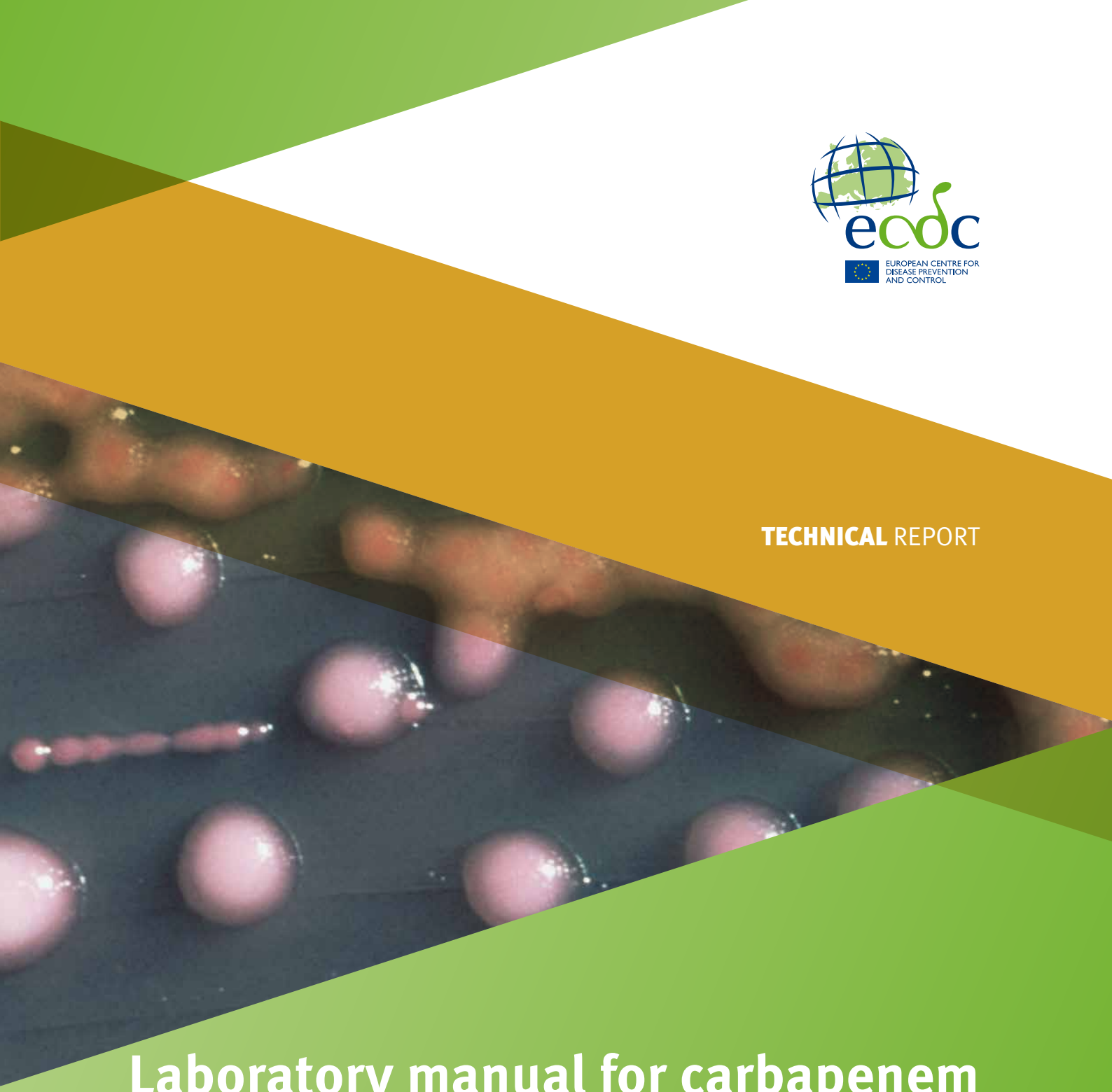

TECHNICAL REPORT

Laboratory manual for carbapenem and colistin resistance detection and characterisation for the survey of carbapenem- and/or colistin-resistant Enterobacteriaceae

Version 2.0

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ECDC TECHNICAL REPORT

Laboratory manual for carbapenem and colistin resistance detection and characterisation for the survey of carbapenem- and/or colistin-resistant Enterobacteriaceae

Version 2.0

This report was commissioned by the European Centre for Disease Prevention and Control (ECDC) [FWC: OJ/2017/OCS/7530], coordinated by Anke Kohlenberg and Barbara Albiger and produced by Sara Byfors and Alma Brolund of the Public Health Agency of Sweden.

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This protocol is based partly on the laboratory manual developed for the European survey of carbapenemase-producing Enterobacteriaceae (EuSCAPE) [1].

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Abbreviations

APBA	Aminophenylboronic acid
bp	Base pairs
CCUG	Culture Collection University of Gothenburg
CDT	Combination disk test
CFU	Colony forming units
DNA	Deoxyribonucleic acid
DPA	Dipicolinic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
E. coli	*Escherichia coli*
EuSCAPE	European survey on carbapenemase-producing Enterobacteriaceae
K. pneumoniae	*Klebsiella pneumoniae*
KPC	K. pneumoniae carbapenemase
MBL	Metallo-β-lactamase
MIC	Minimum inhibitory concentration
MH	Mueller-Hinton
NDM	New Delhi metallo-β-lactamase
PCR	Polymerase chain reaction
QC	Quality control
TE	Tris-EDTA
Tm	Melting temperature
VIM	Verona integron-encoded metallo-β-lactamase

Protocol 1: European Committee on Antimicrobial Susceptibility Testing (EUCAST) – broth microdilution for carbapenem susceptibility testing

For more information, consult the EUCAST recommendation for media preparation [2]: http://www.eucast.org/ast_of_bacteria/media_preparation.

Minimum inhibitory concentration (MIC) determination (broth microdilution according to ISO standard 20776-1)
Medium: Mueller-Hinton broth.
Inoculum: $5x10^5$ colony forming units (CFU)/mL.
Incubation: sealed panels, air, 35±1º C, 18±2h.
Reading: unless otherwise stated, read minimum inhibitory concentrations (MICs) at the lowest concentration of the agent that completely inhibits visible growth.
Quality control: *Escherichia coli* ATCC 25922.

Table 1. EUCAST breakpoints for carbapenems

Carbapenems*	MIC breakpoint (mg/L)		Disk content (µg)	Zone diameter breakpoint (mm)	
	S ≤	R >		S ≥	R <
Ertapenem	0.5	0.5	10	25	5
Imipenem**	2	4	10	22	7
Meropenem	2	8	10	22	16

Breakpoint table used for above values:
http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_9.0_Breakpoint_Tables.pdf.
**: Certain isolates that produce carbapenemase are categorised as susceptible with these breakpoints and should be reported as tested, i.e. the presence or absence of a carbapenemase does not in itself influence the categorisation of susceptibility. Carbapenemase detection and characterisation are recommended for public health and infection control purposes. For carbapenemase screening, a meropenem screening cut-off of >0.125 mg/L (zone diameter <28 mm) is recommended.*
***: Low-level resistance is common in* Morganella *spp.,* Proteus *spp. and* Providencia *spp.*

For a complete list of breakpoints, consult the EUCAST breakpoint table [3]: http://www.eucast.org/clinical_breakpoints.

The latest breakpoint recommendations can be found on the EUCAST website: http://www.eucast.org.

Protocol 2: EUCAST – Kirby-Bauer disk diffusion for carbapenem susceptibility testing

For more information, consult the EUCAST Disc Diffusion Manual [4]: http://www.eucast.org/ast_of_bacteria/disk_diffusion_methodology.

Preparation of media: Prepare Mueller-Hinton (MH) agar according to manufacturer's instructions. The 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). The surface of the agar should be dry before use. Storage and drying conditions determine whether plates require drying and the length of time needed to dry the surface of the agar. Do not overdry plates.

Preparation of inoculum: 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, approximately corresponding to 1-2 x10^8 CFU/mL for *E. coli*. Make the suspension from overnight growth on a 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 cotton swab. Standardise the inoculum suspension to the density of a McFarland 0.5 standard.

Inoculation of agar plates: Optimally, use the adjusted inoculum suspension within 15 minutes of preparation. The suspension must always be used within 60 minutes of preparation. 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. Spread the inoculum evenly over the entire surface of the plate by swabbing in three directions or using an automatic plate rotator. Apply disks within 15 minutes.

Application of antimicrobial disks: 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. 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.

Incubation of plates: Invert plates and incubate them within 15 minutes 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. 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.

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

Measurement of zones and interpretation of susceptibility: 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. Read unsupplemented plates from the back with reflected light and the plate held above a dark background. Read supplemented plates from the front with the lid removed and reflected light.

Do not use transmitted light (plate held up to light) or a magnifying glass unless otherwise stated (see below). Measure the diameters of zones of inhibition to the nearest millimetre with a ruler, caliper or automated zone reader. Interpret zone diameters by reference to breakpoint tables [3]: http://www.eucast.org/clinical_breakpoints. If templates are used for interpreting zone diameters, the plate is placed over the template and zones interpreted according to EUCAST.

Protocol 3: Combination disk test (CDT) for carbapenemase detection (in-house protocol)

This protocol is adapted from Giske et al. [5].

For more information, consult the EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance [6]: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Resistance_mechanisms/EUCAST_detection_of_resistance_mechanisms_170711.pdf.

Test principle

The test is based on the fact that metalloenzymes (i.e., Verona integron-encoded metallo-β-lactamase, VIM, New Delhi metallo-β-lactamase, NDM) are inhibited by dipicolinic acid (DPA), while Class A (serine) enzymes (*Klebsiella pneumoniae* carbapenemase, KPC) are inhibited by aminophenylboronic acid (APBA). Isolates with combined porin loss and AmpC hyperproduction are also inhibited by APBA, but also show cloxacillin synergy. In that respect, meropenem disc zone sizes will increase for metallo-β-lactamase (MBL)-producing strains if DPA is added and for KPC-producing strains if APBA is added. Testing for cloxacillin synergy will further distinguish KPC producers from AmpC-hyperproducing isolates combined with porin loss.

Chemicals/biologicals/consumables

- cation-adjusted MH agar plates
- inoculation loops
- sterile cotton swabs
- meropenem disks (10 µg)
- APBA, 60 mg/mL
- DPA, 100 mg/mL
- cloxacillin, 75 mg/mL

Equipment

- incubator 35° C
- densitometer

Control strains for quality control (QC)

On each occasion the test is performed, well-characterised control strains with (positive control) and without (negative control) the tested resistance mechanism should be included for comparison. See Table 3 for control strains.

Protocol

Preparation of disks

- Dispense on commercially available meropenem disks (disk A)
  - disk B: 10 µL (containing 1 000 µg) of DPA solution
  - disk C: 10 µL (containing 600 µg) of APBA solution
  - disk D: 10 µL (containing 750 µg) of cloxacillin solution
- Disks are allowed to dry at room temperature for 30 minutes before use.

Procedure

- Prepare an inoculum of McFarland 0.5 by suspending a single colony from a pure culture of the test isolate in 0.9% saline according to EUCAST standard.
- Inoculate MH plates with a sterile cotton swab as for the EUCAST disk diffusion method.
- Apply the four types of disks (A, B, C and D).
- Incubate on 35° C±2 for 16–20 hours.

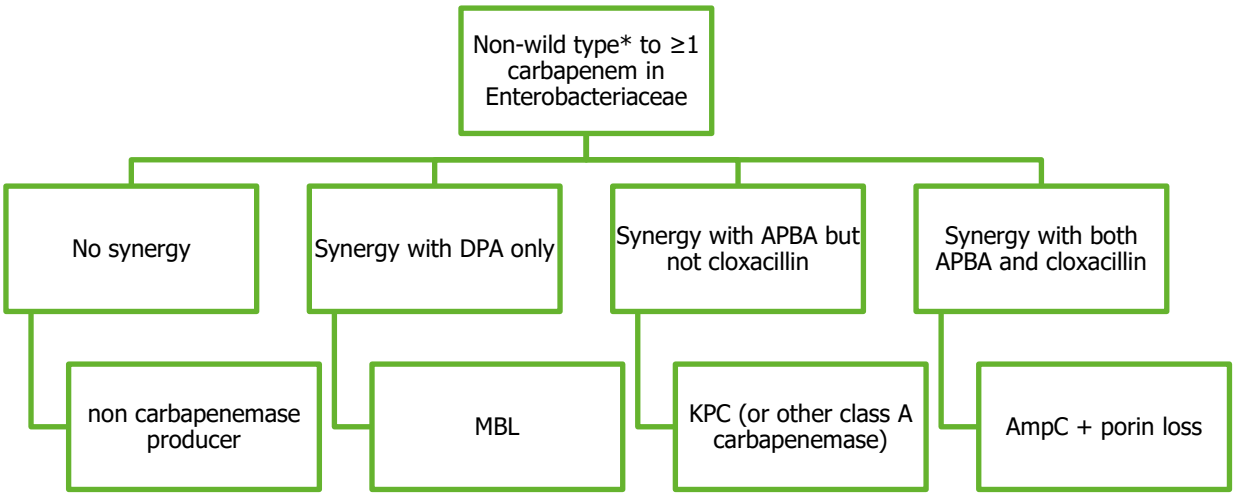
Interpretation

The zone diameters of disks B, C and D are compared to the zone diameter of disk A. The increases in inhibition zone diameters are interpreted according to Table 2. The algorithm for interpretation is further illustrated in Figure 1.

Table 2. Interpretation of the combination disc test (CDT)

Increase in inhibition zone diameters compared to disk A (meropenem)			Interpretation
Disk B (meropenem+DPA)	Disk C (meropenem+APBA)	Disk D (meropenem+cloxacillin)	
< 5 mm	< 5 mm	< 5 mm	Non-carbapenemase producer
≥ 5 mm	< 5 mm	< 5 mm	MBL producer
< 5 mm	≥ 5 mm	< 5 mm	KPC producer
< 5 mm	≥ 5 mm	≥ 5 mm	AmpC + porin loss

Figure 1. Algorithm for interpretation of results with β-lactamase inhibitors



**: MIC above the epidemiological cut-off values defined by EUCAST.*
APBA: aminophenylboronic acid
DPA: dipicolinic acid
MBL: metallo-β-lactamase.

Commercial tests for combination disk testing are available, follow manufacturer's instructions.

Table 3. Suggested quality control strains

Culture Collection University of Gothenburg (CCUG) reference	Genotype
CCUG 59348	*bla*$_{KPC}$
CCUG 60138	*bla*$_{NDM}$
CCUG 58547	*bla*$_{VIM}$
CCUG 64452	*bla*$_{OXA-48}$

For more details regarding these and other control strains, consult the Culture Collection University of Gothenburg website: http://www.ccug.se.

Protocol 4: Polymerase chain reaction (PCR) for detection of four major carbapenemase genes

This protocol is adapted from the EuSCAPE laboratory manual.

Chemicals/biologicals/consumables

- PCR tubes
- primer sequences (Table 4)
- dNTPs (10 mM)
- $MgCl_2$ (25 mM)
- KAPA Taq Polymerase
- crude DNA extracts

Equipment

- thermal cycler (PCR machine)

Control strains for QC

Control strains must always be included:

- negative control – PCR mixture without addition of DNA
- positive control – well-characterised strains positive for the tested resistance genes

Control strains can be obtained from CCUG (Table 3 for recommended strains).

Protocol

- Use a DNA extraction method of choice that is validated for *E. coli* and *K. pneumoniae*. For DNA lysates according to Rebelo et al. [7], suspend a loopful of culture in 100 µl of sterile Tris-EDTA (TE) buffer, boil for 10 min at 100º C, centrifuge for 5 minutes at 6 000 G. Dilute the DNA supernatant 1:10 in TrisHCl buffer.
- For all *bla* genes, PCR mixtures are displayed in Table 4, PCR conditions in Table 5 and specific primer sequences and conditions in Table 6.
- Separate and visualise the PCR products with any suitable method. Consult Table 6 for expected product sizes.

Table 4. Carbapenemase PCR mix

Stock solutions	Quantity	Final concentration
DNA	3 µl	~100 ng
Buffer 5×	5 µl	1×
$MgCl_2$ (25 mM)	1.5 µl	1.5 mM
dNTPs (10 mM)	0.5 µl	200 µM
primer F (10 mM)	1 µl	400 µM
primer R (10 mM)	1 µl	400 µM
KAPA Taq (5 U/µl)	0.2 µl	1 unit
H_2O	12.8 µl	

Table 5. Carbapenemase PCR programme

Cycle step	Temperature (°C)	Time	Number of cycles
Initial denaturation	94	5 minutes	1
Denaturation	94	30 seconds	30
Annealing	Melting temperature (Tm)*	30 seconds	
Extension	72	1 minute	
Hold	72	10 minutes	1

**See Tm of specific genes.*

Table 6. Primer sequences for carbapenemase gene detection

Target gene	Primers and conditions	Melting temperature - Tm (°C)	Product size (bp)	Reference
bla$_{OXA-48}$	OXA_F 5′-TTGGTGGCATCGATTATCGG-3′ OXA_R 5′-GAGCACTTCTTTTGTGATGGC-3′	58	744	[8]
bla$_{NDM}$	NDM_F 5′-TGGCAGCACACTTCCTATC-3′ NDM_R 5′-AGATTGCCGAGCGACTTG-3′	58	488	[1]
bla$_{KPC}$	KPC_F 5′-CTGTCTTGTCTCTCATGGCC-3′ KPC_R 5′-CCTCGCTGTRCTTGTCATCC-3′	60	796	[1,9]
bla$_{VIM}$	VIM_F: 5'-AGTGGTGAGTATCCGACAG-3' VIM_R: 5′-TCAATCTCCGCGAGAAG-3′	52	212	[1,10]

Protocol 5: EUCAST – colistin broth microdilution testing

For more information, consult the EUCAST recommendation for media preparation [2]: http://www.eucast.org/ast_of_bacteria/media_preparation.

EUCAST recommendation

Reference testing of Enterobacteriaceae follows the ISO-standard broth microdilution method (20776-1 or 20776-2 when available).

Note:

- Cation-adjusted MH broth is used.
- No additives may be included in any part of the testing process (particularly no polysorbate-80 or other surfactants).
- Trays must be made of plain polystyrene and not treated in any way before use.
- Sulphate salts of polymyxins must be used (the methanesulfonate derivative of colistin must not be used - it is an inactive pro-drug that breaks down slowly in solution).

Medium: Mueller-Hinton broth
Inoculum: $5x10^5$ CFU/mL
Incubation: Sealed panels, air, 35±1º C, 18±2h
Reading: Unless otherwise stated, read MICs at the lowest concentration of the agent that completely inhibits visible growth.
Quality control: *E. coli* ATCC 25922 and for colistin, add *E. coli* NCTC 13846 with a colistin MIC target value of 4 mg/L (values should mostly be 4 mg/L, but may occasionally be 2 or 8 mg/L). For agents not covered by this strain and for control of the inhibitor component of beta-lactam inhibitor combinations, see EUCAST QC Tables.

Preparation of media: Unsupplemented cation-adjusted MH broth is used for testing of non-fastidious organisms according to the ISO standard 20776-1, 2006. Unsupplemented MH broth may be purchased from commercial sources or prepared locally according to the manufacturers instructions.

Table 7. EUCAST breakpoints for colistin

	MIC breakpoint (mg/L)	
	S ≤	R >
Colistin	2	2

For a complete list of breakpoints, consult the EUCAST breakpoint table [3]: http://www.eucast.org/clinical_breakpoints.

The latest breakpoint recommendations can be found on the EUCAST website: http://www.eucast.org.

Protocol 6: Multiplex PCR for detection of *mcr* genes

This protocol is adapted from Rebelo et al. [7]. There are several additional *mcr* genes identified to date and references to PCR protocols for these genes have been included in Table 12.

Chemicals/biologicals/consumables

- PCR tubes
- primer sequences (Table 11)
- DreamTaq Green PCR Master Mix (2X)
- crude DNA extracts

Equipment

- PCR machine

Control strains for QC

Control strains must always be included:

- negative control – PCR mixture without addition of DNA
- positive control – well characterised strains positive for the tested resistance genes

Commercial control strains can be obtained from NCTC (Table 8 for recommended *mcr*-1 strain).

Protocol

- Use a DNA extraction method of choice that is validated for *E. coli* and *K. pneumoniae*. For DNA lysates according to Rebelo et al. [7]; suspend a loopful of culture in 100 µl of sterile TE buffer, boil for 10 minutes at 100º C and centrifuge for 5 minutes at 6 000 G. Dilute the DNA supernatant 1:10 in TrisHCl buffer.
- PCR mixtures are displayed in Table 9, PCR conditions in Table 10 and specific primer sequences and conditions in Table 11.
- Separate and visualise the PCR products with any suitable method. Consult Table 11 for expected product sizes.

Table 8. Reference strain

Reference isolate	Genotype
E. coli NCTC 13846	*mcr-1*

For more details regarding this control strain and others, consult the National Collection of Type Cultures website: http://www.phe-culturecollections.org.uk/products/bacteria.

Table 9. *mcr* PCR mix

Stock solutions	Quantity	Final concentration
DNA template	2 µl	~100 ng
Dream Taq Green PCR Master Mix (2X)	12.5 µl	1x
Each primer listed in Table 11*	0.5 µl	200 nM
H_2O	5.5 µl	

** Concentration of primer stock solutions should be 10 µM.*

Table 10. *mcr* PCR programme

Cycle step	Temperature (°C)	Time	Number of cycles
Initial denaturation	94	15 minutes	1
Denaturation	94	30 seconds	25
Annealing	58	90 seconds	
Extension	72	1 minute	
Hold	72	10 minutes	1

Table 11 Primer sequences for *mcr* gene detection

Target gene	Genbank	Primers and conditions	Melting temperature - Tm (°C)	Product size (bp)	Reference
mcr-1 (40-359)	KP347127	mcr1_F 5'-AGTCCGTTTGTTCTTGTGGC-3' mcr1_R 5'-AGATCCTTGGTCTCGGCTTG-3'	58	320	[7]
mcr-2 (401-1115)	LT598652	mcr2_F 5'-CAAGTGTGTTGGTCGCAGTT-3' mcr2_R 5'-TCTAGCCCGACAAGCATACC-3'	58	715	[7]
mcr-3 (17-945)	KY924928	mcr3_F5'-AAATAAAAATTGTTCCGCTTATG-3' mcr3_R 5'-AATGGAGATCCCCGTTTTT-3'	58	929	[7]
mcr-4 (38-1153)	MF543359	mcr4_F 5'-TCACTTTCATCACTGCGTTG-3' mcr4_R 5'-TTGGTCCATGACTACCAATG-3'	58	1116	[7]
mcr-5 (1-1644)	KY807921	mcr5_F 5'-ATGCGGTTGTCTGCATTTATC-3' mcr5_R 5'-TCATTGTGGTTGTCCTTTTCTG-3'	58	1644	[11]

Table 12. Additional *mcr* genes not included in protocol

Target gene	Genbank	Primers and conditions	Annealing temperature (°C)	Product size (bp)	Reference
mcr-6 (1617 bp)	NG_055781.1	MCR-6F 5'-GTCCGGTCAATCCCTATCTGT-3' MCR-6R 5'-ATCACGGGATTGACATAGCTAC-3'	55	556	[12]
mcr-7 (1820 bp)	NG_056413	MCR-7F 5'-TGCTCAAGCCCTTCTTTTCGT-3' MCR-7R 5'-TTCATCTGCGCCACCTCGT -3'	55	892	[13]
mcr-8 (1898 bp)	MG736312	MCR-8F 5'-AACCGCCAGAGCACAGAATT-3' MCR-8R 5'-TTCCCCCAGCGATTCTCCAT-3'	60	667	[14]

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