EURL-PH-AMR

EU Reference Laboratory for Public Health on Antimicrobial Resistance in Bacteria

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Document overview

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Deliverable name	Harmonised protocols on phenotypic testing methods for carbapenem and/or colistin resistant Enterobacterales (CCRE) for the members of EURGen-Net
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Purpose

The purpose of this document is to describe the phenotypic testing for the detection of carbapenem-resistance in Enterobacterales at National Reference Laboratories already participating in EURGen-Net. The aim is to harmonise inclusion and reporting of carbapenem resistant isolates for which to initiate European-level genomic surveillance for ECDC standardised surveys (such as the CRE25 survey) and ECDC outbreak investigations. All recommendations are compliant with relevant parts of EUCAST technical guidance for AST. The document is not intended to replace extant general EUCAST guidance (https://www.eucast.org/ast_of_bacteria).

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

For more detailed information, consult the EUCAST Disk Diffusion Manual regularly updated and available at 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 over dry 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 1-2x10^8 colony forming units (CFU)/mL for *Escherichia coli* and *Klebsiella pneumoniae*. 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 plates from the back with reflected light and the plate held above a dark background. Do not use transmitted light (plate held up to light) or a magnifying glass. Measure the diameters of zones of inhibition to the nearest millimetre with a ruler or callipers. Interpret zone diameters by reference to breakpoint tables: http://www.eucast.org/clinical_breakpoints (summarised in table 1).

When carbapenem-resistance is detected by phenotypic methods, report as tested and if appropriate characterize further using sequencing or other available tests (e.g. lateral flow tests).

Protocol 2: EUCAST – broth microdilution for carbapenem susceptibility testing

For more information, consult the EUCAST recommendation for broth microdilution and media preparation at https://www.eucast.org/ast_of_bacteria/mic_determination.

Minimum inhibitory concentration (MIC) determination (broth microdilution according to ISO standard 20776-1)
Medium: Cation-adjusted Mueller-Hinton broth.
Inoculum: $5x10^5$ 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: *E. coli* ATCC 25922.

Detection of carbapenem resistance is in the CRE25-survey mainly performed using EUCAST clinical breakpoints to enable participation of all countries. In agreement with ECDC, laboratories may optionally use both clinical breakpoints and screening cutoff values, in which case this should be clearly stated when reporting results. Detailed instructions in this regard will be provided in the CRE25 survey protocol. Table 1 lists EUCAST carbapenem clinical breakpoints and meropenem screening cut-off values for Enterobacterales. A detection system based on clinical breakpoints will be less sensitive than detection based on the meropenem screening cutoff value.

Table 1. **EUCAST breakpoints for Enterobacterales and carbapenems (v 15.0, 2025).**

Carbapenems	Clinical breakpoint MIC (mg/L)		Clinical breakpoint zone diameter (mm)	Meropenem screening cutoff values	
	S ≤	R >	S ≥ / R <	Meropenem MIC (mg/L), screen positive	Meropenem disk diffusion (10 μg), screen positive
Ertapenem	0.5	0.5	23 / 23	>0.125 mg/L	<28 mm
Imipenem	2	4	22 / 19		
Meropenem	2	8	22 / 16		

For a complete and yearly updated list of breakpoints and screening recommendations, consult the EUCAST breakpoint table at http://www.eucast.org/clinical_breakpoints. For an update of MIC and zone diameter ECOFFs, consult the MIC and zone distribution programme at https://mic.eucast.org.

Protocol 3: EUCAST – colistin broth microdilution testing

For more information, consult the EUCAST recommendation for broth microdilution and media preparation at https://www.eucast.org/ast_of_bacteria/mic_determination and the specific document related to colistin testing (Recommendations_for_MIC_determination_of_colistin_March_2016.pdf).

EUCAST recommendation

Reference testing of Enterobacterales follows the ISO-standard broth microdilution method (20776-1).

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: Cation-adjusted 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 (https://www.eucast.org/ast_of_bacteria/quality_control).

Table 2. **EUCAST MIC breakpoints for Enterobacterales and colistin (v 15.0, 2025)**

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

For a complete and yearly updated list of breakpoints, consult the EUCAST breakpoint table: http://www.eucast.org/clinical_breakpoints.

EUCAST clinical colistin breakpoints are identical to the respective ECOFFs (*E. coli* and *K. pneumoniae* both 2 mg/L). The sensitivity for detection of colistin resistance is therefore absolute. Provided the phenotypic test method is properly controlled (see https://www.eucast.org/ast_of_bacteria) it is not necessary to confirm the phenotypic result by genome sequencing. However, the latter may be deemed necessary for epidemiologic reasons.

For other colistin phenotypic test methods, consult the EUCAST Warning No. 3 (https://www.eucast.org/ast-of-bacteria/warnings) which in summary excludes gradient test and disk diffusion as valid methods.

General aspects on alternative testing and therapy for carbapenem resistant, often multidrug-resistant, *Escherichia coli* and *Klebsiella pneumoniae*

E. coli and *K. pneumoniae* resistant to carbapenems are often resistant also to many other agents. The efficiency of colistin has increasingly been questioned, also when the organism is formally susceptible, and nowadays the drug is only recommended as part of combination therapy. Nevertheless, in some settings, colistin remains the only available agent.

In recent years, several new alternative beta-lactam antibiotics have been introduced to the market, including ceftazidime-avibactam, ceftolozane-tazobactam, cefepime-enmetazobactam, cefiderocol, aztreonam-avibactam, imipenem-relebactam and meropenem-vaborbactam. Many of these agents are potentially useful for treatment of otherwise multi-resistant Gram-negative bacteria and more agents are under development. These agents have different activity profiles, with varying strengths and limitations in relation to the relevant resistance mechanism(s).

EUCAST has issued testing recommendations for all of these agents and the EURL-PH-AMR, via the EUCAST Development Laboratory, can offer help in the form of advice (at eurl@kronoberg.se) on susceptibility testing when needed.