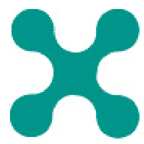

EUCAST EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING

European Society of Clinical Microbiology and Infectious Diseases

Antimicrobial susceptibility testing

EUCAST disk diffusion method

Version 1.0, December 18, 2009

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Document amendments

Version	Amendment	Date
1.0	First edition	December 18, 2009

Abbreviations and terminology

ATCC	American Type Culture Collection http://www.atcc.org
BLNAR	ß-Lactamase negative, ampicillin resistant
CCUG	Culture Collection Universtity of Göteborg http://www.ccug.se
CIP	Collection de Institut Pasteur http://www.cabri.org/CABRI/srs-doc/cip_bact.info.html
DSM	Bacterial cultures from Deutsche Stammsammlung für Mikroorganismen und Zellkulturen (DSMZ) have DSM numbers http://www.dsmz.de/index.htm
ESBL	Extended spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing http://www.eucast.org
MH	Mueller-Hinton agar
MH-F	Mueller-Hinton agar -fastidious organisms (MH supplemented with 5% defibrinated horse blood and 20 mg/L β-NAD)
MRSA	Methicillin resistant *Staphylococcus aureus* (with *mecA* gene)
NCTC	National Collection of Type Cultures http://www.hpacultures.org.uk
ß-NAD	ß-Nicotinamide adenine dinucleotide
Saline	A solution of 0.85% NaCl in water

1	Introduction

Disk diffusion is one of the oldest approaches to antimicrobial susceptibility testing and remains one of the most widely used antimicrobial susceptibility testing methods in routine clinical laboratories. It is suitable for testing the majority of bacterial pathogens, including the more common fastidious bacteria, is versatile in the range of antimicrobial agents that can be tested and requires no special equipment.

In common with several other disk diffusion techniques, the EUCAST method is a standardised method based on the principles defined in the report of the International Collaborative Study of Antimicrobial Susceptibility Testing, 1972, and the experience of expert groups worldwide.

The zone diameter breakpoints in the EUCAST method are calibrated to the harmonised European breakpoints that are published by EUCAST and are freely available from the EUCAST website (http://www.eucast.org).

As with all methods, the described technique must be followed without modification in order to produce reliable results.

2 Preparation of media

2.1 Prepare MH agar according to the manufacturer's instructions, with supplementation for fastidious organisms as indicated in Table 1.

2.2 Medium should have a level depth of 4 mm ± 0.5 mm (25 mL in a 90 mm Petri dish, 70 mL in a 150 mm Petri dish).

2.3 The surface of the agar should be dry before use. Whether plates require drying and the length of time needed to dry the surface of the agar depends on storage and drying conditions. Do not over-dry plates.

2.4 Store plates prepared in-house at 8-10°C. If plates are stored for longer than 7 days, consider storing plates at 4-8°C in sealed plastic bags.

2.5 For plates prepared in-house, plate drying, storage conditions and shelf life should be determined as part of the laboratory quality assurance programme.

2.6 Commercially prepared plates should be stored as recommended by the manufacturer and used within the labelled expiry date.

Table 1 Media for antimicrobial susceptibility testing

Organism	Medium
Enterobacteriaceae	MH agar
Pseudomonas spp.	MH agar
Stenotrophomonas maltophilia	MH agar
Acinetobacter spp.	MH agar
Staphylococcus spp.	MH agar
Enterococcus spp.	MH agar
Streptococcus pneumoniae	MH agar + 5% defibrinated horse blood+ 20 mg/L NAD (MH-F)
Streptococcus Groups A, B, C and G	MH agar + 5% defibrinated horse blood+ 20 mg/L NAD (MH-F)
Other streptococci	MH agar + 5% defibrinated horse blood+ 20 mg/L NAD (MH-F)
Haemophilus spp.	MH agar + 5% defibrinated horse blood+ 20 mg/L NAD (MH-F)
Moraxella catarrhalis	MH agar + 5% defibrinated horse blood+ 20 mg/L NAD (MH-F)
Other fastidious organisms	Pending

3 Preparation of inoculum

3.1 Use the direct colony suspension method to make a suspension of the organism in saline to the density of a McFarland 0.5 turbidity standard (Table 2), approximately corresponding to 1-2 x10^8 CFU/mL for *Escherichia coli*.

The direct colony suspension method is appropriate for all organisms, including fastidious organisms such as *Haemophilus* spp., *Moraxella catarrhalis, Streptococcus pneumoniae*, β-haemolytic and other streptococci.

3.1.1 Make the suspension from overnight growth on non-selective medium. Use several morphologically similar colonies (when possible) to avoid selecting an atypical variant and suspend the colonies in saline with a sterile loop or a cotton swab.

3.2 Standardise the inoculum suspension to the density of a McFarland 0.5 standard. A denser inoculum will result in reduced zones of inhibition and a decreased inoculum will have the opposite effect.

3.2.1 It is recommended that a photometric device is used to adjust the density of the suspension to McFarland 0.5. The photometric device must be calibrated against a McFarland standard according to the manufacturer's instruction.

3.2.2 Alternatively, the density of the suspension can be compared visually to a 0.5 McFarland turbidity standard.

Vigorously agitate the turbidity standard on a vortex mixer before use.

To aid comparison, compare the test and standard against a white background with black lines.

3.2.3 *Streptococcus pneumoniae* is preferably suspended from a blood agar plate to McFarland 0.5. When *Streptococcus pneumoniae* is suspended from a chocolate agar plate, the inoculum must be equivalent to McFarland 1.0.

3.2.4 Adjust the density of the organism suspension to McFarland 0.5 by adding saline or more organisms.

3.3 The suspension should optimally be used within 15 min and always within 60 min of preparation.

Table 2	Preparation of McFarland 0.5 turbidity standard
1	Add 0.5 mL of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2 \cdot 2H_20$) to 99.5 mL of 0.18 mol/L (0.36 N) H_2S0_4 (1% v/v) and mix thoroughly.
2	Check the density of the suspension in a spectrophotometer with a 1 cm light path and matched cuvettes. The absorbance at 625 nm should be in the range 0.08 to 0.13.
3	Distribute the suspension into tubes of the same size as those used for test inoculum adjustment. Seal the tubes.
4	Store sealed standards in the dark at room temperature.
5	Mix the standard thoroughly on a vortex mixer immediately before use.
6	Renew standards or check their absorbance after storage for 6 months.
7	Prepared standards purchased from commercial sources should be checked to ensure that absorbance is within the acceptable range.

4	Inoculation of agar plates
4.1	Optimally, use the adjusted inoculum suspension within 15 min of preparation.
4.2	Dip a sterile cotton swab into the suspension and remove the excess fluid by turning the swab against the inside of the container. It is important to remove excess fluid from the swab to avoid over-inoculation of plates, particularly for Gram-negative organisms.
4.3	Spread the inoculum evenly over the entire surface of the plate by swabbing in three directions or by using an automatic plate rotator.
4.4	Apply disks within 15 minutes. If inoculated plates are left at room temperature for prolonged periods of time before the disks are applied, the organism may begin to grow, resulting in erroneous reduction in sizes of zones of inhibition. Disks should therefore be applied to the surface of the agar within 15 min of inoculation.

5	**Application of antimicrobial disks**

5.1 The required disk contents are listed in the breakpoint and quality control tables at http://www.eucast.org.

5.2 Apply disks firmly to the surface of the inoculated and dried agar plate. The contact with the agar must be close and even. Disks must not be moved once they have been applied to plates as diffusion of antimicrobial agents from disks is very rapid.

5.3 The number of disks on a plate should be limited so that unacceptable overlapping of zones is avoided. The maximum number of disks depends on the organism and the agents tested as some agents consistently give larger zones than others with susceptible isolates. No more than six disks can be accommodated on a 90 mm circular plate or 12 on a 150 mm circular plate.

5.4 Loss of potency of antimicrobial agents in disks results in reduced zone diameters and is a common source of error. The following are essential:

5.4.1 Store disks, including those in dispensers, in sealed containers with a desiccant and protected from light (some agents, including metronidazole, chloramphenicol and the quinolones, are inactivated by prolonged exposure to light).

5.4.2 Store disk stocks at -20°C unless otherwise indicated by the supplier. If this is not possible, store disks at <8°C.

5.4.3 Store working supplies of disks at <8°C.

5.4.4 To prevent condensation, allow disks to warm to room temperature before opening containers.

5.4.5 Discard disks on the manufacturer's expiry date shown on the container.

6	Incubation of plates
6.1	Invert plates and incubate them within 15 min of disk application. If the plates are left at room temperature after disks have been applied, pre-diffusion may result in erroneously large zones of inhibition.
6.2	Stacking plates in the incubator affects results owing to uneven heating of plates. The efficiency of incubators varies and therefore the control of incubation, including appropriate numbers of plates in stacks, should be determined as part of the laboratory's quality assurance programme.
6.3	Incubate plates in the conditions shown in Table 3.
6.4	With glycopeptide susceptibility tests on some strains of *Enterococcus* spp. resistant colonies are not visible until plates have been incubated for a full 24h. However, plates may be examined after 16-20h and any resistance reported, but plates of isolates appearing susceptible must be re-incubated and reread at 24h.

Table 3 Incubation conditions for antimicrobial susceptibility test plates

Organism	Incubation conditions
Enterobacteriaceae	35±1°C in air for 16-20 h
Pseudomonas spp.	35±1°C in air for 16-20 h
Stenotrophomonas maltophilia	35±1°C in air for 16-20 h
Acinetobacter spp.	35±1°C in air for 16-20 h
Staphylococcus spp.	35±1°C in air for 16-20 h
Enterococcus spp.	35±1°C in air for 16-20 h (35±1°C in air for 24 h for glycopeptides)
Streptococcus pneumoniae	35±1°C in 4-6% CO_2 in air for 16-20 h
Streptococcus Groups A, B, C and G	35±1°C in 4-6% CO_2 in air for 16-20 h
Other streptococci	35±1°C in 4-6% CO_2 in air for 16-20 h
Haemophilus spp.	35±1°C in 4-6% CO_2 in air for 16-20 h
Moraxella catarrhalis	35±1°C in 4-6% CO_2 in air for 16-20 h
Other fastidious organisms	Pending

7	Examination of plates after incubation
7.1	A correct inoculum and satisfactorily streaked plates should result in a confluent lawn of growth.
7.2	The growth should be evenly distributed over the plate to achieve uniformly circular (non-jagged) inhibition zones.
7.3	If individual colonies can be seen, the inoculum is too light and the test must be repeated.
7.4	Check that inhibition zones are within quality control limits.

8 Measurement of zones and interpretation of susceptibility

8.1 For all agents, the zone edge should be read at the point of complete inhibition as judged by the naked eye.

8.2 Measure the diameters of zones of inhibition to the nearest millimetre with a ruler, calliper or an automated zone reader.

8.3 Read un-supplemented plates from the back with reflected light and the plate held above a dark background.

8.4 Read supplemented plates from the front with the lid removed and with reflected light.

8.5 Read linezolid susceptibility tests on staphylococci from the back with the plate held up to light (transmitted light).

8.6 When using cefoxitin for the detection of methicillin resistance in *Staphylococcus aureus,* measure the obvious zone, and examine zones carefully in good light to detect colonies within the zone of inhibition. These may be either a contaminating species or the expression of heterogeneous methicillin resistance.

8.7 Discreet colonies growing within the zone of inhibition should be sub-cultured and identified and the test repeated if necessary.

8.8 For *Proteus* spp., ignore swarming and read inhibition of growth.

8.9 Antagonists in the medium may result in faint growth up to the disk within sulphonamide or trimethoprim zones. Such growth should be ignored and the zone diameter measured at the more obvious zone edge.

8.10 For haemolytic streptococci on MH-F medium, read inhibition of growth and not inhibition of haemolysis.

8.11 Interpret zone diameters by reference to breakpoint tables at http://www.eucast.org.

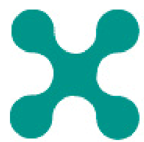
8.12 If templates are used for interpreting zone diameters, the plate is placed over the template and zones interpreted according to the EUCAST breakpoints marked on the template. A program for preparation of templates is freely available from http://www.bsac.org.uk/susceptibility_testing/bsac_disc_diffusion_template_program.cfm

9	Quality control
9.1	Use specified control strains (table 4) to monitor the performance of the test. Principal recommended control strains are typical susceptible strains, but resistant strains can also be used to confirm that the method will detect resistance mediated by known resistance mechanisms (table 5). These strains may be purchased from culture collections or from commercial sources.
9.2	Store control strains under conditions that will maintain viability and organism characteristics. Storage on glass beads at -70°C in glycerol broth (or commercial equivalent) is a convenient method. Non-fastidious organisms can be stored at -20°C. Two vials of each control strain should be stored, one as an in-use supply and the other as an archive for replenishment of the in-use vial when required.
9.3	Each week subculture a bead from the in-use vial on to appropriate non-selective media and check for purity. From this pure culture, prepare one subculture on each day of the week. For fastidious organisms that will not survive on plates for five to six days, subculture the strain daily for no more than one week.
9.4	Acceptable ranges for control strains are shown in EUCAST Quality Control Tables.
9.5	Use the recommended routine quality control strains to monitor test 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. See EUCAST document “Quality assurance of antimicrobial susceptibility testing” for further details.
9.6	Control strains should be tested daily until performance is shown to be satisfactory (no more than 1 in 20 tests outside control limits), at which stage testing frequency may be reduced to once a week. If performance standards are not met, the cause must be investigated.
9.7	In addition to routine QC testing, test each new batch of Mueller-Hinton agar to ensure that all zones are within range. Aminoglycoside disks may disclose unacceptable variation in divalent cations in the medium, tigecycline may disclose variation in magnesium, trimethoprim-sulfamethoxazole will show up problems with the thymine content, erythromycin can disclose an unacceptable pH.

Table 4:	**Quality control organisms for routine testing**	
Organism	**Strain**	**Characteristics**
Escherichia coli	ATCC 25922 NCTC 12241 CIP 7624 DSM 1103 CCUG 17620	Susceptible, wild-type
Pseudomonas aeruginosa	ATCC 27853 NCTC 12934 CIP 76110 DSM 1117 CCUG 17619	Susceptible, wild-type
Staphylococcus aureus	ATCC 29213 NCTC 12973 CIP 103429 DSM 2569 CCUG 15915	Weak β-lactamase producer
Enterococcus faecalis	ATCC 29212 NCTC 12697 CIP 103214 DSM 2570 CCUG 9997	Susceptible, wild-type
Streptococcus pneumoniae	ATCC 49619 NCTC 12977 CIP 104340 DSM 11967 CCUG 66368	Low-level chromosomally mediated penicillin resistant
Haemophilus influenzae	NCTC 8468 CIP 54.94 CCUG 23946	Susceptible, wild type

Table 5:	Additional quality control organisms for detection of specific resistance mechanisms

Organism	Strain	Characteristics
Escherichia coli	ATCC 35218 NCTC 11954 CIP 102181 DSM 5564 CCUG 30600	TEM-1 ß-lactamase, ampicillin resistant
Staphylococcus aureus	NCTC 12493	*mecA*-positive, hetero-resistant MRSA
Haemophilus influenzae	ATCC 49247 NCTC 12699 CIP 104604 DSM 9999 CCUG 26214	ß-lactamase negative, ampicillin resistant (BLNAR)



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