BREAK POINT
2013 - ISSUE 04

WELCOME TO ANOTHER EDITION OF BREAKPOINT

Welcome to the August issue of *Breakpoint*. There has been a need to publish an extraordinary issue.

The National Antimicrobial Committee of ASA (chair: John Turnidge) receives and responds to requests from the CLSI and EUCAST steering committees to comment on documents relevant to antimicrobial susceptibility testing.

In this issue, the first article provided by John Turnidge reports recent CLSI and EUCAST movements. Comments are sought from ASA regarding (i) breakpoints for topical antibiotic agents, (ii), breakpoints for *Corynebacterium spp.,* and (iii) exclusion of *Streptococcus agalactiae* from breakpoint determinations for isoxazoylpenicillins!

The second document is the EUCAST guidelines of detection of resistance mechanisms and specific resistance of clinical and/or epidemiological importance. Version 1.0 July 2013.

A conference calendar completes this issue. A further issue in August, in its usual format, will be published at the end of the month.

The Committee as always, welcomes feedback.

Sharon Chen

ASA Newsletter Editor

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NATIONAL ANTIMICROBIAL SUSCEPTIBILITY TESTING COMMITTEE (NAC) NEWS AND CALL FOR CONSULTATION

Professor John Turnidge
South Australia Pathology

Recent news from both the CLSI and EUCAST fronts are summarised. In addition, the EUCAST committee is inviting comments from the Australian scientific committee see point 2 below.

1. CLSI: insights from the CLSI June 2013 meeting

A. The cefepime breakpoints for Enterobacteriaceae have been revised after extensive analysis, consultation and discussion. They will now be as follows: "Susceptible (S)" ≤2 µg/ml; "Susceptible dose-dependent (SDD)", 4-8 µg/ml; Resistant (R) ≥ 16 µg/ml. Behind the rationale for this decision is evidence that it is clear that there is a wide range of dosing options for this drug and thus these bacteria may be truly "SDD". For those laboratories handicapped by constraints of reporting limited to a "single letter" on their IT systems, the US experience is to use 'D' (for dose) with a comment on the report as to what it means. However, where reporting of "SDD" is possible, it is also recommended that an appropriate comment follows.
EUCAST note: For cefepime, EUCAST have designated categories as follows: S ≤2, I = 4-8, R ≥ 16 µg/ml. There is no "SDD" but an "I" category for isolates classed as "intermediate".

B. The ceftriaxone/Enterobacteriaceae breakpoints were discussed following release of data from the Johns Hopkins Hospital, USA, in paediatric patients. Patients with strains with MICs as high as 4 µg/ml responded to treatment. However, at the CLSI meeting, no changes were made to the breakpoints. The issue was simply that physicians were using doses of ceftriaxone of 75 mg/kg/day to treat non-meningitis infections, 3 times the dose normally recommended in the Australian Antibiotic guidelines (Therapeutic Guidelines-Antibiotics), so the NAC does not recommend adjusting breakpoints for children in Australia

C. Carbapenem breakpoints for *Acinetobacter* spp. were set. They are: for doripenem S ≤1, I 2, R ≥4 ug/ml, and for meropenem and imipenem S ≤2, I 4, R ≥8 ug/ml
EUCAST note: EUCAST breakpoints are similar but not identical; the S breakpoints are the same but the I ranges over two dilutions for all 3 agents i.e doripenem S ≤1, R >4, meropenem and imipenem S ≤2, R >8. ug/ml.

D. A breakpoint for cefazolin has been established as a class representative for oral cephalosporins for the treatment of uncomplicated urinary tract infections (UTIs) caused by Enterobacteriaceae as follwos: S ≤ 16, R ≥32 ug/ml. These cefazolin breakpoints are quite different to those used for systemic infections. No disk diffusion correlates have been developed as yet. Care would need to be taken in implementing these breakpoints. They should probably only ever be used for reporting on urines, and only if that patient has had blood cultures collected (as a potential marker of systemic infection).
EUCAST note: EUCAST has no cefazolin breakpoints at all, and is not currently setting them. The Australian NAC proposed that they should be set in April 2012 but received little support from the EUCAST Steering Committee. In Australia only cephalexin and cefaclor are available orally, with the latter being recommended for treatment of UTIs. However, EUCAST has set breakpoints for cephalexin. In this regard, as EUCAST methods for testing Enterobacteriaceae are essentially identical to CLSI methods, including disc strength, the simplest approach is to test for cephalexin susceptibility by the laboratory's preferred standard!

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2. EUCAST

For consultation

EUCAST have released a document for consultation. Comments from all are warmly welcomed. **We require comments by August 6th, to provide feedback to the EUCAST steering committee.**
Please see (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Consultation/EUCAST_breakpoint_consultation_June_2013f.pdf)
The following three items are out for comment:

A. 'Breakpoints' for topical agents were proposed a while ago, and further comments are requested by consultation. Their original breakpoints were essentially ECOFFs. Their new proposed 'breakpoints' have used systemic breakpoints for those agents where such breakpoints already existed: gentamicin, ciprofloxacin, levofloxacin, ofloxacin, chloramphenicol, colistin and fusidic acid. This seems like a reasonable compromise. The 'breakpoints will not apply to antimicrobial agents administered by inhalation.
CLSI note: CLSI considered setting topical 'breakpoints' a couple of years ago, but as there are essentially no PK-PD or clinical outcome data to support the breakpoints, decided against setting any at all.
Commentary: This is a challenging area in which to set breakpoints. There a few data on the pharmacokinetics of topical applied antimicrobial agents, and a huge number of variables that can impact on local PK. EUCAST have elected to have a breakpoint for topical antimicrobials rather than "nothing", hence the choice of ECOFFs. The message to us and the clinician is that the isolate harbours a resistance mechanism, so response to therapy may be less predictable. NAC is not aware any laboratory in Australia who has formally tackled this issue of testing isolates from sites where topical agents may be used, but would be pleased to hear from any who have. The sites of greatest interest are eyes, ears and skin. Unfortunately EUCAST has chosen to describe these ECOFFs as "breakpoints", which confuses them with breakpoints that have been set using the complete set of laboratory and clinical data.

B. Breakpoints (defaulted to ECOFFs) are proposed for *Corynebacterium* spp. A justification PowerPoint pdf is provided (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Consultation/EUCAST_background_data_Corynebacterium_1306.pdf).
You will see that there is a large amount of laboratory work to support these breakpoints. There are no useful available PK-PD or clinical data.
CLSI note: CLSI have breakpoints for *Corynebacterium* species in the M45 document, and have for some time. They only have MIC breakpoints though; they do not provide zone diameter correlates. The testing medium is not exactly the same either. CLSI uses MHB + 2.5-5% LHB, while EUCAST uses MHB + 5% LHB + 20 mg/L β-NAD (MH-F broth). EUCAST specifies a read a 44 hours of incubation, while CLSI states that resistant results can be at 24 hours, but otherwise read at 48 hours. EUCAST uses MH-F agar for disc diffusion. CLSI breakpoints are considerable higher than those of EUCAST. In short, there is almost nothing interchangeable about the two methods.
Commentary: As can be seen in the background file, there is considerable diversity in the intrinsic and acquired resistances of this genus. What is proposed is a best fit compromise in order to avoid species-specific breakpoints. The great advantage of this dataset is that the organisms have been identified by MALDI-TOF which significantly increases the robustness of the recommendations.

C. Exclusion of *Streptococcus agalactiae* from isoxazoylpenicillin breakpoints? Data on the PK of orally administered flucloxacillin has been generated in order to compare human exposures with MICs at a range of doses. As

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a consequence, it appears that Group B Streptococcus is borderline susceptible at best, or intrinsically resistant at worst. It is assumed that Group B Streptococci are therefore not susceptible to isoxazoylpenicillins generally (or methicillin?). There are currently no breakpoints in EUCAST for any of the isoxazoylpenicillins on the Streptococcus Groups A, B, C and G (see Page 27 of the EUCAST document) . However, Note 1 on this table states that susceptibility to all β-lactam susceptibilities can be predicted from the result for benzylpenicillin. The purpose of this proposal is to exclude isoxazoylpenicillins against *S. agalactiae* (there are 4 listed on the table) from this Note.
CLSI note: CLSI has no breakpoints for β-haemolytic Streptococci for any penicillins other than benzylpenicillin and ampicillin, and make no comment at all about predicting susceptibility to isoxazoylpenicillins.
Commentary: This proposal should not create any particular problems for the laboratory. Isoxazoylpenicillins are not used for testing, and probably never included on the laboratory report. The implications are more clinical. There might be the odd occasion when flucloxacillin is prescribed for Group B streptococcal infection, assuming that the organism is susceptible with or without a laboratory test result showing susceptibility to benzylpenicillin. Medical microbiologists and infectious diseases physicians will have to learn this 'trick of the trade'.

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 1.0
July 2013

EUCAST subcommittee for detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance:
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Contents

1. Introduction

These guidelines have been produced partly in response to frequently asked questions from users of EUCAST guidelines and partly on request from the European Centre for Disease Prevention and Control (ECDC), as expert guidance was needed for updating the EARS-Net microbiological manual.

The remit of the EUCAST 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 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 guidelines have been developed by conducting systematic literature searches, and recommendations are based on multi-centre studies or multiple single centre studies. Several methods currently under development have not been included in the guidelines as multi-centre evaluations or multiple single centre 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. Hence, while detection of these mechanisms may be relevant for infection control and public health, it may not 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 resistant.

Christian G. Giske
Chairman of the subcommittee

Rafael Cantón
Chairman of EUCAST

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 the second half of the 1990s 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 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. 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 may confer resistance to virtually 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 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, 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 are resistant to extended-spectrum (oxyimino) cephalosporins (10). Isolates producing such enzymes may have decreased susceptibility to carbapenems, but with some of these enzymes (OXA-48-like enzymes) the organisms may appear fully susceptible to cephalosporins. However, most of these isolates now also express cephalosporin-hydrolyzing enzymes, such as CTX-Ms, so they are usually also cephalosporin-resistant. Carbapenemases are considered to be of high epidemiological importance, particularly 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

Carbapenem MICs for carbapenemase-producing Enterobacteriaceae may be below the clinical 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 (ESBLs) 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 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	>0.12	≥22	<25[2]
Imipenem[3]	≤2	>1	≥22	<23
Ertapenem[4]	≤0.5	>0.12	≥25	<25

[1]Best balance of sensitivity and specificity

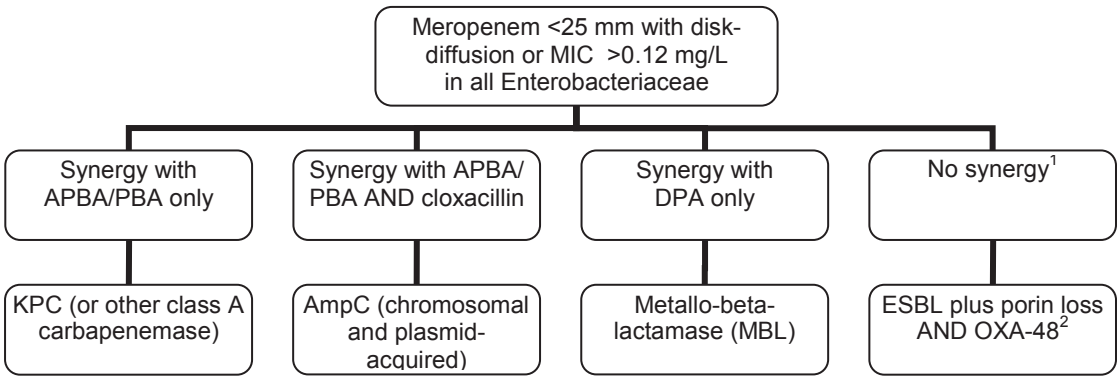
[2]In some cases zone diameters for OXA-48-producers are up to 26 mm, so <27 mm may be used as a screening cut-off during outbreaks caused by OXA-48-producing Enterobacteriaceae, but with reduction in specificity.

[3]With imipenem, the separation between the wild-type and carbapenemase-producers is relatively poor. Imipenem is therefore not recommended for use as a stand-alone screening test compound.
[4]High sensitivity but low specificity, and therefore not recommended for routine use.

2.4.2 Methods for confirmation of carbapenemase-production

Following detection of reduced susceptibility to carbapenems in routine susceptibility tests, phenotypic methods for detection of carbapenemases should be applied. The combination disk test has the advantage of being well-validated in studies and is also commercially available (MAST, Rosco) (15-17). The disks or tablets contain meropenem +/- various inhibitors that are detailed in section 2.4.3. In brief, boronic acid inhibits class A carbapenemases and dipicolinic acid inhibits class B carbapenemases. There is no currently available inhibitor for class D carbapenemases. Cloxacillin 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. The main disadvantage with these methods is that they will take 18 hours to carry out (in practice overnight incubation), for which reason novel rapid methods are now being explored.

Figure 1. Algorithm for carbapenemase detection.



Abbreviations: APBA=aminophenyl boronic acid, PBA=phenyl boronic acid, DPA=dipicolinic acid (all of them β-lactamase inhibitors added to disks or tablets containing meropenem in combination disk testing assays)
[1] Combination of KPC and MBL can also produce no synergy. Normally these isolates will have very high resistance levels to carbapenems. They are easiest to detect with molecular methods.
[2] High-level temocillin resistance (MIC >32 mg/L (12, 18), tentative zone diameter ≤10 mm with temocillin 30 µg disk (17)) is a phenotypic indicator of OXA-48 production.

There are now several faster alternative methods to the combination disk method. Analysis of carbapenem hydrolysis with MALDI-TOF (19) has been reported to confirm carbapenemase production in a few hours, and the Carba NP test (20, 21) can confirm carbapenemase production even more rapidly. However, of these tests there is published evidence only for the Carba NP test beyond the centre where it was developed (21).

A number of genotypic approaches have been reported based on PCR techniques (22). 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 (23), although they cannot overcome general limitations of genotypic techniques. It is recommended that at least reference laboratories have access to genotypic confirmation techniques, although this is not strictly required for surveillance purposes.

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 (MAST, UK) (16) or tablets (Rosco, Denmark) (15-17) are commercially available. 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 >32 mg/L) has been proposed as phenotypic marker for OXA-48-like carbapenemase producers (12, 17, 18). Nevertheless, this marker is not specific for OXA-48-type carbapenemases as other resistance mechanism might confer these phenotypes. The presence of OXA-48-like enzymes therefore has to be confirmed with a genotypic method.

Use of the modified cloverleaf (Hodge) test is not recommended 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 disks or tablets.

β-lactamase	Synergy observed as increase in zone diameter (mm) with 10 µg meropenem disk/tablet				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	-	-	-	-	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] Not applicable. Temocillin is recommended only in cases where no synergy is detected, in order to differentiate between ESBL + porin loss and OXA-48-like enzymes (12, 17, 18).
[2] There is one report supporting the use of commercial tablets containing double inhibitors (DPA or EDTA plus APBA or PBA) (24), but multi-centre studies or multiple single centre studies are lacking. This phenotype is rare outside of Greece and confers high-level resistance to carbapenems.

2.4.4 The Carba NP test

The principle of this test is that carbapenem hydrolysis will give rise to a pH-change which will result in a colour change with phenol red solution (changing colour from red to yellow if the test is positive) (20,21). 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 for carbapenemase producers screening. The Carba NP test cannot be performed with bacterial colonies grown on Drigalski or McConkey agar plates. Details of the different steps in the method should be paid close attention to in order to obtain reproducible results.

2.4.5 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 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 antimicrobial susceptibility categorization	No
Infection control	Yes
Public health	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 not cephamycins or carbapenems. Most ESBLs belong to the Ambler class A of β-lactamases and are inhibited by β-lactamase inhibitors (clavulanic acid, 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 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, followed by SHV- and TEM-derived ESBLs (2-5). Certain class D 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).

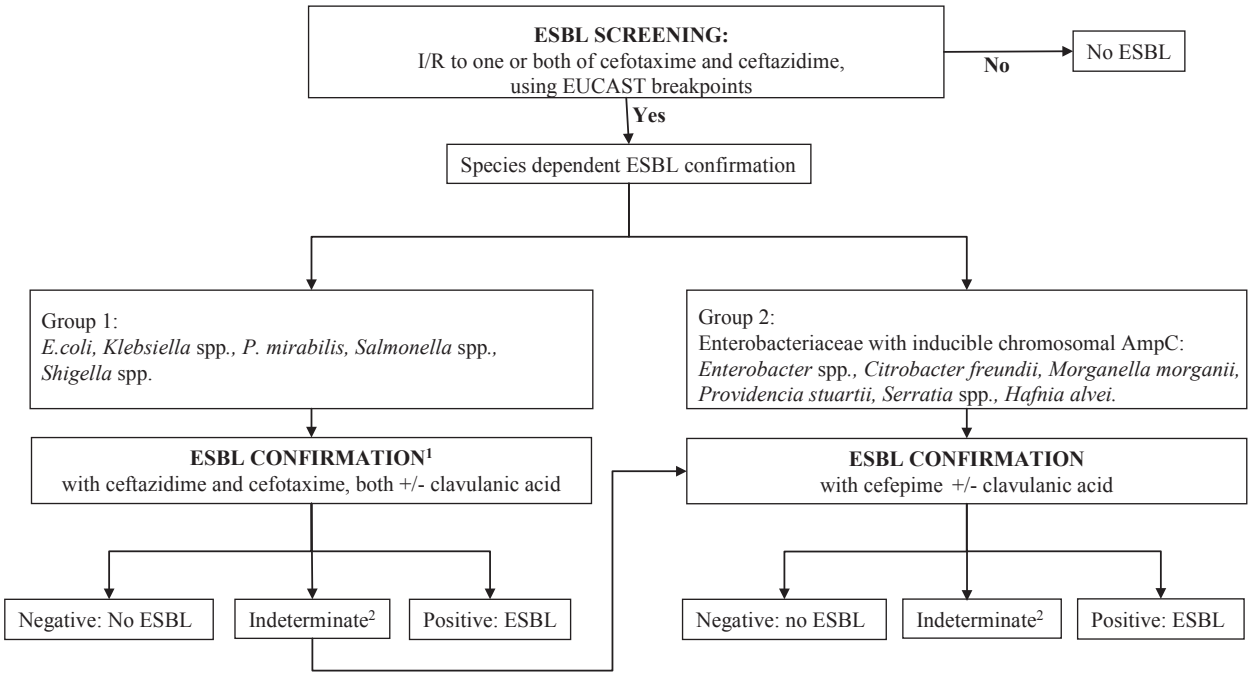
A screening breakpoint of >1mg/L is recommended for cefotaxime, ceftriaxone and ceftazidime, in accordance with the guidelines issued by EUCAST and CLSI (Table 1) (11, 12). The EUCAST clinical breakpoint for Enterobacteriaceae is also S $\leq$ 1 mg/L (11). Cefpodoxime is the most sensitive individual 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 (13) 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 (12-16).

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

[1] With all methods either test cefotaxime or ceftriaxone AND ceftazidime OR cefpodoxime can be tested alone.

Figure 1. Algorithm for phenotypic detection of ESBLs



[1]If cefoxitin MIC > 8 mg/L, perform cefepime+/- clavulanic acid confirmation test
[2]Cannot be determined as either positive or negative (e.g. if the strip cannot be read due to growth beyond the MIC range of the strip.).
[3]Genotypic testing is required.

3.4.1 ESBL-testing in Enterobacteriaceae

A. Screening in group 1 Enterobacteriaceae (*E. coli*, *Klebsiella* 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 (12, 17, 18). 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 (13, 19, 20).

The algorithm 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) (18). However, a very common mechanism of cephalosporin 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 ESBL gradient test, and the broth microdilution test (Tables 2 and 3) (17, 18, 21). The CDT showed a better specificity than the ESBL gradient test and with comparable sensitivity in one multi-centre study (22). Manufacturers of automated susceptibility testing systems have implemented detection 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 the cephalosporin alone (cefotaxime, 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 (Table 3) (23, 24).

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 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; however it may be reduced (15 mm) or expanded (30 mm) for strains with very high or low resistance levels, respectively (17). 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

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. The 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 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 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 1 Enterobacteriaceae (see Figure 1).

Method	Antimicrobial agent (disk content)	Confirmation is positive if
Etest ESBL	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
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 (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 rare 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 usually not hydrolyzed by AmpC β-lactamases. Cefepime may be used in all the CDT, DDST, Etest or broth dilution test formats (25, 31-33). 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 and ceftazidime) with both clavulanic acid and cloxacillin together; and standard CDT or DDST on agar plates supplemented with 200-250 mg/L cloxacillin (17). There are also disks or tablets containing both clavulanic acid and cloxacillin on the market, but multicentre evaluations of these products are lacking.

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 (34, 35). The epidemiological importance of ESBLs in these contexts could be questioned, since the carbapenemase has greater public health importance, 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 currently rare in Enterobacteriaceae.

3.4.4 Genotypic confirmation

For the genotypic confirmation of the presence of ESBL genes use of PCR and ESBL gene sequencing (3) or a DNA microarray-based method are recommended. Recent evaluations of the Check-KPC ESBL microarray (Check-Points, Wageningen, The Netherlands) with different collections of organisms covering the majority of known ESBL genes showed good performance (36-40). 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
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 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 the classical ESBL 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 and horizontal transfer of AmpC genes. 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 acquired AmpCs are *E. coli*, *K. pneumoniae*, *K. oxytoca*, *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. 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

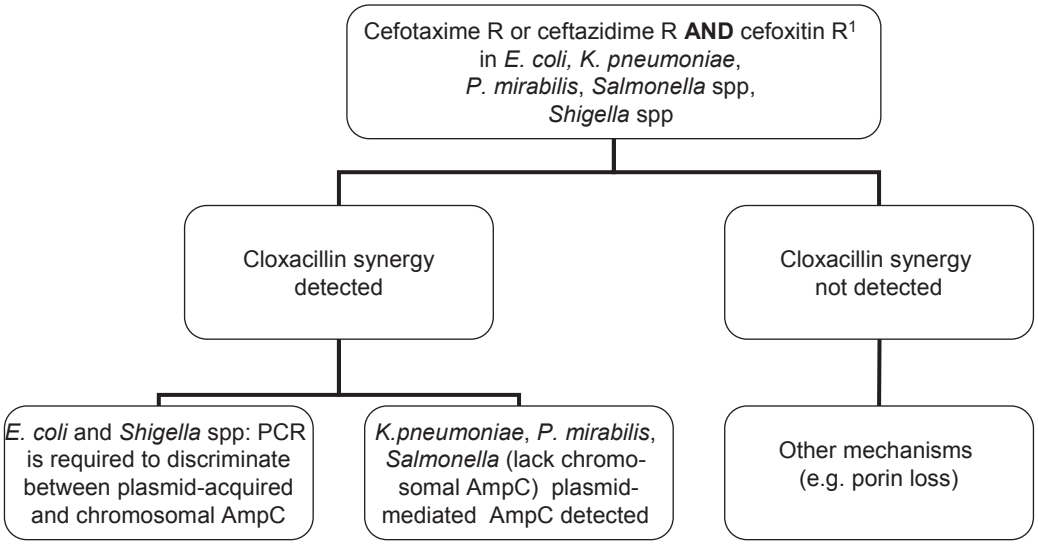
Numerous Enterobacteriaceae and other some other Gram-negative bacilli produce natural AmpCs, either constitutively at the trace level (*e.g. E. coli, Acinetobacter baumannii*) or inducibly (*e.g. Enterobacter* spp., *C. freundii*, *M. morganii*, *P. aeruginosa*). The derepression or hyperproduction of natural AmpCs is due to various genetic changes and confers high-level resistance to cephalosporins and penicillin/β-lactamase inhibitors. The class C cephalosporinases can also occur as acquired enzymes, mainly in Enterobacteriaceae. 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).

Figure 1. Flowchart showing appropriate criteria for AmpC screening.



[1]AmpC can also be present in isolates with positive ESBL-test (clavulanic acid synergy). It is therefore recommended to conduct testing regardless of the result of the ESBL-test.

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 gradient test (currently available only from bioMérieux; sensitivity 84-93%, specificity 70-100%) (11, 12) and Rosco tablets with cefotaxime/cloxacillin and ceftazidime/cloxacillin (sensitivity 96%, specificity 92%) (13,14). For *E. coli* however, AmpC confirmation tests cannot discriminate between acquired AmpC and constitutive hyperproduction of the chromosomal AmpC.

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

Table 1. Appropriate control strains for detection of AmpC.

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

4.5 References

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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 Mechanisms of resistance

The main mechanism of resistance is production of an auxiliary 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 auxiliary 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 marked heterogeneous expression of the *mecA* gene and frequently low MICs of oxacillin hamper the accuracy of susceptibility testing (5). Furthermore, 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 (5).

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

Methicillin/oxacillin resistance can be detected both phenotypically by MIC determination, disk diffusion tests or latex agglutination to detect PBP2a, and genotypically using PCR.

5.4.1 Detection by 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 included in the EUCAST breakpoint table. Strains with increased MICs of oxacillin (MIC >2 mg/L), but which remain 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 for *mecA* or *mecC*.

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

		Cefoxitin result by MIC or disk diffusion	
		S	R
Oxacillin result by MIC	S	Report as oxacillin S	Report as oxacillin R
	R	Report as oxacillin R	Report as oxacillin R

A. Broth microdilution:
Standard methodology (ISO 20776-1) is used and strains with 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) zone diameter <22 mm should be reported as methicillin resistant.

5.4.2 Detection with genotypic and latex agglutination methods

Genotypic detection of the *mecA* gene by PCR and detection of the PBP2a protein with latex agglutination kits is possible using commercial or in-house assays. However, *mecC* and PBP2c can at present not be detected using commercially available genotypic or phenotypic 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

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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

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 glycopeptide resistant *S. aureus* (GRSA) and non-VanA mediated low-level resistant isolates. Hence, the terms glycopeptide intermediate *S. aureus* (GISA) and heteroresistant glycopeptide intermediate *S. aureus* (hGISA) 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 hGISA may also be warranted. Due to the complexity of confirming hGISA, antimicrobial surveillance is focused on detection of GISA and GRSA.

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 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 hGISA is ≤ 2% of MRSA in Europe, with GISA below 0.1% (1). GRSA has not yet been reported in Europe and is currently extremely rare worldwide (1). 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 bloodstream

infections not responding to therapy. Recently there has been increasing evidence that isolates with MICs in the upper part of the susceptible range (MIC >1 mg/L) are associated with poorer outcome and may be linked to increased mortality, at least in bloodstream infections (2-7). It is still uncertain whether the presence of resistant subpopulations is responsible for the poorer outcome, as it could also be a consequence of the slightly elevated vancomycin MICs observed for these strains.

The mechanism of hGISA is complex and detection relies on population analysis (8), which is cumbersome, requires special equipment and needs a high level of technical expertise. Methodology for detection of hGISA will be outlined, but for surveillance reporting is restricted to GISA, which is defined as isolates with an MIC >2mg/L.

6.3 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 (i.e. chromosomal mutations) 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* (1).

6.4 Recommended methods for detection of glycopeptide non-susceptible *S. aureus*

Disk diffusion CANNOT 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 by gradient strip methods, agar dilution or automated systems. 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 (7). The EUCAST breakpoint for resistance to vancomycin in *S. aureus* is MIC >2 mg/L. Isolates with confirmed MICs ≥2 mg/L should be referred to a reference laboratory.

6.4.2 Specific tests 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. Confirmation is by analysing the population profile of the isolate on agar plates containing a range of vancomycin concentrations (PAP-AUC) (8). 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 (9) has shown high sensitivity and specificity, but has so far

only been evaluated in one study, and for that reason not been included. The following methods have been evaluate in a multicentre study (10).

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 hGISA and GISA. The test is set up according to the manufacturer's instructions. Note also that the inoculum is higher (2,0 McFarland) than with standard gradient tests. 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: GISA or hGISA
- Teicoplanin reading 8 mg/L: Test vancomycin. If vancomycin reading is ≥8 mg/L then GISA or hGISA
- Teicoplanin reading <8 mg/L: Not GISA or hGISA

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. 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 more than two colonies at 48h indicates suspected reduced susceptibility to glycopeptides.

D. 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 1. Appropriate control strains for testing glycopeptide susceptibility.

Strain	Mechanism
S. aureus ATCC 29213	Glycopeptide susceptible
S. aureus ATCC 700698	hGISA (Mu3)
S. aureus ATCC 700699	GISA (Mu50)

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

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

7.1 Definition

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

7.2 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 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 MICs of glycopeptides for isolates harbouring VanA and VanB.

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

7.3 Mechanism of resistance

Clinically-relevant resistance is most often mediated by plasmid-encoded VanA and VanB ligases that replace the terminal 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 prevalence 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) but should generally not be considered important from an infection control point of view (5).

7.4 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 accurate, but is seldom used in routine laboratories. Older reports show that detection of *vanB*-mediated resistance is problematic for automated methods (10, 11). Since then updates have been made in 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 5µg vancomycin disk performs well provided the guidelines for reading as specified by EUCAST are followed meticulously.

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 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 MIC methods. EUCAST guidelines should be followed for broth microdilution and the manufacturer's guidelines should be followed for gradient tests.

Broth microdilution is performed according to the ISO standard 20776-1. MIC determination with gradient tests is performed according to the manufacturer's instructions. Please note that MIC gradient strips are sometimes used with a higher inoculum (2.0 McFarland standard) on a rich medium (Brain Heart Infusion agar) to screen for vancomycin resistance but this analysis does not provide an MIC value.

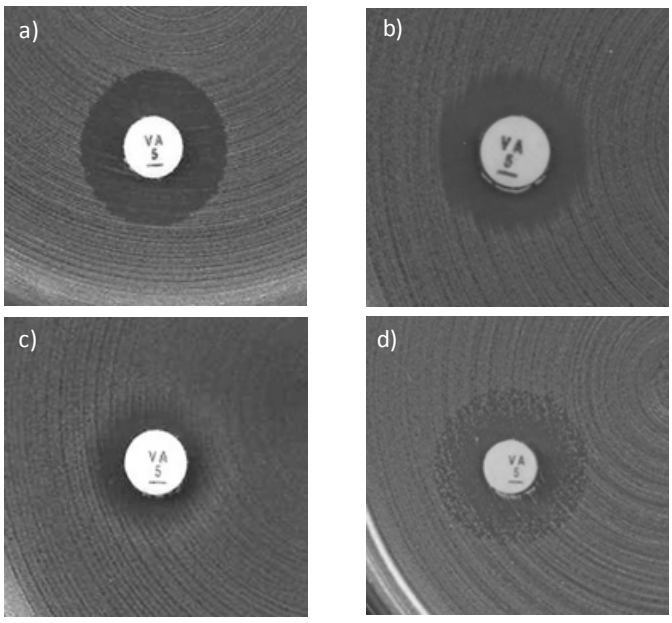
7.4.2 Disk diffusion testing

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 regardless of zone size, should not be reported as susceptible without confirmation by MIC determination (Figure 1).

- 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 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 susceptible.

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 *vanA*- and *vanB*-positive isolates. 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 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

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

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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, i.e. >0.06 mg/L) 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

To be added.

8.3 Mechanism of resistance

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 the number of low-affinity mosaic PBPs present in the isolate, but also on modification of the specific PBPs that 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-meningitis infections when a higher dose of penicillin is used, whereas for meningitis such strains must always be reported as resistant (3).

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

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

8.4.1 Disk diffusion method

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 benzylpenicillin, and the 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, amoxicillin and piperacillin (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.

8.4.2 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 for 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

8.4.3 Quality control

Table 3. Appropriate control strains for testing of benzylpenicillin susceptibility.

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

8.5 References

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9. Transparency declarations

To be added.

2013 - 2014 MEETING CALENDAR

2013

52nd Interscience Conference for Antimicrobial Agents and Chemotherapy
Sept 10-13, Denver, Colorado
Website: www.icaac.org/

Australian College of Infection Prevention and Control
Sept 30- Oct 2, Gold Coast, Queensland
Website: http://www.ashm.org.au/conferences

ID week (IDSA, SHEA, HIVMA, PIDS)
Oct 2-6, San Francisco, California
Website: http://www.idweek.org/idweek2013

8th world congress of Pediatric Infectious diseases
19-22 November, Cape Town, South Africa
Website: http://www2.kenes.com/wspid/

Mycology Master Class VI
31 Oct- 2 Nov, Kingscliffe, NSW
Website: www.asid.net.au

2014

Australian Society for Antimicrobials Annual Meeting
20-22 Feb, Melbourne, Victoria
Website: http://www.asainc.net.au

Royal College of Pathologists Update Meeting
21-23 Feb, Melbourne, NSW
Website: http://www.rcpa.edu.au

Australasia Society for infectious Diseases Annual meeting
26 -29 March, Adelaide, SA
Website: www.asid.net.au

2014

16th International congress on Infectious diseases (ICID)
2-5 April, Cape Town, South Africa
Website: www.isid.org/org/icid

SHEA, Society for Healthcare Epidemiology of America
April 3-6, Denver. USA
Website: http://www.shea-online.org

114th American Society for Microbiology Annual Meeting
17-20 May, Boston, USA
Website: http://www.asm.org

Australian Society for Microbiology, Annual meeting
July 6-9, Melbourne
Website: www.the asm.org.au

54th ICAAC
6-9 Sept, Washington D.C. USA
Website: http://www.asm.org

15th Asia Pacific congress of Clinical Microbiology and Infection.
Nov 26-29, Kuala Lumpur
Website: http:/www.apccmi2014.org/