EUCAST EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING

European Society of Clinical Microbiology and Infectious Diseases

EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance

Version 2.0[1]

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[1] **Based on version 1.0 from December 2013 by the EUCAST subcommittee for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance.** Authors of the original version are acknowledged: Christian G. Giske (Sweden, EUCAST and EARS-Net Coordination Group; chairman), Luis Martinez-Martinez (Spain), Rafael Cantón (Spain, EUCAST), Stefania Stefani (Italy), Robert Skov (Germany), Youri Glupczynski (Belgium), Patrice Nordmann (France), Mandy Wootton (UK), Vivi Miriagou (Greece), Gunnar Skov Simonsen (Norway, EARS-Net Coordination Group), Helena Zemlickova (Czech Republic, EARS-Net Coordination Group), James Cohen-Stuart (The Netherlands), and Marek Gniadkowski (Poland).

Contents

1. Introduction

The current document is an update of the guidelines developed by the EUCAST subcommittee on detection of resistance mechanisms. The EUCAST Steering Committee has carried out the current update. The document has been developed mainly for routine use in clinical laboratories and does not cover technical procedures for identification of resistance mechanisms at a molecular level by reference or expert laboratories. However, much of the content is also applicable to national reference laboratories. Furthermore, it is important to note that the document does not cover screening for asymptomatic carriage (colonization) of multidrug-resistant microorganisms or direct detection of resistance in clinical samples.

All chapters in this document contain a definition of the mechanism or specific resistance, an explanation of the clinical and/or public health need for detection of the mechanism or specific resistance, an outline description of recommended methods of detection, and references to detailed descriptions of the methods. The need for identification of resistance mechanism and the level of identification needed for public health or infection control purposes may vary both geographically and temporally depending on the prevalence and heterogeneity of different resistance mechanisms. The guidelines have been developed by conducting literature searches, and recommendations are based on multi-center studies or more than one single center study. Several methods currently under development have not been included in the guidelines as multi-center evaluations or multiple single center evaluations are yet to be completed. Draft versions of these guidelines were subject to wide consultation through EUCAST consultation contact lists, the EUCAST website and ECDC focal point contacts.

We have as far as possible used generic terms for the products presented in the document, but excluding all specific product names would have made some of the recommendations unclear. It should be noted that some resistance mechanisms do not always confer clinical resistance. This could be due to mechanisms not being expressed or expressed only at a low-level, which will not give rise to phenotypic resistance. Hence, while detection of these mechanisms may be relevant for infection control and public health, it may not be necessary for clinical purposes. Consequently, for some mechanisms, particularly extended-spectrum β-lactamases and carbapenemases in Gram-negative bacilli, detection of the mechanism does not in itself lead to classification as clinically resistant.

Christian G. Giske

Chairman of EUCAST, Past Chairman of the Subcommittee on detection of resistance mechanisms

2. Carbapenemase-producing Enterobacteriaceae

Importance of detection of resistance mechanism	
Required for clinical antimicrobial susceptibility categorization	No
Infection control purposes	Yes
Public health purposes	Yes

2.1 Definition

Carbapenemases are β-lactamases that hydrolyze penicillins, in most cases cephalosporins, and to various degrees carbapenems and monobactams (the latter are not hydrolyzed by metallo-β-lactamases).

2.2 Clinical and/or epidemiological importance

The problem of dissemination of carbapenemases in Europe dates from the second half of the 1990s in several Mediterranean countries, and was observed mainly in *Pseudomonas aeruginosa* (1). In the early 2000s, Greece experienced an epidemic of the Verona integron-encoded metallo-β-lactamase (VIM) among *Klebsiella pneumoniae* (2) followed by an epidemic related to the *K. pneumoniae* carbapenemase (KPC) (1). At present, the OXA-48 carbapenemases comprise the fastest growing group of carbapenemases in Europe (3). In Greece and Italy around 62 and 33%, respectively, of invasive *K. pneumoniae* are now non-susceptible to carbapenems (4). In 2015, 13/38 countries reported inter-regional spread of or an endemic situation for carbapenemase-producing Enterobacteriaceae (CPE), compared with 6/38 in 2013. Only three countries replied that they had not identified one single case of CPE (3). Other particularly problematic carbapenemases are the New Delhi metallo-β-lactamases (NDMs), which are highly prevalent on the Indian subcontinent and in the Middle East and have on several occasions been imported to Europe (3), and in there are also examples of regional dissemination in some countries (5). The IMP-carbapenemases are also common in some parts of the world (6).

Carbapenemases are a source of concern because they may confer resistance to virtually all β-lactams, and are readily transferable. Further, carbapenemase-producing strains frequently possess resistance mechanisms to a wide-range of antimicrobial agents, and infections with carbapenemase-producing Enterobacteriaceae are associated with high mortality rates (7-9).

2.3 Mechanisms of resistance

The vast majority of carbapenemases are acquired enzymes, encoded by genes on transposable elements located on plasmids. Carbapenemases are expressed at various levels and differ significantly in both biochemical characteristics and activity against specific β-lactams. The level of expression and properties of the β-lactamase and the frequent association with other resistance mechanisms (other β-lactamases, efflux and/or altered permeability) result in the wide range of resistance phenotypes observed among carbapenemase-producing isolates (10,11). Decreased susceptibility to carbapenems in Enterobacteriaceae may, however, also be caused by either extended spectrum β-lactamases (ESBL) or AmpC enzymes combined with decreased permeability due to alteration or down-regulation of porins (12), and possibly also penicillin-binding proteins (13).

Carbapenemase-producing Enterobacteriaceae (CPE) usually have decreased susceptibility to carbapenems, and in most cases are resistant to extended-spectrum (oxyimino) cephalosporins (i.e. cefotaxime, ceftriaxone, ceftazidime and/or cefepime) (14). However, with some enzymes (e.g. OXA-48-like) the organisms may be fully susceptible to cephalosporins. Currently, the majority of the CPE isolates also co-express cephalosporin-hydrolyzing enzymes, such as CTX-M-type ESBLs, and are then also resistant to cephalosporins. Carbapenemases are considered to be of high epidemiological importance, particularly when they confer decreased susceptibility to carbapenems (imipenem, meropenem, ertapenem and doripenem), i.e. when the MICs are above the epidemiological cut-off (ECOFF) values defined by EUCAST (15).

2.4 Recommended methods for detection of carbapenemases in Enterobacteriaceae

2.4.1 Screening for carbapenemase-production

Carbapenem MICs for carbapenemase-producing Enterobacteriaceae may be below the clinical breakpoints (14-16). However, the ECOFF values as defined by EUCAST can be used to detect carbapenemase-producers. Meropenem offers the best compromise between sensitivity and specificity in terms of detecting carbapenemase-producers (14, 17). Ertapenem is the most sensitive carbapenem, but has low specificity, especially in species such as *Enterobacter* spp., due to its relatively scarce instability to extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases in combination with porin loss (14). Appropriate cut-off values for detecting putative carbapenemase-producers are shown in Table 1. It should be noted that in order to increase specificity, imipenem and ertapenem screening cut-off values are one-dilution step higher than the currently defined ECOFFs.

Table 1. Clinical breakpoints and screening cut-off values for carbapenemase-producing Enterobacteriaceae (according to EUCAST methodology).

Carbapenem	MIC (mg/L)		Disk diffusion zone diameter (mm) with 10 µg disks	
	S/I breakpoint	Screening cut-off	S/I breakpoint	Screening cut-off
Meropenem[1]	≤2	>0.125	≥22	<28[2]
Ertapenem[3]	≤0.5	>0.125	≥25	<25

[1]Best balance of sensitivity and specificity

[2]Isolates with 25-27 mm only need to be investigated for carbapenemase-production if they are resistant to piperacillin-tazobactam and/or temocillin (temocillin contributes more to the specificity). Investigation for carbapenemases is always warranted if zone diameter of meropenem is <25 mm.

[3]High sensitivity but low specificity. Can be used as an alternative screening agent, but isolates with ESBL and AmpC may be resistant without having carbapenemases.

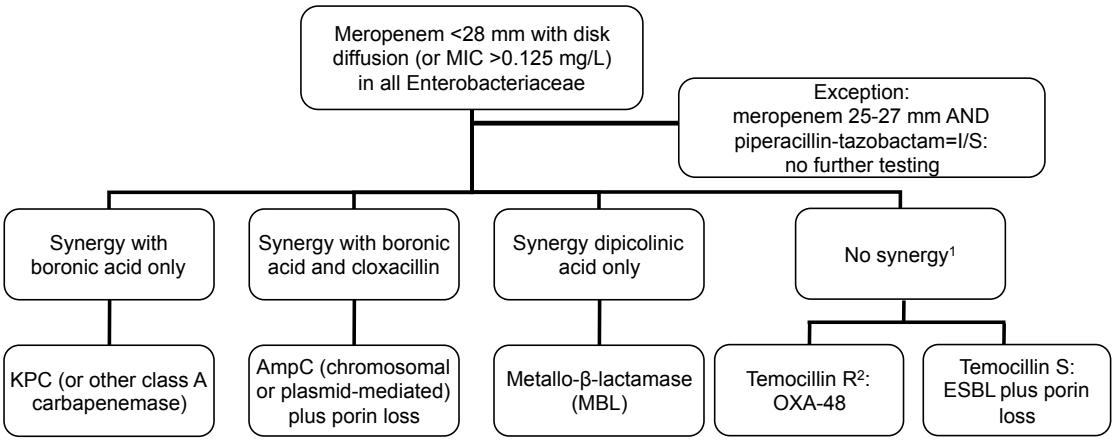
Following detection of reduced susceptibility to carbapenems in routine susceptibility tests, phenotypic methods for detection of carbapenemases should be applied. The main categories of methods are the combination disk test methods, colorimetric assays based on hydrolysis of

carbapenems, other methods detecting carbapenem hydrolysis, and finally lateral flow assays. The various tests are described below.

2.4.2 Combination disk testing

The combination disk test method is commercially available from several manufacturers, and was the first phenotypic test to become available (MAST, UK; Rosco, Denmark) (18-20). The disks or tablets contain meropenem ± various inhibitors. In brief, boronic acid inhibits class A carbapenemases (although data beyond KPC are scarce), and dipicolinic acid and ethylenediaminetetraacetic acid (EDTA) inhibit class B carbapenemases. Moreover, OXA-48 is inhibited by avibactam, which has nevertheless so far not been included in the phenotypic panels (21, 22). Cloxacillin, which inhibits AmpC β-lactamases, has been added to the tests to differentiate between AmpC hyperproduction plus porin loss and carbapenemase-production. The algorithm for interpretation of these inhibitor tests is outlined in Figure 1 and Table 2. The main disadvantage with these methods is that they take 18 hours (in practice overnight incubation), for which reason novel rapid methods have been introduced.

Figure 1. Algorithm for carbapenemase detection.



[1]Combination of several carbapenemases can also contribute to no synergy – e.g. MBL and KPC in combination. Molecular testing is usually necessary in such cases

[2]High-level temocillin resistance (>128 mg/L, zone diameter <11 mm) is a phenotypic marker of OXA-48

The algorithm in Table 2 differentiates between metallo-β-lactamases, class A carbapenemases, class D carbapenemases and non-carbapenemases (ESBL and/or AmpC plus porin loss). The tests can be done with the EUCAST disk diffusion method for non-fastidious organisms. Commercial tests should be set up according to the manufacturer's instructions for each test.

At present there are no available inhibitors for OXA-48-like enzymes. Temocillin high-level resistance (MIC >128 mg/L) has been proposed as a phenotypic marker for putative OXA-48-like carbapenemase producers (23, 24). However, this marker is not specific for OXA-48-type carbapenemases as other resistance mechanisms might confer this phenotype. The presence of OXA-48-like enzymes therefore has to be confirmed with other methods.

Use of the modified cloverleaf (Hodge) test is not currently recommended as results are difficult to interpret, the specificity is poor, and in some cases the sensitivity is also suboptimal (12). Some

novel modifications of the technique have been described, but they are cumbersome for use in routine clinical laboratories and do not solve all problems of sensitivity and specificity.

Table 2. Interpretation of phenotypic tests (carbapenemases in **bold type**) by diffusion methods with disks or tablets. The exact definitions of synergy are provided in package inserts for the various commercial products.

B-lactamase	Synergy observed as increase in zone diameter (mm) with 10 µg meropenem disk/tablet				Temocillin MIC >128 mg/L or zone diameter <11 mm
	DPA/EDTA	APBA/PBA	DPA+APBA	CLX	
MBL	+	-	-	-	Variable[1]
KPC	-	+	-	-	Variable[1]
MBL + KPC[2]	Variable	Variable	+	-	Variable[1]
OXA-48-like	-	-	-	-	Yes
AmpC + porin loss	-	+	-	+	Variable[1]
ESBL + porin loss	-	-	-	-	No

Abbreviations: MBL=metallo-β-lactamase, KPC=*Klebsiella pneumoniae* carbapenemase, DPA=dipicolinic acid, EDTA=ethylenediaminetetraacetic acid, APBA= aminophenyl boronic acid, PBA= phenyl boronic acid, CLX=cloxacillin.

[1] Temocillin susceptibility test is recommended only in cases where no synergy is detected, in order to differentiate between ESBL + porin loss and OXA-48-like enzymes (23, 24). When other enzymes are present the susceptibility is variable and does not provide any further indication of the β-lactamase present.

[2] There is one report supporting the use of commercial tablets containing double inhibitors (DPA or EDTA plus APBA or PBA) (25), but multi-centre studies or multiple single centre studies are lacking. This combination confers high-level resistance to carbapenems and is rare outside Greece.

2.4.3 Biochemical (colorimetric) tests

The CarbaNP test is a rapid (<2 h) test for detection of carbapenem hydrolysis, which will give rise to a pH-change resulting in a colour shift from red to yellow with phenol red solution (26, 27). The Carba NP test has been validated with bacterial colonies grown on Mueller-Hinton agar plates, blood agar plates, trypticase soy agar plates, and most selective media used in screening for carbapenemase producers. The Carba NP test should not be performed with bacterial colonies grown on Drigalski or McConkey agar plates. The different steps in the method must be closely followed in order to obtain reproducible results. Several publications indicate a high sensitivity and specificity of the method (28), whereas one publication observed problems of sensitivity for isolates with a mucoid phenotype and for some Enterobacteriaceae producing OXA-48 (29). One commercial variant of the method has been shown to perform well for detection of carbapenemases in Enterobacteriacae (30, 31). With certain commercial assays, including the commercial version of the CarbaNP test, there are some interpretation issues based on visual reading of the colour shift with doubtful results and a certain proportion (3-5%) of non-interpretable results.

A derivative of the CarbaNP-test, the Blue-Carba test (BCT) is a biochemical test for rapid (<2 h) detection of carbapenemase production (32, 33). It is based on the in vitro hydrolysis of imipenem by bacterial colonies (direct inoculation without prior lysis), which is detected by changes in pH values revealed by the indicator bromothymol blue (blue to green/yellow or green to yellow). In a large evaluation carried out by Pasteran et al. (34), but conducted in only a single laboratory, the test was found to have excellent sensitivity for class A and B enzymes, but suboptimal sensitivity for detection of OXA-48 enzymes.

A third biochemical test is the β CARBA test™, which also can be carried out in <2h. The test is conducted by mixing 1 to 3 colonies in the reagents. Readings should be done after a maximum of 30 min of incubation. Change of color from yellow to orange, red or purple indicated a positive reaction. One study found that the 0.5-hour incubation time recommended by the manufacturer is too short for OXA-48-producing strains. The evaluated strain collection was quite limited, and a larger trial was suggested to investigate how the test compares to other biochemical tests (35). In another evaluation, the β Carba test™ showed excellent performance to detect CPE, and especially OXA-48. However, the ability to detect other class A carbapenemases should be further verified and some false-positive results occurred with other β-lactamases such as overproduction of K1 β-lactamase in *Klebsiella oxytoca* (33).

2.4.4 Carbapenem Inactivation Method

The principle of this method is to detect enzymatic hydrolysis by incubating a carbapenem with a bacterial suspension. The CIM-test utilizes antibiotic susceptibility-testing disks as substrate aliquots. Following two hours of incubation of a full loop of bacteria with a meropenem disk, the disk is placed on an agar inoculated with *Escherichia coli* ATCC 25922. Enzymatic inactivation will produce no zone, whereas no carbapenemase activity will imply there will be a zone, as meropenem in the disk has not been hydrolyzed. The CIM-test has had variable performance in different studies (36-38), but remains a possible alternative, although the negative predictive value of the test is still not clear. One main disadvantage of this technique is that it requires usually at least 18 hours to obtain the results.

2.4.5 Detection of carbapenem hydrolysis with MALDI-TOF

The principle is to detect in a mass spectrometry device (MALDI TOF) the decrease or disappearance of certain specific peaks of carbapenems in a mass spectra when a bacterial suspension is previously incubated with the carbapenem (39, 40). Spectra are measured after drying between m/z 160 and 600, using a Microflex LT mass spectrometer (39). The method has been found in several studies to have good sensitivity and specificity, but except for OXA-48-group enzymes. To amend this problem, NH_4HCO_3 can be added to the reaction, and this modification was shown in one study to improve detection of OXA-48 (41). However, the new method has thus far not been evaluated in a multicenter study design or in multiple single center studies. Another slightly impractical feature is that the settings for the MALDI-TOF need to be altered as compared to what is used for species determination (41).

Lateral flow assays

A new immunochromatographic lateral flow assay was recently described. The test is based on immunological capture of epitopes of OXA-48, using colloidal gold nanoparticles bound to a nitrocellulose membrane within a lateral flow device. The principle of the test is that monoclonal anti-OXA-48 antibodies are selected as specific capture reagents, for direct identification OXA-48-like enzymes (42). The assay takes around four minutes, and has been evaluated both from colonies and from spiked blood culture bottles (43-46). Recently, a similar test for KPC has also

been developed, but its robustness has not been evaluated by multiple single center studies or one multicenter study (46).

2.4.6 Control strains

Below some possible control strains for phenotypic and genotypic experiments have been listed for convenience. Quality control ranges are not available for these strains. Users of commercial methods should study the package insert for information on which control strains to use.

Table 3. Examples of control strains for carbapenemase testing.

Strain	Mechanism
Enterobacter cloacae CCUG 59627	AmpC combined with decreased porin expression
K. pneumoniae CCUG 58547 or *K. pneumoniae* NCTC 13440	Metallo-β-lactamase (VIM)
K. pneumoniae NCTC 13443	Metallo-β-lactamase (NDM-1)
E. coli NCTC 13476	Metallo-β-lactamase (IMP)
K. pneumoniae CCUG 56233 or *K. pneumoniae* NCTC 13438	*Klebsiella pneumoniae* carbapenemase (KPC)
K. pneumoniae NCTC 13442	OXA-48 carbapenemase
K. pneumoniae ATCC 25955	Negative control

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3. Extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae

Importance of detection of resistance mechanism	
Required for clinical antimicrobial susceptibility categorization	No
Infection control purposes	Yes
Public health purposes	Yes

3.1 Definition

ESBLs are enzymes that hydrolyze most penicillins and cephalosporins, including oxyimino-β-lactam compounds (cefuroxime, third- and fourth-generation cephalosporins and aztreonam) but neither cephamycins nor carbapenems. Most ESBLs belong to the Ambler class A of β-lactamases and are inhibited by β-lactamase inhibitors (clavulanic acid, sulbactam and tazobactam) and by diazabicyclooctanones (avibactam) (1).

3.2 Clinical and/or epidemiological importance

The first ESBL-producing strains were identified in 1983, and since then have been observed worldwide. This distribution is a result of the clonal expansion of producer organisms, the horizontal transfer of ESBL genes on plasmids and, less commonly, their emergence *de novo*. By far the most clinically important groups of ESBLs are CTX-M enzymes, emerging since the early 2000s, followed by SHV- and TEM-derived ESBLs (2-5).

ESBL production has been observed mostly in Enterobacteriaceae, first in hospital environments, later in nursing homes, and since around 2000 in the community (outpatients, healthy carriers, sick and healthy animals, food products). The most frequently encountered ESBL-producing species are *E. coli* and *K. pneumoniae.* However, all other clinically relevant Enterobacteriaceae species are also common ESBL-producers. The prevalence of ESBL-positive isolates depends on a range of factors including species, geographic locality, hospital/ward, group of patients and type of infection, and large variations have been reported in different studies (2,3,6,7). The EARS-Net data for 2015 showed that the rate of invasive *K. pneumoniae* isolates non-susceptible to the third-generation cephalosporins exceeded 25% or even 50% in many European countries. Except for Greece and Italy with high proportion of KPC-type carbapenemase-producing isolates, most of these isolates were presumed to be ESBL-producers based on local ESBL test results (8).

3.3 Mechanisms of resistance

The vast majority of ESBLs are acquired enzymes, encoded by genes on plasmids. The acquired ESBLs are expressed at various levels, and differ significantly in biochemical characteristics such as activity against specific β-lactams (*e.g.* cefotaxime, ceftazidime, aztreonam). The level of expression and properties of an enzyme, and the co-presence of other resistance mechanisms (other β-lactamases, active efflux, altered permeability) result in the large variety of resistance phenotypes observed among ESBL-positive isolates (1-4, 9-11).

3.4 Recommended methods for detection of ESBLs in Enterobacteriaceae

In many areas, ESBL detection and characterization is recommended or mandatory for infection control purposes. The recommended strategy for the detection of ESBLs in Enterobacteriaceae is based on non-susceptibility to indicator oxyimino-cephalosporins, followed by phenotypic (and in some cases genotypic) confirmation tests (Table 1, Figure 1).

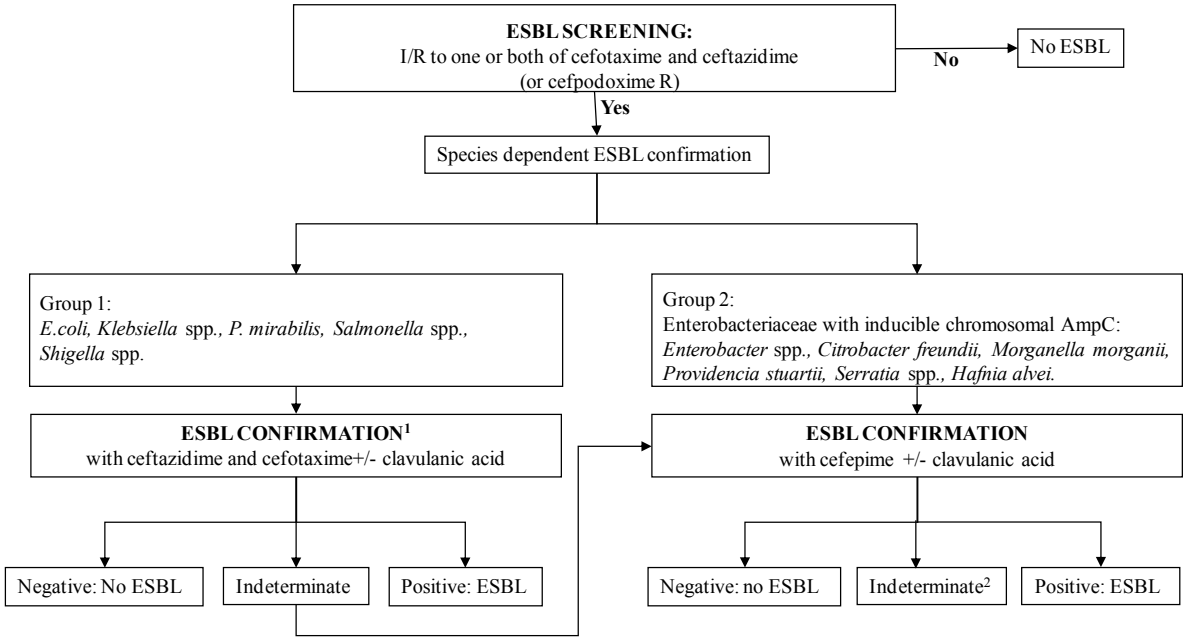
A screening breakpoint of >1mg/L is recommended for cefotaxime, ceftriaxone, ceftazidime, and cefpodoxime, in accordance with the guidelines issued by EUCAST and CLSI (Table 1) (12, 13). The EUCAST clinical breakpoint for Enterobacteriaceae is also Susceptible ≤1 mg/L (12). Cefpodoxime is the most sensitive individual indicator cephalosporin for detection of ESBL production and may be used for screening. However, it is less specific than the combination of cefotaxime (or ceftriaxone) and ceftazidime (14, 15) and only the latter compounds are used in the confirmation testing. Corresponding zone diameters for the indicator cephalosporins are shown in Table 1.

Table 1. ESBL screening methods for Enterobacteriaceae (13-19).

Method	**Antibiotic**	**Conduct ESBL-testing if**
Broth or agar dilution[1]	Cefotaxime/ceftriaxone AND Ceftazidime	MIC >1 mg/L for either agent
	Cefpodoxime	MIC >1 mg/L
Disk diffusion[1]	Cefotaxime (5 µg) or Ceftriaxone (30 µg) AND Ceftazidime (10 µg)	Inhibition zone <21 mm Inhibition zone <23 mm Inhibition zone <22 mm
	Cefpdoxime (10 µg)	Inhibition zone <21 mm

[1] With all methods test either (i) cefotaxime or ceftriaxone AND ceftazidime OR (ii) cefpodoxime alone.

Figure 1. Algorithm for phenotypic detection of ESBLs



[1] If cefoxitin has been tested and has an MIC >8 mg/L, perform cefepime+/- clavulanic acid confirmation test

[2] Cannot be determined as either positive or negative (e.g. if a gradient diffusion strip cannot be read due to growth beyond the MIC range of the strip or there is no clear synergy in combination-disk and double-disk synergy tests). In confirmation with cefepime +/- clavulanic acid is still indeterminate, genotypic testing is required.

3.4.1 ESBL-screening in Enterobacteriaceae

A. Screening in group 1 Enterobacteriaceae (*E. coli*, *Klebsiella* spp., *Raoultella* spp., *P mirabilis*, *Salmonella* spp., *Shigella* spp.)

The recommended methods for ESBL screening in group 1 Enterobacteriaceae are broth dilution, agar dilution, disk diffusion or an automated system (13, 20, 21). It is required that both cefotaxime (or ceftriaxone) and ceftazidime are used as indicator cephalosporins, as there may be large differences in MICs of cefotaxime (or ceftriaxone) and ceftazidime for different ESBL-producing isolates (14, 22, 23).

The algorithm for screening and phenotypic ESBL confirmation methods for group 1 Enterobacteriaceae that are positive in screening tests are described in Figure 1 and Table 2.

B. Screening in group 2 Enterobacteriaceae (*Enterobacter* spp, *Serratia* spp., *Citrobacter freundii*, *Morganella morganii*, *Providencia* spp, *Hafnia alvei*)

For group 2 Enterobacteriaceae it is recommended that ESBL screening is performed according to the methods described above for group 1 *Enterobacteriaceae* (Figure 1 and Table 3) (19). However, a very common mechanism of cephalosporin resistance is derepressed chromosomal AmpC β-lactamase in these species. Since cefepime is stable to AmpC hydrolysis, it can be used in phenotypic testing with clavulanic acid.

3.4.2 Phenotypic confirmation methods

Four of the several phenotypic methods based on the *in vitro* inhibition of ESBL activity by clavulanic acid are recommended for ESBL confirmation: the combination disk test (CDT), the double-disk synergy test (DDST), the ESBL gradient test, and the broth microdilution test (Tables 2 and 3) (20, 21, 24). In one multi-centre study, the CDT showed a better specificity than the ESBL gradient test but with comparable sensitivity (25). Manufacturers of automated susceptibility

testing systems have implemented detection tests based on the inhibition of ESBL enzymes by clavulanic acid. Performance of confirmation methods differs in different studies, depending on the collection of strains tested and the device used (17-19).

A. Combination disk test (CDT)

For each test, disks or tablets containing the cephalosporin alone (cefotaxime, ceftazidime, cefepime) and in combination with clavulanic acid are applied. The inhibition zone around the cephalosporin disk or tablet combined with clavulanic acid is compared with the zone around the disk or tablet with the cephalosporin alone. The test is positive if the inhibition zone diameter is ≥5 mm larger with clavulanic acid than without (Table 3) (26, 27).

B. Double-disk synergy test (DDST)

Disks containing cephalosporins (cefotaxime, ceftazidime, cefepime) are applied to plates next to a disk with clavulanic acid (amoxicillin-clavulanic acid). A positive result is indicated when the inhibition zones around any of the cephalosporin disks are augmented or there is a 'keyhole' in the direction of the disk containing clavulanic acid. The distance between the disks is critical and 20mm centre-to-centre has been found to be optimal for cephalosporin 30 µg disks; however it may be reduced (15 mm) or expanded (30 mm) for strains with very high or low levels of resistance, respectively (20). The recommendations need to be re-evaluated for disks with lower cephalosporin content, as used in the EUCAST disk diffusion method.

C. Gradient test method

ESNL-specific gradient tests are set up, read and interpreted according to the manufacturer's instructions. The test is positive if ≥ 8-fold reduction is observed in the MIC of the cephalosporin combined with clavulanic acid compared with the MIC of the cephalosporin alone or if a phantom zone or deformed ellipse is present (see instructions from the manufacturer for illustrations) (Table 3). The test result is indeterminate if the strip cannot be read due to growth beyond the MIC range of the strip. In all other cases the test result is negative. According to the manufacturer, ESBL gradient test should be used for confirmation of ESBL production only and is not reliable for determination of the MIC.

D. Broth microdilution

Broth microdilution is performed with cation-adjusted Mueller-Hinton broth containing serial two-fold dilutions of cefotaxime, ceftazidime and cefepime at concentrations ranging from 0.25 to 512 mg/L, with and without clavulanic acid at a fixed concentration of 4 mg/L. The test is positive if ≥ 8-fold reduction is observed in the MIC of any of the cephalosporins combined with clavulanic acid compared with the MIC of that cephalosporin alone, otherwise the test result is interpreted as negative (24).

E. Biochemical (colorimetric) testing

The ESBL NDP test was described first in 2012, and uses cefotaxime as indicator antimicrobial, with tazobactam as inhibitor (28). It is carried out in 96-well plates or in separate tubes. Colour change from red to yellow is regarded a positive test. The test has also been used directly on patient samples (29). Excellent sensitivities and specificities have been described, but the test has not been evaluated in any multicenter trial.

The β-LACTA test is a colorimetric test using a chromogenic cephalosporin substrate (HMRZ-86) on isolates and also directly on clinical samples (30). In a prospective multicentric study in Belgium and France, it was found to display an excellent sensitivity and specificity for *E. coli* and

K. pneumoniae (96% and 100%, respectively) while it showed a lower sensitivity (67%) for species producing inducible AmpC β-lactamases. The high negative predictive value for *E. coli* and *K. pneumoniae* (99% in areas with prevalence of C3G resistance ranging between 10-30%) makes this simple test very efficient for the prediction of resistance to third-generation cephalosporins, particularly in extended-spectrum β-lactamase-producing strains.

E. Special considerations in interpretation

ESBL confirmation tests that use cefotaxime as the indicator cephalosporin may be false-positive for *Klebsiella oxytoca* strains with hyperproduction of the chromosomal K1 (OXY-like) β-lactamases (31). A similar phenotype may also be encountered in *Proteus vulgaris, Proteus penneri, Citrobacter koseri* and *Kluyvera* spp. and in some *C. koseri*-related species like *C. sedlakii, C. farmeri* and *C. amalonaticus*, which have chromosomal β-lactamases that are inhibited by clavulanic acid (32, 33). Another possible cause of false-positive results is hyperproduction of SHV-1-, TEM-1- or OXA-1-like broad-spectrum β-lactamases combined with altered permeability (18). Similar problems with false-positive test results for K1-producing *K. oxytoca* or for OXA-1 producing *E. coli* may also arise when using confirmation tests based on cefepime only (34).

Table 2. ESBL confirmation methods for Enterobacteriaceae that are positive in the ESBL screening test (see Table 1). Group 1 Enterobacteriaceae (see Figure 1).

Method	Antimicrobial agent (disk content)	ESBL confirmation is positive if
ESBL gradient test	Cefotaxime +/- clavulanic acid	MIC ratio ≥8 or deformed ellipse present
	Ceftazidime +/- clavulanic acid	MIC ratio ≥8 or deformed ellipse present
Combination disk diffusion test (CDT)	Cefotaxime (30 µg) +/- clavulanic acid (10 µg)	≥5 mm increase in inhibition zone
	Ceftazidime (30 µg) +/- clavulanic acid (10 µg)	≥5 mm increase in inhibition zone
Broth microdilution	Cefotaxime +/- clavulanic acid (4 mg/L)	MIC ratio ≥8
	Ceftazidime +/- clavulanic acid (4 mg/L)	MIC ratio ≥8
	Cefepime +/- clavulanic acid (4 mg/L)	MIC ratio ≥8
Double disk synergy test (DDST)	Cefotaxime, ceftazidime and cefepime	Expansion of indicator cephalosporin inhibition zone towards amoxicillin-clavulanic acid disk

Table 3. ESBL confirmation methods for Enterobacteriaceae that are positive in the ESBL screening (see Table 1). Group 2 Enterobacteriaceae (see Figure 1).

Method	Antibiotic	Confirmation is positive if
ESBL gradient test Etest®® ESBL	Cefepime +/- clavulanic acid	MIC ratio ≥8 or deformed ellipse present
Combination disk diffusion test	Cefepime (30 µg) +/- clavulanic acid (10 µg)	≥5 mm increase in inhibition zone
Broth microdilution	Cefepime +/- clavulanic acid (fixed concentration 4 mg/L)	MIC ratio ≥8
Double disk synergy test (DDST)	Cefotaxime, ceftazidime, Cefepime	Expansion of indicator cephalosporin inhibition zone towards amoxicillin-clavulanic acid disk

3.4.3 Phenotypic detection of ESBL in the presence of other β-lactamases that mask synergy

Indeterminate test results (Etest®) and false-negative test results (CDT, DDST, Etest® and broth microdilution) may result from the high-level expression of AmpC β-lactamases, which mask the presence of ESBLs (20, 34, 35). Isolates with high-level expression of AmpC β-lactamases usually show clear resistance to third-generation cephalosporins. In addition, resistance to cephamycins, e.g. a cefoxitin MIC >8 mg/L, may be indicative of high-level expression of AmpC β-lactamases (34), with the rare exception of ACC β-lactamases, which do not confer cefoxitin resistance (36).

To confirm the presence of ESBLs in isolates with high-level expression of AmpC β-lactamases it is recommended that an additional ESBL confirmation test be performed with cefepime as the indicator cephalosporin, as cefepime is generally not hydrolyzed by AmpC β-lactamases. Cefepime may be used in all the CDT, DDST, gradient test or broth dilution test formats (27, 37-39). Alternative approaches include use of cloxacillin, which is a good inhibitor of AmpC enzymes. Test formats include CDT with disks containing the two cephalosporin indicators (cefotaxime and ceftazidime) with both clavulanic acid and cloxacillin together, and standard CDT or DDST on agar plates supplemented with 200-250 mg/L cloxacillin (19). There are also disks or tablets containing both clavulanic acid and cloxacillin on the market, but there is insufficient published validation of these products.

The presence of ESBLs may also be masked by carbapenemases such as MBLs or KPCs (but not OXA-48-like enzymes) and/or severe permeability defects (40, 41). If detection is still considered relevant in such cases, molecular methods should be used for ESBL detection.

3.4.4 Genotypic confirmation

For the genotypic confirmation of the presence of ESBL genes there are a number of possibilities available ranging from PCR and sequencing to whole-genome sequencing, followed by in silico mapping of resistance genes. There are also different microarrays available. Both commercial and in-house methods exist, but they have not been systematically examined and will therefore not be elaborated upon in this document. Whole-genome sequencing approaches have been described in another EUCAST publication (42).

3.4.5 Quality control

Below some possible control strains for phenotypic and genotypic experiments have been listed for convenience. Quality control ranges are not available for these strains. Users of commercial methods should study the package insert for information on which control strains to use.

Table 4. Examples of control strains for ESBL testing.

Strain	Mechanism
K. pneumoniae ATCC 700603	SHV-18 ESBL
E. coli CCUG62975	CTX-M-1 group ESBL and acquired CMY AmpC
E. coli ATCC 25922	ESBL-negative

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4. Acquired AmpC β-lactamase-producing Enterobacteriaceae

Importance of detection of resistance mechanism	
Required for clinical antimicrobial susceptibility categorization	No
Infection control purposes	Yes
Public health purposes	Yes

4.1 Definition

AmpC-type cephalosporinases are Ambler class C β-lactamases. They hydrolyze penicillins, cephalosporins (including the third-generation but generally not the fourth-generation compounds) and monobactams. In general, AmpC-type enzymes are poorly inhibited by the classical ESBL inhibitors, especially clavulanic acid (1).

4.2 Clinical and/or epidemiological importance

The first isolates producing acquired AmpCs were identified during the 1980s, and since then they have been observed globally as a result of clonal spread and horizontal transfer of AmpC genes (often referred to as plasmid-mediated AmpC). There are several lineages of mobile AmpC genes, originating from natural producers, namely the *Enterobacter* group (MIR, ACT), the *C. freundii* group (CMY-2-like, LAT, CFE), the *M. morganii* group (DHA), the *Hafnia alvei* group (ACC), the *Aeromonas* group (CMY-1-like, FOX, MOX) and the *Acinetobacter baumannii* group (ADC). The most prevalent and most widely disseminated are the CMY-2-like enzymes, although the inducible DHA-like β-lactamases and some others have also spread extensively (1).

The major producer species of acquired AmpCs are *E. coli*, *K. pneumoniae*, *Salmonella enterica* and *P. mirabilis*. Isolates with these enzymes have been recovered from both hospitalized and community patients, and they were recognized earlier than classical ESBL-enzymes in farm animals and in food products (in *E. coli* and *S. enterica*). Although the acquired AmpCs have been spread widely and been recorded in multi-centre studies of enterobacterial resistance to third-generation cephalosporins, their overall frequency has remained far below that of ESBLs, at least in Europe. However, in some local and specific epidemiological settings, the significance of organisms producing these enzymes may substantially increase (1-5).

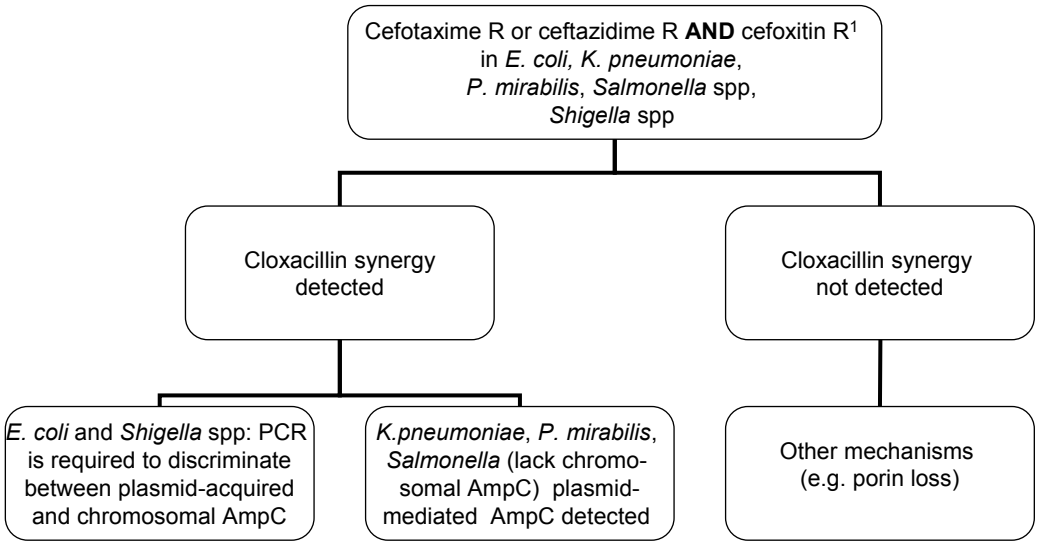
4.3 Mechanisms of resistance

Numerous Enterobacteriaceae and some other Gram-negative bacilli produce natural AmpCs, either constitutively at a trace level (*e.g. E. coli, Shigella spp.*) or inducibly (*e.g. Enterobacter* spp., *C. freundii*, *M. morganii*, *P. aeruginosa*). The derepression or hyperproduction of natural AmpCs is due to various genetic changes and confers high-level resistance to cephalosporins and to penicillin-β-lactamase inhibitor combinations. The class C cephalosporinases can also occur as acquired enzymes, mainly in Enterobacteriaceae. Except for the inducible DHA enzyme, the acquired AmpCs are expressed constitutively, conferring resistance similar to that in the derepressed or hyperproducing mutants of natural AmpC producers. Resistance levels depend on the amounts of enzymes expressed, as well as the presence of other resistance mechanisms. Similar to ESBLs, the acquired AmpC genes are usually found on plasmids (1-3).

4.4 Recommended methods for detection of acquired AmpC in Enterobacteriaceae

A cefoxitin MIC >8 mg/L (zone size <19 mm) combined with phenotypic resistance to ceftazidime and/or cefotaxime (as defined by breakpoints) may be used as phenotypic criteria for investigation of AmpC production in group 1 Enterobacteriaceae, although this strategy will not detect ACC-1, a plasmid-mediated AmpC that does not hydrolyze cefoxitin (6). It should be noted that cefoxitin resistance may also be due to porin deficiency (1).

Figure 1. Algorithm for AmpC detection.



[1] Cefoxitin 'R' is here defined as non-wild type (MIC >8 mg/L or zone diameter <19 mm). For cefotaxime and ceftazidime 'R' is the result obtained using the current EUCAST breakpoints. Investigation of isolates with non-susceptibility to cefotaxime and ceftazidime is an approach with higher sensitivity but lower specificity compared with focusing on cefoxitin resistant isolates (7). AmpC can also be present in isolates with a positive ESBL-test (clavulanic acid synergy). For laboratories not testing cefoxitin, susceptibility to cefepime together with resistance to cefotaxime and/or ceftazidime is another phenotypic indicator of AmpC, although less specific.

Phenotypic AmpC confirmation tests are generally based on inhibition of AmpC by either cloxacillin or boronic acid derivatives. However, boronic acid derivatives also inhibit class A carbapenemases, as well as some class A penicillinases such as K1 in *K. oxytoca*. Although data evaluating these methods is sparse, reasonably accurate detection with in-house methods has been described (8-10) as well as with commercially available tests such as the Mast "AmpC Detection Disc Set" (sensitivity 96-100%, specificity 98%-100%) (11, 12), the AmpC gradient test, currently available only from bioMérieux (sensitivity 84-93%, specificity 70-100%) (12, 13) and Rosco tablets with cefotaxime-cloxacillin and ceftazidime-cloxacillin (sensitivity 96%, specificity 92%) (7,14). For *E. coli,* however, AmpC confirmation tests cannot discriminate between acquired AmpC and constitutive hyperproduction of the chromosomal AmpC.

The presence of acquired AmpCs species may also be confirmed using PCR-based methods (15, 16), or with DNA microarray-based method (17).

Below some possible control strains for phenotypic and genotypic experiments have been listed for convenience. Quality control ranges are not available for these strains. Users of commercial methods should refer to the package insert for information on which control strains to use.

Table 1. Examples of control strains for AmpC testing.

Strain	Mechanism
E. coli CCUG 58543	Acquired CMY-2 AmpC
E. coli CCUG62975	Acquired CMY AmpC and CTX-M-1 group ESBL
K. pneumoniae CCUG 58545	Acquired DHA
E. coli ATCC 25922	AmpC and ESBL negative

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5. Polymyxin resistance in Gram-negative bacilli

Importance of detection of resistance	
Required for clinical antimicrobial susceptibility categorization	Yes
Infection control purposes	Yes
Public health purposes	Yes

Acquired polymyxin resistance in Enterobacteriaceae has emerged in recent years worldwide. Especially the emergence of plasmid-mediated resistance both in animals, food products and humans is worrisome, since it has a great propensity for horizontal dissemination.

Previously, polymyxin resistance was reported always to be chromosomally mediated and usually related to mutations in several genes included in the two- component regulatory system for biosynthesis of lipid A, and thereby regulation of charge in the lipopolysaccharide (LPS) (1,2). In 2015, the first reports on plasmid-mediated colistin resistance appeared, and were related to a plasmid-encoded phosphoethanolamine transferase, which adds a phosphoethanolamine group to lipid A. The resulting effect is a decrease of the net negative charge in the LPS and thereby less interaction with the positively charged polymyxins. The new resistance determinant was named MCR-1 (3). Since then this mechanism of resistance has been documented to be present on all continents. One isolate dates back to the 1980s but it appears that the worldwide emergence has happened the last approximately 5 years (4). During 2016 two new variants of MCR-1 have been described – MCR-1.2 and MCR-2 (5, 6).

In European invasive strains of *K. pneumoniae*, the overall resistance rates to colistin is 8.6%, and can be as high as 29% in carbapenem-resistant isolates (7). It should be noted that the variation in rates between countries is very high, and that methodological issues could contribute to inflated numbers. The majority of the resistance is thought to be due to chromosomal mechanisms, but there have been several reports about carbapenemase-producing Enterobacteriaceae with MCR-1 (4).

There are currently no extensively evaluated methods for phenotypic characterization of the different polymyxin resistance mechanisms other than the MIC itself determined by broth microdilution (gradient diffusion and disk diffusion are unreliable for this drug class). Recently, it was discovered that MCR-enzymes are dependent on zinc for their hydrolysis, and that zinc chelation can therefore inhibit their activity (8). Inhibition tests based on EDTA or dipicolinic acid are therefore expected. However, the current focus is on detecting polymyxin resistance regardless of mechanism. Laboratories are advised to always use broth microdilution for susceptibility testing of colistin, and to always use colistin sulfate (9). Specifically, disk diffusion and gradient tests should not be used, as they are associated with high-risk of both very major and major AST errors (10). Recently, a colorimetric method was also introduced, but it has so far not been tested for robustness in more than one center (11). If further mechanistic studies are warranted, this has to be carried out by molecular methods. The current recommendation is to carry out such further testing only on colistin resistant isolates.

Quality control of colistin must be performed with both a susceptible QC strain (*E. coli* ATCC 25922 or *P. aeruginosa* ATCC 27853) and the colistin resistant *E. coli* NCTC 13846 (*mcr-1* positive). For

E. coli NCTC 13846, the colistin MIC target value is 4 mg/L and should only on occasion be 2 or 8 mg/L.

5.1 References

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6. Carbapenemase producing *P. aeruginosa* and *Acinetobacter*

Importance of detection of resistance mechanism	
Required for clinical antimicrobial susceptibility categorization	No
Infection control purposes	Yes
Public health purposes	Yes

Carbapenemase-producing *P. aeruginosa* and *Acinetobacter baumannii*-group are common in many parts of Europe (1). In *P. aeruginosa* VIM, mainly VIM-2, is the dominant enzyme in Europe but KPC producers have also been noted in Latin American countries (2). In *Acinetobacter* the OXA carbapenemases, mainly OXA-23-, OXA 24/40-, OXA-58-, OXA-143-, OXA-235-like enzymes, are found most commonly (3).

There are currently no specific inhibitors of the class D OXA-carbapenemases and none of the existing phenotypic methods yield satisfactory results for the detection/identification of these carbapenemases in *Acinetobacter*. Colorimetric tests assays have been tried, but have on the whole not proved accurate in this genus (4). *Acinetobacter* may also have carbapenemases of MBL-type, and it is possible that the assays can work better with these enzymes.

For *P. aeruginosa* the MBL Etest® as well as disk-based assays have been used for several decades, but are hampered by poor specificity (5-7). Recently, several authors have also suggested various modifications of the combination disk tests (of either imipenem or meropenem in combination with various class B inhibitory compounds (EDTA or DPA) but these have been validated in single center studies, and their robustness in their settings is difficult to ascertain in other (8, 9). The colorimetric tests have worked better in *P. aeruginosa* than in *Acinetobacter* (10), and are probably the tests with best proven specificity at the moment. Still, no test seems sufficiently specific to be used as a stand-alone test without molecular confirmation.

In general, genotypic approaches should be performed for characterization of putatively carbapenemase-producing *P. aeruginosa* and *Acinetobacter,* but particularly for *P. aeruginosa* some of the above mentioned phenotypic approaches could likely be of value for initial testing.

It should be noted that carbapenemase testing would be most clinically relevant in *P. aeruginosa*, since this species may be carbapenem resistant through multiple chromosomal mechanisms (active efflux, porin alteration or deficiencies). Contrarily, carbapenem resistance in *Acinetobacter* is almost constantly due to production of OXA carbapenemases.

Some suggested control strains are *P. aeruginosa* NCTC 13437 (VIM-10-producing) and *A. baumannii* NCTC 13301 (OXA-23-producing). No QC-ranges exist for these strains.

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7. Methicillin resistant *Staphylococcus aureus* (MRSA)

Importance of detection of resistance	
Required for clinical antimicrobial susceptibility categorization	Yes
Infection control purposes	Yes
Public health purposes	Yes

7.1 Definition

S. aureus isolates with an auxiliary penicillin-binding protein (PBP2a/PBP2c encoded by *mecA* or *mecC* genes) for which β-lactam agents have low affinity, except for the novel class of cephalosporins having anti-MRSA activity (ceftaroline and ceftobiprole).

7.2 Clinical and/or epidemiological importance

Methicillin resistant *S. aureus* is a major cause of morbidity and mortality worldwide (1,2). The mortality of MRSA bloodstream infections is double that of similar infections caused by methicillin susceptible strains due to delayed adequate treatment and inferior alternative treatment regimens (3). MRSA infections are endemic in both hospitals and the community in most parts of the world.

7.3 Mechanisms of resistance

The main mechanism of resistance is production of an auxiliary penicillin-binding protein, PBP2a/PBP2c which renders the isolate resistant to all β-lactams except for the novel class of specific 'anti-MRSA' cephalosporins. These agents have sufficiently high affinity to PBP2a, and probably also the PBP encoded by *mecC*, to be active against MRSA (4). The auxiliary PBPs are encoded by the *mecA* gene or the recently described *mecC* gene (5). The *mec* element is foreign to *S. aureus* and is not present in methicillin susceptible *S. aureus.* Strains with marked heterogeneous expression of the *mecA* gene and frequently low MICs of oxacillin hamper the accuracy of susceptibility testing (5). Some isolates express low-level resistance to oxacillin, but are *mecA* and *mecC* negative and do not produce alternative PBPs [borderline susceptible *S. aureus* (BORSA)]. These strains are relatively rare and the mechanism of resistance is poorly characterized, but may include hyperproduction of β-lactamases or alteration of the pre-existing PBPs (6).

mecA positive *S. aureus* isolates which are susceptible to both cefoxitin and oxacillin (OS-MRSA) due to inactivation of the *mecA* have been described in different parts of the world. These strains are different from the heterogeneously resistant MRSA, which are also oxacillin susceptible but which are resistant to cefoxitin (7, 8). The frequency of such isolates is estimated to be approximately 3% according to combined conventional phenotypic results and PCR results positive for *mecA*. Reversion from methicillin susceptibility to methicillin resistance during prolonged antibiotic therapy has been fully documented in one case (9) but is expected to have happened in other cases, the rate of such reversible resistance is, however, unknown at the present time. Such isolates can by definition only be detected by molecular analysis. It should be noted that this does not apply that molecular analysis should be done with all strains, but the phenomenon can be of importance in case of therapeutic failure. If the *mecA* gene is detected randomly or due to screening because of therapeutic failure, the isolate should always be reported resistant.

7.4 Recommended methods for detection of methicillin resistance in *S. aureus*

Methicillin/oxacillin resistance can be detected phenotypically by MIC determination and by disk diffusion. Agglutination can be used to detect PBP2a, but will not reliably detect PBP2c. Genotypic detection with PCR is reliable.

7.4.1 Detection by MIC determination or disk diffusion

The heterogeneous expression of resistance particularly affects MICs of oxacillin, which can appear susceptible. Cefoxitin is a very sensitive and specific marker of *mecA/mecC*-mediated methicillin resistance including in heterogeneous expressing strains and is the agent of choice. Disk diffusion using oxacillin is discouraged and interpretive zone diameters are no longer included in the EUCAST breakpoint table due to poor correlation with the presence of *mecA*.

A. Broth microdilution:
Standard methodology (ISO 20776-1) is used and strains with cefoxitin MICs >4 mg/L should be reported as methicillin resistant.

B. Disk diffusion**:** The EUCAST disk diffusion method is used. Strains with a cefoxitin (30 µg disk) zone diameter <22 mm should be reported as methicillin resistant.

7.4.2 Detection with genotypic and latex agglutination methods

Genotypic detection of the *mecA* and *mecC* genes by PCR (10, 11) and detection of the PBP2a protein with latex agglutination kits is possible using commercial or in-house assays. PBP2c is not detected by the majority of commercial assays.

7.4.3 Control strains

Below some possible control strains for phenotypic and genotypic experiments have been listed for convenience. Quality control ranges are not available for these strains. Users of commercial methods should study the package insert for information on which control strains to use.

Table 1. Examples of control strains for MRSA-testing.

Strain	Mechanism
S. aureus ATCC 29213	Methicillin susceptible
S. aureus NCTC 12493	Methicillin resistant (*mecA*)
S. aureus NCTC 13552	Methicillin resistant (*mecC*)

7.5 References

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8. Vancomycin-resistant *Staphylococcus aureus*

Importance of detection of resistance	
Required for clinical antimicrobial susceptibility categorization	Yes
Infection control purposes	Yes
Public health purposes	Yes

8.1 Definition

The EUCAST clinical MIC breakpoint for resistance to vancomycin in *S. aureus* is >2 mg/L. In recent years vancomycin breakpoints have been lowered, thereby removing the former intermediate group. However, there are important differences in the mechanism of resistance in VanA-mediated high-level vancomycin resistant *S. aureus* (VRSA) and non-VanA mediated low-level resistant isolates. Hence, the terms vancomycin intermediate *S. aureus* (VISA) and heteroresistant vancomycin intermediate *S. aureus* (hVISA) have been maintained for isolates with non-VanA-mediated low-level resistance to vancomycin. The MIC should always be determined when using vancomycin to treat a patient with severe *S. aureus* infection. In selected cases, e.g. when therapeutic failure is suspected, testing for hVISA may also be warranted. Due to the complexity of confirming hVISA, antimicrobial surveillance is focused on detection of VISA and VRSA.

VRSA: Vancomycin resistant *S. aureus*:
S. aureus isolates with high-level resistance to vancomycin (MIC >8 mg/L).

VISA: Vancomycin intermediate *S. aureus*
S. aureus isolates with low-level resistance to vancomycin (MIC 4 - 8 mg/L).

hVISA: Heterogeneous vancomycin intermediate *S. aureus*.
S. aureus isolates susceptible to vancomycin (MICs ≤2mg/L) but with minority populations (1 in 10^6 cells) with vancomycin MIC >2 mg/L, as judged by population analysis profile investigation.

It should be noted that although these terms still remain, all of the above-mentioned categories should be regarded clinically resistant.

8.2 Clinical and/or epidemiological importance

There are no recent investigations of the prevalence of isolates with reduced susceptibility to glycopeptides in Europe. Based on reports from single institutions it is estimated that the prevalence of hVISA is ≤2% of MRSA in Europe, with VISA below 0.1% (1). VRSA has not yet been reported in Europe (1) and is currently extremely rare worldwide (2). The prevalence of hVISA may be considerably higher locally (1), most often associated with spread of specific clonal lineages (2). The vast majority of isolates with elevated MIC (VISA) or containing resistant subpopulations (hVISA) are MRSA.

The clinical significance of hVISA has been difficult to determine as no well-controlled prospective studies have been performed. However, presence of the hVISA phenotype is believed to be associated with poorer outcome, at least in serious infections (2, 3). It is therefore prudent to investigate for the presence of hVISA in bloodstream infections not responding to therapy. Recently there has been increasing evidence that isolates with MICs in the upper part of the wild-

type range (MIC >1 mg/L) are associated with poorer outcome and may be linked to increased mortality, at least in bloodstream infections (3-8). The possible cause of these observations is unclear but may be due to underexposure to vancomycin (9, 10). Furthermore, the interpretation of the findings these studies is confounded by differences in MICs generated by different testing methods (8, 9).

The mechanism of hVISA is complex and detection relies on population analysis (11), which is cumbersome, requires special equipment and needs a high level of technical expertise. Methodology for detection of hVISA will be outlined, but for surveillance purposes reporting is restricted to VISA and VRSA, which are together defined as isolates with an MIC >2mg/L.

8.3 Mechanism of resistance

For VRSA the resistance is mediated by the *vanA* gene, exogenously acquired from enterococci. For both VISA and hVISA isolates the resistance is endogenous (i.e. chromosomal mutations) and the mechanism highly complex, with no single gene being responsible. The VISA/hVISA phenotype is linked to a thickening of the bacterial cell wall, associated with hyperproduction of glycopeptide binding targets. The hVISA phenotype is often unstable in the laboratory, but hVISA have the capacity to develop into VISA *in vivo* (2).

8.4 Recommended methods for detection of vancomycin non-susceptible *S. aureus*

Disk diffusion cannot be used to test for either hVISA or VISA, but can likely be used to test for VRSA, although there are limited studies to support this (12).

8.4.1 MIC determination

Broth microdilution methodology as recommended by EUCAST (ISO 20776-1) is the gold standard. It should be noted that the results with gradient strip methods may be 0.5-1 two-fold dilution steps higher than the results obtained by broth microdilution (8, 9). The EUCAST breakpoint for resistance to vancomycin in *S. aureus* is MIC >2 mg/L. Isolates with confirmed MICs >2 mg/L (according to broth microdilution) should be referred to a reference laboratory. hVISA will not be detected by MIC determination.

8.4.2 Tests detecting VRSA, VISA and hVISA

Detection of hVISA has proven difficult and detection is therefore divided into screening and confirmation. For screening, a number of specialised methods have been developed. Confirmation is obtained by analysing the population profile of the isolate on agar plates containing a range of vancomycin concentrations (PAP-AUC) (11). This method is technically challenging without extensive experience and consequently is mostly performed by reference laboratories. A method based on a vancomycin and casein screening agar (13) has shown high sensitivity and specificity, but has so far only been evaluated in one study, and is for that reason cannot yet be recommended. The following methods will all detect VRSA and VISA, and have been evaluated in multicentre studies (14, 15).

A. Macro gradient test:
This test gives an indication of reduced vancomycin susceptibility but note that the readings are not MICs. Furthermore, the test does not differentiate between hVISA, VISA and VRSA. The test must be set up according to the manufacturer's instructions. Note also that the inoculum is higher (2.0 McFarland) than with standard gradient tests, and the gradient test is applied on Broth Heart Infusion (BHI) agar instead of Mueller Hinton. Moreover, the test is finally read after 48 h. A positive result is indicated by readings ≥8mg/L for both vancomycin and teicoplanin, OR ≥12mg/L for teicoplanin alone.

As both criteria include teicoplanin, testing of vancomycin could be dependent on the result of the teicoplanin test. The algorithm would then be:

- Teicoplanin reading ≥12 mg/L: VRSA, VISA or hVISA
- Teicoplanin reading 8 mg/L: Test vancomycin. If vancomycin reading is ≥8 mg/L then VRSA, VISA or hVISA
- Teicoplanin reading <8 mg/L: Not VRSA, VISA or hVISA

B. Glycopeptide resistance detection (GRD) gradient test:
Test according to the manufacturer's instructions. The test is considered positive if the GRD strip result is ≥8 mg/L for either vancomycin or teicoplanin.

C. Teicoplanin screening agar:
A Mueller-Hinton plate containing 5 mg/L teicoplanin is used (14). Several colonies are suspended in 0.9% saline to obtain an inoculum with equivalent turbidity to a 2.0 McFarland standard. Ten microliters of inoculum is delivered as a spot on the surface of the agar, and the plate incubated at 35°C in air for 24 to 48 h. Growth of colonies at 48h indicates suggests reduced susceptibility to glycopeptides.

D. Confirmatory testing for hVISA/VISA:
Any isolate screening positive for reduced susceptibility and not identified as VRSA or VISA by MIC determination may be hVISA and may be investigated by population analysis profile-area under curve (PAP-AUC) (9), typically by referral to a reference laboratory.

8.4.3 Control strains

Below some possible control strains for phenotypic experiments have been listed for convenience. Quality control ranges are not available for these strains. Users of commercial methods should examine the package insert for information on which control strains to use.

Table 1. Examples of control strains for testing of vancomycin resistance in *S. aureus*.

Strain	Mechanism
S. aureus ATCC 29213	Glycopeptide susceptible
S. aureus ATCC 700698	hVISA (Mu3)
S. aureus ATCC 700699	VISA (Mu50)

8.5 References

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9. Vancomycin resistant *Enterococcus faecium* and *Enterococcus faecalis*

Importance of detection of resistance	
Required for clinical antimicrobial susceptibility categorization	Yes
Infection control purposes	Yes
Public health purposes	Yes

9.1 Definition

Enterococcus faecium or *Enterococcus faecalis* with resistance to vancomycin (VRE) (vancomycin MIC >4 mg/L).

9.2 Clinical and/or epidemiological importance

Enterococci, especially *E. faecium*, are generally resistant to most clinically available antimicrobial agents. Treatment of infections caused by vancomycin-resistant enterococci (VRE) is therefore difficult, with few treatment options. VRE are known to spread efficiently and persist in the hospital environment, and can colonize many individuals of which only a few may develop enterococcal infections (1, 2). Isolates harbouring VanB are usually phenotypically susceptible to teicoplanin. There are two case reports of selection of teicoplanin resistance during treatment of enterococci harbouring *vanB* (3, 4), and recently four cases of therapeutic failure have been described (5), indicating that teicoplanin should be used with caution against enterococci with VanB. Typical MIC values for the clinically most important Van enzymes are shown in Table 1.

Table 1. Typical MICs of glycopeptides for enterococci expressing VanA or VanB.

Glycopeptide	MIC (mg/L)	
	VanA	VanB
Vancomycin	64-1024	4-1024
Teicoplanin	8-512	0.06-1

9.3 Mechanism of resistance

Clinically relevant resistance is most often mediated by plasmid-encoded VanA and VanB ligases that replace the terminal D-Ala(nine) in the peptidoglycan with D-Lac(tate). This substitution reduces the binding of glycopeptides to the target. VanA-producing strains exhibit resistance to both vancomycin and teicoplanin, whereas VanB-producing strains usually remain susceptible to teicoplanin due to lack of induction of the resistance operon. Other Van enzymes of lower prevalence are VanD, VanE, VanG, VanL, VanM and VanN (6-9), although *vanM* was recently shown to increase significantly in China in *E. faecium* (10).

Additional enterococcal species (i.e. *E. raffinosus, E. gallinarum* and *E. casseliflavus)*, may contain *vanA, vanB* or other *van* genes encoding enzymes listed above, but these strains are relatively rare. Chromosomally-encoded VanC enzymes are found in all *E. gallinarum* and *E. casseliflavus*

isolates. VanC mediates low-level vancomycin resistance (MIC 4-16 mg/L) but should generally not be considered important from an infection control point of view (11).
Vancomycin variable enterocci is a term used for VRE where the expression of *van* genes is phenotypically silenced by genetic rearrangements, which may be reversed under glycopeptide selection pressure (12, 13). Moreover, low-MIC VRE is a term used for *vanB* isolates that due to initial poor ability to be induced by vancomycin have a low expression of *vanB* genes giving MIC levels below the clinical breakpoint. These low-MIC VRE may increase their MIC to above the breakpoint upon longer exposure to vancomycin (14). Both VVE and low-MIC VRE strains can often only be detected by molecular analysis. Their current prevalence in different geographical regions is unknown.

9.4 Recommended methods for detection of glycopeptide resistance in *E. faecium* and *E. faecalis*

Vancomycin resistance can be detected by MIC determination, disk diffusion and the breakpoint agar method. For all three methods, it is essential that plates are incubated for a full 24 h in order to detect isolates with inducible resistance.

All three methods readily detect *vanA*-mediated resistance. Detection of *vanB*-mediated resistance is more challenging. MIC determination by agar or broth dilution is not always reliable for VanB, (15-17). Older reports show that detection of *vanB*-mediated resistance is problematic for automated methods (18). Since then updates have been made to the automated methods, but more recent studies on whether the performances of these methods for detection of *vanB*-mediated resistance have improved are lacking. Disk diffusion with a 5 µg vancomycin disk can be difficult but the test performs well provided the guidelines for reading as specified by EUCAST are followed meticulously (19).

When interpreting the MIC or disk diffusion test results it is important to ensure that the isolate is not *E. gallinarum* or *E. casseliflavus,* which may be erroneously identified as *E. faecium* due to a positive arabinose test. MALDI-TOF mass spectrometry is in this context very useful for species identification of enterococci (20). In settings where MALDI-TOF is not available, the MGP (methyl-alpha-D-glucopyranoside) test or a motility test can be used to distinguish *E. gallinarum /E. casseliflavus* from *E. faecium* (MGP negative, non-motile).

9.4.1 MIC determination

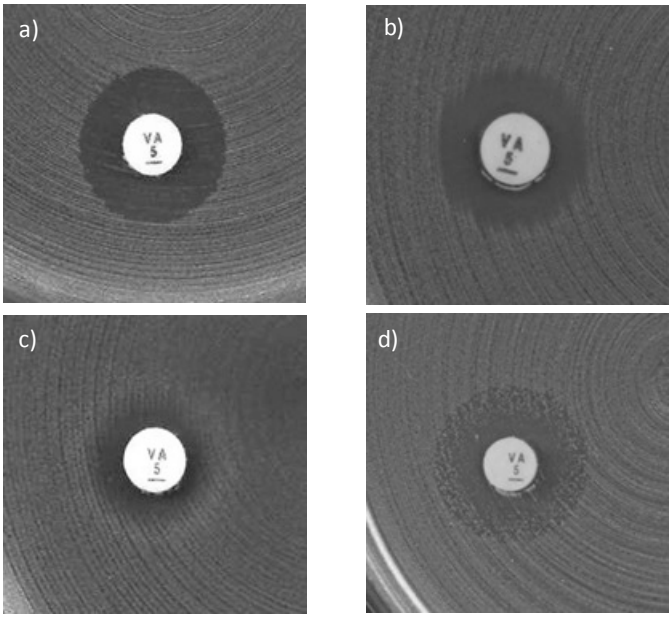
MIC determination may be performed by agar dilution, broth microdilution or gradient MIC methods.
Broth microdilution is performed according to the ISO standard 20776-1 as recommended by EUCAST.

9.4.2 Disk diffusion testing

For disk diffusion, the method specified by EUCAST must be followed meticulously. Inspect zones for fuzzy edges and/or microcolonies with transmitted light. Sharp zone edges indicate that the isolate is susceptible and isolates with sharp zones and zone diameters above the breakpoint can be reported as vancomycin susceptible. Isolates with fuzzy zone edges or colonies within the zone (Figure 1) may be resistant regardless of zone size and should not be reported as susceptible without confirmation by MIC determination. In a recent multicenter study the disk diffusion

method worked better than VITEK2 for detection of *vanB*-producing enterococci, particularly in laboratories experienced with ready of fuzzy zone edges (19). Disk diffusion is performed according to the EUCAST disk diffusion methodology for non-fastidious organisms. Incubation for 24 h is needed in order to detect resistance in some isolates with inducible resistance.

Figure 1. Reading of vancomycin disk diffusion tests on *Enterococcus* spp.



a) Sharp zone edges and zone diameter ≥12 mm. Report as susceptible.

b-d) Fuzzy zone edges and/or colonies within the zone. Report as resistant regardless of zone diameter.

9.4.3 Breakpoint agars

Breakpoint agar tests with Brain Heart Infusion agar and 6 mg/L vancomycin are reliable for detection of *vanA*- and *vanB*-positive isolates (19). Breakpoint plates can be obtained from commercial manufacturers or made in-house. The breakpoint agar test is performed by application of 1 x 10^5 - 1 x 10^6 cfu (10 µl of a 0.5 McFarland suspension) on Brain Heart infusion agar with 6 mg/L vancomycin. Incubation for 24 h at 35 ± 1°C in ambient air is needed in order to detect resistance in some isolates with inducible resistance. Growth of more than one colony is scored as a positive test.

9.4.4 Genotypic testing

Detection of vancomycin resistance by the use of PCR targeting *vanA* and *vanB* can also be performed using in-house or commercial methods (20-22).

9.4.5 Quality control

Below some possible control strains for phenotypic and genotypic experiments have been listed for convenience. Quality control ranges are not available for these strains. Users of commercial methods should study the package insert for information on which control strains to use.

Table 2. Examples of control strains for testing of enterococci.

Strain	Mechanism
E. faecalis ATCC 29212	Vancomycin-susceptible
E. faecalis ATCC 51299	Vancomycin-resistant (*vanB*)
E. faecium NCTC 12202	Vancomycin-resistant (*vanA*)

9.5 References

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10. Penicillin non-susceptible (non-wild type) *Streptococcus pneumoniae*

Importance of detection of resistance	
Required for clinical antimicrobial susceptibility categorization	Yes
Infection control purposes	No
Public health purposes	Yes

10.1 Definition

S. pneumoniae isolates with reduced susceptibility to penicillin (MICs above those of the wild-type, i.e. >0.06 mg/L) due to the presence of modified penicillin-binding proteins (PBPs) with lower affinity for β-lactams.

10.2 Clinical and/or epidemiological importance

S. pneumoniae is the most common cause of pneumonia worldwide. Morbidity and mortality are high and approximately three million people are estimated to die each year of pneumococcal infections. Low-level penicillin non-susceptibility is associated with increased mortality when meningitis is treated with benzylpenicillin (1). In other infection types no increased mortality is observed with low-level resistance if higher dosages are given. Many countries carry out vaccination programmes against several pneumococcal serotypes, and this may affect resistance levels observed in invasive isolates (2). However, penicillin non-susceptible *S. pneumoniae* remain a major clinical problem from a public health point of view, although these microorganisms are not associated with spread in healthcare institutions, unlike many of the other pathogens described in this document.

10.3 Mechanism of resistance

S. pneumoniae contains six PBPs, of which PBP 2x is the primary target of penicillin (3). The presence of "mosaic genes" encoding low-affinity PBPs is the result of horizontal gene transfer from commensal viridans streptococci (3). The level of β-lactam resistance depends not only on low-affinity mosaic PBPs present in the isolate, but also on modification of the specific PBPs that are essential for *S. pneumoniae* (4). Strains with MICs of benzylpenicillin in the range 0.12 to 2 mg/L are considered susceptible in non-meningitis infections when a higher dose of penicillin is used, whereas for meningitis such strains must always be reported as resistant (5).

10.4 Recommended methods for detection of penicillin non-susceptible *S. pneumoniae*

Penicillin non-susceptibility can be detected phenotypically by MIC or disk diffusion methods.

10.4.1 Disk diffusion method

The disk diffusion method with 1 µg oxacillin disks is an effective screening method for the detection of penicillin non-wild type pneumococci (6-8). The method is very sensitive, but is not highly specific because strains with zone diameters of ≤19 mm may have variable susceptibility to benzylpenicillin, and the benzylpenicillin MIC should be determined for all isolates that are non-wild type with the screening method (8).

For β-lactams other than benzylpenicillin the oxacillin zone diameter can be used to predict susceptibility as in Figure 1.

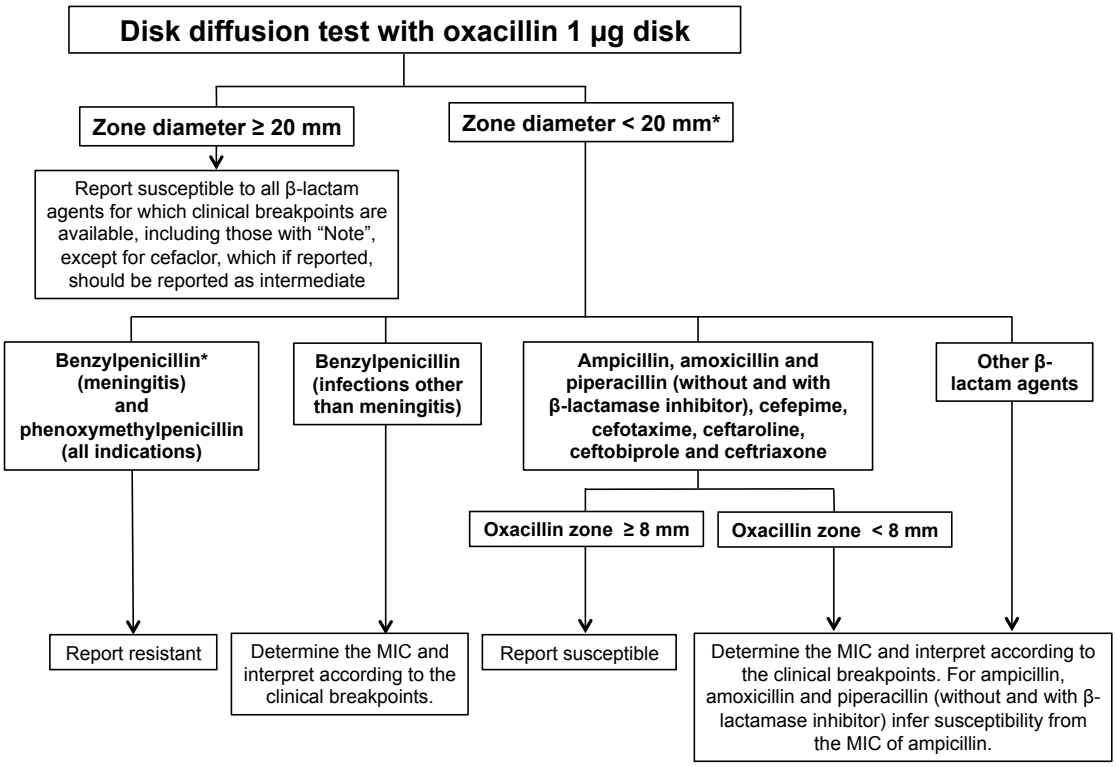

Figure 1. Screening for β-lactam resistance in *S. pneumoniae*

*Oxacillin 1 µg <20 mm: Always determine the MIC of benzylpenicillin but do not delay reporting of other β-lactams as recommended above and do not delay reporting of benzylpenicillin in meningitis.

10.4.2 Clinical breakpoints

The penicillin breakpoints were initially designed to ensure the success of therapy for pneumococcal meningitis. However, clinical studies demonstrated that the outcome of pneumococcal pneumonia caused by most strains with elevated MICs to penicillin and treated with parenteral penicillin was no different to that for patients treated with other agents. Considering microbiological, pharmacokinetic and pharmacodynamic data, the clinical breakpoints for benzylpenicillin for non-meningitis isolates were revisited (4) and current EUCAST breakpoints are as listed in Table 1 and in the last version of the EUCAST breakpoint table.

Table 1. Reporting of benzylpenicillin susceptibility in meningitis and non-meningitis.

Indications	**MIC breakpoint (mg/L)**		**Notes**
	S ≤	R >	
Benzylpenicillin (non-meningitis)	0.06	2	**In pneumonia**, when a dose of 1.2 g x 4 is used, isolates with **MIC ≤0.5 mg/L** should be regarded as susceptible to benzylpenicillin. **In pneumonia**, when a dose of 2.4 g x 4 or 1.2 g x 6 is used, isolates with **MIC ≤1 mg/L** should be regarded as susceptible to

			benzylpenicillin. **In pneumonia**, when a dose of 2.4 g x 6 is used, isolates with **MIC ≤2 mg/L** should be regarded as susceptible.
Benzylpenicillin (meningitis)	0.06	0.06	

10.4.3 Quality control

Below a possible control strain for phenotypic testing has been listed for convenience.

Table 2. Example of control strain for benzylpenicillin testing.

Strain	Mechanism
S. pneumoniae ATCC 49619	Mosaic PBP, benzylpenicillin MIC 0.5 mg/L

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