Antimicrobial susceptibility testing
issues relating to EARSS

Prepared by the Irish EARSS Steering Group.
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1. Introduction

This document was prepared in response to some important antimicrobial susceptibility testing (AST) issues that have been highlighted over the past four years as a result of Ireland’s participation in the European Antimicrobial Resistance Surveillance System (EARSS). It is not intended to be a comprehensive guide to susceptibility testing and is confined to the current EARSS pathogens (Staphylococcus aureus, Streptococcus pneumoniae, Escherichia coli and Enterococcus faecalis/ffiæcium). For further details on performing disc diffusion susceptibility testing or MIC determinations, please refer to the testing guidelines being used in your laboratory. As laboratories in Ireland have agreed to adopt the National Committee for Clinical Laboratory Standards (NCCLS) guidelines for routine susceptibility testing, NCCLS recommendations are included where possible. Where there are conflicting viewpoints on particular issues in the literature, this document has included as many as possible of the differing opinions proposed in an attempt to present a more balanced picture of the current state of knowledge. This may be helpful in making laboratories aware that in a number of instances no one view has been accepted as definitive.

2. S. aureus

2.1. Methicillin/Oxacillin

A number of different resistance phenotypes can occur:

2.1.1. MRSA/ORSA (methicillin MIC ≥16 mg/L; oxacillin MIC ≥4 mg/L)

Methicillin resistance proper occurs when isolates produce an additional penicillin-binding protein, PBP2a (also known as PBP2’), which is a product of the mecA gene, and confers resistance to all beta-lactam antibiotics. NCCLS guidelines recommend testing oxacillin to detect resistance to the penicillinase-stable beta-lactams (which include oxacillin, methicillin, flucloxacillin, cloxacinil, dicloxacillin and nafcillin).

Resistance may be classified as:

- homogeneous resistance, where all cells in the inoculum population express resistance, or
- heterogeneous resistance, where only some (often a minority) of cells in the inoculum population express resistance. The proportion of cells expressing resistance may be as low as 1 in 10⁴ to 1 in 10⁸ cells. Very small numbers of resistant cells may not be detected readily by standard susceptibility test methods hence the need for using lower incubation temperatures and/or salt-containing media for the detection of methicillin resistance.

Note: It is recommended that an oxacillin MIC be performed on a patient’s first isolate of MRSA, or alternatively, that the isolate be tested for mecA gene carriage by PCR or for PBP-2a production using one of a number of commercially available kits. Demonstration of the mecA gene is considered by many to be the gold standard for confirming the presence of methicillin resistance.

Less commonly, reduced susceptibility to oxacillin or methicillin may be due to other mechanisms:
2.1.2. BORSA (borderline *S. aureus*; methicillin MIC 2 – 4 mg/L, oxacillin MIC 1 – 2 mg/L)

These isolates do not carry the *mecA* gene but typically hyper-produce penicillinase, which confers low-level resistance.

2.1.3. MODSA (modified-PBP *S. aureus*; methicillin MIC 2 – 4 mg/L, oxacillin MIC 1 – 2 mg/L)

These isolates do not carry the *mecA* gene but produce a modified PBP or express different levels of other PBPs.

Both BORSA and MODSA strains remain susceptible to beta-lactam/beta-lactamase inhibitor combinations, are infrequently isolated and their clinical importance is doubtful or unknown.

2.1.4. Cefoxitin testing as an alternative to methicillin/oxacillin testing

A number of recent studies have shown that cefoxitin disc testing has higher sensitivity and specificity than previously recommended tests for detecting methicillin/oxacillin resistance in *S. aureus*. This test does not require a special inoculum, lower incubation temperature, longer incubation period (the plates can also be read at 16 – 20 hours as opposed to 24 hours) or media containing higher salt concentrations which means that it is easier to apply in most routine laboratories than many other recommended tests.

For NCCLS guidelines using a confluent inoculum on MHA and 30 µg discs, EARSS recommends that the following breakpoints should be applied: susceptible (S), ≥20 mm; resistant (R), <20 mm. (Actual NCCLS breakpoints are: S, ≥18 mm; intermediate (I), 15 – 17 mm; R, ≤14 mm). For European guidelines using a semi-confluent inoculum on ISA and 10µg discs, EARSS recommends that the following breakpoints should be applied: S, ≥18 mm; R, <18 mm.

Any laboratory intending to switch over to cefoxitin testing should test cefoxitin in parallel with their current method for testing resistance to oxacillin or methicillin.

2.2. Glycopeptides (vancomycin/teicoplanin)

In *S. aureus*, three types of glycopeptide resistance have been reported to date:

2.2.1. Vancomycin- or Glycopeptide-resistant *S. aureus* (VRSA or GRSA; vancomycin MIC ≥32 mg/L, teicoplanin ≥32 mg/L)

In July 2002, the first *in vivo* isolates of VRSA were reported from a patient with a diabetic foot ulcer from Michigan in the US. This isolate was shown to have the *vanA* gene that was previously associated with some enterococci. *E. faecalis* carrying the *vanA* gene was also isolated from the same site suggesting that transfer of this gene had occurred. The patient was successfully treated with a combination of wound debridement and co-trimoxazole. No further spread of this strain was reported among staff, family members or patient contacts.

A second epidemiologically unrelated isolate of VRSA was reported from Pennsylvania in September 2002.
2.2.2. Vancomycin- or Glycopeptide-intermediate *S. aureus* (VISA or GISA; vancomycin MIC 8 – 16 mg/L; teicoplanin 16 mg/L)

VISA/GISA have been reported from Japan, the USA, France, Germany, Spain, the UK, Hong Kong and Korea. Glycopeptide-intermediate *S. aureus* is perhaps more correctly termed *S. aureus* with reduced susceptibility to glycopeptide. This type of resistance is unrelated to the high-level resistance seen in enterococci due to *van* genes. Reduced susceptibility to glycopeptide has been attributed to hyper-production of cell wall precursors and the enzymes that process them, which results in a thicker peptidoglycan layer and decreased activity of autolytic enzymes. To date, GISAs have only been identified from MRSA infections that respond poorly to treatment with glycopeptides. Haemodialysis and continuous ambulatory peritoneal dialysis patients are particularly at risk because (a) they have a high risk of developing MRSA infections as they frequently carry MRSA and (b) they often receive long-term glycopeptide therapy. GISA should be suspected if a *S. aureus* infection is failing to respond to treatment with a glycopeptide.

2.2.3. Hetero-VISA or hetero-GISA (h-VISA or h-GISA; vancomycin MIC of resistant sub-population 8 mg/L)

With h-GISA, only a small proportion of cells in the bacterial population express resistance. The MIC of the resistant subpopulation is 8 mg/L and may occur at a frequency of one cell in about $10^5 – 10^6$. This phenomenon is similar to the methicillin/oxacillin resistance referred to above.

Different researchers and centres vary in their approaches to both detection of GISA/h-GISA and the interpretation of their clinical significance:

2.2.4. Detection of VISA/GISA

Standard disc diffusion is not recommended, as this will not detect GISA (and by extension h-GISA) isolates.

Present recommendations for the detection of VISA/GISA from the Centers for Disease Control [(CDC), Atlanta, Georgia, USA], the Antimicrobial Resistance Monitoring and Reference Laboratory [(ARMRL), Colindale, London, UK] and the Irish National MRSA Reference Laboratory require that three criteria must be satisfied:

- Growth on Brain Heart Infusion (BHI) agar with 6 mg/L vancomycin inoculated with 10 µl of a bacterial suspension equivalent to a 0.5 McFarland standard
- Etest® MIC >4 mg/L after 24 hours incubation on Mueller-Hinton (MH) agar with an inoculum density equivalent to 0.5 McFarland standard
- Vancomycin MIC of >4 mg/L by broth microdilution following NCCLS methodology (in cation-adjusted MH broth using an inoculum of $10^4$ CFU and examined after 24 hours incubation) or by agar dilution following British Society of Antimicrobial Chemotherapy (BSAC) methodology [on Diagnostic Sensitivity Test (DST) agar with 5% lysed horse blood using an inoculum of $10^4 – 10^5$ CFU and examined after 24 hours incubation]

Currently, the NCCLS breakpoints for vancomycin are: S, ≤4 mg/L; I, 8 – 16 mg/L; R, ≥32 mg/L; and for teicoplanin: S, ≤8 mg/L; I, 16 mg/L; R, ≥32 mg/L.

The equivalent BSAC breakpoints for both vancomycin and teicoplanin are: S, ≤4 mg/L; R, ≥8 mg/L. There is no intermediate category.
It is important to note that GISA isolates will most likely be associated with treatment failure and that all GISA isolates detected so far have been amongst MRSA isolates. Increased vigilance is required both clinically and in the laboratory to detect such isolates and, in the event they do occur, to limit their further spread.

2.2.5. Detection of h-VISA/h-GISA

The standard disc diffusion method described in Section 2.2.4. will not detect h-GISA. AB-Biodisk has developed the Etest macromethod to screen for glycopeptide resistance (including h-GISA).

The Etest macromethod involves inoculating a BHI agar plate with a heavy inoculum of the organism (equivalent to a 2 McFarland turbidity standard). Following application of vancomycin and teicoplanin Etest strips, the plates are incubated for a full 48 hours. Isolates yielding Etest values of 8 mg/L against both vancomycin and teicoplanin or of 12 mg/L against teicoplanin alone require further investigation. (Please note: these macromethod Etest values are not MICs and should not be reported as such).

Confirmation of h-GISA requires population analysis profiling: area under the curve (PAP-AUC) analysis, whereby MICs are performed using different concentrations of inocula to determine whether the isolate contains a resistant sub-population. This is considered by many to be the most appropriate method for detecting GISA/h-GISA, however, this service is not widely provided even amongst reference laboratories.

Practical approach for laboratories

The National MRSA Reference Laboratory has suggested that, as an interim measure, laboratories should consider screening all MRSA using BHI agar with 6 mg/L vancomycin (BHIV6) as described in Section 2.2.4. MRSA from patients who fail to respond to glycopeptide therapy, especially patients with chronic renal failure, should be screened by the Etest macromethod.

Other considerations

According to CDC, screening using the Etest macromethod can be done as part of research protocols but results generated should not be reported. Until further clinical data are available to assess the significance of hetero-resistance, routine screening for h-GISA is not warranted in the US. The Bristol Centre for Antimicrobial Research and Evaluation (BCARE) recommends the Etest macromethod as an initial screening method to detect vancomycin resistance due to its speed and relatively low rate of false positives, however, in their opinion the modified-PAP is a more accurate method. BSAC considers PAP-AUC analysis to be the most reliable method for detecting heterogeneous resistance to vancomycin and recommends sending any suspect isolates, based on clinical grounds, to BCARE in Bristol or to ARMRL in Colindale for further investigation (however, ARMRL does not currently offer this service). The Irish National MRSA Reference Laboratory is not yet in a position to offer PAP-AUC analysis but to date has had five h-GISA isolates confirmed by BCARE, where a commercial PAP-AUC analysis service is offered.

2.3. Mupirocin

Mupirocin is a topical antibiotic used to eradicate colonisation by MRSA from skin and mucosal surfaces. It works by preventing protein synthesis by binding to isoleucyl tRNA synthetase (IRS). Resistance to mupirocin may be low-level or high-level.
2.3.1. Low-level mupirocin resistance (MIC 8-256 mg/L)

Low-level resistance is due to a mutation affecting the IRS target site (chromosomal-mediated).

2.3.2. High-level mupirocin resistance (MIC ≥512 mg/L)

High-level resistance is due to the acquisition of a second IRS that is encoded by the \textit{mupA} gene (plasmid-mediated). This gene is transferable between strains and is often associated with particular epidemic strains of MRSA.

2.3.3. Mupirocin susceptibility testing

There are no NCCLS guidelines for performing or interpreting susceptibility testing for mupirocin or other topical antimicrobial agents. New tentative guidelines using mupirocin 5 \(\mu\)g discs have been produced by BSAC. Isolates with a zone diameter <21 mm should be further examined by MIC determination to distinguish between those isolates with intermediate/low-level resistance [(LLR), MIC 8 – 256 mg/L] and high-level resistance [(HLR), MIC >512 mg/L] due to carriage of the \textit{mupA} gene. Only the latter are considered clinically significant.

Testing with mupirocin 5 \(\mu\)g discs will distinguish between susceptible and resistant isolates but will not distinguish between low-level and high-level resistance. In a study by Palepou \textit{et al}, the 200 \(\mu\)g disc was not considered to be a reliable means of detecting all HLR or for distinguishing LLR from HLR.

2.4. Fusidic acid

Preparations of fusidic acid are marketed for systemic and topical use. Fusidic acid inhibits the transfer of the growing protein molecule (peptidyl-transfer RNA) from the ribosomal acceptor site to the donor/peptidyl site. This requires elongation factor G (EF-G) and hydrolysis of guanosine triphosphate (GTP). Resistance can be due to chromosomal mutation (\textit{fusA}) of the target site, which lowers the affinity of EF-G for fusidic acid, or can be plasmid-mediated (\textit{fusB}) resulting in reduced antibiotic permeability/uptake.

There are no NCCLS guidelines for the performance or interpretation of susceptibility testing for fusidic acid but the literature suggests that MICs of ≥4 mg/L should be considered resistant. There is debate whether values of 2 mg/L should be considered intermediate or resistant.

According to BSAC, MICs of ≤1 mg/L are considered to be susceptible while MICs of ≥2 mg/L are resistant. The breakpoint interpretive criteria for disk diffusion susceptibility testing are tentative. French guidelines advocate breakpoints of ≤2 mg/L (susceptible) and ≥16 mg/L (resistant).

3. \textit{S. pneumoniae}

3.1. Penicillin (intermediate, MIC 0.1 – 1.0 mg/L; high-level resistant, 2.0 mg/L)

Disc diffusion susceptibility testing using an oxacillin 1 \(\mu\)g disc can only distinguish between isolates that are susceptible and non-susceptible to penicillin. MIC determination (by Etest, for instance) is required on all non-susceptible isolates to
determine if they are intermediate or high-level resistant. The MIC, in association with the site of infection, will influence the choice of treatment regimen (i.e. whether penicillin will be effective or not). Penicillin non-susceptible pneumococci are categorised according to NCCLS interpretive criteria as follows:

- **0.1 – 1.0 mg/L = penicillin-intermediately resistant** (treatment of pneumonia/septicaemia possible but **not** meningitis)
- **≥2.0 mg/L = penicillin-high-level-resistant** (do **not** use to treat invasive infections)

The oxacillin-screening test for pneumococci is very sensitive, i.e. it rarely fails to detect penicillin non-susceptible isolates. The screening test is not very specific, as some isolates that appear to be non-susceptible may give an MIC below the susceptible breakpoint. Hence, it is important to examine all isolates giving a zone diameter ≤19 mm using an MIC method to determine the level of susceptibility. Note that when determining the zone diameter in the oxacillin-screen test, the zone of inhibition of growth **not** the zone of inhibition of alpha-haemolysis should be measured. Alpha haemolysis may extend inside the zone of inhibition of growth.

Penicillin reduced susceptibility/resistance is due to the presence of altered PBPs (penicillinases have not been found in pneumococci thus far). High-level resistance is due to changes in three PBPs (1a, 2b and 2x) while intermediate or moderate resistance is due to changes in two of these. Changes to 1a and 2x alone can result in high-level resistance to cefotaxime and ceftriaxone, while changes to 2x alone can result in resistance to oxacillin. This highlights the importance of verifying susceptibility to cefotaxime or ceftriaxone when these agents are used to treat invasive infection with penicillin non-susceptible isolates. PBP-2b is not a target for cefotaxime or ceftriaxone hence resistance to these agents does not necessarily correlate with resistance to penicillin. Virtually all penicillin-susceptible or penicillin-intermediate strains are also susceptible to cefotaxime and ceftriaxone.

NCCLS recommends that penicillin, cefotaxime or ceftriaxone and meropenem MICs should be determined for isolates with oxacillin zone sizes of ≤19 mm as there is no reliable disc diffusion method for these antibiotics. NCCLS also recommends that MICs for these antibiotics should be performed routinely for all blood and CSF isolates from patients with life-threatening infections. Isolates with oxacillin zone sizes of ≥20 mm are susceptible to penicillin and can be considered susceptible to ampicillin, cefaclor, cefuroxime, cefotaxime, ceftriaxone, imipenem and meropenem. Such isolates are also susceptible to amoxicillin/clavulanic acid. However it would not be rational to use a combination of amoxicillin and a beta-lactamase inhibitor specifically to treat a *S. pneumoniae* infection given that beta-lactamase production has not been reported in *S. pneumoniae*.

### 3.2. Cefotaxime MIC testing (intermediate, MIC 2.0 mg/L; resistant, ≥4.0 mg/L)

According to NCCLS guidelines, disc diffusion is not reliable for detecting resistance to cefotaxime or ceftriaxone. These antibiotics should be examined using an MIC method to determine their *in vitro* activity.
3.3. Ciprofloxacin MIC testing (No NCCLS breakpoints)

The EARSS Advisory Board in the Netherlands recommended ciprofloxacin MIC testing on all penicillin-non-susceptible pneumococci in light of the number of newer fluoroquinolone agents with greater anti-pneumococcal activity becoming available (moxifloxacin, sparfloxacin, etc). Laboratories reporting to EARSS in Ireland are not expected to perform ciprofloxacin MICs if they do not test ciprofloxacin routinely. There are no NCCLS criteria for interpreting ciprofloxacin MICs, however, there are NCCLS interpretive criteria for ofloxacin (S, ≤2 mg/L; R, ≥8 mg/L) and for a number of the newer fluoroquinolones.

4. E. coli

4.1. Extended-Spectrum Beta-Lactamases

Extended-spectrum beta-lactamases (ESBLs) attack third-generation cephalosporins (3GCs), e.g. cefotaxime, ceftriaxone and ceftazidime, and monobactams, e.g. aztreonam, but individual enzymes vary greatly in their spectrum of activity, hence for some 3GCs/monobactams they may appear to be susceptible in vitro. Certain ESBLs have greater activity against cefotaxime while others are more active against ceftazidime, hence the necessity to examine more than one of these compounds. ESBLs are generally susceptible to inhibition by beta-lactamase inhibitors (clavulanic acid, tazobactam, sulbactam) and this provides the basis for their detection in the double-disc test, in which a 3GC or monobactam disc is placed 30 mm from a disc containing a beta-lactamase inhibitor. Enlargement of the zone of inhibition around the 3GC/monobactam disc on the side closest to the disk containing beta-lactamase inhibitor represents a positive test for ESBL. Organisms suspected of harbouring ESBLs should be reported as resistant to all 3GCs and monobactams. The easiest way to detect ESBLs has been outlined in the document produced by Dr Dearbhaile Morris and Prof Martin Cormican at NUI Galway (available on the NDSC website: http://www.ndsc.ie/Publications/AntimicrobialResistance-EARSSReports/d573.PDF). In short, this involves testing isolates against cefpodoxime (CPD) and cefpodoxime/clavulanic acid (CPC) using established NCCLS methodologies. A zone diameter for CPC that is 5 mm or greater than the zone diameter for CPD alone is indicative of the presence of an ESBL. Isolates that are found to be positive for ESBL on the CPD/CPC screen test should be confirmed by other means, e.g. Etest, or sent to a reference laboratory. Correct storage of discs is important as some agents, especially clavulanic acid, are susceptible to moisture, temperature fluctuations and/or exposure to light.

4.2. Imipenem

Isolates that appear to be resistant to imipenem or meropenem on disc diffusion should be re-checked by repeating the test using a disc from a fresh cartridge and/or testing a susceptible control organism in tandem. Imipenem discs are known to lose their activity on prolonged storage at +4°C. If still found to be resistant, isolates should be further examined MIC determination and sent to a reference laboratory for confirmation.
5. Enterococci

5.1. Ampicillin

*E. faecium* isolates are typically, but not always, ampicillin-resistant (and predominantly susceptible to quinupristin/dalfopristin, or “Synercid®”, which could serve as a useful marker) while *E. faecalis* is more likely to be ampicillin-susceptible (and always Synercid-resistant). The identity of ampicillin-sensitive *E. faecium* isolates should be re-checked as they often turn out to be *E. faecalis* (speciation of enterococci is notoriously problematic). Ampicillin resistance is most likely due to beta-lactamase production in *E. faecalis* (and this is always associated with high-level gentamicin resistance) and to over-production of PBP-5 in *E. faecium*. Beta-lactamase activity in enterococci may be missed on routine ampicillin disc diffusion susceptibility testing and so a rapid test for beta-lactamase, such as nitrocefin, is recommended by NCCLS. This test is rarely, if at all, carried out in Irish laboratories, however it could be useful to test all invasive enterococcal isolates that are ampicillin susceptible.

5.2. Aminoglycosides

Enterococci are inherently resistant to low-levels of aminoglycosides due to poor uptake of the antibiotic, hence treatment with these agents alone is not indicated even if they appear susceptible *in vitro*.

High-level resistance (HLR) to gentamicin (or streptomycin) is due to the acquisition of enzymes that inactivate aminoglycosides. An isolate may exhibit high-level resistance to gentamicin but remain susceptible to streptomycin. Testing for high-level resistance to gentamicin (and streptomycin, if gentamicin HLR) is indicated to predict if therapeutic synergy of gentamicin (or streptomycin) with other cell wall-active antibiotics, such as ampicillin or vancomycin, is likely to be effective. This can be done by disc diffusion using a high-potency disc (gentamicin 120 µg according to NCCLS guidelines, or 200 µg if following BSAC guidelines). Only isolates with a zone diameter of 6 mm (growth up to the disc edge) are considered to be HLR by NCCLS, while any isolates with zone sizes of 7 – 9 mm are inconclusive and require an MIC to confirm if they are HLR or not. Alternatively, NCCLS have described an agar screening method using a plate containing 500 mg/L of gentamicin. Isolates with a zone size of ≤9 mm are considered to be HLR by BSAC.

Using NCCLS methodology, one laboratory in Ireland has found isolates that give zone diameters of 6 mm when tested by disc diffusion but exhibited an MIC of <500 mg/L when examined using Etests. Such isolates would not be classified as high-level gentamicin resistant by NCCLS interpretive criteria.

5.3. Glycopeptides

Identification of enterococci to species level is notoriously problematic but is essential for proper interpretation of antimicrobial susceptibility test patterns. Susceptibility to glycopeptides provides an important example of this. Certain species are intrinsically resistant to low levels of both vancomycin and teicoplanin (*E. casseliflavus* and *E. gallinarum*). High-level resistance to glycopeptides in *E. faecalis* and *E. faecium* is related to a change in the building blocks of the cell wall cross-bridges. The two terminal
residues of the cross-linked chain are altered from D-Ala-D-Ala to D-Ala-D-Lac. Glycopeptides are unable to bind to the altered cross-linked chain and therefore lose their effectiveness.

There are two main glycopeptide resistance phenotypes observed, VanA (high-level resistance to both vancomycin and teicoplanin and VanB (high-level resistance to vancomycin only, teicoplanin remains susceptible). For disc diffusion susceptibility test reading, an apparent zone of inhibition should be examined carefully for a light haze of growth within the apparent zone. If a zone of lighter growth is present the zone diameter must be measured from the margin of the light growth. With both NCCLS and BSAC methods, plates should be incubated for a full 24 hours before reporting vancomycin and teicoplanin susceptibilities. NCCLS guidelines recommend MIC testing on any isolate yielding a zone diameter in the intermediate range (15 – 16 mm).

6. References


**Disclaimer**

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