EUCAST EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING

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

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1. Introduction

In 2012 the EUCAST steering committee established a subcommittee for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance. The subcommittee was established partly in response to frequently asked questions from users of EUCAST guidelines on this issue, and partly on request from the European Centre for Disease Control and Prevention (ECDC), as expert guidance was needed for updating the EARS-Net microbiological manual.

The remit of the subcommittee was to develop practical guidelines for detection of specific antimicrobial resistance mechanisms of clinical and/or epidemiological importance. All chapters in this document contain a definition of the mechanisms or resistance, an explanation of the clinical and/or public health need for detection of the mechanisms or resistances, an outline description of recommended methods of detection, and references to detailed descriptions of the methods. The document has been developed by conducting systematic literature searches, and most recommendations are based on multi-centre studies, as these provide the best measure of robustness of the methods. Prior to publication of these guidelines, they were subjected to wide consultation through the EUCAST consultation contact lists, the EUCAST website and ECDC focal point contacts.

It should be noted that some resistance mechanisms do not always confer clinical resistance according to EUCAST clinical breakpoints. Hence, while detection of these mechanisms may be relevant from an infection control and public health point of view, for several of the mechanisms current evidence does not support editing of susceptibility results if they appear susceptible after applying clinical breakpoints. Consequently for many of the mechanisms, particularly extended-spectrum β-lactamases and carbapenemases in Gram-negative bacilli, detection of the mechanism does not in itself lead to classification as resistant.

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2. Carbapenemase-producing Enterobacteriaceae

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

2.1 Definition

Carbapenemases are β-lactamases that hydrolyze penicillins, in most cases cephalosporins, and to varying 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 to around 2000 in several Mediterranean countries, and was observed mainly in *P. aeruginosa* (1). Later on, Greece experienced an epidemic of the Verona integron-encoded metallo-β-lactamase (VIM) among *K. pneumoniae* (2), which was followed by an epidemic related to the *K. pneumoniae* carbapenemase (KPC), which is presently the most common carbapenemase in Europe among Enterobacteriaceae (1). In Greece and Italy around 60 and 15%, respectively, of invasive *K. pneumoniae* are now non-susceptible to carbapenems (3). In other European countries several outbreaks have been reported, but the problem has not been widely observed in invasive isolates (1). Other particularly problematic carbapenemases are the New Dehli metallo-β-lactamase (NDM), which is highly prevalent on the Indian subcontinent and in the Middle East, and has on several occasions been imported to Europe. The OXA-48-like enzymes have caused outbreaks in several European countries and are now spreading rapidly (1).

Carbapenemases are a source of concern because they confer resistance to essentially all β-lactams, strains producing carbapenemases frequently possess resistance mechanisms to a wide-range of antimicrobial agents, and infections with

carbapenemase-producing Enterobacteriaceae are associated with high mortality rates (4-6).

2.3 Mechanisms of resistance

The vast majority of carbapenemases are acquired enzymes, encoded by plasmids or other mobile genetic elements. 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 an enzyme, and the frequent association with other resistance mechanisms (other β-lactamases, efflux, altered permeability), result in the wide range of resistance phenotypes observed among carbapenemase-producing isolates (7, 8). Decreased susceptibility to carbapenems in Enterobacteriaceae may, however, also be caused by either ESBL or AmpC enzymes combined with decreased permeability due to alteration or down-regulation of porins (9).

Most carbapenemase-producers, with the exception of OXA-48-like producers, are resistant to broad-spectrum (oxyimino) cephalosporins (10). Isolates producing such enzymes may have decreased susceptibility to carbapenems, but with some of these enzymes the organisms may appear fully susceptible to cephalosporins. However, most of these isolates now also express cephalosporinase enzymes, such as CTX-M, so they are usually also cephalosporin resistant. Carbapenemases are considered to be of high epidemiological importance when they confer decreased susceptibility to any of the carbapenems (imipenem, meropenem, ertapenem and doripenem), i.e. when the MICs are above the epidemiological cut-off values (ECOFFs) defined by EUCAST (11).

2.4 Recommended methods for detection of carbapenemases in Enterobacteriaceae

2.4.1 Phenotypes to screen for carbapenemase-production

Carbapenemase-producing Enterobacteriaceae often have MICs below the clinical carbapenem breakpoints (10, 11, 13). 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 (10, 14). Ertapenem has excellent sensitivity, but poor specificity, especially in species such as *Enterobacter* spp., due to its relative instability to extended-spectrum β-lactamases and AmpC β-lactamases in combination with porin loss (10). Appropriate cut-off values for detecting putative carbapenemase-producers are shown in Table 1. It should be noted that for imipenem and ertapenem screening cut-off values are one-dilution step higher than the currently defined ECOFFs. This change has been done to increase specificity.

Table 1. Clinical breakpoints and screening cut-off values for carbapenemase-producing Enterobacteriaceae.

Carbapenem	MIC (mg/L)		Disk diffusion zone diameter (mm)	
	S/I breakpoint	Screening cut-off	S/I breakpoint	Screening cut-off
Meropenem[1]	≤2	>0.125	≥22	<25[2]
Imipenem	≤2	>1	≥22	<23
Ertapenem[3]	≤0.5	>0.125	≥25	<25

[1]Best balance of sensitivity and specificity.

[2]In rare cases OXA-48-producers have zone diameters of 24-26 mm, so 27 mm may be used as a screening cut-off during outbreaks, but with significant reduction in specificity.

[3]High sensitivity, but low specificity and therefore not recommended.

2.4.2 Methods for confirmation of carbapenemase-production

Analysis of carbapenem hydrolysis with MALDI-TOF (15) or the Carba NP test (16, 17) are methods that can be used to confirm carbapenemase-production in a few hours. However, these methods have so far been evaluated in only a few laboratories, and their performance in laboratories lacking extensive experience with β-lactamase detection remains to be determined.

A number of genotypic approaches have been reported based on PCR techniques. These methods, however, have the disadvantage of not being able to identify new β-lactamase variants, and might be considered expensive in some settings (10). Commercial DNA microarray methods are marketed and may increase the user-friendliness of these tests (18). The classical phenotypic methods are slow, but are the most extensively evaluated, and for this reason remain the recommended methods for laboratories without special expertise in β-lactamase detection.

2.4.3 Interpretation of phenotypic detection methods

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 methodology for non-fastidious organisms. Disks can either be made in-house or commercial tablets (Rosco, Denmark) can be used (19). Other manufactures are expected to market similar tests in the future.

At present there are no available inhibitors for OXA-48-like enzymes. Temocillin high-level resistance (MIC >32 mg/L) is a good phenotypic marker for differentiating between OXA-48-like carbapenemases and the combination of ESBL and porin loss as OXA-48-like enzymes confer high-level resistance to temocillin (12). Also, piperacillin-tazobactam resistance in the absence of phenotypic evidence of AmpC is another indicator of production of OXA-48-like enzymes (12).

It is not recommended to use the modified cloverleaf (Hodge) test as results are difficult to interpret and sensitivity and specificity are poor (10). 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**) with in-house or commercial disks or tablets.

β-lactamase	Synergy observed as increase in meropenem zone diameter (mm) with 10µg disk				Temocillin MIC > 32 mg/L
	DPA/EDTA	APBA/PBA	DPA+APBA	CLX	
MBL	≥5	-	-	-	NA[1]
KPC	-	≥4	-	-	NA[1]
MBL+KPC[2]	Variable	Variable	≥5	-	NA[1]
OXA-48-like[3]	-	-	-	-	Yes
AmpC + porin loss	-	≥4	-	≥5	NA[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 is recommended only in cases where no synergy is detected, in order to differentiate between ESBL + porin loss and OXA-48-like enzymes.

[2] There are no published reports with commercial disks or tablets containing double inhibitors (DPA or EDTA plus APBA or PBA), and in-house tests have not been evaluated in multi-centre studies. This phenotype is rare outside of Greece and confers high-level resistance to carbapenems.

[3] In the absence of a temocillin MIC, high-level resistance to piperacillin-tazobactam (MIC>32 mg/L) may indicate OXA-48 as ESBLs tend to confer lower MICs.

2.4.4 Control strains

Table 3. Appropriate 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 13439	Metallo-β-lactamase (VIM)
K. pneumoniae CCUG 56233 or *K. pneumoniae* NCTC 13438	*Klebsiella pneumoniae* carbapenemase (KPC)
Klebsiella pneumoniae NCTC 13442	OXA-48 carbapenemase
K. pneumoniae ATCC 25955	Negative control

2.5 References

1. Cantón R, Akóva M, Carmeli Y, Giske CG, Glupczynski Y, et al. Rapid evolution and spread of carbapenemases among Enterobacteriaceae in Europe. Clin Microbiol Infect. 2012 May;18(5):413-31
2. Vatopoulos A. High rates of metallo-β-lactamase-producing *Klebsiella pneumoniae* in Greece – a review of the current evidence. Euro Surveill. 2008 Jan 24;13(4). doi:pii: 8023
3. European Centres for Disease Prevention and Control (ECDC). Antimicrobial resistance surveillance in Europe 2011. Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net)
4. Souli M, Galani I, Antoniadou A, Papadomichelakis E, Poulakou G et al. An outbreak of infection due to β-lactamase *Klebsiella pneumoniae* carbapenemase 2-producing *K. pneumoniae* in a Greek University Hospital: molecular characterization, epidemiology, and outcomes. Clin Infect Dis 2010;50:364–73.
5. Bratu S, Landman D, Haag R, Recco R, Eramo A, Alam M, Quale J. Rapid spread of carbapenem-resistant *Klebsiella pneumoniae* in New York City: a new threat to our antibiotic armamentarium. Arch Intern Med. 2005;165(12):1430-5.
6. Marchaim D, Navon-Venezia S, Schwaber MJ, Carmeli Y. Isolation of imipenem-resistant Enterobacter species: emergence of KPC-2 carbapenemase, molecular characterization, epidemiology, and outcomes. Antimicrob Agents Chemother. 2008 Apr;52(4):1413-8

7. Queenan AM, Bush K. Carbapenemases: the versatile β-lactamases. Clin Microbiol Rev 2007;20:440–58
8. Falcone M, Mezzatesta ML, Perilli M, Forcella C, Giordano A et al. Infections with VIM-1 metallo β-lactamase-producing *Enterobacter cloacae* and their correlation with clinical outcome. J Clin Microbiol 2009;47: 3514–9.
9. Doumith M, Ellington MJ, Livermore DM, Woodford N. Molecular mechanisms disrupting porin expression in ertapenem-resistant *Klebsiella* and *Enterobacter* spp. clinical isolates from the UK. J Antimicrob Chemother. 2009;63:659-67
10. Nordmann P, Gniadkowski M, Giske CG, Poirel L, Woodford N, Miriagou V. Identification and screening of carbapenemase-producing Enterobacteriaceae. Clin Microbiol Infect. 2012;18(5):432-8.
11. European Committee on Antimicrobial Susceptibilty Testing (EUCAST). Website with MIC-distributions. (http://mic.eucast.org/, accessed on 23 December 2012)
12. Glupczynski Y, Huang TD, Bouchahrouf W, Rezende de Castro R, Bauraing C et al. Rapid emergence and spread of OXA-48-producing carbapenem-resistant Enterobacteriaceae isolates in Belgian hospitals. Int J Antimicrob Agents. 2012 Feb;39(2):168-72
13. Tato M, Coque TM, Ruíz-Garbajosa P, Pintado V, Cobo J et al. Complex clonal and plasmid epidemiology in the first outbreak of Enterobacteriaceae infection involving VIM-1 metallo-β-lactamase in Spain: toward endemicity? Clin Infect Dis. 2007;45(9):1171-8.
14. Vading M, Samuelsen Ø, Haldorsen B, Sundsfjord AS, Giske CG. Comparison of disk diffusion, Etest and VITEK2 for detection of carbapenemase-producing *Klebsiella pneumoniae* with the EUCAST and CLSI breakpoint systems. Clin Microbiol Infect. 2011;17(5):668-74.
15. Hrabák J, Studentová V, Walková R, Zemlicková H, Jakubu V et al. Detection of NDM-1, VIM-1, KPC, OXA-48, and OXA-162 carbapenemases by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol. 2012;50(7):2441-3
16. Nordmann P, Poirel L, Dortet L. Rapid detection of carbapenemase-producing Enterobacteriaceae. Emerg Infect Dis. 2012;18(9):1503-7.
17. Dortet L, Poirel L, Nordmann P. Rapid Identification of Carbapenemase Types in Enterobacteriaceae and *Pseudomonas* spp. by Using a Biochemical Test. Antimicrob Agents Chemother. 2012;56(12):6437-40.
18. Cuzon G, Naas T, Bogaerts P, Glupczynski Y, Nordmann P. Evaluation of a DNA microarray for the rapid detection of extended-spectrum β-lactamases (TEM, SHV and CTX-M), plasmid-mediated cephalosporinases (CMY-2-like, DHA, FOX, ACC-1, ACT/MIR and CMY-1-like/MOX) and carbapenemases (KPC, OXA-48, VIM, IMP and NDM). J Antimicrob Chemother. 2012;67(8):1865-9.
19. Giske CG, Gezelius L, Samuelsen Ø, Warner M, Sundsfjord A, Woodford N. A sensitive and specific phenotypic assay for detection of metallo-β-lactamases and KPC in *Klebsiella pneumoniae* with the use of meropenem disks supplemented with aminophenylboronic acid, dipicolinic acid and cloxacillin. Clin Microbiol Infect. 2011;17(4):552-6.

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

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

3.1 Definition

ESBLs are enzymes hydrolyzing most penicillins and cephalosporins, including oxyimino-β-lactam compounds (cefuroxime, third- and fourth-generation cephalosporins and aztreonam) but not cephamycins and carbapenems. Most ESBLs belong to the Ambler class A of β-lactamases and are inhibited by β-lactamase inhibitors (clavulanate, sulbactam and tazobactam) (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 has been due to the clonal expansion of producer organisms, the horizontal transfer of ESBL genes and their emergence *de novo*. By far the most important groups of ESBLs are CTX-M enzymes, followed by SHV- and TEM-derived ESBLs (2-5). Certain OXA-derived enzymes are also included within ESBLs, although inhibition by class A-β-lactamase inhibitors is weaker than for other ESBLs.

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 *Escherichia 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 2011 showed that the rate of invasive *K. pneumoniae* isolates non-susceptible to the third-generation cephalosporins exceeded 10% in the majority of European countries, with some reporting resistance rates higher than 50%. 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 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, efflux, altered permeability) result in the large variety of resistance phenotypes observed among ESBL-positive isolates (1-4, 9, 10).

3.4 Recommended methods for detection of ESBLs in Enterobacteriaceae

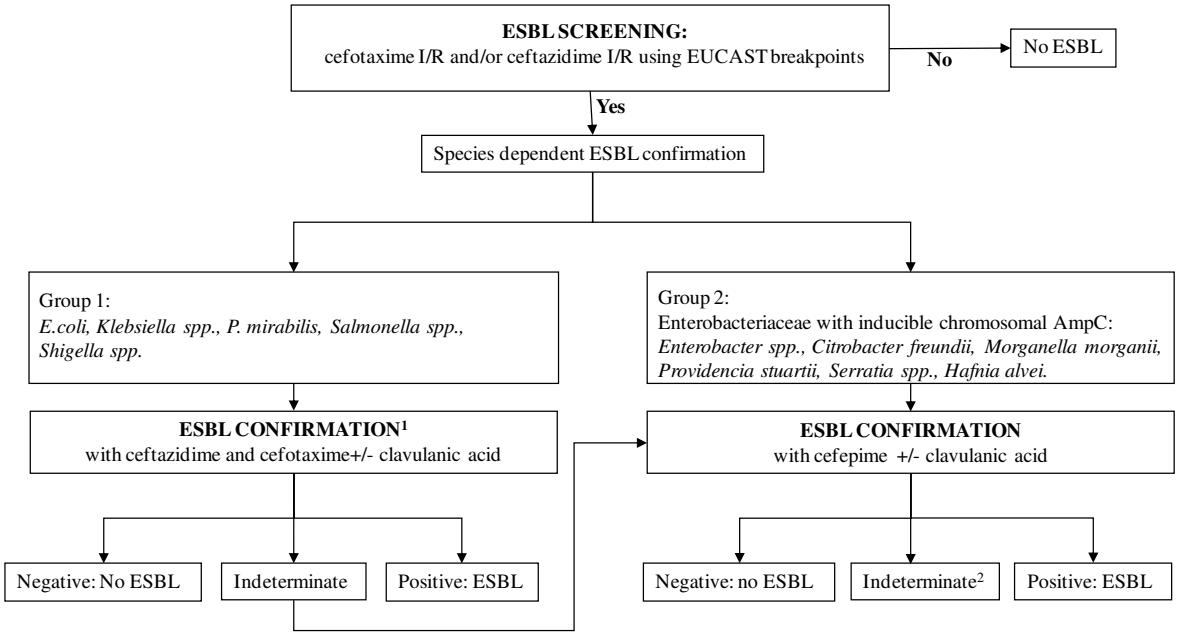
In many areas, ESBL detection and characterization is recommended or mandatory for infection control purposes. The recommended strategy for the detection of ESBL in Enterobacteriaceae is based on non-susceptibility to indicator oxyimino-cephalosporins, followed by phenotypic (and in some cases genotypic)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

A screening breakpoint of >1mg/L is recommended for both cefotaxime (and ceftriaxone) and ceftazidime, in accordance with the guidelines issued by EUCAST and CLSI (Table 1) (11, 12). The EUCAST clinical breakpoint for 'susceptible' Enterobacteriaceae is also S $\leq$ 1 mg/L (11). Corresponding zone diameters for the indicator cephalosporins are shown in Table 1.

Table 1. ESBL screening methods for Enterobacteriaceae (12-16).

Method	**Antibiotic**	**Conduct ESBL-testing if**
Broth dilution	cefotaxime	MIC > 1 mg/L
	ceftazidime	MIC > 1 mg/L
Agar dilution	cefotaxime	MIC > 1 mg/L
	ceftazidime	MIC > 1 mg/L
Disk diffusion	cefotaxime (5 µg)	Inhibition zone < 21 mm
	ceftriaxone (30 µg)	Inhibition zone < 23 mm
	ceftazidime (10 µg)	Inhibition zone < 22 mm
Automated systems	cefotaxime	MIC > 1 mg/L
	ceftazidime	MIC > 1 mg/L

Figure 1. Algorithm for phenotypic ESBL detection.



[1]If cefoxitin MIC > 8 mg/L, perform cefepime+/- clavulanic acid confirmation test
[2]Genotypic testing is required.

3.4.1 ESBL-testing in Enterobacteriaceae

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

The recommended methods for ESBL screening in group I Enterobacteriaceae are broth dilution, agar dilution, disk diffusion or an automated system, such as Microscan (Siemens), Phoenix (Becton-Dickinson), or VITEK 2 (bioMérieux) (Table 2) (12, 17, 18). It is recommended to use both cefotaxime (or ceftriaxone) and ceftazidime as indicator cephalosporins, as there may be large differences in MICs of cefotaxime (or ceftriaxone) and ceftazidime for different ESBL-producing isolates (13, 19, 20).

Cefpodoxime is the most sensitive indicator cephalosporin for detection of ESBL production may be used for screening. However, it is less specific than the combination of cefotaxime (or ceftriaxone) and ceftazidime (13) and only the latter compounds are used in the confirmation testing.

For automated systems the combination of indicator cephalosporins for ESBL screening is dependent on the choice of the manufacturer, but should be in accordance with the recommendations on indicator cephalosporins provided in this guideline.

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

For group II Enterobacteriaceae it is recommended that ESBL screening is performed according to the methods described above for group I *Enterobacteriaceae* (Figure 1 and Table 2) (18). However, the most common mechanism of resistance is derepressed chromosomal AmpC β-lactamase in these species.

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 Etest ESBL, and the broth microdilution test (Table 3) (17, 18, 21). The combination disk diffusion test showed a better specificity than the Etest ESBL and with comparable sensitivity in one multicentre study (22). According to the manufacturer's instructions for each test, a Mueller-Hinton agar plate is inoculated with a bacterial suspension (density of 0.5 McFarland) and the cephalosporin disks/tablets/strips are applied.

Manufacturers of automatic susceptibility testing systems have implemented detections tests based on the inhibition of ESBL enzymes by clavulanic acid. Results vary in different studies, depending on the collection of strains tested and the device used (14-16).

A. Combination disk test (CDT)

For each test disks containing cephalosporin alone (cefotaxime or ceftriaxone, ceftazidime, cefepime) and in combination with clavulanic acid are applied. The

inhibition zone around the cephalosporin disk/tablet combined with clavulanic acid is compared with the zone around the disk/tablet with the cephalosporin alone. The test is positive if the inhibition zone diameter is ≥ 5 mm larger with clavulanic acid than without (and the MIC for the isolate is >1 mg/L for the cephalosporin tested, i.e. ESBL screening is positive) (Table 3) (23, 24). In all other cases the test result is negative.

B. Double-disk synergy test (DDST)

Disks containing cephalosporins (cefotaxime or ceftriaxone, ceftazidime, cefepime) are applied next to a disk with clavulanic acid (amoxicillin-clavulanic acid or ticarcillin-clavulanic acid). Positive result is indicated when the inhibition zones around any of the cephalosporin disks are augmented 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 30µg disks (17). This might need to be re-evaluated for disks with lower cephalosporin content, as recommended by EUCAST.

C. Etest ESBL method

The Etest ESBL (bioMérieux) is applied, and the test is read according to the manufacturer's instructions. The test is positive if a ≥ 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 deformed ellipse is present (and the MIC of the cephalosporin tested is >1 mg/L, i.e. ESBL screening was positive) (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. The Etest ESBL MIC 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 Mueller-Hinton broth containing serial two-fold dilutions of cefotaxime (or ceftriaxone), 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. A bacterial suspension is inoculated into each well of the microtitre plate (21). The microtitre plate is incubated at 37°C for 18 to 24 hours. The test is positive if a $\geq$ 8-fold reduction is observed in the MIC of the cephalosporin combined with clavulanic acid compared with the MIC of the cephalosporin alone. In all other cases the test result is negative (21).

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 or OXY-like β-lactamases (25). A similar phenotype may also be encountered in *Proteus vulgaris, 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 (26, 27). 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 (15).

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

Method	**Antimicrobial agent (disk content)**	**Confirmation is positive if**
Etest ESBL	Cefotaxime +/- clavulanic acid	MIC ratio[1] ≥ 8 or deformed ellipse present
	Ceftazidime +/- clavulanic acid	MIC ratio[1] ≥ 8 or deformed ellipse present
Combination disk diffusion test (CDT)	Cefotaxime (30 µg) +/- clavulanic acid (10 µg)	≥ 5 mm increase in inhibition zone[2]
	Ceftazidime (30 µg) +/- clavulanic acid (10 µg)	≥ 5 mm increase in inhibition zone[2]
Broth microdilution	Cefotaxime +/- clavulanic acid (4 mg/L)	MIC ratio[1] ≥ 8
	Ceftazidime +/- clavulanic acid (4 mg/L)	MIC ratio[1] ≥ 8
	Cefepime +/- clavulanic acid (4 mg/L)	MIC ratio[1] ≥ 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 II Enterobacteriaceae (see Figure 1)

Method	Antibiotic	Confirmation is positive if
Etest ESBL	Cefepime +/- clavulanic acid	MIC ratio[1] ≥ 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 (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 masks the presence of ESBLs (17, 28, 29). Isolates with high-level expression of AmpC β-lactamases usually show clear resistance to third-generation cephalosporins, and also resistance to cephamycins, e.g. a cefoxitin MIC >8 mg/L, may be indicative of high-level expression of AmpC β-lactamases (28), with the exception of ACC β-lactamases (30).

To confirm presence of ESBLs in isolates with high-level expression of AmpC β-lactamases it is recommended that an additional ESBL confirmation test is performed with cefepime as the indicator cephalosporin, as cefepime is not hydrolyzed by AmpC β-lactamases. Cefepime may be used in all the CDT, DDST, Etest or broth dilution test formats. Alternative approaches include use of cloxacillin, which is a good inhibitor of

AmpC enzymes. Test formats are CDT with disks containing the two cephalosporin indicators (cefotaxime or ceftriaxone and ceftazidime) with clavulanic acid and cloxacillin together; and standard CDT or DDST on agar plates supplemented with 100-250mg/L cloxacillin (17).

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 (31, 32). The epidemiological importance of ESBLs in these contexts could be questioned, but if detection is still considered relevant it is recommended to use molecular methods for ESBL-detection.

It should be remembered that the class D (OXA-type) ESBLs are poorly inhibited by clavulanic acid and therefore cannot be detected by the methods described above (4, 17). These enzymes are rare in Enterobacteriaceae.

3.4.4 Genotypic confirmation

For the genotypic confirmation of the presence of ESBL genes it is recommended to use PCR and ESBL gene sequencing (3) or a DNA microarray-based method. Recent evaluations of the Check-KPC ESBL microarray (Check-Points, Wageningen, The Netherlands) using different collections of organisms covering the majority of known ESBL genes showed good performance (33-37). Test results are usually obtained within 24 hours. It should be noted that sporadically occurring ESBL genes and new ESBL genes are not detected by this microarray.

3.4.5 Quality control

Table 4. Appropriate strains for quality control of ESBL detection tests.

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

3.5 References

1. Bush K, Jacoby GA, Medeiros AA. A functional classification scheme for β -lactamases and its correlation with molecular structure. Antimicrob Agents Chemother. 1995;39:211-1233
2. Livermore DM. β-Lactamases in laboratory and clinical resistance. Clin Microbiol Rev. 1995;8:557-584
3. Bradford PA. Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin Microbiol Rev. 2001;14:933-951
4. Naas T, Poirel L, Nordmann P. 2008. Minor extended-spectrum β-lactamases. Clin Microbiol Infect. 2008;14(Suppl1):42-52
5. Cantón R, Novais A, Valverde A, Machado E, Peixe L, Baquero F, Coque TM. Prevalence and spread of extended-spectrum β-lactamase-producing Enterobacteriaceae in Europe. Clin Microbiol Infect. 2008;14(Suppl1):144-153
6. Livermore DM, Cantón R, Gniadkowski M, Nordmann P, Rossolini GM, Arlet G, Ayala J, Coque TM, Kern-Zdanowicz I, Luzzaro F, Poirel L, Woodford N. CTX-M: changing the face of ESBLs in Europe. J Antimicrob Chemother. 2007;59:165-174
7. Carattoli A. Animal reservoirs for extended-spectrum β-lactamase producers. Clin Microbiol Infect. 2008;14(Suppl1):117-123
8. European Centres for Disease Prevention and Control (ECDC). Antimicrobial resistance surveillance in Europe 2011. Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net)
9. Gniadkowski M. 2008. Evolution of extended-spectrum β-lactamases by mutation. Clin Microbiol Infect. 2008;14(Suppl1):11-32
10. Livermore DM. Defining an extended-spectrum β-lactamase. Clin Microbiol Infect. 2008;14(Suppl1):3-10
11. European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. EUCAST; 2013. Version 3.0 http://www.eucast.org/clinical_breakpoints/ (last accessed 23 December 2012).
12. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Twenty-first Informational Supplement M 100-S21. Wayne, PA, USA: CLSI; 2011.
13. Hope R, Potz NA, Warner M, Fagan EJ, Arnold E, Livermore DM. Efficacy of practised screening methods for detection of cephalosporin-resistant Enterobacteriaceae. J Antimicrob Chemother. 2007;59(1):110-3.
14. Leverstein-van Hall MA, Fluit AC, Paauw A, Box AT, Brisse S, Verhoef J. Evaluation of the Etest ESBL and the BD Phoenix, VITEK 1, and VITEK 2 automated instruments for detection of extended-

spectrum β-lactamases in multiresistant *Escherichia coli* and *Klebsiella* spp. J Clin Microbiol. 2002;40(10):3703-11.

15. Spanu T, Sanguinetti M, Tumbarello M, D'Inzeo T, Fiori B, Posteraro B, Santangelo R, Cauda R, Fadda G. Evaluation of the new VITEK 2 extended-spectrum β-lactamase (ESBL) test for rapid detection of ESBL production in Enterobacteriaceae isolates. J Clin Microbiol. 2006;44(9):3257-62.
16. Thomson KS, Cornish NE, Hong SG, Hemrick K, Herdt C, Moland ES. Comparison of Phoenix and VITEK 2 extended-spectrum-β-lactamase detection tests for analysis of *Escherichia coli* and *Klebsiella* isolates with well-characterized β-lactamases. J Clin Microbiol. 2007;45(8):2380-4.
17. Drieux L, Brossier F, Sougakoff W, Jarlier V. Phenotypic detection of extended-spectrum β-lactamase production in Enterobacteriaceae: review and bench guide. Clin Microbiol Infect. 2008;14 Suppl 1:90-103.
18. Paterson DL, Bonomo RA. Extended-spectrum β-lactamases: a clinical update. Clin Microbiol Rev. 2005;18(4):657-86
19. Biedenbach DJ, Toleman M, Walsh TR, Jones RN. Analysis of *Salmonella* spp. with resistance to extended-spectrum cephalosporins and fluoroquinolones isolated in North America and Latin America: report from the SENTRY Antimicrobial Surveillance Program (1997-2004). Diagn Microbiol Infect Dis. 2006;54(1):13-21.
20. Hirakata Y, Matsuda J, Miyazaki Y, Kamihira S, Kawakami S et al. Regional variation in the prevalence of extended-spectrum β-lactamase-producing clinical isolates in the Asia-Pacific region (SENTRY 1998-2002). Diagn Microbiol Infect Dis. 2005;52(4):323-9.
21. Jeong SH, Song W, Kim JS, Kim HS, Lee KM. Broth microdilution method to detect extended-spectrum β-lactamases and AmpC β-lactamases in Enterobacteriaceae isolates by use of clavulanic acid and boronic acid as inhibitors. J Clin Microbiol. 2009;47(11):3409-12.
22. Platteel TN, Cohen Stuart JW, de Neeling AJ, Voets GM, Scharringa J et al. Multi-centre evaluation of a phenotypic extended spectrum β-lactamase detection guideline in the routine setting. Clin Microbiol Infect. 2011. Epublished.
23. M'Zali FH, Chanawong A, Kerr KG, Birkenhead D, Hawkey PM. Detection of extended-spectrum β-lactamases in members of the family Enterobacteriaceae: comparison of the MAST DD test, the double disc and the Etest ESBL. J Antimicrob Chemother. 2000;45(6):881-5.
24. Towne TG, Lewis JS 2nd, Herrera M, Wickes B, Jorgensen JH. Detection of SHV-type extended-spectrum β-lactamase in *Enterobacter* isolates. J Clin Microbiol. 2010;48(1):298-9.
25. Stürenburg E, Sobottka I, Noor D, Laufs R, Mack D. Evaluation of a new cefepime-clavulanate ESBL Etest to detect extended-spectrum β-lactamases in an Enterobacteriaceae strain collection. J Antimicrob Chemother. 2004;54(1):134-8.
26. Nukaga M, Mayama K, Crichlow GV, Knox JR. Structure of an extended-spectrum class A β-lactamase from *Proteus vulgaris* K1. J Mol Biol. 2002;317(1):109-17.

27. Petrella S, Renard M, Ziental-Gelus N, Clermont D, Jarlier V, Sougakoff W. Characterization of the chromosomal class A β-lactamase CKO from *Citrobacter koseri*. FEMS Microbiol Lett. 2006 Jan;254(2):285-92.
28. Jacoby GA. AmpC β-lactamases. Clin Microbiol Rev. 2009 Jan;22(1):161-82
29. Munier GK, Johnson CL, Snyder JW, Moland ES, Hanson ND, Thomson KS. Positive extended-spectrum-β-lactamase (ESBL) screening results may be due to AmpC β-lactamases more often than to ESBLs. J Clin Microbiol. 2010;48(2):673-4.
30. Bauernfeind A, Schneider I, Jungwirth R, Sahly H, Ullmann U. A novel type of AmpC β-lactamase, ACC-1, produced by a *Klebsiella pneumoniae* strain causing nosocomial pneumonia. Antimicrob Agents Chemother. 1999;43(8):1924-31.
31. Tsakris A, Poulou A, Themeli-Digalaki K, Voulgari E, Pittaras T, Sofianou D, Pournaras S, Petropoulou D. Use of boronic acid disk tests to detect extended- spectrum β-lactamases in clinical isolates of KPC carbapenemase-possessing Enterobacteriaceae. J Clin Microbiol. 2009;47(11):3420-6.
32. March A, Aschbacher R, Dhanji H, Livermore DM, Böttcher A et al. Colonization of residents and staff of a long-term-care facility and adjacent acute-care hospital geriatric unit by multiresistant bacteria. Clin Microbiol Infect. 2010 Jul;16(7):934-44.
33. Cohen Stuart J, Dierikx C, Al Naiemi N, Karczmarek A, Van Hoek AH et al. Rapid detection of TEM, SHV and CTX-M extended-spectrum β-lactamases in Enterobacteriaceae using ligation-mediated amplification with microarray analysis. J Antimicrob Chemother. 2010;65(7):1377-81
34. Endimiani A, Hujer AM, Hujer KM, Gatta JA, Schriver AC et al. Evaluation of a commercial microarray system for detection of SHV-, TEM-, CTX-M-, and KPC-type β-lactamase genes in Gram-negative isolates. J Clin Microbiol. 2010;48(7):2618-22.
35. Naas T, Cuzon G, Truong H, Bernabeu S, Nordmann P. Evaluation of a DNA microarray, the Check-Points ESBL/KPC array, for rapid detection of TEM, SHV, and CTX-M extended-spectrum β-lactamases and KPC carbapenemases. Antimicrob Agents Chemother. 2010;54(8):3086-92.
36. Platteel TN, Stuart JW, Voets GM, Scharringa J, van de Sande N et al. Evaluation of a commercial microarray as a confirmation test for the presence of extended-spectrum β-lactamases in isolates from the routine clinical setting. Clin Microbiol Infect. 2011;17(9):1435-8.
37. Willemsen I, Overdevest I, Al Naiemi N, Rijnsburger M, Savelkoul P et al. New diagnostic microarray (Check-KPC ESBL) for detection and identification of extended-spectrum β-lactamases in highly resistant Enterobacteriaceae. J Clin Microbiol. 2011;49(8):2985-7.

3. Acquired AmpC β-lactamase-producing Enterobacteriaceae

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

4.1 Definition

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

4.2 Clinical and/or epidemiological importance

The first isolates producing acquired AmpCs were identified at the end of 1980s, and since then they have been observed globally as a result of clonal spread, horizontal transfer of AmpC genes, and their emergence *de novo*. 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 (ABA). 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 the acquired AmpCs are *E. coli*, *K. pneumoniae*, *Klebsiella oxytoca*, *Salmonella enterica* and *Proteus 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. 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 (*e. g. Enterobacter* spp., group *Citrobacter freundii*, *Morganella morganii*, *E. coli*) or other Gram-negatives (*e.g. P. aeruginosa*) produce natural AmpCs, either constitutively at the trace level (*e.g. E. coli*) or inducibly (*e.g. E. cloacae*, *C. freundii*, *M. morganii*, *P. aeruginosa*). The derepression or hyperproduction of natural AmpCs that are due to various genetic events and that confer resistance to their substrates and inhibitor combinations are highly relevant. The class C cephalosporinases have been observed also as acquired enzymes, mainly in enterobacteria. Except for a few inducible types (*e.g.* DHA), 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 AmpCs are usually encoded by plasmid-mediated genes (1-3).

4.4 Recommended methods for detection of acquired AmpC in Enterobacteriaceae

A cefoxitin MIC >8 mg/L combined with a ceftazidime and/or cefotaxime MIC >1mg/L 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).

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. Although data evaluating these methods is sparse, reasonably accurate detection with in-house methods has been described (7-9) as

well as with commercially available tests such as the Mast AmpC Detection Disc Set (sensitivity 96-100%, specificity 98%-100%) (10, 11), the AmpC Etest (sensitivity 84-93%, specificity 70-100%) (11, 12) and Rosco tablets with cefotaxime/cloxacillin and ceftazidime/cloxacillin (sensitivity 96%, specificity 92%) (13). For *E. coli* however, AmpC confirmation tests cannot discriminate between acquired AmpC and constitutive hyperproduction of the chromosomal AmpC.

The presence of acquired AmpCs may also be confirmed using PCR-based methods (14, 15), or with a DNA microarray-based method (Check-Points, Wageningen, The Netherlands) (16).

Table 1. Appropriate control strains for detection of AmpC.

Strain	**Mechanism**
Escherichia coli CCUG 58543	Acquired CMY-2 AmpC
Escherichia coli CCUG62975	Acquired CMY AmpC and CTX-M-1 group ESBL
E. coli ATCC 25922	AmpC negative.

4.5 References

1. Jacoby GA. AmpC beta-lactamases. Clin Microbiol Rev. 2009 Jan;22(1):161-82
2. Philippon A, Arlet G, Jacoby GA. Plasmid-determined AmpC-type β-lactamases. Antimicrob Agents Chemother. 2002;46:1-11
3. Beceiro A, Bou G. Class C β-lactamases: an increasing problem worldwide. Rev Med Microbiol. 2004;15:141-152
4. Empel J, Hrabák J, Kozińska A, Bergerová T, Urbášková P, Kern-Zdanowicz I, Gniadkowski M. DHA-1-producing *Klebsiella pneumoniae* in a teaching hospital in the Czech Republic. Microb Drug Resist. 2010;16:291-295
5. D'Andrea MM, Literacka E, Zioga A, Giani T, Baraniak A, Fiett J, Sadowy E, Tassios PT, Rossolini GM, Gniadkowski M, Miriagou V. Evolution of a multi-drug-resistant *Proteus mirabilis* clone with chromosomal AmpC-type cephalosporinases spreading in Europe. Antimicrob Agents Chemother. 2011;55:2735-2742

6. Bauernfeind A, Schneider I, Jungwirth R, Sahly H, Ullmann U. A novel type of AmpC β-lactamase, ACC-1, produced by a *Klebsiella pneumoniae* strain causing nosocomial pneumonia. Antimicrob Agents Chemother. 1999;43(8):1924-31.
7. Yagi T, Wachino J, Kurokawa H, Suzuki S, Yamane K et al. Practical methods using boronic acid compounds for identification of class C beta-lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli*. J Clin Microbiol. 2005;43(6):2551-8.
8. Tenover FC, Emery SL, Spiegel CA, Bradford PA, Eells S et al. Identification of plasmid-mediated AmpC β-lactamases in *Escherichia coli*, *Klebsiella* spp., and *Proteus* spp. can potentially improve reporting of cephalosporin susceptibility testing results. J Clin Microbiol. 2009;47(2):294-9.
9. Tan TY, Ng LS, He J, Koh TH, Hsu LY. Evaluation of screening methods to detect plasmid-mediated AmpC in *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*. Antimicrob Agents Chemother. 2009;53(1):146-9
10. Halstead FD, Vanstone GL, Balakrishnan I. An evaluation of the Mast D69C AmpC Detection Disc Set for the detection of inducible and derepressed AmpC β-lactamases. J Antimicrob Chemother. 2012;67(9):2303-4.
11. Ingram PR, Inglis TJ, Vanzetti TR, Henderson BA, Harnett GB, Murray RJ. Comparison of methods for AmpC β-lactamase detection in Enterobacteriaceae. J Med Microbiol. 2011;60(Pt 6):715-21.
12. Peter-Getzlaff S, Polsfuss S, Poledica M, Hombach M, Giger J et al. Detection of AmpC β-lactamase in *Escherichia coli*: comparison of three phenotypic confirmation assays and genetic analysis. J Clin Microbiol. 2011;49(8):2924-32.
13. Hansen F, Hammerum AM, Skov RL, Giske CG, Sundsfjord A, Samuelsen O. Evaluation of ROSCO Neo-Sensitabs for phenotypic detection and subgrouping of ESBL-, AmpC- and carbapenemase-producing Enterobacteriaceae. APMIS. 2012;120(9):724-32.
14. Pérez-Pérez FJ, Hanson ND. Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. J Clin Microbiol. 2002;40(6):2153-62.
15. Brolund A, Wisell KT, Edquist PJ, Elfström L, Walder M, Giske CG. Development of a real-time SYBRGreen PCR assay for rapid detection of acquired AmpC in Enterobacteriaceae. J Microbiol Methods. 2010 Sep;82(3):229-33.
16. Cuzon G, Naas T, Bogaerts P, Glupczynski Y, Nordmann P. Evaluation of a DNA microarray for the rapid detection of extended-spectrum β-lactamases (TEM, SHV and CTX-M), plasmid-mediated cephalosporinases (CMY-2-like, DHA, FOX, ACC-1, ACT/MIR and CMY-1-like/MOX) and carbapenemases (KPC, OXA-48, VIM, IMP and NDM). J Antimicrob Chemother. 2012;67(8):1865-9.

5. Methicillin resistant *Staphylococcus aureus* (MRSA)

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

5.1 Definition

S. aureus isolates with an auxiliary penicillin binding protein (PBP2a or the recently discovered PBP2c) for which β-lactam agents, except for the novel class of cephalosporins having anti-MRSA activity, have low affinity.

5.2 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 doubled compared to similar infections caused by methicillin susceptible strains due to delayed adequate treatment and inferior alternative regimens (1,2). MRSA infections are endemic in both hospitals and the community in all parts of the world.

5.3 Mechanism of resistance

The main mechanism of resistance is production of an auxillary penicillin binding protein, PBP2a or the recently discovered PBP2c, which render the isolate resistant to all β-lactams except for the novel class of cephalosporins, which have sufficiently high affinity to PBP2a and probably also PBP2c to be active against MRSA (3). The auxillary PBPs are encoded by the *mecA* gene or the recently described *mecC* (formerly known as $mecA_{LGA251}$) (4) respectively. The *mec* element is foreign to *S. aureus* and is not present in methicillin susceptible *S. aureus.* Strains with heterogeneous expression of the mecA gene and therefore low MICs to oxacillin hamper the accuracy of susceptibility testing (5). Furthermore, some isolates express low-level resistance but are *mecA* negative and do not produce alternative PBPs (borderline susceptible *S. aureus* (BORSA)). These strains are relatively rare. The

mechanism of resistance in these isolates is often poorly characterized but may include hyperproduction of β-lactamases or alteration of the pre-existing PBPs (5).

5.4 Recommended methods for detection

Methicillin/oxacillin resistance can be detected both phenotypically by MIC-testing, disk diffusion testing or latex agglutination, or genotypically using PCR.

5.4.1 Detection with MIC determination or disk diffusion

The heterogeneous expression of resistance particularly affects MICs of oxacillin. Cefoxitin is a very sensitive and specific marker of *mecA/mecC* mediated methicillin resistance and is the substance of choice for disk diffusion. Disk diffusion using oxacillin is discouraged and interpretive zone diameters are no longer presented in the EUCAST breakpoint table. Strains with increased MIC of oxacillin (MIC >2 mg/L) but susceptible to cefoxitin (zone diameter ≥ 22 mm, MIC ≤ 4 mg/L) are uncommon. If oxacillin is tested and gives a different interpretation than with cefoxitin the interpretation should be as shown below. It is recommended to subject such strains to phenotypic or genotypic investigations of MecA or MecC.

Table 1. Interpretation when oxacillin and cefoxitin results are discrepant.

	Cefoxitin MIC or disk diffusion	
	S	R
Oxacillin MIC R	Report as R	Report as R
Oxacillin MIC S	Report as S	Report as R

A. Broth microdilution:

Standard methodology (ISO 20776-1) is used with cefoxitin in two-fold dilutions in cation-supplemented Mueller-Hinton broth. Trays are incubated for 18 h ± 2 h at a maximum of 35°C. Stains with MICs > 4 mg/L should be regarded methicillin resistant.

Quality Control: *S. aureus* ATCC 29213 (methicillin susceptible) is included at least daily and whenever a new batch/lot of antibiotic or reagents is used. One well for each strain is incubated without addition of antibiotics and one well in each plate should include neither antibiotics nor bacteria.

B. Disk diffusion**:** The EUCAST disk diffusion method is used. The inoculated agar is incubated at maximum 35°C in ambient air for 18 ± 2 h. Strains with a cefoxitin (30 µg) zone diameter <22 mm should be regarded as methicillin resistant.

Quality Control: *S. aureus* ATCC 29213 (methicillin susceptible) is included daily at least until performance has been shown to be adequate and then at least weekly and whenever a new batch/lot of antibiotic or reagents is used.

5.4.2 Detection with genotypic and latex agglutination methods

Genotypic detection of the *mecA* gene by PCR and detection of the PBP2a protein using latex agglutination kits is possible using commercial or in-house assays, but it should be noted that these methods are neither 100 % sensitive nor 100% specific. Moreover, *mecC* and PBP2c can at present not be detected using commercially available genotypic methods. Primers and methods for detection of *mecC* have recently been published (6, 7).

5.4.3 Control strains

Table 2. Appropriate control strains for testing of methicillin susceptibility.

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

5.5 References

1. Cosgrove SE, Sakoulas G, Perencevich EN, Schwaber MJ, Karchmer AW, Carmeli Y. Comparison of mortality associated with methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* bacteremia: a meta-analysis. Clin Infect Dis. 2003;36(1):53-9.
2. de Kraker ME, Wolkewitz M, Davey PG, Koller W, Berger J, et al. Clinical impact of antimicrobial resistance in European hospitals: excess mortality and length of hospital stay related to methicillin-resistant *Staphylococcus aureus* bloodstream infections. Antimicrob Agents Chemother. 2011;55(4):1598-605.
3. Chambers HF, Deleo FR. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat Rev Microbiol. 2009;7(9):629-41.
4. de Kraker ME, Davey PG, Grundmann H; BURDEN study group. Mortality and hospital stay associated with resistant *Staphylococcus aureus* and *Escherichia coli* bacteremia: estimating the burden of antibiotic resistance in Europe. PLoS Med. 2011;8(10):e1001104.
5. García-Álvarez L, Holden MT, Lindsay H, Webb CR, Brown DF, et al. Meticillin-resistant *Staphylococcus aureus* with a novel *mecA* homologue in human and bovine populations in the UK and Denmark: a descriptive study. Lancet Infect Dis. 2011;11(8):595-603
6. Chambers HF. Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. Clin Microbiol Rev. 1997;10(4):781-91.
7. Stegger M, Andersen PS, Kearns A, Pichon B, Holmes MA, Edwards G, Laurent F, Teale C, Skov R, Larsen AR. Rapid detection, differentiation and typing of methicillin-resistant *Staphylococcus aureus* harbouring either *mecA* or the new *mecA* homologue $mecA_{LGA251}$. Clin Microbiol Infect. 2012; 4:395-400.
8. Pichon B, Hill R, Laurent F, Larsen AR, Skov RL, Holmes M, Edwards GF, Teale C, Kearns AM. Development of a real-time quadruplex PCR assay for simultaneous detection of *nuc*, Panton-Valentine leucocidin (PVL), *mecA* and homologue $mecA_{LGA251}$. J Antimicrob Chemother. 2012; 67:2338-41.

6. Glycopeptide non-susceptible *Staphylococcus aureus*

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

6.1 Definition

GRSA: Glycopeptide resistant *S. aureus*

S. aureus isolates with high-level resistance to vancomycin (MIC > 8 mg/L).

GISA: glycopeptide intermediate *S. aureus*

S. aureus isolates with low-level resistance to vancomycin (MIC 4 - 8 mg/L).

hGISA: Heterogeneous glycopeptide 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.

6.2 Mechanism of resistance

For GRSA the resistance is mediated by the *vanA* gene exogenously acquired from enterococci. For both GISA and hGISA isolates the resistance is endogenous and the mechanism highly complex with no single gene being responsible. The GISA/hGISA phenotype is linked to a thickening of the bacterial cell wall with hyperproduction of glycopeptide binding targets. The hGISA phenotype is often unstable in the laboratory but hGISA have the capacity to develop into GISA *in vivo* (8).

6.3 Clinical and/or epidemiological importance

There are no recent investigations of the prevalence of isolates with reduced susceptibility to glycopeptides across Europe. Based on reports from single

institutions it is estimated that the prevalence of hGISA is ≤ 2% of MRSA in Europe, with GISA below 0.1% (1). GRSA has not yet been reported in Europe. The prevalence of hGISA may be considerably higher locally (1), most often associated with spread of specific clonal lineages (2). Almost all isolates with elevated MIC (GISA) or containing resistant subpopulations (hGISA) are MRSA.

The clinical significance of hGISA has been difficult to determine as no well-controlled prospective studies have been performed. However, presence of the hGISA phenotype is believed to be associated with poorer outcome at least in serious infections (1, 2). It is therefore prudent to detect hGISA in clinically relevant scenarios as determined locally. Recently there has been increasing evidence that even hGISA isolates with MICs in the upper susceptible range (MIC 1 - 2 mg/L) are associated with poorer outcome and may be linked to increased mortality, at least in bloodstream infections (2,3, 4, 5, 6, 7). It is still uncertain whether the presence of resistant subpopulations is responsible for the poorer outcome, as it could also depend on the slightly elevated vancomycin MICs observed in these strains.

6.4 Recommended methods for detection

Disk diffusion can **NOT** be used to test for either hGISA or GISA.

6.4.1 MIC determination

Broth microdilution using methodology recommended by EUCAST (ISO 20776-1) is the gold standard, but MICs may also be determined with gradient strips, agar dilution or automated systems. It should be noted that the results with gradient strip methods may be 0.5 - 1 twofold dilution steps higher than the results obtained by broth microdilution (7). The EUCAST breakpoint for resistance to vancomycin in *S. aureus* is MIC > 2 mg/L.

6.4.2 Specific tests for hGISA

GISA are detected by determining the MIC, but this is not the case for hGISA. Detection of hGISA has proven difficult and detection is therefore divided into screening and confirmation. For screening a number of specialised methods have been developed.

When choosing a screening method it is important to use a method with a high negative predictive value to ensure a low number of false negatives. The macro gradient test and the glycopeptide resistance detection (GRD) gradient test are the best tests for screening for hGISA (1). Confirmation is by analysing the population profile of the isolate on agar plates containing a range of vancomycin concentrations (PAP-AUC) (9). This method is technically challenging without extensive experience and consequently is mostly performed by reference laboratories.

A. Macro gradient test:

Please note that the test merely gives an indication of reduced vancomycin susceptibility and that the readings are not MICs. Furthermore, the test does not differentiate between hGISA and GISA.

Inoculate a 90 mm Brain Heart Infusion (BHI) agar plate evenly with 0.1mL of a 2 McFarland suspension. Apply vancomycin and teicoplanin gradient strips and incubate at 35 °C in ambient air and read at 24h and 48h. The macro gradient test has been widely evaluated with Etests.Evaluations of the test with other commercial gradient strips are not available. Read endpoints at the point of complete inhibition, tilting the plate to look for hazes, microcolonies and isolated colonies with the aid of a magnifying glass and oblique light. A positive result is indicated by readings ≥ 8mg/L for both vancomycin and teicoplanin, OR ≥ 12mg/L to teicoplanin alone.

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

- Teicoplanin ≥ 12 mg/L: GISA or hGISA

- Teicoplanin 8 – 12 mg/L: Test vancomycin
- Teicoplanin < 8mg/L: No GISA or hGISA

B. Glycopeptide resistance detection (GRD) gradient test:

Test according to the manufacturers' instructions. A 0.5 McFarland inoculum is swabbed onto MHA with 5% sheep blood. The ellipse of inhibition should be read at 24 and 48 h after incubation at 35°C. The test isolate is considered positive if the GRD strip result is ≥8mg/L for either vancomycin or teicoplanin.

C. Confirmatory testing for hGISA/GISA:

Any isolate screening positive for hGISA should be investigated by population analysis profile area under curve (PAP-AUC) (8), typically by referral to a reference laboratory.

6.4.3 Control strains

Table 2. Appropriate control strains for testing of glycopeptide susceptibility.

Strain	Mechanism
S. aureus ATCC 29213	Glycopeptide susceptible
S. aureus Mu3	hGISA
S. aureus Mu50	GISA

6.5 References

1. Howden BP, Davies JK, Johnson PDR, Stinear TP, Grayson ML. Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection and clinical implications. Clin Microbiol Rev 2010;1: 99-139
2. Van Hal SJ, Lodise TP, Paterson DL. The clinical significance of vancomycin minimum inhibitory concentration in *Staphylococcus aureus* infections: a systematic review and meta-analysis. Clinical Infectious Diseases 2012; 54: 755-771.
3. Chang HJ, Hsu PC, Yang CC, Siu LK, Kuo AJ, et al. Influence of teicoplanin MICs on treatment outcomes among patients with teicoplanin-treated methicillin resistant *Staphylococcus aureus*

bacteraemia: a hospital based retrospective study. J. Antimicrob Chemother 2012, Vol 67(3):736-41.

4. Honda H, Doern CD, Michael-Dunne W Jr, Warren DK. The impact of vancomycin susceptibility on treatment outcomes among patients with methicillin resistant *Staphylococcus aureus* bacteremia. BMC Infect Dis. 2011; 5:11:335.
5. Lodise TP, Graves J, Evans A, Graffunder E, Helmecke M, et al. Relationship between vancomycin MIC and failure among patients with methicillin-resistant *Staphylococcus aureus* bacteremia treated with vancomycin. *Antimicrob Agents Chemother* 2008; 52*:* 3315-20
6. Rojas L, Bunsow E, Munoz P, Cercenado E, Rodrigueuz-Creixems, Bouza E. Vancomycin MICs do not predict the outcome of methicillin-resistant *Staphylococcus aureus* bloodstream infections in correctly treated patients. J. Antimicrob Chemother 2012; 7: 1760-8.
7. Sader HS, Jones RN, Rossi KL, Rybak MJ. Occurrence of vancomycin tolerant and heterogeneous vancomycin resistant strains (hVISA) among *Staphylococcus aureus* causing bloodstream infections in nine USA hospitals. Antimicrob Chemother. 2009; 64: 1024-8.
8. Cui L, Ma X, Sato K, Okuma K, Tenover F C, et al. Cell wall thickening is a common feature of vancomycin resistance in *Staphylococcus aureus.* J Clin Microbiol. 2003; 41: 5-14.
9. Wootton M, Howe RA, Hillman R, Walsh TR, Bennett PM, MacGowan AP. A modified population analysis profile (PAP) method to detect hetero-resistance to vancomycin in *Staphylococcus aureus* in a UK hospital. J Antimicrob Chemother. 2001; 47: 399-403

7. Detection of vancomycin resistance in *Enterococcus faecalis* and *Enterococcus faecium*

Importance of detection of resistance	
Required for antimicrobial susceptibility categorization	Yes
Infection control/public health	Yes
Public health	Yes

7.1 Definition

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

7.2 Mechanism of resistance

Clinically relevant resistance is most often mediated by plasmid-encoded VanA and VanB ligases that confer replacement of D-Ala in the peptidoglycan with D-Lac. This substitution reduces the binding of glycopeptides to the target. VanA strains exhibit resistance to both vancomycin and teicoplanin, whereas VanB strains usually remain susceptible to teicoplanin due to lack of induction of the resistance operon. Other Van enzymes of lower clinical significance are VanD, VanE, VanG, VanL, VanM and VanN (1-4).

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) and should not be considered important from an infection control point of view (5).

7.3 Clinical and/or epidemiological importance

Enterococci, and especially *E. faecium*, are generally resistant to most clinically available antimicrobials. Treatment of VRE infections is therefore difficult, with few treatment options. Vancomycin resistant enterococci are known to spread efficiently in the hospital environment, and can colonize a very high number of individuals of which only a few may develop enterococcal infections (6, 7). 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 (8, 9), but reports of clinical failures are lacking and the current EUCAST recommendation is to report the result for teicoplanin as found. Typical MIC-values for the clinically most important Van enzymes are shown in Table 1.

Table 1. Typical MICsof glycopeptides for isolates harbouring VanA and VanB.

Glycopeptides	**MIC-values**	
	VanA	**VanB**
Vancomycin	64-1,024 mg/L	4-1,024mg/L
Teicoplanin	16-512mg/L	0.06-1mg/L

7.4 Recommended methods for detection

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 highly accurate but is seldom used in routine laboratories. Reports show that detection of *vanB*-mediated resistance is problematic for automated methods (10-12). Disk diffusion with a 5µg vancomycin disk performs well provided the guidelines for reading as specified by EUCAST are followed meticulously.

When interpreting the MIC/disk results it is important to ensure that the isolate is not *E. gallinarum* or *E. casseliflavus,* which may be erroneously perceived as *E. faecium* due to a positive arabinose test. 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). MALDI TOF mass spectrometry is also useful for species identification in enterococci (13).

7.4.1 MIC determination

MIC determination may be performed by agar dilution, broth microdilution or gradient strips. EUCAST guidelines should be followed for broth microdilution and the manufacturer's guidelines should be followed for gradient strips.

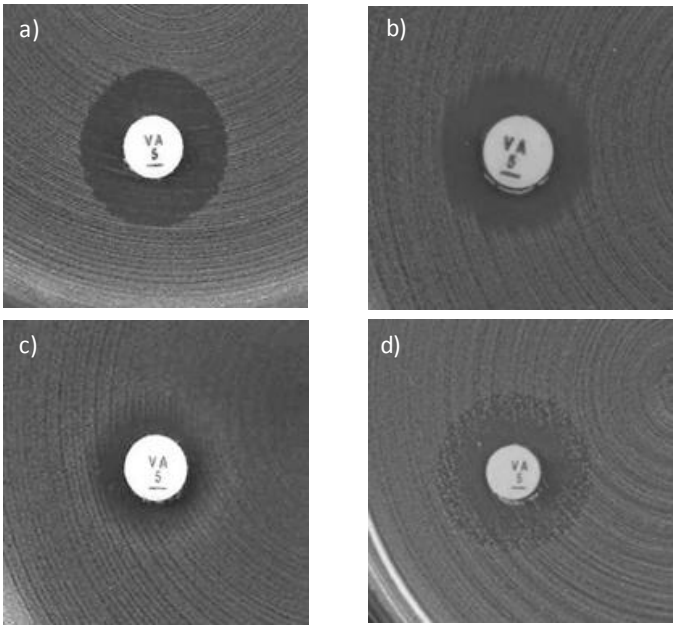
- Broth microdilution is performed according to the ISO standard 20776-1, 2006 with cation-supplemented MH broth for non-fastidious organisms (and a final inoculum of 5×10^5 cfu/ml. Sealed broth microdilution plates are incubated at 35±1°C in ambient air for a full 24 h.
- MIC determination with gradient tests is performed using a 0.5 McFarland inoculum on MH agar according to the manufacturer's instructions. Please note that MIC gradient strips are sometimes used with a higher inoculum (2 McFarland) on a rich medium (Brain Heart Infusion agar) to screen for vancomycin resistance but this analysis does not provide an MIC value.

7.4.2 Disk diffusion testing

For disk diffusion the guidelines specified by EUCAST have to 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 may be resistant and should regardless of zone size not be reported as susceptible without confirmation by MIC determination (Figure 1).

- Disk diffusion is performed on MH agar according to the EUCAST disk diffusion methodology for non-fastidious organisms. Incubation for 24 h is needed in order to detect isolates with inducible resistance.

Figure 1. Reading examples for the combination *Enterococcus* spp. and vancomycin.



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

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

7.4.3 Breakpoint agars

Breakpoint agar tests with Brain Heart Infusion agar and 6 mg/l vancomycin are reliable for detection of *vanB* positive isolates. Breakpoint plates can be obtained from commercial manufacturers or made in-house. Quality control is particularly important with plates 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 isolates with inducible resistance. Growth of more than one colony is scored as a positive test.

7.4.4 Genotypic testing

Vancomycin-resistance by the use of PCR targeting *vanA* and *vanB* can also be performed using in-house or commercial methodologies (14-16).

7.4.5 Quality control

E. faecalis ATCC 29212 (wild-type) and *E. faecalis* ATCC 51299 (VanB) are included whenever a new batch/lot of antibiotics or reagents is used. Routine quality control is performed daily (or weakly when accuracy of test is sufficiently documented) or every time the test is set up.

Table 2. Appropriate control strains for testing of vancomycin susceptibility.

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

7.5 References

1. Depardieu F, Perichon B, Courvalin P. Detection of the *van* alphabet and identification of enterococci and staphylococci at the species level by multiplex PCR. J Clin Microbiol. 2004;42(12):5857-60.
2. Boyd DA, Willey BM, Fawcett D, Gillani N, Mulvey MR. Molecular characterization of *Enterococcus faecalis* N06-0364 with low-level vancomycin resistance harboring a novel D-Ala-D-Ser gene cluster, *vanL*. Antimicrob Agents Chemother. 2008;52(7):2667-72.
3. Xu X, Lin D, Yan G, Ye X, Wu S, Guo Y, Zhu D, Hu F, Zhang Y, Wang F, Jacoby GA, Wang M. *vanM*, a new glycopeptide resistance gene cluster found in *Enterococcus faecium*. Antimicrob Agents Chemother. 2010;54(11):4643-7.
4. Lebreton F, Depardieu F, Bourdon N, Fines-Guyon M, Berger P, Camiade S, Leclercq R, Courvalin P, Cattoir V. D-Ala-d-Ser VanN-type transferable vancomycin resistance in *Enterococcus faecium*. Antimicrob Agents Chemother. 2011;55(10):4606-12.
5. Ramotar K, Woods W, Larocque L, Toye B. Comparison of phenotypic methods to identify enterococci intrinsically resistant to vancomycin (VanC VRE). Diagn Microbiol Infect Dis. 2000;36(2):119-24.

6. Mazuski JE. Vancomycin-resistant enterococcus: risk factors, surveillance, infections, and treatment. Surg Infect. 2008;9(6):567-71.
7. Tenover FC, McDonald LC. Vancomycin-resistant staphylococci and enterococci: epidemiology and control. Curr Opin Infect Dis. 2005;18(4):300-5.
8. Hayden MK, Trenholme GM, Schultz JE, Sahm DF. In vivo development of teicoplanin resistance in a VanB *Enterococcus faecium* isolate. J Infect Dis. 1993;167:1224-7.
9. Kawalec M, Gniadkowski M, Kedzierska J, Skotnicki A, Fiett J, Hryniewicz W. Selection of a teicoplanin-resistant *Enterococcus faecium* mutant during an outbreak caused by vancomycin-resistant enterococci with the *vanB* phenotype. J Clin Microbiol. 2001;39(12):4274-82.
10. Swenson JM, Clark NC, Sahm DF, Ferraro MJ, Doern G, Hindler J, Jorgensen JH, Pfaller MA, Reller LB, Weinstein MP, et al. Molecular characterization and multilaboratory evaluation of *Enterococcus faecalis* ATCC 51299 for quality control of screening tests for vancomycin and high-level aminoglycoside resistance in enterococci. J Clin Microbiol. 1995;33(11):3019-21.
11. Endtz HP, Van Den Braak N, Van Belkum A, Goessens WH, Kreft D, Stroebel AB, Verbrugh HA. Comparison of eight methods to detect vancomycin resistance in enterococci. J Clin Microbiol. 1998;36(2):592-4.
12. Klare I, Fleige C, Geringer U, Witte W, Werner G. Performance of three chromogenic VRE screening agars, two Etest vancomycin protocols, and different microdilution methods in detecting *vanB* genotype *Enterococcus faecium* with varying vancomycin MICs. Diagn Microbiol Infect Dis. 2012;74(2):171-6.
13. Dutka-Malen S, Evers S, Courvalin P. Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. J Clin Microbiol. 1995;33(5):1434.
14. Fang H, Ohlsson AK, Ullberg M, Özenci V. Evaluation of species-specific PCR, Bruker MS, VITEK MS and the VITEK2 system for the identification of clinical *Enterococcus* isolates. Eur J Clin Microbiol Infect Dis. 2012; 31: 3073-7
15. Dahl KH, Simonsen GS, Olsvik O, Sundsfjord A. Heterogeneity in the *vanB* gene cluster of genomically diverse clinical strains of vancomycin-resistant enterococci. Antimicrob Agents Chemother. 1999;43(5):1105-10.
16. Gazin M, Lammens C, Goossens H, Malhotra-Kumar S; MOSAR WP2 Study Team. Evaluation of GeneOhm VanR and Xpert *vanA*/*vanB* molecular assays for the rapid detection of vancomycin-resistant enterococci. Eur J Clin Microbiol Infect Dis. 2012;31(3):273-6.

8. Penicillin non-susceptible *Streptococcus pneumoniae*

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

8.1 Definition

S. pneumoniae isolates with reduced susceptibility (MICs above those of the wild-type) to penicillin due to the presence of modified penicillin binding proteins (PBPs) with lower affinity to β-lactams.

8.2 Clinical and/or epidemiological importance

S. pneumoniae contains six PBPs, of which PBP 2x is the primary target of penicillin (1). The presence of "mosaic genes" encoding low-affinity PBPs is the result of horizontal gene transfer from commensal viridans streptococci (1). The level of β-lactam resistance depends not only on number of low-affinity mosaic PBPs present in the isolate but also on modification of the specific PBPs which are essential for *S. pneumoniae* (2). Strains with MICs of benzylpenicillin in the range ≥0.12 to 2 mg/l are considered susceptible in non-CNS infections when a higher dose of penicillin is used, whereas for meningitis such strains must always be reported as resistant (3).

8.3 Recommended methods for detection

Penicillin non-susceptibility can be detected phenotypically by MIC or disk diffusion methods. The disk diffusion method with 1µg oxacillin disks is an effective screening method for the detection of penicillin non-susceptible pneumococci (4, 5, 6). The method is very sensitive but is not highly specific as strains with zone diameters of ≤19 mm may have variable susceptibility to penicillin, and benzylpenicillin MIC should be determined for all isolates that are non-susceptible with the screening method (6).

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

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

Zone diameter (mm) with oxacillin (1µg)	**Antimicrobial agents**	**Further testing and/or interpretation**
≥ 20 mm	All β -lactam agents for which clinical breakpoints are listed (including those with "Note")	Report susceptible irrespective of clinical indication.
< 20 mm*	Benzylpenicillin (meningitis) and phenoxymethylpenicillin (all indications)	Report resistant.
	Ampicillin and amoxicillin (with and without β -lactamase inhibitor), cefotaxime, ceftriaxone and cefepime.	Oxacillin zone diameter ≥ 8 mm: Report susceptible.
		Oxacillin zone diameter < 8 mm: determine the MIC of the β -lactam agent intended for clinical use but for ampicillin, amoxicillin and piperacillin (without and with β -lactamase inhibitor) infer susceptibility from the MIC of ampicillin.
	Other β -lactam agents (including benzylpenicillin for infections other than meningitis)	Test by an MIC method for the agent considered for clinical use and interpret according to the clinical breakpoints

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

8.4 Clinical breakpoints

The penicillin breakpoints were primarily designed to ensure the success of therapy for pneumococcal meningitis. However, clinical studies demonstrated that the outcome of pneumococcal pneumonia caused by strains with intermediate susceptibility to penicillin and treated with parenteral penicillin was no different to that in patients treated with other agents. Considering microbiological,

pharmacokinetic and pharmacodynamic data, the clinical breakpoints for benzylpenicillin for non-meningitis isolates were revisited (3) and current EUCAST breakpoints are as listed in Table 2

Table 2. 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	

Note: 1.2 g of benzylpenicillin is equal to 2 MU (million units) of benzylpenicillin

8.5 Methodology according to EUCAST

8.5.1 Test media

Disk diffusion and gradient tests: Mueller-Hinton-fastidious (MH-F) agar (MH agar with 5% defibrinated horse blood and 20 mg/l β-NAD).

Broth microdilution: Mueller-Hinton-fastidious (MH-F) broth (MH broth with 5% defibrinated horse blood and 20 mg/l β-NAD).

8.5.2 Inoculum preparation and incubation

Disk diffusion and gradient test: Plates are inoculated with a bacterial suspension prepared from fresh overnight cultures. If the inoculum is from 5% blood agar plates a suspension equivalent to a 0.5 McFarland standard is used. If the inoculum is from chocolate agar plates a McFarland 1 standard is used. Tests are incubated at 35 ± 1°C in 5% CO_2 for 18± 2 h.

Broth microdilution: Trays are inoculated with a bacterial suspension prepared from fresh overnight cultures, as for disk diffusion, and diluted to give a final inoculum of 5×10^5 cfu/ml. Plates are incubated at 35 ± 1°C in air for 18± 2 h.

8.5.3 Quality control

S. pneumoniae ATCC 49619 (bensylpenicillin 0.5 mg/L) is included whenever a new batch/lot of antimicrobial agents or reagents are used. Routine quality control is performed daily (or weekly when accuracy of the test is sufficiently documented) or every time the test is used if tests are infrequent.

8.6 References

1. Hakenbeck R, Kaminski K, König A, van der Linden M, Paik J, Reichmann P, Zähner D. Penicillin-binding proteins in beta-lactam-resistant *Streptococcus pneumoniae*. Microb Drug Resist 1999; 5: 91-99.

2. Grebe T, Hakenbeck R. Penicillin-binding proteins 2b and 2x of *Streptococcus pneumoniae* are primary resistance determinants for different classes of beta-lactam antibiotics. Antimicrob Agents Chemother 1996; 40: 829-834.
3. Weinstein MP, Klugman KP, Jones RN. Rationale for revised penicillin susceptibility breakpoints versus Streptococcus pneumoniae: Coping with antimicrobial susceptibility in an era of resistance. Clin Infect Dis 2009; 48: 1596 – 1600.
4. Dixon JMS, Lipinski AE, Graham MEP. Detection and prevalence of pneumococci with increased resistance to penicillin. Can Med Assoc J 1977; 117: 1159-61.
5. Swenson JM, Hill BC, Thornsberry C. Screening pneumococci for penicillin resistance. J Clin Microbiol 1986; 24: 749-52.
6. Jetté LP and C Sinave. Use of an oxacillin disk screening test for detection of penicillin- and ceftriaxone-resistant pneumococci. J Clin Microbiol 1999; 37: 1178-81.