates from the incubator and measure the zone diameter, in mm, using a set of callipers/ruler. To measure: the callipers have to be held on the back of the inverted plate over a dark, non-reflecting background, and illuminated from above (except oxacillin and vancomycin, which should be read with transmitted light i.e. plate held up to light source and any diskernible growth within the zone of inhibition taken as indicative of resistance). Figure 1 shows where the measurement should be made.

 The diameter of the zone of inhibition includes the diameter of the disk. The end of the zone should be taken as the area showing no obvious visible growth that can be detected with unaided eyes. Ignore faint growth of tiny colonies that can only be detected with a magnifying lens at the edge of the zone of inhibited growth

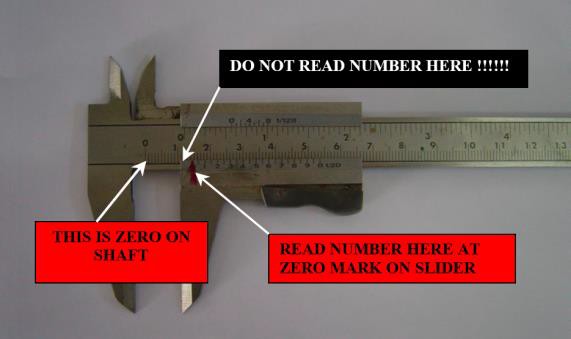
 When measuring zones on Mueller-Hinton plates with blood, the zone of growth inhibition should be measured NOT the zone of haemolysis inhibition. The zones should be measured from the upper surface of the agar, illuminated with reflected light, with the cover removed.

 The growth on the plates must be even and near confluent. If there are only isolated colonies, the test must be repeated.

 Staphylococci that are resistant to oxacillin or cefoxitin should be reported as resistant to all beta-lactams.

**Figure 1**

Use of callipers



11. Compare the measured zone size with that for the species and antibiotic combination in Table 2 or 3. Record the zone diameter and the category taken from the table. If the organism and zone size is not included in the table then refer to the CLSI manuals for further information. Results can usually be put into one of the categories below:

 **Susceptible (S)**

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This implies that isolates are inhibited by the usually achievable concentrations of antimicrobial agent when the dosage recommended to treat the site of infection is used.

 **Intermediate (I)**

This category includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels, and for which response rates may be lower than for susceptible isolates. The intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated (e.g. quinolones and -lactams in urine) or when a higher than normal dosage of a drug can be used (e.g. -lactams). The “intermediate” category also includes a “buffer zone” which should prevent small, uncontrolled technical factors from causing major diskrepancies in interpretation, especially for drugs with narrow pharmacotoxicity margins.

 **Resistant (R)**

Resistant strains are isolates that are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules and/or that demonstrate MICs or zone diameters that fall in the range where specific microbial resistance mechanisms are likely (e.g. -lactamases) and clinical efficacy of the agent against the isolate has not been reliably shown in treatment studies.

 **Nonsusceptible**

A category used for isolates for which only a susceptible interpretative criterion has been designated because of the absence or rare occurrence of resistant strains. Isolates that have MICs above or zone diameters below the value indicated for the susceptible breakpoint should be reported as nonsusceptible. This does not necessarily mean that the isolate has a resistance mechanism – it is possible that isolates with MICs above the susceptible breakpoint that lack resistance mechanisms may be encountered within the wild-type distribution after the susceptible only breakpoint is set.

**4. ESBL testing**

*E. coli* and *Klebsiella* spp. that have cefpodoxime zones ≤ 17 mm or ceftriaxone zones ≤ 25 mm or cefotaxime zones ≤ 27 mm or ceftazidime zones ≤ 22 mm and *Proteus mirabilis* that have cefpodoxime zones ≤ 22 mm or cefotaxime zones ≤ 27 mm or ceftazidime zones ≤ 22 mm should be tested for ESBL production.

1. Inoculate a Mueller Hinton plate as described above.

2. Place on the inoculated plate disks of the following:

a. Ceftazidime 30 μg

b. Ceftazidime-clavulanic acid 30/10 μg c. Cefotaxime 30 μg

d. Cefotaxime-clavulanic acid 30/10 μg

**3.** Incubate at 35-37°C in air for 16-18h.

**4.** Measure zone diameters as described above.

**5.** A ≥5mm increase in a zone diameter for either agent in the presence of clavulanic

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acid is a positive result for ESBL.

**5. Quality assurance**

Regular testing of the correct quality control strains is essential to provide assurance that the media and disks being used most frequently are performing satisfactorily. Test the organisms and agents shown in Appendix 1 every week (preferably on a Monday) and record the results on the sheets in Appendix 1. If any of the results fall outside the ranges given on the sheets, inform the Deputy Laboratory Manager. If testing an unusual antibiotic which is not on this list, check the relevant CLSI documents and perform relevant quality control at the same time as doing the test.

If ESBL testing is being performed regularly, *K. pneumoniae* ATCC 700603 should be used for QC – see M100-S23 for further details. If testing large numbers of isolates of *Haemophilus* sp. or *Strep. pneumoniae*, additional QC strains may need to be obtained and tested (e.g. *H. influenzae* ATCC 49247, *H. influenzae* ATCC 49766, *Strep. pneumoniae* ATCC 49619).

Additional quality assurance is provided by participation in an accredited external quality assurance scheme.

**6. Limitations**

Although the quality control carried out during manufacture of antibiotic disks is usually of a high standard, disk content may deteriorate during storage. This is one reason why regular quality control is essential. Correct storage and rotation of disk stocks is essential to maintain this quality. The main stock of disks should be stored at -20°C with a small quantity for current use being kept at 4°C. The disks required for the day’s work should be brought to room temperature before opening the container. Desiccant should be changed regularly and kept in sealed containers. The oldest disks must be used first and always before their expiry date.

Tests on mixed cultures (as judged by different zone patterns or colonial types) may be unreliable and should be repeated.

There are a number of characteristics of particular organisms that mean that their zones must be read and interpreted with caution. It is difficult to list all of these but some of the commoner issues that can cause problems are listed below. For further details see the CLSI documents.

 **Penicillin & *Staphylococcus aureus***

When a Staphylococcus produces the enzyme penicillinase it will be resistant to penicillin. This phenomenon can be observed by examining the zone edge. If there is evidence of a built up or 'heaped up' edge to the zone of inhibition, regardless of the zone size, then the strain should be called resistant. Equivocal strains should be tested for β-lactamase (penicillinase) production.

 **Sulphonamides and Trimethoprim**

Must only be tested on PABA free media as this sulphonamide inhibitor is present in other media such as blood agar.

Heavy inoculation may invalidate tests with sulphonamides as enough PABA is contained in the inoculum to inactivate the drug.

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Disregard any slight growth (20% or less of the lawn of growth) and measure the more obvious margin to determine the zone diameter.

 ***Proteus* species**

Swarming Proteus can cause problems if too heavily inoculated or insufficiently dried plates are used. There is frequently swarming back on the chloramphenicol and trimethoprim zones. When carefully examined a zone edge can be diskerned with swarming growth inside it.

Measurement should be from the zone edge and the swarming growth disregarded.

 **Enterococci**

Lancefield Group D streptococci such as *E. faecalis* are only moderately sensitive (MIC 2μg/ml) to penicillin and ampicillin and will therefore give no zone to 1μg or a small zone to a 1.5μg disk so l0μg disks are used. They are always resistant to cephalosporins and aminoglycosides and should be reported as such (although enterococci that do not have high level resistance to aminoglycosides may exhibit synergy with cell wall acting agents and may be used in combination with them for severe infections such as endocarditis).

 **Streptococci**

Aminoglycosides (e.g. gentamicin) have only moderate activity against streptococci (MIC 4-6μg/ml) and is therefore not recommended clinically. These should be reported as resistant irrespective of zone diameter.

**7. References**

1. Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard –

Tenth Edition. M02-A11. Clinical and Laboratory Standards Institute, January 2012.

2. Performance Standards for Antimicrobial Disk Susceptibility Tests; Twenty-First Informational Supplement. M100-S23. Clinical and Laboratory Standards Institute, January 2013.

3. Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Approved Guideline. M45-A2. Clinical and Laboratory Standards Institute 2010.

4. Standard Operating Procedures (SOP), Wellcome-Mahidol-Oxford Melioidosis project laboratory, Sapasitthiprasong Hospital, Ubon Ratchatani. Version: 1.1, June 2003.

5. Bridson EY (1998). The Oxoid Manual. Oxoid Ltd

6. Bridson EY (2006). The Oxoid Manual, 9th Edition. Oxoid Ltd.

7. Murray P.R., Baron E.J., Pfaller M.A., Tenover F.C., Yolken R.H. Manual of Clinical

Microbiology 7th Edition (1999) Am.Sc.Micro.

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| **8. COSHH risk assessment - COSHH Assessment**  **Form** | |
| **Description of procedure**  Susceptibility testing by disk diffusion | **Substances used**  Pathogenic bacteria Various agars Antibiotic disks |
| **Quantities used**  5 ml of bacterial suspensions | **Frequency of use**  Daily |
| **Hazards identified**  Infection risk with certain organisms | **Could a less hazardous substance be used**  **instead?**  No |
| **What measures have you taken to control risk?**  Routine laboratory practice and PPE (blue gowns, protective glasses etc.). All suspensions to be prepared and plates inoculated in a Class II BSC. | |
| **Checks on control measures**  Observation and supervision by senior staff. | |
| **Is health surveillance required?**  No | **Training requirements:**  All staff to be trained in this SOP and in the use of a BSC before being allowed to work unsupervised |
| **Emergency procedures**:  Report any incidents to line manager and Area Safety Adviser | **Waste disposal procedures**:  All cultures autoclaved prior to disposal. |

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**Appendix 1**

**Form for recording zone diameters of weekly disk diffusion quality control testing**

|  |  |  |  |
| --- | --- | --- | --- |
| Antibiotic | Disk content | Acceptable  range (mm) (CLSI)\* | Zone diameter  (mm) |

|  |  |  |  |
| --- | --- | --- | --- |
| **Name of organism**-*Escherichia coli* ATCC 25922 | | | |
| Amikacin | 30g | 19-26 |  |
| Amoxicillin-clavulanic acid | 30g | 18-24 |  |
| Ampicillin | 10g | 16-22 |  |
| Ampicillin-sulbactam | 10/10g | 19-24 |  |
| Cefixime | 5g | 23-27 |  |
| Cefpodoxime | 10g | 23-28 |  |
| Ceftriaxone | 30g | 29-35 |  |
| Ceftazidime | 30g | 25-32 |  |
| Cephalothin | 30g | 15-21 |  |
| Chloramphenicol | 30g | 21-27 |  |
| Ciprofloxacin | 5g | 30-40 |  |
| Doxycycline | 30g | 18-24 |  |
| Fosfomycin | 200g | 22-30 |  |
| Gentamicin | 10g | 19-26 |  |
| Nalidixic acid | 30g | 22-28 |  |
| Nitrofurantoin | 300g | 20-25 |  |
| Ofloxacin | 5g | 29-33 |  |
| Trimethoprim-sulfamethoxazole | 1.25/23.75g | 23-29 |  |

|  |  |  |  |
| --- | --- | --- | --- |
| **Name of organism**-*Escherichia coli* ATCC 35218 | | | |
| Amoxicillin-clavulanic acid | 30g | 17-22 |  |
| Ampicillin | 10g | 6 |  |
| Ampicillin-sulbactam | 10/10g | 13-19 |  |

|  |  |  |  |
| --- | --- | --- | --- |
| **Name of organism**-*Staphylococcus aureus* ATCC 25923 | | | |
| Chloramphenicol | 30g | 19-26 |  |

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|  |  |  |  |
| --- | --- | --- | --- |
| Cefoxitin | 30g | 23-29 |  |
| Erythromycin | 15g | 22-30 |  |
| Gentamicin | 10g | 19-27 |  |
| Oxacillin | 1g | 18-24 |  |
| Penicillin | 10 Units | 26-37 |  |
| Tetracycline | 30g | 24-30 |  |
| Trimethoprim-sulfamethoxazole | 1.25/23.75g | 24-32 |  |
| Vancomycin | 30g | 17-21 |  |

|  |  |  |  |
| --- | --- | --- | --- |
| **Name of organism**-*Pseudomonas aeruginosa* ATCC 27853 | | | |
| Amikacin | 30g | 18-26 |  |
| Ceftazidime | 30g | 22-29 |  |
| Ciprofloxacin | 5g | 25-33 |  |
| Colistin | 10g | 11-17 |  |
| Gentamicin | 10g | 17-23 |  |
| Imipenem | 10g | 20-28 |  |
| Meropenem | 10g | 27-33 |  |

|  |  |  |  |
| --- | --- | --- | --- |
| **Name of organism**-*Neisseria gonorrhoeae* ATCC 49226 | | | |
| Penicillin | 10 units | 26-34 |  |
| Ciprofloxacin | 5g | 48-58 |  |
| Ceftriaxone | 30g | 39-51 |  |
| Tetracycline | 30g | 30-42 |  |
| Spectinomycin | 100g | 23-29 |  |

\* If any zones are outside the acceptable range, inform a senior member of staff.

Date: Signature

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| Competency Assessment Form: | Employee/Position: | | | | | | | |
| Supervisor/Position: | | | | | | | |
| **Assessment methods:**  O = Observation  V = Verbal  W = Written | **Level of competency:**  1 = Not competent  2 = Competent if supervised  3 = Competent and can perform independently  4 = Competent and can perform independently and is able to assess the competency of others | | | | | | | |
| **Activity** | | **Date** | **Method** | | | **Level** | **Employee**  **signature** | **Employer**  **signature** |
| **O** | **V** | **W** |
| 1. Has read and understood the SOP | |  |  |  |  |  |  |  |
| 2. Has read and understood the risk assessment and all  aspects of health and safety related to this SOP | |  |  |  |  |  |  |  |
| 3. Is competent in describing and recording CSF  macroscopic appearance. | |  |  |  |  |  |  |  |
| 4. Is competent in carrying out CSF cell counts using a  chamber, including differentiating between WBC and  RBC. | |  |  |  |  |  |  |  |
| 5. Is competent in performing Gram stain on CSF samples  and interpreting the results. | |  |  |  |  |  |  |  |
| 6. Knows when and how to concentrate a CSF sample. | |  |  |  |  |  |  |  |
| 7. Is competent in setting up culture for CSF samples. | |  |  |  |  |  |  |  |
| 8. Is competent in interpreting and knowing how to follow-  up CSF cultures. | |  |  |  |  |  |  |  |
| 9. Is competent in reporting CSF results on the laboratory  system. | |  |  |  |  |  |  |  |
| **CAF Comments:** | | | | | | | | |