EUCAST EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING

European Society of Clinical Microbiology and Infectious Diseases

Antimicrobial susceptibility testing

EUCAST disk diffusion method

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Contents

Changes from previous version (v. 6.0)

Section	Change
Table 1, Table 3, 8.9.3	New taxonomy: Enterobacteriaceae changed to Enterobacterales.
Table 1, Table 3	*Aeromonas* spp. added
8.9.2	Instructions for reading of trimethoprim-sulfamethoxazole for *Aeromonas* spp. added.

Abbreviations and terminology

ATCC	American Type Culture Collection http://www.atcc.org
CCUG	Culture Collection University of Gothenburg http://www.ccug.se
CECT	Colección Española de Cultivos Tipo http://www.cect.org
CFU	Colony Forming Unit
CIP	Collection de l`Institut Pasteur https://www.pasteur.fr/en/public-health/crbip/collections/collection-institut-pasteur-cip NEW ADDRESS
DSM	Bacterial cultures from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) have DSM numbers https://www.dsmz.de
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 for Fastidious organisms (MH supplemented with 5% defibrinated horse blood and 20 mg/L β-NAD)
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin Resistant *Staphylococcus aureus* (with *mecA* or *mecC* gene)
NCTC	National Collection of Type Cultures https://www.phe-culturecollections.org.uk/collections/nctc NEW ADDRESS
ß-NAD	ß-Nicotinamide Adenine Dinucleotide
QC	Quality Control
Saline	A 0.85% solution of NaCl in water (8.5 g/L)

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 disk diffusion method are calibrated to the harmonised European MIC breakpoints that are published by EUCAST and are freely available from the EUCAST website (http://www.eucast.org).

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

2 Preparation and storage of media

2.1 Prepare Mueller-Hinton (MH) agar according to the manufacturer's instructions, with supplementation for fastidious organisms as indicated in **Table 1**. Preparation and addition of supplements are described in detail at http://www.eucast.org.

2.2 The medium should have a level depth of 4.0 ± 0.5 mm (approximately 25 mL in a 90 mm circular plate, 31 mL in a 100 mm circular plate, 71 mL in a 150 mm circular plate, 40 mL in a 100 mm square plate). Ascertain that a correct volume, based on the true dimensions of the Petri dish in use, is calculated. Plate dimensions may differ between manufacturers.

2.3 The surface of the agar should be dry before use. No drops of water should be visible on the surface of the agar or inside the lid. If necessary, dry plates either at 20-25°C overnight, or at 35°C, with the lid removed, for 15 min. Do not over-dry plates.

2.4 Store plates prepared in-house at 4-8°C.

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.

2.7 For agar plates (commercially or in-house prepared) stored in plastic bags or sealed containers, it may be necessary to dry the plates prior to use (see section 2.3). This is to avoid excess moisture, which may result in problems with fuzzy zone edges and/or haze within zones.

Table 1	Media for antimicrobial susceptibility testing
Organism	**Medium**
Enterobacterales	MH agar
Pseudomonas spp.	MH agar
Stenotrophomonas maltophilia	MH agar
Acinetobacter spp.	MH agar
Staphylococcus spp.	MH agar
Enterococcus spp.	MH agar
Streptococcus groups A, B, C and G	MH-F agar[1]
Streptococcus pneumoniae	MH-F agar[1]
Viridans group streptococci	MH-F agar[1]
Haemophilus influenzae	MH-F agar[1]
Moraxella catarrhalis	MH-F agar[1]
Listeria monocytogenes	MH-F agar[1]
Pasteurella multocida	MH-F agar[1]
Campylobacter jejuni and *coli*	MH-F agar[1] (see Appendix A)
Corynebacterium spp.	MH-F agar[1]
Aerococcus sanguinicola and *urinae*	MH-F agar[1]
Kingella kingae	MH-F agar[1]
Aeromonas spp.	MH agar
Other fastidious organisms	Pending

[1] MH + 5% mechanically defibrinated horse blood + 20 mg/L β-NAD

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 0.5 McFarland turbidity standard (**Table 2**), approximately corresponding to 1-2 x 10^8 CFU/mL for *Escherichia coli*.

The direct colony suspension method is appropriate for all organisms, including fastidious organisms in **Table 1**.

3.2 Use a sterile loop or a cotton swab to pick colonies from an overnight culture on non-selective media. Use several morphologically similar colonies (when possible) to avoid selecting an atypical variant. Suspend the colonies in saline and mix to an even turbidity.

3.3 Adjust the density of the organism suspension to 0.5 McFarland by adding saline or more bacteria. A denser inoculum will result in reduced zones of inhibition and a decreased inoculum will have the opposite effect.

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

3.3.2 Alternatively, the density of the suspension can be compared visually to a 0.5 McFarland turbidity standard. To aid comparison, compare the test and standard against a white background with black lines.

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

3.4 The suspension should optimally be used within 15 min[1] and always within 60 min of preparation.

[1] Part of the 15-15-15 minute rule: use the inoculum suspension within 15 minutes of preparation, apply disks within 15 minutes of inoculation and incubate plates within 15 minutes of disk application.

Table 2	Preparation of 0.5 McFarland 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 bacterial inoculum suspensions. 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.

4	Inoculation of agar plates
4.1	Make sure that agar plates are at room temperature prior to inoculation.
4.2	Optimally, use the adjusted inoculum suspension within 15 min[1] of preparation. The suspension must always be used within 60 min of preparation.
4.3	Dip a sterile cotton swab into the suspension.
4.3.1	To avoid over-inoculation of Gram-negative bacteria, remove excess fluid by pressing and turning the swab against the inside of the tube.
4.3.2	For Gram-positive bacteria, do not press or turn the swab against the inside of the tube.
4.4	When inoculating several agar plates with the same inoculum suspension, repeat the procedure in section 4.3 for each agar plate.
4.5	Plates can be inoculated either by swabbing in three directions or by using an automatic plate rotator. Spread the inoculum evenly over the entire agar surface ensuring that there are no gaps between streaks.
4.5.1	For Gram-positive bacteria, take particular care to ensure that there are no gaps between streaks.
4.6	Apply disks within 15 min[1] of inoculation. 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 inhibition zone diameters.

[1] Part of the 15-15-15 minute rule: use the inoculum suspension within 15 minutes of preparation, apply disks within 15 minutes of inoculation and incubate plates within 15 minutes of disk application.

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 Allow disks to reach room temperature before opening cartridges or containers used for disk storage. This is to prevent condensation, leading to rapid deterioration of some agents.

5.3 Apply disks firmly to the surface of the inoculated agar plate within 15 minutes of inoculation[1]. Disks must be in close and even contact with the agar surface and must not be moved once they have been applied as the initial diffusion of antimicrobial agents from disks is very rapid.

5.4 The number of disks on a plate should be limited to avoid overlapping of zones and interference between agents. It is important that zone diameters can be reliably measured. The maximum number of disks depends on the organism and the selection of disks. Normally 6 and 12 disks are the maximum possible number on a 90 and 150 mm circular plate, respectively.

5.4.1 To be able to detect inducible clindamycin resistance in staphylococci and streptococci, the erythromycin and clindamycin disks must be placed at a distance of 12-20 mm from edge to edge for staphylococci and 12-16 mm from edge to edge for streptococci.

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

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

5.5.2 Store disk stocks according the manufacturers' instructions. Some agents are more labile than others (*e.g.* amoxicillin-clavulanic acid, cefaclor and carbapenems) and specific recommendations may be available from the manufacturers.

5.5.3 Store working supplies of disks according to the manufacturers' instructions. Once disk containers have been opened, disks should be used within the time limit specified by the manufacturer.

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

5.5.5 Perform frequent quality control (see Section 9) of working supplies to control that the antimicrobial disks have not lost potency during storage.

[1] Part of the 15-15-15 minute rule: use the inoculum suspension within 15 minutes of preparation, apply disks within 15 minutes of inoculation and incubate plates within 15 minutes of disk application.

6 Incubation of plates

6.1 Invert agar plates and make sure disks do not fall off the agar surface. Incubate plates within 15 min[1] 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 may affect results due to uneven heating. The efficiency of incubators varies and therefore the control of incubation, including appropriate number of plates in any one stack, should be determined as part of the laboratory's quality assurance programme. For most incubators, a maximum of five plates per stack is appropriate.

6.3 Incubate plates in the conditions shown in **Table 3**.

6.3.1 Incubation beyond the recommended time limits should not be performed as this may result in growth within inhibition zones and reporting isolates as false resistant.

6.3.2 With glycopeptide susceptibility tests on *Enterococcus* spp. resistant colonies may not be visible until plates have been incubated for 24 h. However, plates may be examined after 16-20 h and any resistance reported, but plates of isolates appearing susceptible must be re-incubated and reread at 24 h.

[1] Part of the 15-15-15 minute rule: use the inoculum suspension within 15 minutes of preparation, apply disks within 15 minutes of inoculation and incubate plates within 15 minutes of disk application.

Table 3	Incubation conditions for antimicrobial susceptibility test plates
Organism	**Incubation conditions**
Enterobacterales	35 ± 1°C in air for 18 ± 2 h
Pseudomonas spp.	35 ± 1°C in air for 18 ± 2 h
Stenotrophomonas maltophilia	35 ± 1°C in air for 18 ± 2 h
Acinetobacter spp.	35 ± 1°C in air for 18 ± 2 h
Staphylococcus spp.	35 ± 1°C in air for 18 ± 2 h
Enterococcus spp.	35 ± 1°C in air for 18 ± 2 h (24 h for glycopeptides)
Aeromonas spp.	35 ± 1°C in air for 18 ± 2 h
Streptococcus groups A, B, C and G	35 ± 1°C in 4-6% CO_2 in air for 18 ± 2 h
Streptococcus pneumoniae	35 ± 1°C in 4-6% CO_2 in air for 18 ± 2 h
Viridans group streptococci	35 ± 1°C in 4-6% CO_2 in air for 18 ± 2 h
Haemophilus influenzae	35 ± 1°C in 4-6% CO_2 in air for 18 ± 2 h
Moraxella catarrhalis	35 ± 1°C in 4-6% CO_2 in air for 18 ± 2 h
Listeria monocytogenes	35 ± 1°C in 4-6% CO_2 in air for 18 ± 2 h
Pasteurella multocida	35 ± 1°C in 4-6% CO_2 in air for 18 ± 2 h
Campylobacter jejuni and *coli*	See **Appendix A**
Corynebacterium spp.	35 ± 1°C in 4-6% CO_2 in air for 18 ± 2 h. Isolates with insufficient growth after 16-20 h are re-incubated immediately and inhibition zones read after a total of 40-44 h incubation.
Aerococcus sanguinicola and *urinae*	35 ± 1°C in 4-6% CO_2 in air for 18 ± 2 h. Isolates with insufficient growth after 16-20 h are re-incubated immediately and inhibition zones read after a total of 40-44 h incubation.
Kingella kingae	35 ± 1°C in 4-6% CO_2 in air for 18 ± 2 h. Isolates with insufficient growth after 16-20 h are re-incubated immediately and inhibition zones read after a total of 40-44 h incubation.
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.

7.1.1 If individual colonies can be seen, the inoculum is too light and the test must be repeated.

7.2 The growth should be evenly distributed over the agar surface to achieve uniformly circular (non-jagged) inhibition zones.

7.3 Check that inhibition zones for quality control strains are within acceptable ranges (http://www.eucast.org).

8 Measurement of zones and interpretation of susceptibility

8.1 For all agents (unless otherwise stated in section 8.9), the zone edge should be read at the point of complete inhibition as judged by the naked eye with the plate held about 30 cm from the eye.

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

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

8.4 Do not use transmitted light (plate held up to light) or a magnifying glass, unless otherwise stated (see section 8.9).

8.5 Measure the inhibition zone diameters to the nearest millimetre with a ruler or a calliper.

8.5.1 If an automated zone reader is used, it must be calibrated to manual reading.

8.6 Interpret zone diameters into susceptibility categories according to the current breakpoint tables at http://www.eucast.org.

8.7 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. Make certain that the breakpoints used are in accordance with the latest version of the EUCAST breakpoint tables. A program for preparation of templates is freely available from http://bsac.org.uk/susceptibility/template-program.

8.8 Several examples of pictures showing reading of inhibition zone diameters are available in the Reading Guide at http://www.eucast.org. This document also includes reading instructions for specific organism-antimicrobial agent combinations.

8.9 Specific reading instructions:

8.9.1 In case of double zones, or distinct colonies within zones, check for purity and repeat the test if necessary. If cultures are pure, colonies within zones should be taken into account when measuring the diameter.

8.9.2 For trimethoprim and trimethoprim-sulfamethoxazole, faint growth up to the disk may appear due to antagonists in the medium. Such growth should be ignored and the zone diameter measured at the more obvious zone edge.

For *Stenotrophomonas maltophilia* with trimethoprim-sulfamethoxazole, an isolate showing any sign of inhibition zone ≥ the susceptible breakpoint should be reported susceptible. Note that there may be substantial growth within zones. Read as no zone only if there is growth up to the disk and no sign of an inhibition zone.

For *Aeromonas* spp. with trimethoprim-sulfamethoxazole, read the obvious zone edge and disregard haze or growth within the inhibition zone. If there is an obvious inner zone edge, read the inhibition zone as the inner zone.

8.9.3	For Enterobacterales with ampicillin, ampicillin-sulbactam and amoxicillin-clavulanic acid, ignore growth that may appear as a thin film producing an inner zone on some batches of Mueller-Hinton agar.
8.9.4	For *Escherichia coli* with mecillinam, ignore isolated colonies within the inhibition zone.
8.9.5	For *Proteus* spp., ignore swarming and read inhibition of growth.
8.9.6	For *Staphylococcus aureus* with benzylpenicillin, examine the zone edge closely from the front of the plate with the plate held up to light (transmitted light). Isolates with inhibition zone diameters ≥ the susceptible breakpoint, but with sharp zone edges should be reported resistant.
8.9.7	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.
8.9.8	Read linezolid susceptibility tests on staphylococci from the back with the plate held up to light (transmitted light).
8.9.9	For enterococci with vancomycin, examine the zone edge closely from the front of the plate with the plate held up to light (transmitted light). Fuzzy zone edges and colonies within zone indicate vancomycin resistance and should be investigated further. Isolates must not be reported susceptible before 24 h incubation.
8.9.10	For haemolytic streptococci, read inhibition of growth and not inhibition of haemolysis. β-Haemolysis is usually free from growth, whereas α-haemolysis and growth usually coincide. Tilt the plate back and forth to better differentiate between haemolysis and growth.
8.9.11	For *Escherichia coli* with fosfomycin, ignore isolated colonies within the inhibition zone and read the outer zone edge.

9 Quality control

9.1 Use the quality control (QC) strains specified in **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 (Extended QC, **Table 5**). QC strains may be purchased from culture collections or from commercial sources.

9.1.1 To control the inhibitor component of β-lactam-β-lactamase inhibitor combination disks, specific β-lactamase-producing strains are recommended (**Table 4**). This should be part of the routine QC. The active component is checked with a susceptible QC strain.

9.2 Store control strains under conditions that will maintain viability and organism characteristics. Storage on 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 onto 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.

When subculturing a control strain, use several colonies to avoid selecting a mutant.

9.4 Check that results for control strains are within acceptable ranges in EUCAST QC Tables at http://www.eucast.org.

9.4.1 In EUCAST quality control tables, both ranges and targets are listed. Repeat testing of EUCAST QC strains should yield zone diameter values randomly distributed within the recommended ranges. If the number of tests is ≥10, the mean zone diameter should be close to the target value (±1 mm from the target value).

9.5 Use the recommended routine QC strains to monitor test performance.

Control tests should be set up and checked daily, or at least four times per week 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 target. If two or more of 20 tests are out of range investigation is required.

9.6 In addition to routine QC testing, test each new batch of Mueller-Hinton agar to ensure that all zones are within range.

Aminoglycosides 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 CECT 434	Susceptible, wild-type
Escherichia coli	ATCC 35218 NCTC 11954 CIP 102181 DSM 5564 CCUG 30600 CECT 943	TEM-1 ß-lactamase, ampicillin resistant (for control of the inhibitor component of β-lactam-β-lactamase inhibitor combination disks)
Klebsiella pneumoniae	ATCC 700603 NCTC 13368 CCUG 45421 CECT 7787	ESBL-producing strain (SHV-18) (for control of the inhibitor component of β-lactam-β-lactamase inhibitor combination disks)
Pseudomonas aeruginosa	ATCC 27853 NCTC 12934 CIP 76110 DSM 1117 CCUG 17619 CECT 108	Susceptible, wild type
Staphylococcus aureus	ATCC 29213 NCTC 12973 CIP 103429 DSM 2569 CCUG 15915 CECT 794	Weak β-lactamase producer
Enterococcus faecalis	ATCC 29212 NCTC 12697 CIP 103214 DSM 2570 CCUG 9997 CECT 795	Susceptible, wild type
Streptococcus pneumoniae	ATCC 49619 NCTC 12977 CIP 104340 DSM 11967 CCUG 33638	Reduced susceptibility to benzylpenicillin
Haemophilus influenzae	ATCC 49766 NCTC 12975 CIP 103570 DSM 11970 CCUG 29539	Susceptible, wild type
Campylobacter jejuni	ATCC 33560 NCTC 11351 CIP 702 DSM 4688, CCUG 11284	Susceptible, wild type For testing conditions, see Appendix A

Table 5:	Additional quality control organisms for detection of specific resistance mechanisms (extended QC)	
Organism	**Strain**	**Characteristics**
Klebsiella pneumoniae	ATCC 700603 NCTC 13368 CCUG 45421 CECT 7787	ESBL-producing strain (SHV-18)
Staphylococcus aureus	NCTC 12493	*mecA* positive, hetero-resistant MRSA
Enterococcus faecalis	ATCC 51299 NCTC 13379 CIP 104676 DSM 12956 CCUG 34289	High-level aminoglycoside resistant (HLAR) and vancomycin resistant (*vanB* positive)
Haemophilus influenzae	ATCC 49247 NCTC 12699 CIP 104604 DSM 9999 CCUG 26214	Reduced susceptibility to β-lactam agents due to PBP mutations (ß-lactamase negative, ampicillin resistant, BLNAR)

Appendix A

Disk diffusion testing of *Campylobacter jejuni* and *coli*

The following methodology (Table A1) must be adhered to when performing disk diffusion testing of *Campylobacter jejuni* and *coli* according to EUCAST.

Table A1	**Disk diffusion methodology for *Campylobacter jejuni* and *coli***
Medium	Mueller-Hinton agar supplemented with 5% defibrinated horse blood and 20 mg/L β-NAD (MH-F) In order to reduce swarming, the MH-F plates should be dried prior to inoculation (at 20-25°C overnight, or at 35°C, with the lid removed, for 15 min).
Inoculum	0.5 McFarland
Incubation	Microaerobic environment 41±1°C 24 h Incubation should result in confluent growth. Some *C. coli* isolates may not have sufficient growth after 24 h incubation. These are re-incubated immediately and inhibition zones read after a total of 40-48 h incubation. An incubation temperature of 41±1°C was chosen to create favourable conditions for growth of *Campylobacter* spp.
Reading	Standard EUCAST reading instructions are used: Read MH-F plates from the front with the lid removed and with reflected light. Zone edges should be read at the point of complete inhibition as judged by the naked eye with the plate held about 30 cm from the eye.
Quality Control	*Campylobacter jejuni* ATCC 33560

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