EUCAST EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING

European Society of Clinical Microbiology and Infectious Diseases

Antimicrobial susceptibility testing

EUCAST disk diffusion method

Version 5.0

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Contents

Document amendments

Version	Amendment	Date
5.0	*E. coli* ATCC 35218 moved from extended QC (**Table 5**) to routine QC (**Table 4**).	January 2015
5.0	**Table 4:** Note on removal of *H. influenzae* NCTC 8468 added.	January 2015
5.0	**Table 4:** *H. influenzae* ATCC 49766 added.	January 2015
4.0	**Section 2.7:** New section on storage and handling of MH-F agar plates.	June 2014
4.0	**Tables 1 and 3:** *Corynebacterium* spp. added.	June 2014
4.0	**Section 5.3.1:** Placement of disks to detect inducible clindamycin resistance in streptococci revised.	June 2014
3.0	**Sections 2.1 and 2.4**: Clarification regarding preparation and storage of media.	April 2013
3.0	**Tables 1 and 3**: *Pasteurella multocida*, *Campylobacter jejuni* and *coli* added.	April 2013
3.0	**Section 3.2.2**: Clarification regarding the use of turbidity standards.	April 2013
3.0	**Section 4.1**: Clarification regarding the use of inoculum suspensions.	April 2013
3.0	**Section 5.3**: Clarification regarding the number of antibiotic disks on each agar plate.	April 2013
3.0	**Section 5.3.1**: New section. Placement of disks to detect inducible clindamycin resistance.	April 2013
3.0	**Section 8.8.2**: Specific information on reading of trimethoprim-sulfamethoxazole zones for *Stenotrophomonas maltophilia*.	April 2013
3.0	**Table 4**: *Campylobacter jejuni* ATCC 33560 added.	April 2013
3.0	**Table 5**: DSM and CCUG numbers added for *E. faecalis* ATCC 51299.	April 2013
3.0	**Appendix A**: New section. EUCAST methodology for disk diffusion testing of *Campylobacter jejuni* and *coli*.	April 2013

Version	Amendment	Date
2.1	**Sections 8**: Revised numbering. New/revised sections: 8.1 and 8.4.	February 2012
2.0	**Section 2.2**: Clarification regarding agar depth.	January 2012
2.0	**Tables 1 & 3**: New terminology (Viridans group streptococci). *Listeria monocytogenes* added.	January 2012
2.0	**Section 8**: Revised numbering. New/revised sections: 8.1, 8.7, 8.7.3, 8.7.4, 8.7.6, 8.7.9 and 8.7.10.	January 2012
2.0	**Table 5**: *K. pneumoniae* ATCC 700603 and *E. faecalis* ATCC 51299 added.	January 2012
2.0	**Tables 4 & 5** and **Abbreviations**: Spanish Culture Collection numbers added.	January 2012
1.0	First edition	December 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
CECT	Colección Española de Cultivos Tipo. http://www.cect.org
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 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 -fastidious organisms (MH supplemented with 5% defibrinated horse blood and 20 mg/L β-NAD)
MRSA	Methicillin resistant *Staphylococcus aureus* (with *mecA* or *mecC* 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 and storage of media

2.1 Prepare 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 Medium should have a level depth of 4 mm ± 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).

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, alternative storage, such as storing plates at 4-8°C in sealed plastic bags, may be required.

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 MH-F[1] agar plates (commercial or in-house prepared) stored in plastic bags or sealed containers, it may be necessary to dry the plates prior to use. This is to avoid excess moisture, which may result in problems with fuzzy zone edges and/or haze within zones.

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

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 groups A, B, C and G	MH-F agar[1]
Streptococcus pneumoniae	MH-F agar[1]
Viridans group streptococci	MH-F agar[1]
Haemophilus spp.	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]
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 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. The photometric device must be calibrated against a McFarland 0.5 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 (some commercial standards are gel-based and should not be mixed, so follow the instructions of the supplier).

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

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. The suspension must always be used within 60 minutes 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 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.

5.3.1 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.

5.4 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 fluoroquinolones, 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 groups A, B, C and G	35±1°C in 4-6% CO_2 in air for 16-20 h
Streptococcus pneumoniae	35±1°C in 4-6% CO_2 in air for 16-20 h
Viridans group 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
Listeria monocytogenes	35±1°C in 4-6% CO_2 in air for 16-20 h
Pasteurella multocida	35±1°C in 4-6% CO_2 in air for 16-20 h
Campylobacter jejuni and *coli*	See Appendix A
Corynebacterium spp.	35±1°C in 4-6% CO_2 in air for 16-20 h. Isolates with insufficient growth after 16-20 h incubation are reincubated immediately and inhibition zones read after a total of 40-48 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.
7.2	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 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 below).

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

8.6 Interpret zone diameters by reference to 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 Table. A program for preparation of templates is freely available from http://bsac.org.uk/susceptibility/template-program.

8.8 Specific reading instructions:

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

8.8.2 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.

For *Stenotrophomonas maltophilia* with trimethoprim-sulfamethoxazole, growth within zones may be substantial. Such growth should be ignored and an inhibition zone read if any zone edge can be seen. Read as no zone only if there is growth up to the disk and no sign of an inhibition zone.

8.8.3 For Enterobacteriaceae with ampicillin, ignore growth that may appear as a thin film producing an inner zone on some batches of Mueller-Hinton agar.

8.8.4 For *E. coli* with mecillinam, ignore isolated colonies within the inhibition zone.

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

8.8.6 For staphylococci with benzylpenicillin, inspect the zone edge closely 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.

8.8.7	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.
8.8.8	Read linezolid susceptibility tests on staphylococci from the back with the plate held up to light (transmitted light).
8.8.9	For enterococci with vancomycin, inspect the zone edge closely with the plate held up to light (transmitted light). Fuzzy zone edges and colonies within zone indicate vancomycin resistance and should be investigated further.
8.8.10	For haemolytic streptococci on MH-F medium, read inhibition of growth and not inhibition of haemolysis. β-Haemolysis is usually free from growth, whereas α-haemolysis and growth usually coincide.

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 at http://www.eucast.org.

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 target. If two or more of 20 tests are out of range investigation is required.

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 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
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	NCTC 8468[1]	Susceptible, wild type
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

[1] *H. influenzae* NCTC 8468 exhibits unusual growth characteristics and will be excluded from 2016.

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	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 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	McFarland 0.5
Incubation	Microaerobic environment 41±1°C 24 hours Incubation should result in confluent growth. Some *C. coli* isolates may not have sufficient growth after 24 h incubation. These are reincubated 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	Inhibition zones for *Campylobacter jejuni* ATCC 33560 must be within the defined range (http://www.eucast.org).

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