



**EUCAST**

EUROPEAN COMMITTEE  
ON ANTIMICROBIAL  
SUSCEPTIBILITY TESTING

European Society of Clinical Microbiology and Infectious Diseases

# EUCAST disk diffusion method for antimicrobial susceptibility testing

Version 1.1

June 2010

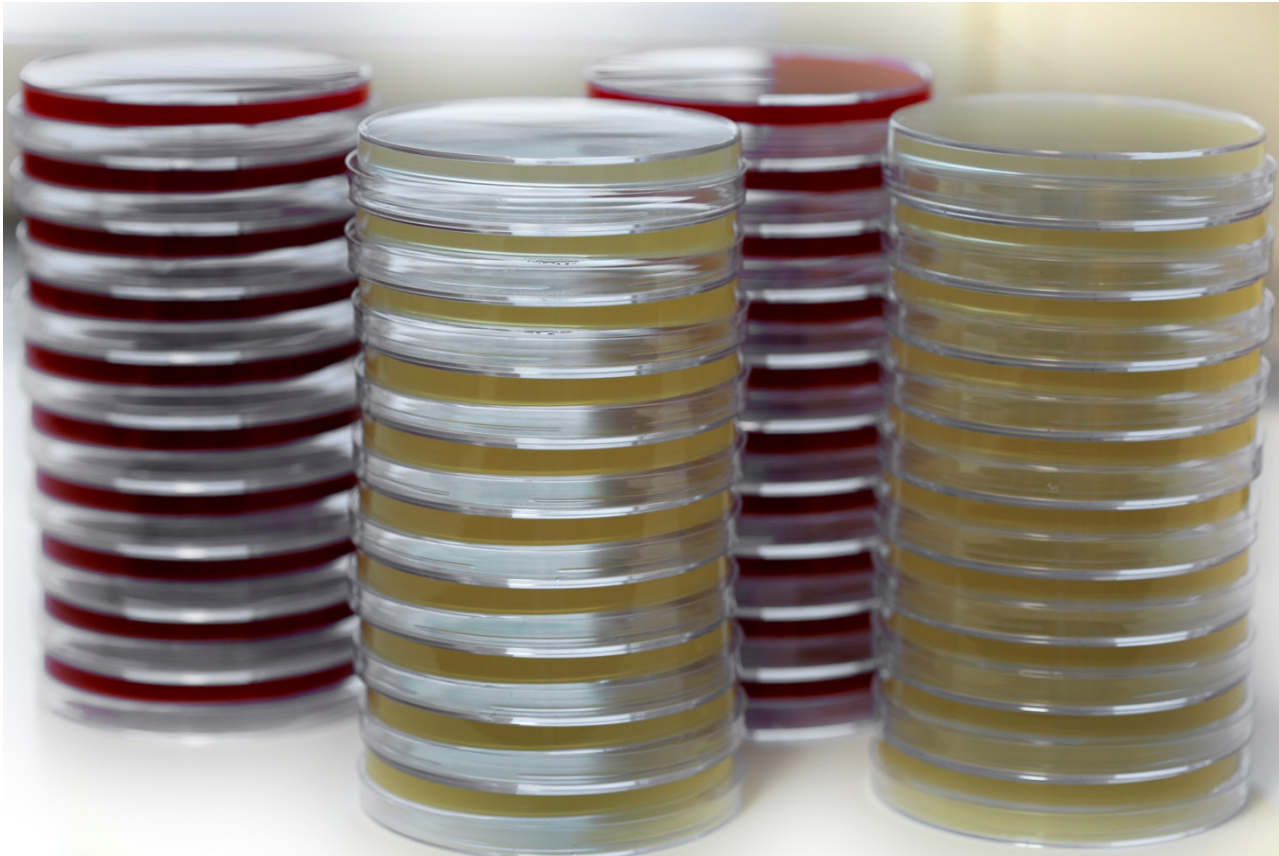
# Contents

- Media
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# Modifications to EUCAST disk diffusion method slide show

- 2009-12-18: EUCAST disk diffusion method slide show first published on EUCAST website (v1.0)
- 2010-06-01: Clarification regarding agar depth on slide 7, Spanish Culture Collection Numbers added on slides 29 and 30 (v. 1.1)

# Susceptibility testing media



# Susceptibility testing media

- Use only Mueller-Hinton agar (MH)
- Medium for fastidious organisms (MH-F, **Mueller-Hinton Fastidious**) is MH supplemented with 5% defibrinated horse blood and 20 mg/L  $\beta$ -nicotinamide adenine dinucleotide (NAD).

# Media for different organisms

| Organisms   | Medium  |
|---|---|
| Enterobacteriaceae<br><i>Pseudomonas</i> spp.<br><i>Stenotrophomonas maltophilia</i><br><i>Acinetobacter</i> spp.<br><i>Staphylococcus</i> spp.<br><i>Enterococcus</i> spp. | Mueller-Hinton agar   |
| <i>Streptococcus pneumoniae</i><br><i>Streptococcus</i> Groups A, B, C and G<br>Other streptococci<br><i>Haemophilus</i> spp.<br><i>Moraxella catarrhalis</i>               | Mueller-Hinton agar + 5% defibrinated horse blood + 20 mg/L $\beta$ -NAD (MH-F) |
| Other fastidious organisms  | Pending   |

# In-house preparation of media

- Prepare media as directed by the manufacturer.
- Do not add blood or NAD until medium has cooled to 42-45°C (ensure that media preparators have accurate temperature settings), mix well and pour plates immediately.
- Pour plates on a level surface to give a uniform depth of  $4.0 \pm 0.5$  mm. If repeat measurements show the depth to be reproducibly above or below 4 mm, adjust the volume even when the agar depth is within 3.5 - 4.5 mm.

Commonly used plate sizes are 90 mm circular plate (~25 mL), 100 mm circular plate (~31 mL), 150 mm circular plate (~71 mL) 100 mm square plate (~40 mL).

# Quality control of Mueller-Hinton agar

Check that all Mueller-Hinton batches are within control limits for all bacteria-antimicrobial agent combinations.

## Particular problems:

- High or low concentrations of divalent cations may be indicated by inhibition zones for aminoglycosides with *P. aeruginosa* ATCC 27853 above/below quality control limits.
- Excess thymine and thymidine may be indicated by inhibition zones for trimethoprim-sulfamethoxazole and *E. faecalis* ATCC 29212 below quality control limits.

# Drying and storage of agar plates

- No drops of water should be visible on the surface of the agar when the plates are used.
- Plates must not be over-dried.
- Drying and storage conditions for home-produced media will depend on available equipment and should be determined locally.
- For commercially prepared media, storage requirements defined by the manufacturer should be followed.

# Inoculum

- The method requires an inoculum suspension equivalent to a 0.5 McFarland standard\*.

\* Approximately corresponding to  $1-2 \times 10^8$  CFU/mL for *E. coli*.



# Select well-isolated colonies

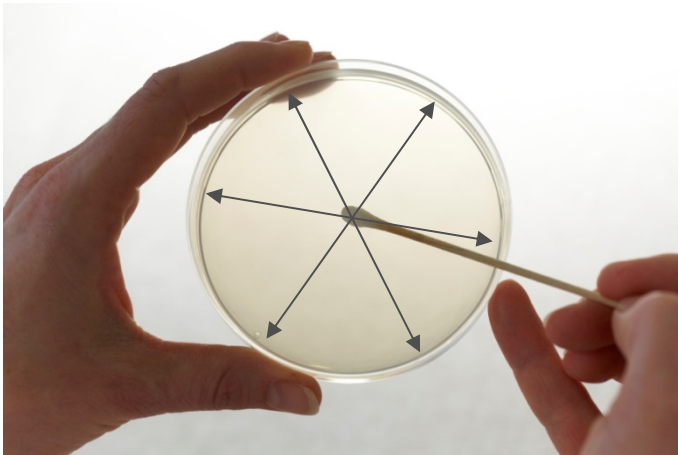


# Inoculum preparation

- Suspend one to several colonies in 0.85% saline to produce an even, visible turbidity equal to the density of a 0.5 McFarland standard.
  - Exception: *Streptococcus pneumoniae* is suspended to McFarland 0.5 from a blood agar plate, but to McFarland 1.0 from a chocolate agar plate.
- Adjust the turbidity by adding more bacteria or saline solution, preferably by measuring to McFarland 0.5 with a photometric device.

# Inoculation of plates

- Optimally, use the adjusted suspension within 15 minutes of preparation and always within 60 minutes.
- Dip a cotton swab in the suspension and remove excess fluid by turning the swab against the inside of the tube.
- Spread the inoculum evenly over the entire surface by inoculating in three directions or by using a plate rotator.



# Avoid heavy inoculation of plates

- It is important not to inoculate plates too heavily.
- Check that zone diameters for control strains are within range. Heavy inocula will give smaller zone diameters.
- Remove excess fluid from the swab by turning it gently against the inside of the tube (but do not excessively drain the swab, particularly with Gram-positive organisms) before inoculation of the plate.

# Storage of antimicrobial disks

- Store stocks of disks under conditions recommended by the manufacturer.
- Store disks in current use at 4-8°C in sealed containers with an indicating desiccant and protected from light.
- To avoid condensation of water on disks, allow them to warm to room temperature before opening containers. It is better to keep disks at room temperature during the day than to transfer repeatedly to and from cold storage.
- Do not use disks beyond the manufacturer's expiry date shown on the container.

# Application of antimicrobial disks

- Disks should be applied within 15 min of inoculation.
- Disks should be in firm, even contact with the surface of the medium.
- Disks should be spaced so that zones of inhibition in susceptible isolates do not overlap. Overlapping will impede the measurement of zone diameters.



# Summary of inoculation process

- Suspend isolated colonies from an overnight culture on a non-selective medium.
- Adjust to a density equivalent to McFarland 0.5, preferably with a photometric device. Optimally, use the inoculum within **15 minutes**.
- Dip a sterile swab into the solution and remove excess fluid by turning the swab against the inside of the tube.
- Apply the inoculum with even strokes over the entire agar surface.
- Apply antibiotic disks within **15 minutes** of inoculating the plate and start incubation within another **15 minutes**.

# Incubation of plates

- Incubate plates within 15 minutes of disk application. This limits pre-diffusion which may otherwise result in large zone sizes.
- Keep stacks of plates as small as possible as uneven heating of plates may affect zone sizes (depends on efficiency of incubator).
- MH is incubated in air and MH-F in air with 4-6% CO<sub>2</sub>.

# Incubation of plates

| <b>Organism</b>                           | <b>Incubation conditions</b>                        |
|---|---|
| Enterobacteriaceae                        | 35±1 °C in air for 16-20h                           |
| <i>Pseudomonas</i> spp.                   | 35±1 °C in air for 16-20h                           |
| <i>Stenotrophomonas maltophilia</i>       | 35±1 °C in air for 16-20h                           |
| <i>Acinetobacter</i> spp.                 | 35±1 °C in air for 16-20h                           |
| <i>Staphylococcus</i> spp.                | 35±1 °C in air for 16-20h                           |
| <i>Enterococcus</i> spp.                  | 35±1 °C in air for 16-20h                           |
| <i>Streptococcus</i> Groups A, B, C and G | 35±1 °C in air with 4-6% CO <sub>2</sub> for 16-20h |
| Other streptococci                        | 35±1 °C in air with 4-6% CO <sub>2</sub> for 16-20h |
| <i>Streptococcus pneumoniae</i>           | 35±1 °C in air with 4-6% CO <sub>2</sub> for 16-20h |
| <i>Haemophilus</i> spp.                   | 35±1 °C in air with 4-6% CO <sub>2</sub> for 16-20h |
| <i>Moraxella catarrhalis</i>              | 35±1 °C in air with 4-6% CO <sub>2</sub> for 16-20h |
| Other fastidious organisms                | Pending   |

# The 15-15-15 minute rule

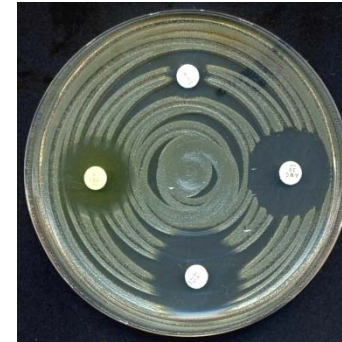
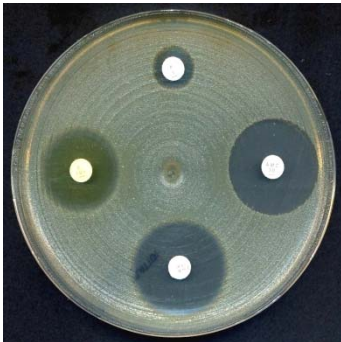
Prepare plates so that you:

- Use the inoculum within **15 minutes** of preparation – and never beyond 60 minutes.
- Apply disks within **15 minutes** of inoculating plates.
- Start incubation within **15 minutes** of application of disks.

# Examining plates after incubation

- A correct inoculum and satisfactorily streaked plates will result in a confluent lawn of growth.
- It is important that there is an even lawn of growth to achieve uniformly circular inhibition zones (see next slide).
- If individual colonies can be seen, the inoculum is too light and the test must be repeated.

The growth should be confluent and evenly spread over the plate



**Plates should look like this..**

**..and NOT like this!**

# Reading zones

- Zone edges should be read at the point of complete inhibition as judged by the naked eye.

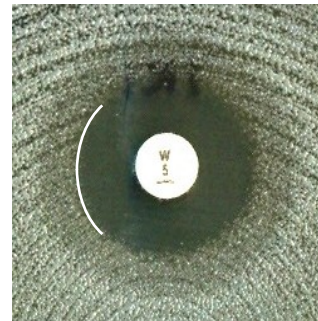
Examples:



*E. coli*  
Ciprofloxacin



*S. aureus*  
Erythromycin



CoNS  
Trimethoprim



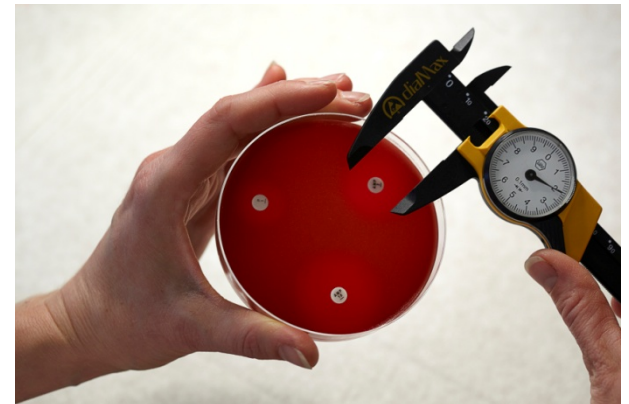
*S. pneumoniae*  
Rifampicin

# Reading zones

- Measure zone diameters with a ruler, calliper or automated zone reader.
- In case of distinct colonies within zones, subculture the colonies, check purity and repeat test if necessary.

# Reading zones

- Read **MH** plates from the back against a black background illuminated with reflected light.
- Read **MH-F** plates from the front with the lid removed illuminated with reflected light.



# Reading zones – exceptions

| <b>Organism</b>            | <b>Antimicrobial agent</b>                    | <b>Reading inhibition zones</b>  |
|----------------------------|---|--|
| <i>Proteus</i> spp.        | Any   | Ignore swarming.   |
| <i>Streptococcus</i> spp.  | Any   | Read inhibition of growth and not the zone of haemolysis.                        |
| Any                        | Trimethoprim<br>Trimethoprim-sulfamethoxazole | Ignore a fine haze of growth up to the disk within zones with an obvious margin. |
| <i>Staphylococcus</i> spp. | Linezolid                                     | Examine with transmitted light (plate held up to light).                         |
| <i>Enterococcus</i> spp.   | Vancomycin                                    | Examine with transmitted light (plate held up to light).                         |
| Enterobacteriaceae         | Ampicillin                                    | Ignore fine growth that may appear as an inner zone on some batches of MH agar.  |

# Interpreting zones

- Check that zone diameters for control strains are within acceptable ranges before interpreting tests.
- Measured zone diameters (to the nearest millimeter) are interpreted into categories of susceptibility (S, I and R) according to published tables ([www.eucast.org](http://www.eucast.org)). Alternatively, a template with EUCAST breakpoints may be used.

# Control of susceptibility testing

- Use the recommended routine quality control strains to monitor test performance (see [EUCAST Quality Control Tables](#)).
- Quality control strains with defined resistance mechanisms may be used to confirm the ability to detect resistance.
- Quality control strains may be purchased from culture collections or from commercial sources.

# EUCAST routine quality control strains

| Organism             | Culture collection numbers   | Characteristics                  |
|----------------------|--|----------------------------------|
| <i>E. coli</i>       | ATCC 25922; NCTC 12241; CIP 7624<br>DSM 1103; CCUG 17620, CECT 434   | Susceptible, wild-type           |
| <i>P. aeruginosa</i> | ATCC 27853; NCTC 12903; CIP 76110<br>DSM 1117; CCUG 17619; CECT 108  | Susceptible, wild-type           |
| <i>S. aureus</i>     | ATCC 29213; NCTC 12973; CIP 103429<br>DSM 2569; CCUG 15915; CECT 794 | Weak $\beta$ -lactamase producer |
| <i>E. faecalis</i>   | ATCC 29212; NCTC 12697; CIP 103214<br>DSM 2570; CCUG 9997; CECT 795  | Susceptible, wild-type           |
| <i>S. pneumoniae</i> | ATCC 49619; NCTC 12977; CIP 104340<br>DSM 11967; CCUG 33638          | Penicillin intermediate          |
| <i>H. influenzae</i> | NCTC 8468; CIP5494, CCUG 23946                                       | Susceptible, wild-type           |

ATCC, American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA.

NCTC, National Collection of Type Cultures, Health Protection Agency Centre for Infections, 61 Colindale Avenue, London NW9 5HT, UK.

CIP, Collection de Institut Pasteur, 25–28 Rue du Docteur Roux, 75724 Paris Cedex 15 France.

DSMZ, Deutsche Stammsammlung für Mikroorganismen und Zellkulturen, Mascheroder Weg 16, D-38124 Braunschweig, Germany.

CCUG, The Culture Collection University of Gothenburg <http://www.ccug.se/>

CECT. Colección Española de Cultivos Tipo. Universidad de Valencia. 46100. Burjassot. Valencia. Spain. <http://www.cect.org>

# EUCAST strains for detection of specific resistance mechanisms (under development)

| Organism             | Culture collection numbers   | Characteristics  |
|----------------------|--|--|
| <i>E. coli</i>       | ATCC 35218; NCTC 11954;<br>CIP 102181; DSM 5564;<br>CCUG 30600; CECT 943 | TEM-1 $\beta$ -lactamase producer                            |
| <i>S. aureus</i>     | NCTC 12493   | Oxacillin hetero-resistant,<br><i>mecA</i> positive          |
| <i>H. influenzae</i> | ATCC 49247; NCTC 12699;<br>CIP 104604; DSM 9999<br>CCUG 26214            | $\beta$ -lactamase negative,<br>ampicillin-resistant (BLNAR) |

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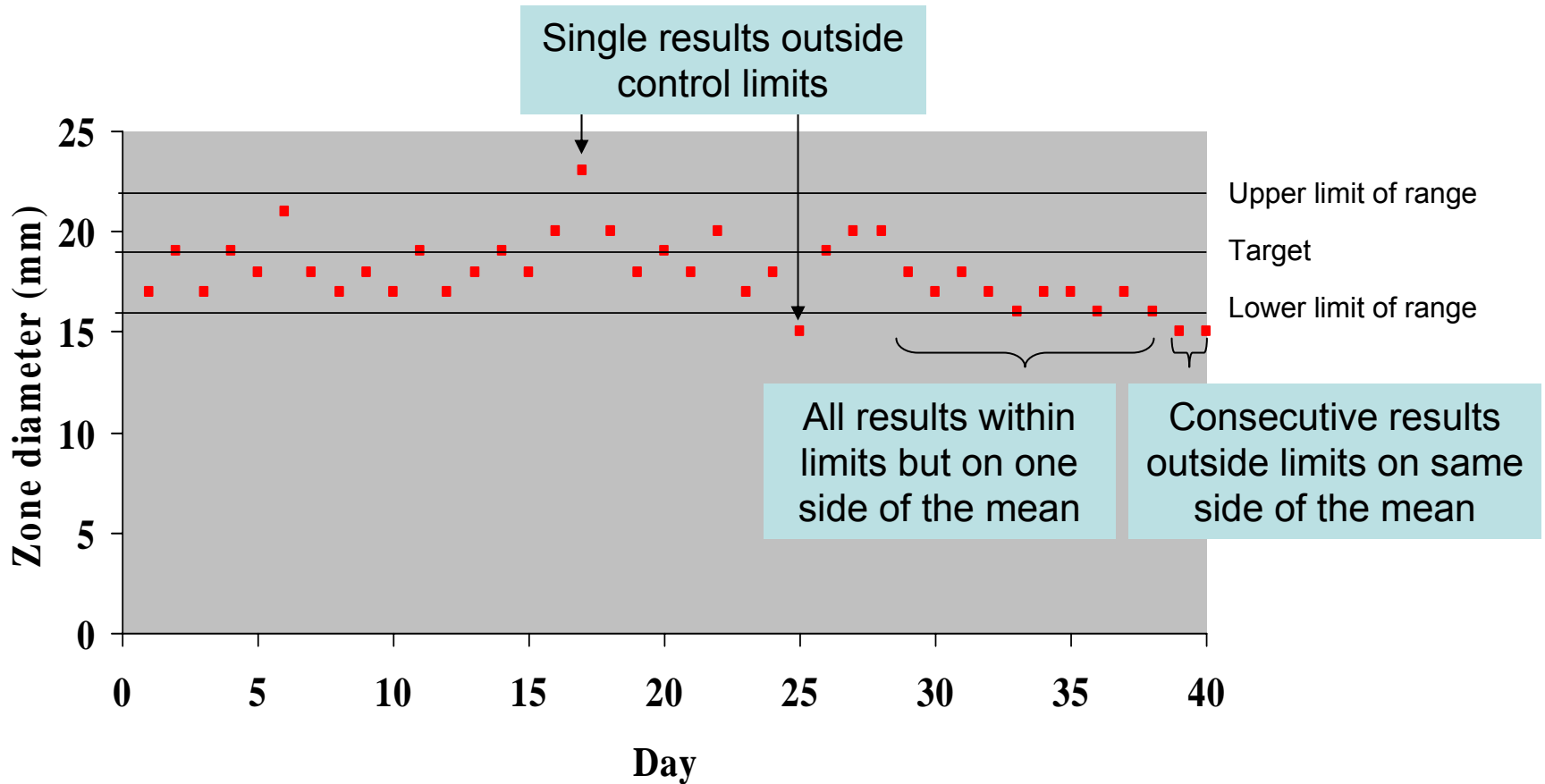
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CECT. Colección Española de Cultivos Tipo. Universidad de Valencia. 46100. Burjassot. Valencia. Spain. <http://www.cect.org>

# Use routine quality control strains to assess general performance

- Control tests should be set up and checked daily, at least for antibiotics which are part of routine panels.
- Each day that tests are set up, examine the results of the last 20 consecutive tests.
- Examine results for trends and for zones falling consistently above or below the mean.
- If two or more of 20 tests are out of range investigation is required.

# Monitoring test performance



# Response to QC results out of range

- If two non-consecutive control zone diameters of 20 tests are outside the acceptable range – then report susceptibility test results and investigate.
- If two consecutive control zone diameters of 20 tests are outside the acceptable range – then investigate before reporting susceptibility test results. The tests may have to be repeated.
- If multiple disks (>2) are out of range on one day – then investigate before reporting susceptibility test results. The tests may have to be repeated.
- If resistance in a resistant control strain is not recognised – then suppress susceptibility test results, investigate and retest.

# Storage and subculture of control strains

- Store strains at -70 °C in glycerol broth on beads: one “in-use” vial, one “archive”. Alternatively, use freeze dried cultures or commercial storage systems.
- Subculture weekly from the in-use vial onto appropriate non-selective media and check for purity.
- Subculture from the purity plate each day for up to 7 days.
- Fastidious organisms only may be serially sub-cultured for 6 days.
- When the in-use vial is depleted, subculture from the archive vial and prepare another in-use vial from the subculture.

# Potential sources of error (1)

|                        |   |
|------------------------|---|
| <b>Medium</b>          | Storage of plates   |
|                        | Not prepared to instructions  |
|                        | Batch to batch variation or change of supplier of agar  |
|                        | Supplements (batch to batch variations, incorrect amount or expired)  |
|                        | pH  |
|                        | Agar depth/Agar volume  |
|                        | Expiry date   |
| <b>Test conditions</b> | “15-15-15”-rule not adhered to (suspension used within 15 min, disks applied within 15 min, incubation within 15 min) |
|                        | Incubation (temperature, atmosphere and time)   |
|                        | Incorrect inoculation (too light, too heavy or uneven)  |
|                        | Reading conditions  |
|                        | Reading zone edges  |

# Potential sources of error (2)

|                          |   |
|--------------------------|---|
| <b>Disks</b>             | Incorrect disk (wrong agent or wrong disk strength)         |
|                          | Disk potency (incorrect storage, labile agent, expiry date) |
|                          | Disks not at room temperature when containers opened        |
|                          | Too many disks on plate (interference between agents)       |
| <b>Control organisms</b> | Incorrect QC strain   |
|                          | Mutation  |
|                          | Contamination   |
|                          | Age of culture  |

# EUCAST website

- Check the EUCAST website regularly for updates on methodology, QC ranges and breakpoints.

[www.eucast.org](http://www.eucast.org)

- Please send any comments and suggestions to [erika.matuschek@ltkronoberg.se](mailto:erika.matuschek@ltkronoberg.se) or to the EUCAST secretariat (see website).



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