# Aim

To measure the susceptibilities of pathogenic bacteria to appropriate antimicrobials by a standardised gradient diffusion method.

# Principle

It is not possible to determine antimicrobial susceptibilities for all organism-drug combinations by disk diffusion testing (SOP MIC-001). Formal estimation of the minimum inhibitory concentration (MIC) is occasionally required. The MIC of a drug is the lowest concentration (usually expressed in µg/ml) required to inhibit the growth of an isolate. Notable requirements for MIC testing include:

* Beta-lactam testing of *Streptococcus pneumoniae*, where disk testing is unreliable and breakpoints for penicillin and ceftriaxone differ by site of infection (meningitis vs. non-meningitis)
* Azithromycin testing of *Salmonella* Typhi, where neither disk zone diameter nor formal MIC breakpoints have been defined
* Testing of fastidious or uncommonly isolated organisms (e.g. non-fermenting Gram negatives), were disk zone diameter breakpoints are not yet defined
* Vancomycin testing of *Staphylococcus* spp., where disk testing is unreliable
* Co-trimoxazole testing of *Burkholderia pseudomallei,* where disk testing is unreliable

MIC testing by broth dilution or agar incorporation is time consuming and technically demanding. Etest strips provide a simpler method for MIC determination. Etests consist of a plastic strip impregnated with a gradient of antimicrobial (providing an MIC range of 15 two-fold dilutions). Once applied to an isolate-inoculated agar plate, the antimicrobial diffuses out and results in a stable concentration gradient in the medium. After incubation, the point of intersection between organism growth and the Etest device enables the MIC value to be determined from the range printed on the plastic strip.

# Method

## Reagents and equipment

* Agar plates (3.5-4.5 mm deep)
* Etest strips
* Sterile saline or appropriate broth (2-5ml)
* Sterile cotton tipped swabs
* Forceps
* Incubator with correct atmosphere at appropriate temperature

## 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 Etest package / storage tube from the -20°C freezer and allow the strips to reach room temperature for approximately 30 minutes. Replace the Etests and container in the freezer as soon as you have finished using them. Do not use Etests past their expiry date.
3. Using a straight wire or loop, touch at least six individual colonies from the pure culture.
4. Emulsify the colonies in sterile saline or broth to give an equivalent turbidity to the appropriate McFarland Standard (Table 1).
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.
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 should be left to dry for 10-15 minutes after streaking to allow for any excess surface moisture to be absorbed.

**Table 1.** Incubation media, inoculum details, atmospheres and times

|  |  |  |
| --- | --- | --- |
| **Organism** | **Inoculum / Medium** | **Incubation conditions** |
| Aerobes (e.g. *B. pseudomallei, S.* Typhi) | Saline; 0.5 McFarland\*Mueller Hinton | 35-37°C in air for 16-20h |
| Staphylococci | Saline; 0.5 McFarlandMueller Hinton | 35-37°C in air for 24h (*S. aureus*) / 48h (CoNS) |
| *Neisseria meningitidis* | Saline / Mueller Hinton broth / BHI broth; 0.5 McFarland\*Mueller Hinton with 5% sheep blood | 35-37°C in 5% CO2 for 20-24h |
| *Streptococcus pneumoniae* and other α-haemolytic streptococci | Saline / Mueller Hinton broth / BHI broth; 0.5 McFarland\*Mueller Hinton with 5% sheep blood | 35-37°C in 5% CO2 for 20-24h |

\*Use 1 McFarland suspension if mucoid; also use 1 McFarland for *B. pseudomallei*

## Application of Etests

1. Place Etests of the appropriate antibiotics for the species in question (Appendix 1 of SOP MIC-001) on the plate manually using forceps.
* Do not touch the antimicrobial impregnated side of the Etest strip with your fingers.
* One or two Etests can be applied per 90mm agar plate (See Figure 1).
* Etests need to be applied evenly on the dry agar surface with the MIC scale facing upward; press gently on the strip after application. Small bubbles will not affect the results
* Because some antibiotics diffuse almost instantaneously, an Etest should not be relocated once it has come into contact with the agar surface.
* Etests should be applied no later than 15 minutes after the plates have been inoculated. Similarly, once the Etests are applied, they should be put in the incubator within a 15 minutes interval to prevent pre-diffusion of the antimicrobial at room temperature.
1. Invert the plates and incubate in the correct atmosphere for the appropriate time as indicated in Table 1.
* Agar plates should not be placed in stacks of more than 5 because the middle plates will take longer to reach the incubator temperature. This delay could cause overlarge inhibition zones.

**Figure 1.** Correct application of two Etest strips to a 90mm agar plate

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## Reading and interpreting results

1. After the incubation is complete, remove the plates from the incubator and measure the MIC, where the edge of the inhibition ellipse interests the side of the strip (see Appendix 1).
* If there is growth all along the strip and no visible ellipse, record the MIC as greater than (>) the highest value on the scale
* If the inhibition ellipse is below the strip, then report the MIC as less than (<) the lowest value on the scale.
* For bactericidal drugs (e.g. beta-lactams), read the MIC at the point of complete inhibition of all growth including hazes, micro-colonies, and isolated colonies. Tilt the plate and use a magnifying glass if necessary.
* For bacteriostatic drugs (e.g. co-trimoxazole), read the 80% inhibition end-point: this is the first point of significant inhibition as judged by the naked eye.
* 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, or a mixed growth, the test must be repeated.
1. An Etest value which falls between two-fold MIC values (i.e. 0.06, 0.12, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256µg/ml etc.) on the strip should be rounded-up to the next two-fold value before categorization.
* For example, a pneumococcal isolate with an Etest MIC of 0.094µg/ml should be rounded up to 0.12µg/ml before interpretation.
1. Compare the rounded-up MIC with that for the species and antibiotic combination in SOP MIC-001. Record the MIC and the category taken from the table. If the organism and MIC is not included in the table then refer to the CLSI documents for further information. Results can usually be put into one of the categories below:
* **Susceptible (S)** includes 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)** 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 β-lactams in urine) or when a higher than normal dosage of a drug can be used (e.g. β-lactams). The “intermediate” category also includes a “buffer zone” which should prevent small, uncontrolled technical factors from causing major discrepancies in interpretation, especially for drugs with narrow pharmacotoxicity margins.
* **Resistant (R)** includes 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. β-lactamases) and clinical efficacy of the agent against the isolate has not been reliably shown in treatment studies.
* **Nonsusceptible (NS)** is 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 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.

# Quality assurance

Regular testing of the correct quality control strains is essential to provide assurance that the media and Etests being used most frequently are performing satisfactorily. Test the organisms and agents shown in Appendix 1 every month (preferably on the first Monday) and record the results on the worksheet. If any of the results fall outside the ranges given on the sheets, inform the 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.

# Limitations

Although the quality control carried out during manufacture of Etests is usually of a high standard, antimicrobial content may deteriorate during storage. This is one reason why regular quality control is essential. Correct storage at -20°C and rotation of Etest stocks is essential to maintain this quality. The oldest Etests 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 demonstrated in Appendix 2.

# References

1. Etest Package Insert (9302553C – 2012/01). bioMerieux 2012.
2. Etest Performance, Interpretive Criteria and QC Ranges (15211E – 2012/03). bioMerieux 2012.

# Risk assessment

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| **COSHH risk assessment - University of Oxford COSHH Assessment Form** |
| **Description of procedure**Determination of bacterial antimicrobial susceptibilities by Etest | **Substances used**1. Cultured bacteria2. Antimicrobial impregnated Etest strips |
| **Quantities of chemicals used**N/A | **Frequency of SOP use**Daily |
| **Hazards identified**1. Autoclaved liquid2. Potentially infectious material in sample 3. Potentially pathogenic bacteria | **Could a less hazardous substance be used instead?** No |
| **What measures have you taken to control risk?** 1. Training in good laboratory practices (GLP)2. Appropriate PPE (lab coat, gloves, eye protection)3. Use of biosafety cabinet for reading of plates / follow-up of BSL-3 organisms (e.g. *B. pseudomallei*) |
| **Checks on control measures**Observation and supervision by senior staff |
| **Is health surveillance required?**No | **Training requirements:**GLP |
| **Emergency procedures**:1. Report all incidents to Safety Adviser2. Use eyewash for splashes3. Clean up spills using 1% Virkon or chemical spill kit | **Waste disposal procedures**:1. Sharps discarded into appropriate rigid containers for incineration2. Infectious waste discarded into autoclave bags or 1% Virkon solution prior to autoclaving and subsequent incineration3. Chemical waste disposed of according to manufacturer’s instructions |

# Appendix 1: Etest QC procedure

**Etest QC sheet**

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| --- | --- |
| **QC strains:**  | *E. coli* ATCC 25922; *P. aeruginosa* ATCC 27853;*S. pneumoniae* ATCC 49619 |
| **Date MH(B) prepared:** |  |
| **Date MIC measured:** |  |
| **QC performed by:** |  |

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| --- | --- |
| **Etest** | **MIC (µg/ml)** |
| ***E. coli*****ATCC 35218****MH agar** | ***P. aeruginosa*****ATCC 27853****MH agar** | ***S. pneumoniae*****ATCC 49619****MHB agar** |
| **Code** | **Expected** | **Measured** | **Expected** | **Measured** | **Expected** | **Measured** |
| **AZ** | - | - | - | - | 0.5 - 2 |  |
| **CI** | - | - | 0.25 - 1 |  | - | - |
| **PG** | - | - | - | - | 0.25 - 1 |  |
| **TS** | 0.064 – 0.25 |  | - | - | - | - |
| **TX** | - | - | - | - | 0.032 - 0.125 |  |
| **VA** | - | - | - | - | 0.125 – 0.5 |  |

# Appendix 2: Etest reading guide