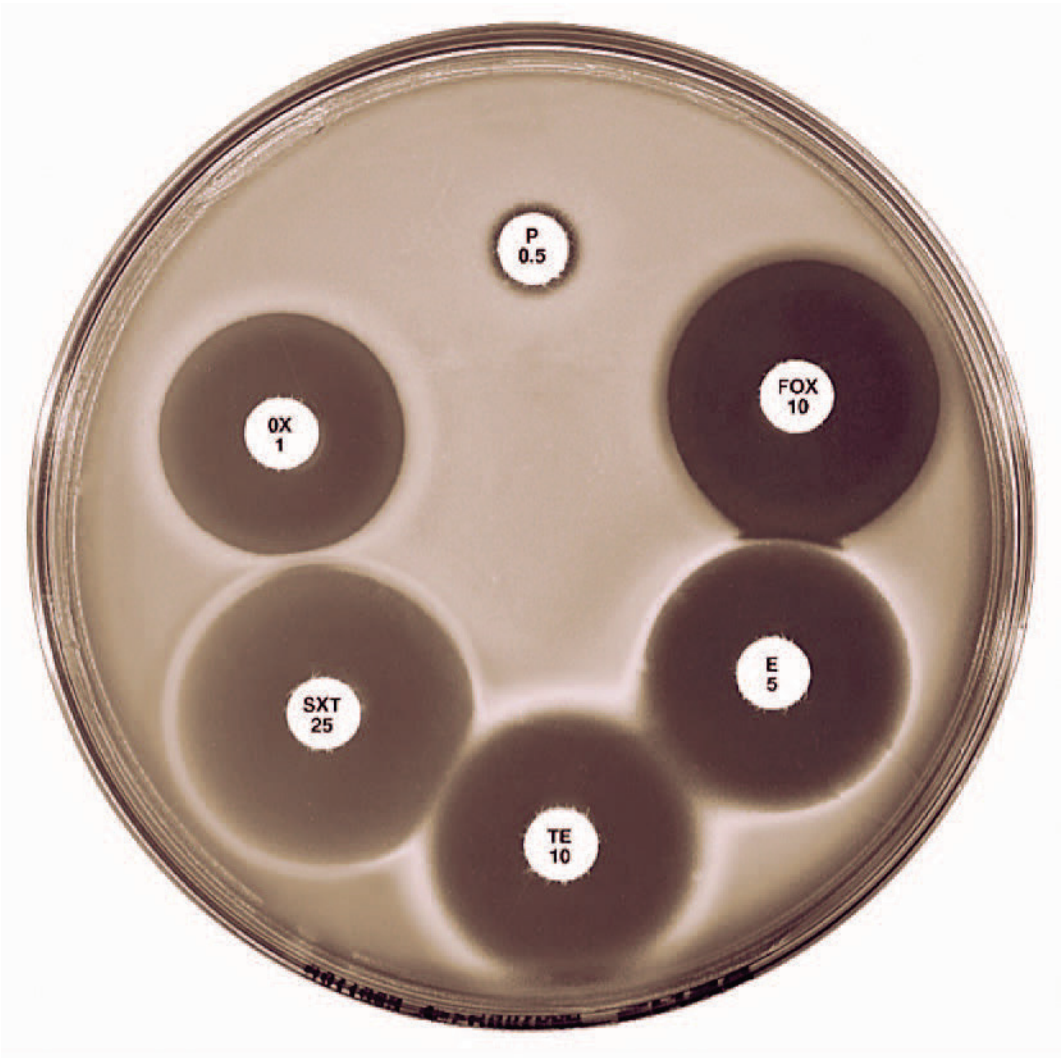
ANTIBIOTIC SUSCEPTIBILITY TESTING BY THE CDS METHOD



A MANUAL FOR MEDICAL AND VETERINARY LABORATORIES 2016

Eighth Edition

S. M. Bell, J. N. Pham, D.L. Rafferty, J.K. Allerton

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Preface to the Eighth Edition

There have been some major changes in the management of the CDS Laboratory since the publication of the 7th edition. However despite these changes the development of the method and support to the CDS Users has continued uninterrupted. Sadly Dr. Jeannette Pham has decided to retire but her role has been taken up by Dianne Rafferty who has returned to the CDS Laboratory after an absence of a number of years. Dianne has been joined by Julie Allerton, an experienced microbiologist and this team is providing a valuable service to the CDS users as well as undertaking a major project in reconciling the results of MIC determination by different techniques. The laboratory has been relocated to SEALS Microbiology at Kogarah and is supported bv A/Prof. Peter Taylor, the Director of Kogarah Microbiology and Chinmoy Mukerjee, the Laboratory Manager. Sadly, Dr. Peter Newton was forced to withdraw as an author because of pressure of his other duties in Wollongong but on a brighter note Dr. Pham is staying on as an author despite being on LSL.

All sections of the Manual have been reviewed and a number of minor corrections, additions and deletions have been made. Major changes include a review of the section on quality assurance to ensure that CDS Test Method complies fully with TGA's latest legislative requirements (2016) for *in vitro* devices (IVD). There has been a major change in the section on Neisseria. We have divided it into 2 sections, the first is aimed at routine laboratories and the second aimed at reference laboratories, the latter kindly provided by Prof. Monica Lahra. Note the section on testing yeasts has been deleted entirely because we no longer have the resources to update it.

Syd Bell

October 2016

1. Introduction

The first published description of the CDS Test appeared in "Pathology" in 1975[1]. Diagnostic laboratories in Australasia soon adopted the CDS and it became the most commonly used method of susceptibility testing in this country. Some years ago the CDS Users Group was formed and the feedback from this group stimulated and assisted in further development of the test. Since the original description of the CDS Test there have been, in addition to the published updates of the method, thirty-five CDS Newsletters that have been distributed to members of the CDS Users Group.

Over time, several refinements have been introduced into the method and the scope of the CDS Test has been broadened to enable the vast majority of organisms encountered in a diagnostic laboratory to be tested using all of the currently available antimicrobials. Despite these changes the principles and requirements underlying the test remain the same. These includes: firstly, the requirement that before any antibiotic can be tested by the method, it must be calibrated, that is, the size of the zones of inhibition observed with each species must be correlated with quantitative values (MIC) and secondly, that in the performance of the test, the operator must adhere closely to the method, as described, thereby reproducing the conditions that pertained at the time of calibration.

Whilst a section on quality assurance is included in the method, the operator should remember that the most effective single quality assurance measure is to follow the prescribed technique assiduously. There is no doubt that somebody will find a simpler or more effective way of performing one or more of the steps in the CDS Test. However, before any improvement can be incorporated into the method it is necessary to confirm that it does not disturb the correlation between zone size and MIC.

1.1. Unique features and basis of the CDS Test

Readers are referred to the original monograph on the CDS[1], (reproduced on the CDS website), for a description of the theoretical basis of antibiotic disc testing. Some of the unique features of the CDS Test are described here and in the course of this it is hoped that the derivation of the CDS name will become evident.

1.1.1. Calibration (C) of the Test

As the "gold standard" of the antibiotic susceptibility of an organism is the minimum inhibitory concentration (MIC) of the antibiotic under test all the methods of susceptibility testing must relate to this value. Moreover, the MIC must be determined by an internationally standardised technique. Before any antibiotic or bacterial species is included in the CDS Test the test must be calibrated for that particular antibiotic and the targeted species. Calibration consists of plotting the zone sizes observed with a large number of strains of the species included in the CDS Test against the log MIC of each antibiotic.

The agreed gold standard test is the agar dilution technique originally proposed by Ericsson and Sherris in 1971[2]. The agar dilution method is still accepted by the WHO as the gold standard and it has considerable advantages over broth dilution, a technique that is used by other methods to determine the MIC. More recently some other methods of susceptibility testing have been using broth dilution as the quantitative technique to which the disc test is calibrated. This is a convenient technique, lends itself to greater automation and is supplied by commercial resources that have obtained an ISO 20776-1 standard for the method. We

continue to calibrate the CDS test using agar dilution because it can be applied to a much broader range of organisms and does not suffer from the drawbacks associated with broth dilution that Ericsson and Sherris clearly demonstrated[2].

In this 8th edition we have included a detailed description of the agar dilution technique that is used to calibrate the CDS test. This is done for two reasons, first it is an essential part of the regulatory requirements set out by TGA for Australian laboratories use of an in-house IVD (see page 21). Also it will be useful for overseas users so that they may calibrate antibiotics that for one reason or another are not calibrated by the CDS Reference Laboratories. For those laboratories who do not have access to a Steers' replicator to inoculate the agar plates a satisfactory technique is to use a wire loop calibrated to deliver 4μL in a spot inoculation. The agar technique is described in detail below:

Determination of MIC by Agar Dilution (WHO)

Preparation of Media

Sensitest Agar
Sensitest Agar is reconstituted with distilled water according to the manufacturers instructions and steamed at 100°C to dissolve the agar-agar component. Forty-nine mL aliquots of the medium are dispensed into 100 mL capacity glass bottles with their caps loose and are autoclaved at 121°C for 15 min and then placed in a water bath at 50°C.

Sensitest Blood Agar
To 46.5 mL Sensitest Agar prepared as described above and cooled to 50°C in a water bath, add 2.5mL of defibrinated horse blood (final concentration of 5%).

Columbia Blood Agar Base chocolate agar
To 45 mL Columbia Blood Agar Base prepared as described above and cooled to 70°C in a water bath, add 4 mL of defibrinated horse blood (final concentration of 8%). The contents are mixed and kept at 70°C for 15 min to obtain 'chocolate agar'. The medium is then cooled to 50°C.

Haemophilus Test Medium
To 47 mL of Haemophilus Test Medium base (Oxoid CM898) sterilised and cooled to 50°C, add 1 mL of fresh or deep frozen of each of haematin and nicotinamide adenine dinucleotide (NAD) solutions to reach a final concentration of 15 mg/L of each. This medium has a shortened storage life and must be used within 2 weeks of preparation.

Supplemented Brucella Medium Base
To 44.5 mL Brucella Medium Base (Oxoid CM0169) sterilised and cooled to 50°C, add 2.5 mL of defibrinated horse blood (final concentration of 5%) and 1mL of a fresh or deep frozen solution of haemin to a final concentration of 5 mg/L, and 1 mL of vitamin K to a final concentration of 1 mg/L.

Preparation of antibiotic solutions

Depending upon the antibiotic and the concentrations required, the antibiotic is weighed and dissolved in the appropriate solvent. The antibiotic solutions are sterilised using 0.45 μm Millipore filters and two-fold dilutions of the antibiotic are prepared in McCartney bottles using

the appropriate sterile diluent. The antibiotic dilutions should cover the range appropriate for each antibiotic and for the organisms tested.

Preparation of antibiotic agar plates

After the medium has cooled to 50°C, a 1 mL volume of the antibiotic solution is added to the 49 mL aliquot of molten medium. The bottle is gently inverted to thoroughly mix the contents before dispensing 25 mL into each of two Petri dishes which have been labelled with the appropriate antibiotic concentration. Plates may be used on the same day or stored at 4°C for one day. Prior to use, the plates are surface dried for 30 min at 35°C in an incubator.

Preparation of the inoculum

Bacterial strains are grown overnight on horse blood agar (chocolate agar is used for *H.influenzae*, Brucella agar is used for fastidious anaerobes*)* incubated at 35°C. Cell suspensions are prepared in sterile 0.9% saline *(*saline containing 20% peptone water is used for fastidious bacteria) and the turbidity of the suspensions is adjusted to 0.8 using a spectrophotometer set at a wavelength of 640 nm. These suspensions thus contain 10^9cfu/mL. Each suspension is diluted 100 fold using a standard 40 dropper Pasteur pipette that delivers one drop (0.025 mL) of the initial suspension into 2.5 mL diluent.

Replication of the inoculum

The two suspensions prepared as described above are dispensed into wells of a Steer's replicator. The probes deliver 0.004 mL of the suspensions to the surface of the agar plates. For each organism, the inocula used contain 10^6 and 10^4 cfu. The control plate containing no antibiotic is inoculated first, followed by the remaining agar plates beginning at the lowest and progressing to the highest concentration of antibiotic. The inoculated plates are incubated in appropriate conditions for eighteen hours or longer, depending on the growth requirement of tested organisms. Appropriate reference strains are also included as controls.

Reading of the antibiotic minimum inhibitory concentration (MIC)

The MIC is the concentration of antibiotic at which there is complete inhibition of growth of 10^{-4} inoculum except when the antibiotic is known to select resistant variants at a high frequency. A fine haze is ignored in the interpretation of the result.

1.1.2. Dichotomous (D) separation

The CDS Test divides and reports antibiotic susceptibilities simply into two categories, "susceptible" and "resistant". We do not recognise "intermediate" as a valid category in the CDS Test. The reasons that we advanced early in the development of the test and which are still valid, were that, when it varied, the susceptibility of the common pathogens to the then available antibiotics was distributed bi-modally. In those rare cases where some strains were less resistant than others we were able to demonstrate that no method of disc testing had sufficient precision to reliably define an "intermediate" group. Also in the present era of Evidence Based Medicine (EBM) the strongest case against reporting "intermediate"

susceptibility is the dearth of evidence relating to the response to antibiotic therapy of infection caused by these isolates. As far as the CDS Test is concerned, they are classed as resistant because we regard the role of susceptibility testing is to act as a guide to the clinician in the choice of the most appropriate antibiotic.

1.1.3. Susceptibility (S) and break points

Over time we have adopted the term "susceptibility and susceptible" in preference to "sensitivity and sensitive" when these relate to CDS testing. The reason for this was the introduction of statistical analyses into CDS testing along with most other tests we perform in the clinical laboratory (see below). So as to avoid confusion between "antibiotic sensitivity" and "statistical sensitivity" we changed the former to "antibiotic susceptibility" and the categories of susceptibility to "resistant" and "susceptible".

With many bacterial species if susceptibility to a particular antibiotic varies it naturally divides into one or two groups. In these cases the MICs are bi-modally distributed into widely separated values and this is no problem in defining susceptible and resistant categories. With other species and particularly with many newer antibiotics the distribution of MIC's is continuous and separation into categories of susceptibility is made on the basis of an arbitrary break point, irrespective of the method used.

Although there may be some supporting evidence such as clinical response, accepted tissue levels and extrapolation from experience and studies with closely related antibiotics in the majority of cases break points still are arbitrary values. The break point MICs of the CDS Test generally are similar to those of other methods. Where we do differ is that we tend to have a more conservative approach and we will select the lower end of the range of break point MICs as the CDS break point.

Even so, argument about a twofold difference in break points in different methods can only be considered as pseudo-exactitude when it is remembered that the values are determined by a gold standard method of MIC determination, which uses doubling dilutions. Doubling dilution irrespective of the technique will have an inbuilt error in excess of the difference of the values under discussion.

1.1.4. Interpretation of results

Where possible the CDS Test uses a uniform zone size to define susceptible strains. The susceptible zone size of 6 mm annular radius (18 mm diameter) was not chosen at random but was that point of the diffusion sigmoid curve that enabled the greatest discrimination between susceptible and resistant strains with the majority of antibiotics having a similar diffusion constant. It is worthwhile revisiting here the Humphrey and Lightbown's formula[3] describing diffusion in agar that is reproduced in the original CDS monograph

$$r^2 = 9.21\ Dt\ (\log M - \log 4\pi hDtc)$$

Where, for our purposes, **r** is the radius of the inhibitory zone, **t** is time from start, **c** is the MIC, **D** is the diffusion constant, **M** is the disc potency and **h** is depth of agar.

The simplest interpretation of this is that the zone size is directly proportional to the diffusion constant and the log of the disc potency and inversely proportional to the log of the MIC.

It can be seen from this formula that with antibiotics of a similar diffusion constant an appropriate adjustment of the disc potency with each antibiotic will result in isolates with different susceptible MIC to each of the antibiotics yielding a uniform zone size for all

susceptible strains. On the other hand, if the diffusion constant of the antibiotic is markedly reduced and it is not possible to increase the disc potency, e.g., vancomycin and polymyxin, then the zone cut-off point will need to be reduced.

Where one species has a susceptible MIC different from that of the predominant species when tested against a particular antibiotic, the designer of the test has two choices. Either the susceptible zone size can be adjusted, e.g., gentamicin with *Pseudomonas* and the *Enterobacteriaceae* (4 mm), or the potency of the disc can be changed for that species alone, e.g., ampicillin with *Haemophilus influenzae* versus the *Enterobacteriaceae* (5 µg v/s 25 µg).

1.1.5. Performance characteristics of the CDS Test

In common with other laboratory tests an assessment can be made of the performance characteristics of the CDS Test. Statistics such as sensitivity, specificity and the predictive value of the CDS Test can be calculated by relating the test results to those obtained with a standard quantitative method. In susceptibility testing statistical sensitivity measures how well the test correctly identifies “true susceptible” strains whereas specificity refers to the ability of the test to correctly categorise “true resistant” strains. The CDS Test is designed to achieve maximum specificity, i.e., the conditions of the test are set to avoid reporting a resistant strain as susceptible.

Laboratory tests rarely can achieve 100 per cent sensitivity and specificity. Similarly with the CDS Test it may be necessary to sacrifice some statistical sensitivity to achieve maximum specificity. In practical terms this means that with some calibrations a few marginally susceptible strains may not be correctly identified as such by susceptibility testing. With each calibration we also calculate the positive predictive value (PPV) of the test that measures the percentage of "true susceptibles" versus all susceptibles (true plus false) reported by the test. An acceptable calibration is one where the positive predictive value is over 98 per cent.

References

1 **Bell, S.M.** 1975. The CDS disc method of antibiotic sensitivity testing (Calibrated Dichotomous Sensitivity Test). *Pathology.* **7**: No. 4. *Suppl*. 1-48.

2 **Ericsson, H.M. & Sherris, J.C.** 1971. Antibiotic Sensitivity Testing *Acta Path. Microbiol. Scand.* 217: *Suppl*.

3 **Humphrey, J.H., & Lightbown, J.W.** 1952. A general theory for plate assay of antibiotics with some practical applications. *J. Gen. Microbiol.* **7**,129-43.

2. Material and Methods

2.1. Materials

- The basic medium used in the CDS Test for the majority of organisms is Sensitest Agar (Oxoid CM409). Fastidious organisms will require the use of an enriched medium: Sensitest agar with 5% horse blood; Supplemented Haemophilus Test Medium Base (Oxoid CM898); Chocolate Columbia Blood Agar (Oxoid CM331); Supplemented Brucella Medium Base (Oxoid CM0169); Casitone complex agar medium (see section 2.2.1). Organisms requiring enriched media are specified in chapters 4, 5, 6, 7, 9 and 12.

- "90 mm diameter" plastic Petri dishes.

- Tubes with 2.5 mL (±5%) of sterile isotonic saline.

- 10 cm of 0.574 mm diameter nichrome wire (gauge B&S 23 or AWG 23) in a loop holder. The wire tip must be cut or filed square and must be replaced if it becomes worn or distorted. Available in lengths of one or more meters from:

SYDNEY	BRISBANE	MELBOURNE
Heatrex Electric Elements pty ltd	**Cynebar**	**Hotco**
17 Herbert St. Mortlake NSW 2137 Phone: (02) 9743 3646 Fax: (02) 9743 5020	164 Abbotsford Rd. Bowen Hills QLD 4006 PO Box 213 Albion QLD 4010 Phone: (07) 3252 4257 Fax: (07) 3252 3720	5 Park Rd Cheltenham VIC 3192 Phone: (03) 9585 1944 Fax: (03) 9585 0268 Contact: Ron

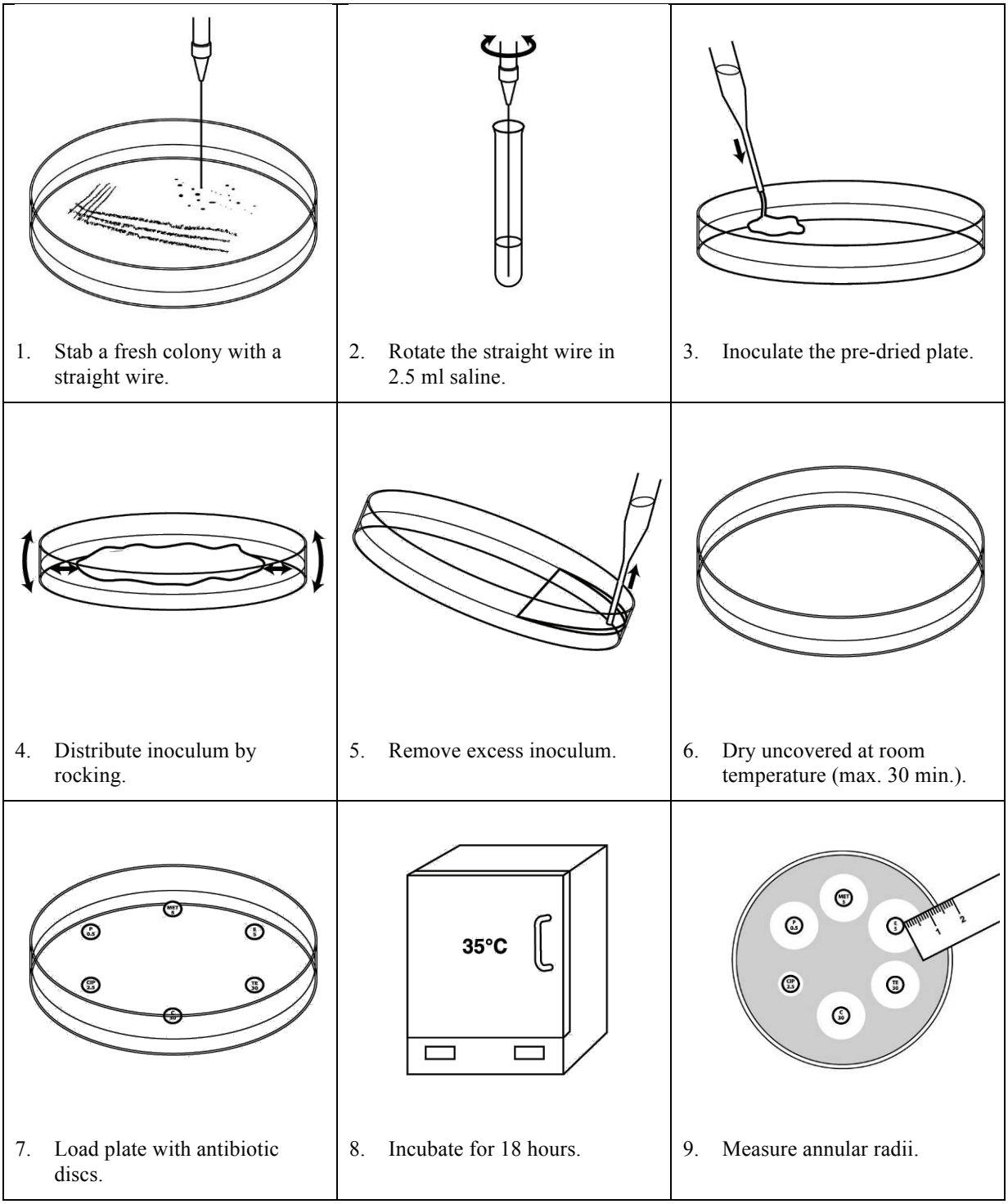
On-Line (rolls of 100g only)	Pre-cut straight nichrome wires
Wiltronics Research Pty Ltd	**bioMérieux Australia Pty Ltd**
Website: www.wiltronics.com.au Navigate to: Components and Supplies Cables and Wires (Under category) Nichrome Wire (Under Sub-category)	Pre-cut Nichrome straight wires (0.57 mm diameter) are available either unattached (MW197) or permanently attached to Microstreaker handles (MW186) Phone: 1800 333 421 Fax: 1800 065 421

Flame free laboratories can use disposable plastic inoculating needles from Copan Innovations (Supplied within Australia by Interpath Services – Catalogue No: 176CS20, Phone 1800 626 369 or 03 9457 6277). These needles yield a slightly heavier, but acceptable, suspension.[1]

- Pasteur pipettes.
- 6 mm diameter antibiotic discs supplied by Oxoid Pty Ltd (Thermofisher), Mast or other specified sources.
- Disc dispenser (maximum of 6 discs) available from Oxoid Pty Ltd or Mast.
- Max/min thermometer.
- Clear plastic ruler, marked in millimetres.
- 0.5 and 2 McFarland standards available from BD.
- Spectrophotometer (optional).

2.2. Methods

The nine steps followed in performing the CDS Test are represented diagrammatically in Figure 2.1. Further details of particular aspects of the method, including preparations necessary before the performance of the actual test, are set out below:



1. Stab a fresh colony with a straight wire.
2. Rotate the straight wire in 2.5 ml saline.
3. Inoculate the pre-dried plate.
4. Distribute inoculum by rocking.
5. Remove excess inoculum.
6. Dry uncovered at room temperature (max. 30 min.).
7. Load plate with antibiotic discs.
8. Incubate for 18 hours.
9. Measure annular radii.

Figure 2.1 Performance of the CDS Test.

2.2.1. Agar plates

Media

Prepare and handle dehydrated media strictly according to the manufacturer's instructions.

Sensitest Agar: Oxoid Sensitest agar (CM409).

Blood Sensitest Agar: Prepare and sterilise Oxoid Sensitest agar (CM409). Cool to 50°C and add defibrinated horse blood to a final concentration of 5% (e.g. 100 mL defibrinated horse blood to 2 L prepared agar).

Haemophilus Test Medium: Prepare and sterilise Haemophilus Test Medium base (Oxoid CM898). Cool to 50°C and add fresh or deep frozen solutions of haematin and nicotinamide adenine dinucleotide (NAD) to a final concentration of 15 mg/L of each. *Note: In-house prepared HTM must be used within 2 weeks of preparation.*

Chocolate Columbia Blood Agar: Prepare and sterilise Columbia Blood Agar Base (Oxoid CM331). Cool to 70°C and add defibrinated horse blood to a final concentration of 8% (e.g. 180 mL defibrinated horse blood to 2 L prepared agar base). Hold at 70°C for 30 min to allow blood to chocolate. Cool to 50°C before pouring.

Supplemented Brucella Medium Base: Prepare and sterilise Brucella Medium Base (Oxoid CM0169). Cool to 50°C and add defibrinated horse blood to a final concentration of 5%, a fresh or deep frozen solution of haemin to a final concentration of 5 mg/L, and vitamin K to a final concentration of 1 mg/L.

Alternatively, small laboratories can purchase Brucella supplemented agar plate (PP2459, Oxoid) to test anaerobes.

Plate preparation and storage

1. Dispense 20 mL of agar into “90 mm diameter” Petri dishes.
2. Store agar plates at 2-8°C for a maximum of 4 weeks in sealed containers, plastic bags or shrink-wrapped.
3. Prior to use, surface dry the plates face down with the lid removed in an incubator at 35-37°C. This will take approximately 1 hour in a fan-forced incubator or 2 hours in an ordinary incubator.
4. Store dried plates at 4°C and use within 2 days of drying.

NOTE: *If commercial preparations, that comply with the specification of the above media, are used the manufacturers instructions must be followed in all respects.*

2.2.2. Preparation of the inoculum

The preferred method

1. Use an overnight culture, preferably grown on blood agar, to prepare the CDS inoculum. With the straight wire, stab 1 colony (1 to 2 mm in diameter). Bacterial material should be visible on the tip of the straight wire.
2. Inoculate the saline by rotating the straight wire at least 10 times with the tip in contact with the bottom of the tube.
3. Mix up and down at least 10 times using a Pasteur pipette. With practice you should be able to see a faint turbidity in the saline suspension after mixing.

Using a Spectrophotometer

Prepare a suspension in 0.9% saline to achieve an absorbance of 0.15 at 640 nm (0.5 McFarland Standard). Dilute the suspension 1 in 5 (1 part suspension, 4 parts saline) in normal saline to obtain the CDS suspension.

Disposable plastic inoculating needles

The CDS method of antimicrobial susceptibility testing stipulates that a nichrome wire with a diameter of 0.574 mm (B&S 23) be used to collect the inoculum and create the CDS suspension. Open flames and other heat sources for sterilisation of metal inoculating needles and loops are not always available in microbiology laboratories. Gas may not be available in small laboratories, and open flames may be a fire hazard and a source of particulate pollution, which may have consequences for occupational health and safety. Other methods of sterilisation have similar disadvantages. Disposable plastic inoculating loops are widely used in microbiology laboratories for convenience and/or safety. Sterile disposable plastic inoculating needles (regular size) from Copan Innovation™ were compared with the recommended nichrome wire[1]. The Copan inoculating needle has a protruding cylindrical tip with a diameter of 0.64 mm. The Copan needles, in general, produced a marginally higher inoculum than the wire, but the CDS method was sufficiently robust to accommodate this increase and no major discrepancies in zone sizes were found.

Alternative methods for difficult organisms

With some organisms, using the preferred method may not yield visible bacterial material on the tip of the wire. In these situations one of the following acceptable alternative methods can be used.

SMALL COLONIES

Stab 3-5 colonies (suitable for small colonies such as streptococci, haemophilus etc.).

STICKY COLONIES

Tease the colony apart and pick up bacterial material.

TINY OR PINHEAD COLONIES

Holding the straight wire at an angle of approximately 45°, move it in one direction along the edge of confluent growth until cellular material is just visible on the tip of the wire.

This is the least desirable method as the resulting inoculum may not be pure, but it may be necessary with *Streptococcus milleri* (*anginosus*) and *Streptococcus pneumoniae*.

SCANTY SMALL / TINY COLONIES

In situations where very few colonies of *Streptococcus pneumoniae* have grown on the primary culture media, CDS sensitivity testing can be performed by growing the pneumococcal suspension in a tube containing 3 mL peptone water (10 g of peptone plus 5 g sodium chloride in 1 L). To obtain the CDS inoculum of 10^7 cfu/mL, suspend 3 colonies (1 mm in diameter) or 6 colonies (0.5 mm in diameter) or 8 colonies (< 0.5 mm in diameter) in 3 mL peptone water and incubate at 35°C for 4 hours. The turbidity of the bacterial suspension should be visible to the naked eye.

Inoculum preparation for specific organisms

ANAEROBES

The inoculum is prepared from a pure culture of the organism grown for 24-48 hours in an anaerobic atmosphere on any agar medium that will support its growth. Organisms are harvested from the surface of the agar and a bacterial suspension is prepared in 0.9% saline. The turbidity of the suspension is adjusted to an equivalent 0.5 McFarland standard. Alternatively the suspension can be adjusted to an absorbance of 0.15 using a spectrophotometer set at a wavelength of 640 nm.

HELICOBACTER PYLORI

The inoculum is prepared in Brain Heart Infusion broth (NOT SALINE) using a 48 – 72 hour culture of *Helicobacter pylori* grown on blood agar or chocolate agar (chocolate Columbia blood agar) in a microaerophilic atmosphere at 35-37°C. The bacterial suspension should be adjusted to an equivalent 2.0 McFarland standard. Also, the suspension can be prepared by adjusting the absorbance to 0.3 using a spectrophotometer set at a wavelength of 640 nm. The inoculum gives a lawn of confluent growth (not semi-confluent).

NEISSERIA

The inoculum is prepared using an 18 to 24 hour culture from a suitable Neisseria growth medium. Suspend one 1.0 to 1.5 mm colony in 2.5 ml saline. If colonies are small (<1.0 mm in diameter) suspend 3 to 5 colonies.

2.2.3. Inoculation of plates and application of discs

1. Flood agar plate, rock the plate to distribute the suspension and remove excess with a Pasteur pipette.
2. Remove the lid and place the plate, uncovered, on the bench to dry. This will usually take 5 to 10 min. Plates must not be left longer than 30 min.
3. Apply no more than 6 antibiotic/antifungal discs usually. In some circumstances an extra disc may be applied as in the case of testing erythromycin and clindamycin for inducible clindamycin resistance.

NOTE: *When testing Helicobacter pylori, apply only 3 discs per plate. With anaerobes, the inhibitory zone sizes around Timentin, meropenem and metronidazole might be very large and interfere with the reading of the inhibitory zones around neighbouring antibiotic discs. If necessary, repeat the test with fewer discs.*

See Chapter 12 (Tables) for correct disc potencies and Section 3.3 (CDS-QANTAS checklist) for correct storage and handling of stock and in use antibiotic discs.

2.2.4. Incubation of plates

Plates are placed immediately in the incubator so that there is no prediffusion and incubated upside down to prevent dehydration of the agar. Most susceptibility tests are performed either on Sensitest agar at 35-37°C in air overnight, or on blood Sensitest agar at 35-37°C in 5% CO_2 overnight. However there are a few exceptions:

Anaerobes: Supplemented Brucella medium base at 35-37°C anaerobically for 24 hours. Slow growing organisms will require 48 hours of incubation.

Campylobacter species: Blood Sensitest agar at 42°C in microaerophilic conditions.

Haemophilus species: HTM agar at 35-37°C in 5% CO_2

Helicobacter pylori: Chocolate Columbia blood agar at 35-37°C in microaerophilic conditions for 72 hours.

Neisseria gonorrhoeae: Chocolate Columbia blood agar at 35-37°C in 5% CO_2 and > 80% humidity.

Yersinia enterocolitica: Sensitest agar at 30°C in air.

2.2.5. Organisms with special growth requirements

Cysteine, thymidine or glutamine requiring strains of Enterobacteriaceae and pyridoxal requiring streptococci (named *Abiotrophia defectives* and *Abiotrophia adiacens*) can be tested by adding 5 drops of a sterile aqueous solution containing one of the following: cysteine (2000 mg/L), thymidine (5000 mg/L), glutamine (1000 mg/L) or pyridoxal (1000 mg/L) to 2.5 mL of saline before inoculation. Sulphonamide and trimethoprim cannot be tested in the presence of thymidine.

CO_2 dependent staphylococci can be incubated in 5% CO_2 at 35-37°C. The effect of CO_2 on the zone sizes is not sufficient to influence the susceptibility test results.

2.2.6. Reading the zones

1. Measure the zones from the back of the plate where possible.
2. Measure the annular radius (the shortest distance from the edge of the disc to the edge of confluent growth). This usually corresponds to the sharpest edge of the zone (Figure 2.2).

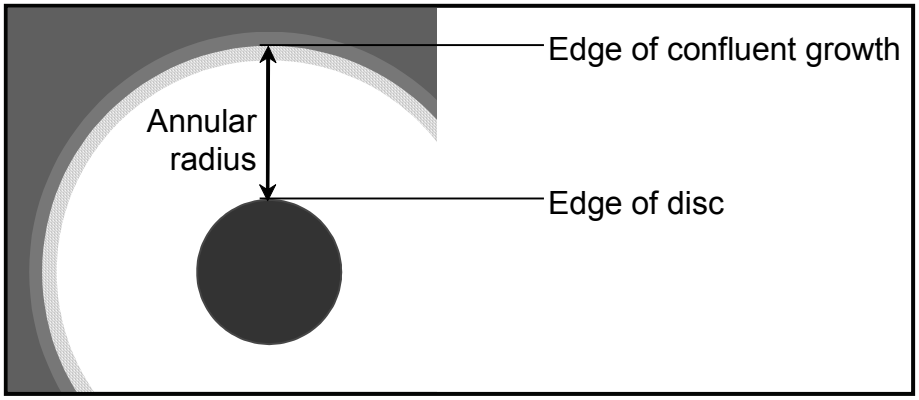


Figure 2.2 Diagram showing the annular radius of the zone of inhibition.

2.3. Interpretation of results

See Chapter 12 (Tables) for the MIC breakpoints and the annular radius cut-offs for susceptibility to calibrated antibiotics, Surrogate antibiotics for antibiotics not calibrated, and specifics for the testing and reporting of β-lactam antibiotics.

Standard Interpretation:

Annular radius	≥ 6 mm	=	SUSCEPTIBLE
	< 6 mm	=	RESISTANT

Exceptions to the Standard Interpretation:

Exceptions to the standard 6 mm annular radius cut off are flagged in Tables 12.1 Calibrations.

2.3.1. Assessment of inhibitory zone morphology

In the CDS test we have adopted the practice of examining the morphology of the inhibitory zone when this may enhance the assessment of an organism's susceptibility. The very oldest example of this is the observation of the sharp edge of the penicillin inhibitory zone with lactamase producing *Staphylococcus aureus*. Throughout the manual attention is drawn to those circumstances where assessment of susceptibility is enhanced by observing difference in zone morphology between susceptible and resistant isolates. In many cases, also, useful changes in zone morphology may be induced by the presence of an adjacent antibiotic disc so that disc placement becomes an essential component of the CDS test.

References

1 **Outhred, A., Bell, S., Pham, J., Varettas, K., Rafferty, D.** 2006. Performance of a Disposable Plastic Inoculating Needle in the Preparation of the CDS Inoculum. Australian Society for Microbiology. Annual Conference, Gold Coast, Australia.

3. Quality assurance

Quality assurance (QA) measures in the CDS Test are incorporated in the method and references to these measures are included in each section of the description. The most important QA measures are *strict compliance* with the described CDS method and with the manufacturer's directions for the use and storage of proprietary consumables. Many of the QA checks used in the CDS test since its introduction are now part of the general routine of NATA accredited laboratories; monitoring and documentation of refrigerator and incubator temperatures, stock batch numbers and receipt times. In this chapter we have included a trouble shooting guide or checklist to which we gave the catchy name "CDS-QANTAS" hoping that this would encourage laboratories to copy it and use it where necessary.

3.1. Regulatory requirements for in-house *in vitro* diagnostic medical device (IVD)

In the 7th edition we reported that, according to NATA, antibiotic susceptibility testing by the disc method is regarded as an in house IVD and as such Australian laboratories performing antibiotic susceptibility testing need to comply with the regulatory framework as laid down by the Therapeutic Goods Administration (TGA) by 30th June 2014. More recently, in March 2016, TGA has issued an update "Regulatory requirements for in-house IVDs Version 2.0." In this 8th edition we have retained as Appendix 1 the detailed explanation of the situation by NATA included in the 7th edition but added the update by TGA as Appendix 2 on the on-line version of the Manual. . In essence the susceptibility test must be contained in the list of in-house IVDs put forward for accreditation and include the approximate date of introduction and a copy of the corporate validation procedure. Any modification of the procedure introduced since 2007 must be supported with a full validation report. More details of the regulatory requirements can be found on the TGA website at https://www.tga.gov.au/, then search for "in-house IVDs".

The CDS fully complies with these requirements as the method was introduced before 2007, the validation procedure is included in this edition (page 8) and any modification (application to a new antibiotic) is fully validated by the CDS Reference Laboratory. To ensure compliance with the TGA regulatory framework laboratories must maintain meticulous records of all aspects of testing including the results of the QC requirements of the CDS test as set out in this manual.

3.2. Reference strains

The performance of a CDS Test with appropriate reference strains is a critical QA measure. An unsatisfactory result with a reference strain invalidates the results obtained with test strains. It is highly recommended that antibiotic susceptibility testing, with the relevant reference strain, be performed on the same day isolates are tested. In laboratories where antibiotic susceptibility tests are performed infrequently, all discs "in use" should be tested with the relevant reference strain at least once weekly.

Table 3.1. Reference Strains

CDS Quality Assurance reference strains for aerobic, micro-aerophilic, and anaerobic bacteria and yeast susceptibility testing and their Type Culture Reference Number.

Reference Strain	Type Culture Reference Number
Bacteroides fragilis	ATCC 25285 (β-lactamase positive)
Campylobacter jejuni	NCTC 11168
Candida parapsilosis	ATCC 22019
Clostridium perfringens	POW 2006
Enterococcus faecalis	POW 1994
Escherichia coli	NCTC 10418
Escherichia coli	NCTC 11560 (β-lactamase positive)
Haemophilus influenza	NCTC 4560
Haemophilus influenza	NCTC 11315 (β-lactamase positive)
Neisseria gonorrhoeae	WHO C
Neisseria gonorrhoeae	WHO O (β-lactamase positive)
Neisseria gonorrhoeae	WHO L
Neisseria gonorrhoeae	WHO P
Pseudomonas aeruginosa	NCTC 10662
Staphylococcus aureus	NCTC 6571
Streptococcus pneumonia	ARL 10582

3.2.1. Obtaining reference strains

The Australian Collection of Micro-organisms (ACM) has suspended its activity and ACM numbering of reference strains is no longer used. However registered CDS users can obtain the equivalent typed reference strains (listed above) from CDS reference laboratory. A certificate of quality is supplied with each strain. Note also that the reference strains POW 2006, POW1994, NCTC 11560 and ARL 10582 have been independently characterised by 16S gene sequencing.

There is no charge for the supply of reference strains to registered CDS users and the address for the laboratory is:

The Antibiotic Reference Laboratory
Department of Microbiology
Clinical Services Building
St. George Hospital
Kogarah NSW 2217Australia
Tel: (02) 9113 3346
Fax: (02) 9113 3349
E-mail: NSWPATH-SealsCDS@health.nsw.gov.au
dianne.rafferty@health.nsw.gov.au
julie.allerton@health.nsw.gov.au

Neisseria gonorrhoeae WHO C, WHO L, WHO L and WHO P may be obtained from:

The WHO Collaborating Centre for STD
and Neisseria Reference Laboratory
Department of Microbiology
The Prince of Wales Hospital
Randwick NSW 2031
Australia.
Tel: (02) 9382 9084
Fax: (02) 9398 4275
Email:
WHOCC.SYDNEY@health.nsw.gov.au

3.2.2. Handling and storage of reference strains

Upon receipt, immediately subculture reference strains onto suitable media as indicated below.

Reference Strain	Media
Haemophilus influenzae	Chocolate agar
Neisseria gonorrhoeae	Chocolate agar
Candida parapsilosis	Sabouraud Dextrose agar
All other reference strains	Blood agar

Compliance with NATA Requirements

The handling and storage of reference strains decribed below complies fully with NATA's Technical Circular 24-January 2016,"The Maintenance of Microbiological Reference Culture Collections (MRCC)". This document is included in the on-line version of the Manual as Appendix 3.

-70°C Storage

Storage at or below -70°C is the preferred method for maintaining a reliable stock of the reference organisms. Reference cultures used for quality assurance testing ideally should be no more than six passages removed from the received strain.

PREPARATION

On receipt, culture each reference strain on a suitable medium and incubate overnight. Following overnight incubation, prepare a heavy suspension of the organism in sterile nutrient broth supplemented with 20% glycerol in a cryogenic vial for storage at -70°C or in liquid nitrogen. Multiple suspensions may be created and stored concurrently as backups. These suspensions are one passage removed from the received reference strain.

RECOVERY

To recover a reference strain from storage, aseptically scrape a small sample of the in-use frozen suspension using the tip of a Pasteur pipette. Inoculate the scraping onto a suitable

medium and immediately return the frozen suspension to -70°C storage. After overnight incubation the recovered culture is now two passages removed from the received reference strain.

The recovered culture, with the exception of *Neisseria* (see below) and anaerobes, may be used as a temporary stock culture for one week provided it is held at 4°C. Storage at 4°C lessens, but does not suspend, the probability of genetic change. Under no circumstances should 4°C stock cultures be re-incubated or allowed to stand at room temperature for a prolonged period following the initial overnight incubation. Fresh 4°C stock cultures can be prepared, by successive re-passaging of the 4°C stock culture at the end of each storage week, a maximum of three more times.

After one week the expired 4°C stock culture should be discarded. These 4°C stock cultures are always between two and five passages removed from the received reference strain.

FRESH WORKING CULTURES

Quality assurance testing should always be performed from fresh 18 to 24 hours cultures. These can be prepared by subculturing from the 4°C stock cultures as required. These fresh working cultures will be at most six passages removed from the received QC reference strain.

4°C storage

Many smaller laboratories do not have -70°C or liquid nitrogen storage capability. With the exception of *Neisseria gonorrhoeae* (see below) and anaerobes, it is permissible for these laboratories to maintain reference strain stock cultures at 4°C with successive weekly re-passaging for a maximum of 30 weeks.

Fresh working subcultures for quality assurance testing may be made from the 4°C stock culture throughout the week, but these should be discarded after use and must not be used for the re-passaging of 4°C stock culture.

After 30 weeks the laboratory must reacquire the reference organisms from an external -70°C or below storage facility.

Storage at 4°C will reduce bacterial metabolism and so will lessen, but not suspend, the probability of genetic change. Laboratories following this protocol need to be aware that this method is not optimal and they must be vigilant in monitoring the cultures for changes that may affect their reliability as reference organisms.

Neisseria gonorrhoeae

Neisseria gonorrhoeae will not remain viable if stored at 4°C, but can be stored at -70°C or below, as described above. The Neisseria Reference Laboratory (above) recommends that in the absence of -70°C storage facilities, *Neisseria gonorrhoeae* be stored on chocolate agar slopes under paraffin oil at 30 to 37°C for one month. Culture the received strain onto a chocolate agar slope and incubate in CO_2 at 35 to 36°C. After 24 hours incubation cover the slope with sterile paraffin oil and store at 30 to 36°C for one month. Fresh working subcultures for quality assurance testing can be made as required throughout the month.

New storage slopes can be prepared, from the previous slope at the start of each month for up to six months. After six months the laboratory must reacquire the reference organism from an external -70°C or below storage facility. As with 4°C storage, this method is not optimal and laboratories must be vigilant in monitoring the culture for changes that may affect its reliability as a reference organism.

3.2.3. Testing reference strains

It is recommended that quality assurance testing with appropriate reference organisms be performed concurrently (on the same day) with the susceptibility testing of the organisms under investigation to ensure that all components of the test are in good working condition. Quality assurance testing of frequently used discs should be performed at least once weekly. An unsatisfactory result with a reference strain invalidates the results obtained for the organism under investigation. For antibiotic discs used infrequently, perform quality assurance testing concurrently with the testing of the organism under investigation.

Quality assurance testing must be performed with each new batch of antibiotic discs and with each new batch of agar plates. It is not necessary to simultaneously test a given cartridge of antibiotic discs against more than one reference organism. For example, if a cartridge of gentamicin 10 µg is tested against *Escherichia coli* NCTC 10418, there is no need to test the same cartridge against *Pseudomonas aeruginosa* NCTC 10662 on the same day.

The -70°C stock culture must be accessed whenever a reference strain consistently fails to give zone sizes within the recommended ranges as specified in Table 12.3.a to d.

Bacteroides fragilis ATCC25285 and *Clostridium perfringens* POW 2006

When performing susceptibility testing on *Helicobacter pylori* isolates but not anaerobes, *Bacteroides fragilis* ACTC 25285 is the reference strain used for the quality assurance testing of metronidazole 5 µg.

When performing susceptibility testing on anaerobes, *Bacteroides fragilis* ATCC 25825 (a β-lactamase producer) is used for the quality assurance testing of Augmentin 3 µg, Tazocin 55 µg and Timentin 85 µg and *Clostridium perfringens* for the quality assurance testing of all other calibrated antibiotics.

See Chapter 7 (Anaerobes), and Table 12.1.c and Table 12.3. for more information on the testing of anaerobes.

3.2.4. Measuring and recording reference strain results

CDS Users should record the actual measurement of the annular radius of each zone of inhibition each time a reference strain is tested. If the records are kept in a cumulative fashion they will draw attention to changing conditions that in their early stages may not lead to results outside the acceptable range but will eventually do so. Disc potency deterioration is the most common example of this and it is possible to detect this before it becomes a problem by observing the gradual reduction in zone sizes on successive observations.

When testing an antibiotic against the reference strains, if the annular radii lie outside the acceptable interval on two consecutive testings, retest using a new cartridge from the same batch. If the annular radii from the new cartridge also lie outside the acceptable interval, discontinue the use of this batch. The reason being that with 95% confidence limits, there is a chance of the reading to "occasionally" land outside the acceptable range but is unlikely on two consecutive testings.

Figure 3.1 shows an example of the method of recording quality assurance zone sizes used in the Microbiology Department at SEALS, Randwick and is recommended for use by laboratories using the CDS test. The antibiotic disc, its potency and the acceptable zone sizes are shown in bold type. This data can be used in estimating the Measurement of Uncertainty (MoU) for CDS testing by the laboratory. Further information on MoU including its calculation can be found in Newsletter 17.

***Staphylococcus aureus* NCTC 6574**									
Date	**Annular radii (mm)**[1,2]						**Sign**	**Batch No.**	**Expiry Date**
	P 0.5 8.7–13.5	**FOX 10 7.1–10.1**	**E 5 8.0–10.8**	**TE 10 11.3–14.4**	**OX 1 7.4–10.4**	**CIP 2.5 9.2–12.4**			
2.11.06	12	9	9.5	12	9	11	JP	12345	7/7/07
9.11.06	11	8.5	10	12.5	9.5	11.5	GF	12345	7/7/07

1. Please circle zone sizes outside the acceptable interval.

2. If an annular radius lies outside the acceptable interval on two consecutive testings, retest using a new cartridge from the same batch. If the annular radii from the new cartridge also lie outside the acceptable interval discontinue use of this batch.

Figure 3.1 Sample quality assurance sheet for antibiotic discs.

3.3. CDS-QANTAS checklist

The CDS Quality Assurance Notations when Testing Antimicrobial Susceptibility checklist is used in "trouble shooting" to define problems revealed by the results observed with the appropriate reference strains in internal QA i.e. the annular radius of the zone of inhibition is not within the acceptable range (see Section 12.3, Table 12.3.a to d). QA is performed with the reference organisms under the conditions described in Section 3.1. If the QA fails, go through the checklist carefully to define the problem.

CDS-QANTAS CHECKLIST [Y / N]

Organism tested……

Medium	Appropriate medium used	[ ]
	"90 mm diameter" Petri dish used	[ ]
	Dehydrated media used within expiry date	[ ]
	Manufacturer's instructions followed	[ ]
	20 ml of medium in Petri dish	[ ]
	4.0 ± 0.2 mm depth of medium (measured externally below meniscus)	[ ]
	Poured plates are stored at 2-8°C	[ ]
	Plates used within 4 weeks of preparation	[ ]
Inoculum	0.56 mm diameter wire used	[ ]
	Plastic inoculating needle from Copan Innovations (Cat No: 176CS20)	[ ]
	Colony sampled less than 36 hours old	[ ]
	Material visible on tip of wire	[ ]
	Tip of wire not pointed	[ ]
	Tip of wire not corroded	[ ]
	Wire allowed to cool before stabbing colony	[ ]
	Homogeneous suspension	[ ]
	Suspension turbidity visible	[ ]
	Whole plate flooded	[ ]
	Excess suspension removed	[ ]
	Flooded plate should dry within 15 min	[ ]
Antibiotic discs	Stock discs stored at or below -20°C	[ ]
	Discs in use stored at 4°C with active desiccant	[ ]
	Packaging of discs not damaged	[ ]
	Discs used within expiry date	[ ]
	Dispenser at room temperature before opening	[ ]
	Desiccant in dispenser active*	[ ]
	Positions in dispensers not shared	[ ]
	Correct disc potencies	[ ]
	No more than 6 discs on plate (usually)	[ ]
	Antibiotic discs applied within 30 min of flooding	[ ]
	Discs flat on medium	[ ]
Incubation conditions	Correct incubation temperature	[ ]
	Correct atmosphere of incubation and humidity	[ ]
	Incubated overnight (minimum16 hours)	[ ]
	No more than 5 plates per stack when possible	[ ]
Measuring zones of inhibition	Homogeneous lawn of growth	[ ]
	Satisfactory growth of organism	[ ]
	Measured from edge of disc	[ ]
	Measured to edge of confluent growth	[ ]
	Measured from back of plate (where possible)	[ ]
	Not measured adjacent to another antibiotic disc	[ ]
	Check antibiotics with 2 or 4 mm cut-off	[ ]

* Timentin, Augmentin, and Tazocin discs are highly susceptible to inactivation by humidity and ambient temperature. These discs need to be stored at a low temperature (4°C or -20°C) with an active desiccant (Bacto Lab. Ph: 02 9602 5499, Merck Microbiology. Ph: 1800 335 571).

3.4. External quality assurance program

CDS Users are reminded to follow the guidelines listed below when participating in the Royal College of Pathologists of Australasia Quality Assurance program (RCPA, QAP).

1. Do not test antibiotics or use discs that have NOT been calibrated for use with the CDS Test.
2. If the antibiotic required is not calibrated, look up Table 12.2.a or b 'Surrogate Disc Testing' for the surrogate disc and report S or R based on the results obtained with the surrogate disc.
3. Do not report the susceptibility of any antibiotic that is not calibrated (Table 12.1.a to d) or is not in the 'Surrogate Disc Testing' tables (Table 12.2.a to b).
4. Read the section relevant to the type of organism or mechanism of resistance when dealing with uncommon mechanisms of resistance.

Example: If the organism is a member of the Enterobacteriaceae such as *Enterobacter cloacae* (member of the EEC group) expressing an inducible β-lactamase (flattened zone between cefotaxime 5 μg and imipenem 10 μg). It is known that resistant mutants producing large amounts of the enzyme are present at a high frequency. The report should be resistant for penicillins, penicillin/inhibitor combinations, cephalosporins (except cefpirome and cefepime), cephamycins and monobactams irrespective of the size of the inhibitory zone. Test and report cefpirome, cefepime, imipenem, meropenem and ertapenem, the antibiotics marked as T in Table 12.4.a.

5. If requested to test an organism not calibrated for the testing by the CDS, reluctantly we can use the method of testing used for an organism with similar growth requirement and behaviour (see Section 9). For example, the testing and reporting of antibiotic susceptibility of *Chromobacterium* sp. may be performed using the method used for that of non-fastidious Gram negative bacilli such as *Pseudomonas* sp. A rider should be added indicating that the CDS has not been calibrated for this organism.

4. Application of the CDS to Gram positive species

4.1. *Corynebacterium* species

Corynebacterium susceptibility testing is performed on Blood Sensitest Agar at 35-37°C, in an atmosphere of 5% CO_2. Slow growing isolates are incubated for 48h. Isolates resistant to benzylpenicillin 0.5 units can be tested against ampicillin 5 µg. An annular radius of < 6 mm with benzylpenicillin 0.5 units and ≥ 4 mm with ampicillin 5 µg indicates reduced susceptibility to penicillin - Report as: "There is decreased susceptibility to penicillin/amoxycillin/ampicillin with the MIC between 0.25 mg/L and 2 mg/L."

4.2. Enterococci

4.2.1. Ampicillin

Most enterococci (excluding *E. faecium*) are susceptible to ampicillin and will have zone of inhibition of annular radius ≥ 4 mm and a diffuse edge around an ampicillin 5 µg disc. The corresponding MIC for these isolates is ≤ 4 mg/L (Table 12.1.a).

β-Lactamase producing *Enterococcus faecalis*

The occurrence of rare β-lactamase producing isolates of *Enterococcus faecalis* makes it essential that CDS Users assiduously adhere to the protocol for testing ampicillin 5 µg against enterococci. The reference strain, *E. faecalis* POW 1994, has a zone of inhibition with a hazy edge around ampicillin 5 µg (Plate 13.1.A). β-Lactamase producing strains give a sharp edged zone of inhibition around ampicillin 5 µg (Plate 13.1.B).

NOTE: β-Lactamase producing isolates may have an inhibitory zone > 4 mm in annular radius, but will still have a sharp edge to the zone. Perform a nitrocefin-based test to confirm the presence of β-lactamase and report the isolate resistant to ampicillin if β-lactamase is detected.

***Enterococcus faecium* and ampicillin**

The majority of *E. faecium* are resistant to ampicillin with growth up to the 5 µg disc (Plate 13.1.C). The resistance to ampicillin/benzylpenicillin in *E. faecium* is associated with low affinity penicillin binding proteins[1].

4.2.2. Doxycycline

Doxycycline 30 µg (Oxoid, DO 30, CT0018B) has been calibrated for the use in the CDS to test organism isolated from localised urinary tract infections[2]. *Enterococci* species are tested on Blood Sensitest agar, 35°C, 5% CO2. The MIC of susceptible strain is ≤ 16 mg/L and the annular radius of susceptible strain is ≥ 4 mm.

For quality control use *Enterococcus faecalis* POW 1994 acceptable range of annular radius is 7.0 – 11.0 mm.

4.2.3. Fosfomycin

Fosfomycin/Trometamol 200 µg disc (Oxoid, FOT 200, CT0758) has been calibrated for the use in the CDS to test organisms isolated from uncomplicated UTI only (urine antiseptic). *Enterococcus* species are tested on Blood Sensitest agar, 35°C, 5% CO2. The MIC of susceptible strains is ≤ 64mg/L and the annular radius of susceptible strain is ≥ 6 mm.

For the quality control, *Enterococcus faecalis* POW 1994 acceptable range of annular radius is 7.1 – 10.7 mm.

4.2.4. Nitrofurantoin

The inclusion of a nitrofurantoin 200 µg disc when performing susceptibility testing of enterococci can assist in the differentiation of *E. faecium* from other enterococci when the strain under investigation is susceptible to nitrofurantoin (annular radius ≥ 4 mm)

A hazy edged zone of inhibition around a nitrofurantoin 200 µg disc with an annular radius of 5 to 7 mm indicates the identity is most likely to be *E. faecium* (Plate 13.1.C).

4.2.5. Trimethoprim

With the CDS Test, the testing of enterococci against trimethoprim is not recommended. *In vitro*, enterococci may appear to be susceptible to trimethoprim but this may not be the case *in vivo*. Enterococci can utilise exogenous dihydrofolate, folinic acid, tetrahydrofolate and thymidine that may be present in the urine and these compounds may antagonise the antibacterial activity of cotrimoxazole or trimethoprim. This may result not only in the failure of therapy of urinary infections caused by enterococci but also in these cases development of bacteraemia has been reported[3].

4.2.6. Quinupristin/Dalfopristin

E. faecalis is inherently resistant to quinupristin and dalfopristin but the combination is usually active against *E. faecium*. Some recent isolates of *E. faecium* are resistant to these agents and to vancomycin.

4.2.7. Vancomycin-resistant enterococci (VRE)

As a result of the emergence of "low level" vancomycin-resistance in enterococci, important modifications have been introduced into the CDS Test for determining the susceptibility to vancomycin. "Low-level" vancomycin-resistant enterococci are those where greater than 90% of cells are inhibited at a concentration of 1 – 2 mg/L of vancomycin whilst the remaining 5-10% is inhibited at a concentration of 8 mg/L. As a result, there is a marked inoculum effect i.e., the higher the inoculum, the higher the MIC. For these reasons, a low inoculum may lead to an error when determining the susceptibility to vancomycin. Therefore, it is essential that the correct CDS inoculum be used (10^7 cfu/mL – ensure cellular material is visible on the tip of the wire or prepare a 1 in 5 dilution of a suspension equivalent to McFarland standard 0.5).

It is mandatory that the strain under investigation be compared with the reference strain (*E. faecalis* POW 1994 which has a sharp edged inhibitory zone of > 2 mm in annular radius around the vancomycin 5 µg disc). The interpretation of the susceptibility is based on the characteristics of the inhibitory zone edge as well as the size of the zone.

The following patterns are seen when testing vancomycin 5 µg against enterococci.

- Susceptible to vancomycin:

1. ***E. faecalis*** generally have a zone > 2 mm with a sharp edge similar to that of the *E. faecalis* POW 1994 (Plate 13.1.A)
2. ***E. faecium*** have a vancomycin zone up to 4 to 6 mm with a sharp edge (Plate 13.1.C).

- Resistant to vancomycin of **vanA phenotype**:

  These strains grow up to the edge of the vancomycin disc and the organism is also resistant to teicoplanin although some strains may have a zone of less than 2 mm around the teicoplanin 15 μg disc but strains with heterogeneous resistance may have a zone up to 6 mm with a very fine growth advancing towards the disc (Plate 13.2.B).

- Resistant to vancomycin of **vanB phenotype**:

  1. ***E. faecalis*** of **vanB phenotype** usually have a reduced zone of less than 2 mm with a hazy edge (growth at the edge of the inhibitory zone) and therefore should be easily recognised.
  2. ***E. faecium*** of **vanB phenotype** may have an inhibitory zone of up to 3 mm in annular radius with a light growth advancing near Van 5 disc when measured from the edge of confluent growth (Plate 13.2.A). Note that incubation in 5% CO_2 enhances the fine growth thus the resistance will be more obvious at 24 hours than the incubation in air. In all cases, if there is doubt on interpreting the result at 24 hours, the plate is incubated for a further 24 hours to observe the light growth advancing towards Van 5 disc.

- Resistant to vancomycin of **vanC phenotype**

  The organism has a sharp edged zone considerably smaller than that of the control strain. This is typical of *Enterococcus gallinarum* and *Enterococcus casseliflavus* possessing the natural vanC type resistance (Plate 13.2.D). These strains are considered resistant to vancomycin although this status is still under discussion. *E. gallinarum*, *E. casseliflavus* and *E. faecium* do not utilise pyruvate; *E. faecalis* does.

NOTE: *The term VRE refers only to E. faecalis and E. faecium that have acquired resistance to vancomycin. Leuconostoc and Pediococcus species are inherently resistant to vancomycin (Plate 13.2.D). VRE can be distinguished from Leuconostoc and Pediococcus by its pyrrolidonyl arylamidase (PYR) activity (See section 4.4).*

4.2.8. High level aminoglycoside resistance in *E. faecalis:*

All enterococci are known to be resistant to the aminoglycosides and all isolates would have a zone < 6 mm with a gentamicin 10 ug disc (CN 10). Therefore enterococci are not calibrated against CN 10. However CN 200 and S 300 discs have been calibrated in the CDS to detect high level resistance to these aminoglycosides in isolates of *E. faecalis* from blood cultures in patients with suspected endocarditis. If the isolate does not have high level resistance, gentamicin or streptomycin may used to provide synergy to ampicillin. Note that the high level resistance is mediated by a different mechanism in each of the two aminoglycosides. Therefore if need be both should be tested.

4.3. *Erysipelothrix* species

Erysipelothrix species are included in the Table of Calibrations with streptococci (Table 12.1.a).

4.4. *Leuconostoc* and *Pediococcus*

Leuconostoc and *Pediococcus* species have high inherent resistance to both vancomycin and teicoplanin, i.e., there is no zone of inhibition observed around either a vancomycin 5 µg or a teicoplanin 15 µg disc (Plate 13.2.D). *Leuconostoc* and *Pediococcus* species lack pyrrolidonyl arylamidase (PYR) activity and this can be used to differentiate them from VRE and other enterococci.

***Nocardia* :** Note this species has been removed from this Manual **as** testing of Nocardia by disc testing is no longer recommended. (see Newsletter 32)

4.5. Staphylococci

4.5.1. Cefoxitin & oxacillin (methicillin)

Methicillin-resistant (*mecA* gene positive) *Staphylococcus aureus* can now be identified using cefoxitin 10 µg discs. Coagulase-negative staphylococci (excluding *Staphylococcus saprophyticus*) are tested against oxacillin 1 µg discs.

When the identification is not available at the time of susceptibility testing both cefoxitin 10 µg and oxacillin 1 µg should be used and the appropriate result reported once the identification is known.

4.5.2. Ceftaroline

Ceftaroline 5 µg disc (Oxoid, CPT 5, CT1942B) has been calibrated for the use in the CDS to test MRSA. *S. aureus* are tested on Sensitiest Agar in air at 35-37°C for 24 hours. The MIC of susceptible strain is ≤ 1mg/L and the annular radius of susceptible strain is ≥ 6 mm.

For the quality control, *S. aureus* NCTC 6571 acceptable range of annular radius is 10.5 – 14.1 mm.

4.5.3. Borderline oxacillin resistant *Staphylococcus aureus* (BORSA)

Some *mecA* gene negative isolates of *S. aureus* may produce large amounts of β-lactamase, which make them appear resistant to oxacillin because this agent is less resistant to hydrolysis by staphylococcal β-lactamase than many other β-lactams. These strains are termed borderline oxacillin-resistant *S. aureus* (BORSA). Cefoxitin is not affected by staphylococcal β-lactamase to the same extent and the use of a cefoxitin 10 µg disc in the CDS test allows a better differentiation of *mecA* gene negative from *mec*A gene positive *S. aureus.*

4.5.4. *Staphylococcus aureus* with low β-lactamase activity

The annular radius of the inhibitory zone around benzylpenicillin 0.5 units (P 0.5u) with β-lactamase negative *S. aureus,* such as *S. aureus* NCTC 6571, is about 12 mm (Plate 13.3.A) and the edge of the inhibitory zone is hazy.

There are rare strains of *S. aureus* that produce low levels of β-lactamase and for these strains, the annular radius of the zone of inhibition around benzylpenicillin 0.5 units (P 0.5u) may be as large 4 to 5 mm the zones of inhibition will still have a sharp edge (Plate 13.3.B). If the inoculum is too light it may result in semi-confluent lawn of growth with an inhibitory zone annular radius of up to 7 mm. However, the edge of the inhibitory zone is still sharp and these strains must be reported as resistant to penicillin and the test should be repeated. If there is any doubt, perform a β-lactamase detection test (eg. Nitrocefin test).

4.5.5. Methicillin susceptible (*mecA* gene negative) *Staphylococcus aureus* (MSSA)

Methicillin susceptible (*mecA* gene negative) strains of *S. aureus* have a zone of inhibition around cefoxitin 10 µg with an annular radius of > 6 mm (usually between 8 and 9 mm). These isolates are reported as susceptible to methicillin (Plate 13.3.C).

Some strains of *S. aureus* are *mecA* gene negative and yet show multiple resistance to other antibiotics. These strains are often loosely referred to as 'Ex-Methicillin-Resistant *S. aureus*'. The annular radius of the zone of inhibition around a cefoxitin 10 µg disc is clearly > 6 mm (Plate 13.3.D). They are reported as susceptible to methicillin.

4.5.6. Methicillin resistant *Staphylococcus aureus* (MRSA)

Although methicillin discs are no longer available*, S. aureus* strains possessing the *mecA* gene are still referred to as methicillin resistant *S. aureus* or MRSA. The annular radius of the inhibitory zone around a cefoxitin 10 µg disc is < 6 mm (usually 0 to 3 mm) for these organisms. (Plate 13.4.A, Plate 13.4.B and Plate 13.4.C). Report these isolates as resistant to methicillin and all other β-lactams.

Multi-resistant MRSA

Multi-resistant MRSA strains are often isolated from institutionalised patients and are resistant to a large number of antibiotics, such as erythromycin, tetracycline, ciprofloxacin, cotrimoxazole and gentamicin.

Non multi-resistant MRSA

MRSA strains that are resistant only to benzylpenicillin and methicillin i.e., non multi-resistant MRSA (NMR-MRSA) or Community Acquired MRSA (CA-MRSA) are now being isolated with increasing frequency from patients in the community and now may be implicated in hospital acquired infections. Typically, these strains have no zone of inhibition or a reduced zone of between 2 and 4 mm in annular radius around a cefoxitin 10 µg disc (Plate 13.4.B). These strains may also be resistant to erythromycin, tetracycline and/or ciprofloxacin.

When cefoxitin 10 µg discs are used neither Mannitol Salt Agar nor incubation at 30°C is required to detect methicillin resistance.

4.5.7. Vancomycin resistance in *Staphylococcus aureus*

Vancomycin resistant *Staphylococcus aureus* (VRSA)

VRSA was first isolated in the USA in 2002[4]. The isolate was resistant to oxacillin/methicillin and vancomycin (MIC > 128 mg/L). A second VRSA strain was isolated

in New York in 2004. These strains will exhibit either no zone or a zone of light growth right up to the edge of a vancomycin 5 μg disc.

Vancomycin intermediate *Staphylococcus aureus* (VISA/GISA)

MRSA with reduced susceptibility to vancomycin and teicoplanin, known as VISA or GISA (vancomycin or glycopeptide intermediate *S. aureus*) have been described overseas[5]. These strains do not have the same mechanism of resistance to glycopeptides that occurs in *Enterococcus faecalis* and *Enterococcus faecium*. Electron microscopy has revealed a thickened cell wall that traps glycopeptide molecules thereby blocking access to the target site. The MIC of vancomycin determined by agar dilution for such strains was between 4 and 8 mg/L.

Vancomycin susceptible staphylococci have a sharp edged zone of inhibition of > 2 mm in annular radius around vancomycin 5 μg and teicoplanin 15 μg discs. VISA strains produce a hazy edged zone of inhibition of < 2 mm in annular radius around vancomycin and teicoplanin discs. i.e., there is fine growth at the edge of the zones of inhibition. This effect is more apparent with teicoplanin than vancomycin. If in doubt, incubate the plates for a further 24 hours (Plate 13.4.C).

h-VISA

A small percentage of cells in the bacterial population of some MRSA isolates have reduced susceptibility to vancomycin but the MIC for the population, as determined by the standard agar dilution technique, remains low (2 mg/L[6]). These strains are referred to as hetero or h-VISA. Detection of this sub-population with reduced susceptibility can be difficult. H-VISA strains will not be readily detected during routine laboratory susceptibility testing using the CDS. Test after 24 or 48 hours of incubation. Exposed to a vancomycin concentration gradient, cells with reduced susceptibility may multiply and become visible after 48 to 72 hours of incubation. It is important to be aware that if treatment with a glycopeptide has failed, h-VISA may be present. In this situation, isolates should be referred to a specialised laboratory.

4.5.8. Erythromycin, clindamycin and *Staphylococcus aureus*

S. aureus resistant to erythromycin are primarily (98%) of the MLS_B phenotype (either constitutive or inducible) and are therefore also resistant to clindamycin and lincomycin (Plate 13.4.D). The remaining 2% possess an efflux mechanism that does not confer resistance to clindamycin. See section 4.7.

4.5.9. Coagulase negative staphylococci (CNS)

In determining methicillin susceptibility for coagulase-negative staphylococci (excluding *S. saprophyticus*) consider only the zone around the oxacillin 1 μg disc; ignore the zone around the cefoxitin 10 μg disc. Note: *S. saprophyticus is excluded from the following discussion.*

Methicillin susceptible CNS

Methicillin susceptible (*mecA* gene negative) coagulase negative staphylococci (excluding *S. saprophyticus*) have a zone of inhibition around oxacillin 1 μg with an annular radius of > 6 mm (usually between 7 and 10 mm). These isolates are reported as susceptible to methicillin.

Methicillin resistant CNS

The majority of methicillin resistant (*mecA* gene positive) coagulase negative staphylococci (excluding *S. saprophyticus*) have a zone of inhibition around oxacillin 1 µg with an annular radius of < 6 mm (usually between 0 and 4 mm). These isolates are reported as resistant to methicillin.

4.5.10. Rifampicin/sodium fusidate

Rifampicin and sodium fusidate are frequently used in combination to treat infections caused by methicillin resistant *S. aureus* and coagulase negative staphylococci. The mutation rate to resistance for each antibiotic is high, in the order of 10^{-5} to 10^{-7} and colonies will often be observed within the inhibitory zones around both rifampicin 1 µg and fusidate 2.5 µg. If the zones of inhibition around rifampicin and fusidate are ≥ 6 mm report the isolate susceptible to the individual antibiotics.

It is advisable that a warning such as "Rifampicin and fusidate must be given in combination since resistance will develop rapidly to either agent if used alone" be issued when reporting the susceptibility of these two antibiotics.

4.5.11. *Staphylococcus saprophyticus* from urine

It is recommended that a novobiocin 5 µg disc is included for testing staphylococci isolated from urine specimens. Urine isolates of coagulase negative staphylococci resistant to novobiocin (annular radius < 4 mm) may be presumptively identified as *S. saprophyticus*. *S. saprophyticus* is a special case where benzylpenicillin 0.5 units and oxacillin 1 µg discs are not used for testing. The MICs of benzylpenicillin and oxacillin are relatively high with wild strains of *S. saprophyticus* isolated from urine when compared with other staphylococci, i.e. they are intrinsically less susceptible to all penicillins and cephalosporins. Also, some isolates produce very low levels of a non-inducible penicillinase. For these reasons, the annular radius of the inhibitory zone around benzylpenicillin 0.5 units and oxacillin 1 µg discs recorded with susceptible strains of *S. saprophyticus* may be < 6 mm and therefore these two discs are not used for testing this species.

Ampicillin 5 µg (instead of penicillin 0.5 u) and cephalexin 100 µg (instead of oxacillin 1 µg) discs are therefore used for the testing of this species and cephalexin is used as the surrogate disc for reporting the susceptibility to Augmentin.

S. saprophyticus may possess a non-inducible or an inducible β-lactamase or no β-lactamase at all. In addition, the *mecA* gene may or may not be present.

Results that may be obtained from testing ampicillin and cephalexin against *S. saprophyticus* are:

- Susceptible to both ampicillin and cephalexin.

  The isolate does not have the *mecA* gene and possesses either no β-lactamase or a non-inducible β-lactamase (Plate 13.5.A).

- Resistant to ampicillin but susceptible to cephalexin.

  The isolate does not have the mecA gene but does possess an inducible β-lactamase (Plate 13.5.B).

- Resistant to ampicillin and cephalexin.

  The isolate has the *mecA* gene (Plate 13.5.C).

4.6. Streptococci

4.6.1. *Streptococcus pneumoniae*

Five enzymes in the cell wall of *S. pneumoniae*, the penicillin-binding proteins (PBP 1A, 1B, 2A, 2B and 2X) are the target sites for β-lactam antibiotics. Increases in the MIC of benzylpenicillin and cefotaxime/ceftriaxone are the result of changes in one or more of the PBPs. Although *S. pneumoniae* strains resistant to cefotaxime/ceftriaxone are often resistant to benzylpenicillin, the correlation is not perfect. A similar situation applies with oxacillin and benzylpenicillin. For these reasons, both benzylpenicillin (not oxacillin) and cefotaxime/ceftriaxone should be tested. Testing and interpretation of susceptibility results are dependent on the site of isolation.

CSF

- Benzylpenicillin: Isolates are tested using a benzylpenicillin 0.5 u disc (P 0.5u). Only isolates with an annular radius of the zone of inhibition ≥ 6 mm are reported susceptible to benzylpenicillin. The MIC of benzylpenicillin of susceptible strains is ≤ 0.125 mg/L.
- Cefotaxime or ceftriaxone: Isolates are tested using a cefotaxime or a ceftriaxone 0.5 µg disc. Only isolates with an annular radius of the zone of inhibition ≥ 4 mm are reported susceptible to cefotaxime or ceftriaxone The MIC is ≤0.5 mg/L.

Sites other than CSF

Isolates from sites other than CSF (sputum, ear, eye and blood cultures not associated with meningitis) are tested against an ampicillin 5 µg disc and a higher potency cefotaxime or ceftriaxone 5 µg disc (Table 12.1.a) in addition to those used for CSF isolates.

- Benzylpenicillin 0.5 u/ampicillin 5 µg: If the inhibitory zone is < 6 mm with a benzylpenicillin 0.5 u disc and ≥ 4 mm with an ampicillin 5 µg disc, report the susceptibility as follows: "There is decreased susceptibility to penicillin, ampicillin and amoxycillin with the MIC between 0.25 mg/L and 2.0 mg/L" (Table 12.1.a).
- Cefotaxime or ceftriaxone 0.5 µg /cefotaxime or ceftriaxone 5 µg: if the inhibitory zone is < 4 mm with a cefotaxime or a ceftriaxone 0.5 µg disc and ≥ 6 mm with a cefotaxime or a ceftriaxone 5 µg disc, report the susceptibility as follows – "There is decreased susceptibility to cefotaxime (or ceftriaxone) with the MIC between 1.0 mg/L and 2.0 mg/L".

Note : The "susceptible" breakpoints of ≤ 0.125 mg/L for penicillin and ≤ 0.5 mg/L for cefotaxime/ceftriaxone were established with wild type isolates of pneumococci and correlated well with clinical response. Strains with a diminished susceptibility to these agents appeared after the initial calibrations and there is a dearth of strong clinical evidence to indicate that infections with these strains would respond to treatment with the antibiotics concerned. This should be kept in mind when interpreting susceptibility testing reports of "decreased susceptibility" for *S. pneumoniae.*

4.6.2. Other streptococci

The susceptibility of β-haemolytic streptococci of groups A, C, G to the penicillins and cephalosporins (except ceftazidime as Gram-positive organisms are resistant to this cephalosporin) is extrapolated from benzylpenicillin 0.5 u (Table 12.2.a). Other streptococci including group B streptococci, *S. mitis*, *S. sanguis* etc and *S. milleri* group showing resistance to penicillin 0.5 u or CTX 0.5 µg can be tested against ampicillin, cefotaxime and ceftriaxone 5 µg discs and interpreted as for *S. pneumoniae* (Section 4.6.1).

NOTE: If infective endocarditis is present, the MIC should be determined.

4.7. Erythromycin and clindamycin

Three mechanisms have been found amongst Gram positive bacteria that confer resistance to the macrolides (erythromycin, roxithromycin, clarithromycin, azithromycin) and the lincosamides (clindamycin, lincomycin[7,8]).

- Methylation of the 23S ribosomal subunit (the target site of action of macrolides and clindamycin) confers resistance to all macrolides and clindamycin. This is the MLS_B phenotype (M = macrolide, L = lincosamide, SB = streptogramin B). Resistance may be constitutive ($cMLS_B$ phenotype) or inducible ($iMLS_B$ phenotype).
- An efflux pump promotes the efflux of macrolides but not clindamycin from the cell. This is the M phenotype.
- The third mechanism of resistance is unknown. It confers resistance to clindamycin but not to erythromycin. This is the LSA (L=lincosamide, SA= streptogramin A) phenotype described for *Streptococcus agalactiae.*[9]

When erythromycin and clindamycin are tested against Gram-positive organisms one of five susceptibility profiles is seen.

1. The isolate is susceptible to both erythromycin and clindamycin.

   The isolate is reported as susceptible to clindamycin, erythromycin and all other macrolides.

   When *S. aureus* was exposed to erythromycin, resistant mutants arose at a high frequency of 10^{-5} to 10^{-6}. In contrast, no clindamycin resistant mutants arose when 10^9 cfu of *S. aureus* were exposed to clindamycin. It is likely that clinical infections with organisms of this phenotype will respond to clindamycin.

   NOTE: *We recommend that in this case the susceptibility of staphylococci to erythromycin, roxithromycin or clarithromycin is not reported – report clindamycin instead.*
   If a Gram-positive organism is susceptible to erythromycin then, in all probability, it will be susceptible to clindamycin (*except on rare occasions where the LSA phenotype might be present - S. agalactiae*).

2. The isolate shows resistance to both erythromycin and clindamycin.

   The isolate is reported resistant to all macrolides and clindamycin ($cMLS_B$ phenotype).

3. The isolate shows resistance to erythromycin but appears susceptible to clindamycin. Adjacent disc testing with clindamycin 2 µg and erythromycin 5 µg positioned 13 mm

apart (edge to edge) shows a flattening of the clindamycin inhibitory zone adjacent to the erythromycin disc (Plate 13.4.D)

The isolate has inducible clindamycin resistance (ICR positive) and is reported as resistant to erythromycin and clindamycin. ICR positive streptococci, corynebacteria, anaerobes and staphylococci can be detected in this way.

Exposing *S. aureus* with this inducible $iMLS_B$ phenotype to clindamycin gave rise to a high frequency of resistant mutants (10^{-5} to 10^{-6}). The MIC of clindamycin for these mutants was 16 mg/L.

4. The isolate shows resistance to erythromycin but appears susceptible to clindamycin with no flattening of the inhibitory zone adjacent to an erythromycin disc.

   The isolate is of the M phenotype and is ICR negative. There is efflux of erythromycin and other macrolides but not clindamycin from the cell. The isolate is reported as resistant to erythromycin and all other macrolides but susceptible to clindamycin.

   Clindamycin therapy may be successful under certain clinical circumstances.

   The M phenotype is uncommon in *S. aureus* and MRSA, occurring in only 1 to 2% of erythromycin resistant strains, the remainder being of the $cMLS_B$ or $iMLS_B$ phenotypes and therefore resistant to all macrolides and clindamycin. Consequently, there is no need to routinely test for the M phenotype (by adjacent disc testing as described above) with *S. aureus* and MRSA isolates. However, adjacent disc testing can be performed where the mechanism of resistance is of interest ($cMLS_B$, $iMLS_B$ or M phenotype).

5. The isolate appears susceptible to erythromycin but resistant to clindamycin.

   This is the rare LSA phenotype, described in *S. agalactiae*. The mechanism of resistance is unknown[9].

   Further information on the phenotypes of clindamycin susceptibility can be found in Newsletter 17.

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5. Application of the CDS to Gram negative species

5.1. *Acinetobacter* species

Acinetobacter species can be separated into two groups on the basis of their susceptibility to ampicillin and cephalexin.

- The ampicillin and cephalexin susceptible *A. lwoffi and A. lwoffi* like group.

  This group has an inhibition zone of approximately 8 mm in annular radius around ampicillin 25 µg discs, and smaller zones around cephalexin 100 µg (7 - 8 mm) and cefotaxime 5 µg (6mm). They are resistant to trimethoprim. See Plate 13.6.A.

- The ampicillin and cephalexin resistant *A. baumannii* and *A. baumannii* like group.

  This group possesses a non-inducible cephalosporinase of the AmpC type. Derepressed β-lactamase producing mutants are not found. They are resistant to ampicillin and cephalosporins. Urinary isolates may be recognised by their susceptibility to Augmentin and resistance to cephalexin and trimethoprim. See Plate 13.6.B.

  There have been reports of multi-resistant strains of *A. baumannii* or *A. baumannii* like organisms possessing plasmid mediated zinc-metallo-carbapenemases (IMP-4, inhibited by EDTA but not by clavulanic acid) or OXA types of β-lactamase not inhibited by EDTA and variably inhibited by clavulanic acid[1,2]). Functionally similar enzymes have been found in the Enterobacteriaceae, *P. aeruginosa* and other organisms (See Section 5.5.8).

Both groups (even the β-lactamase negative *A. lwoffi* like group) have some degree of resistance to cephalosporins. The cephalosporinase produced by *Acinetobacter* species is not inhibited by clavulanic acid. Augmentin (amoxicillin combined with clavulanate potassium) and Timentin (Ticarcillin combined with clavulanic acid) belong to the β-lactam/ β-lactam inhibitor combination group of antimicrobials. These combination groups contain a β-lactam and a second agent that has minimal activity of its own but potentiates the activity of the β-lactam to act as an inhibitor of some β-lactamases. The MIC of clavulanic acid ranges from 1 to 2 mg/L with the *A. lwoffi* / *A. lwoffi* like group and from 4 to 32 mg/L with the *A. baumannii* / *A. baumannii* like group. Susceptibilities are reported according to the standard 6 mm annular radius interpretation.

Some *Acinetobacter* species (NOT *A. lwoffi* or *A. lwoffi*-like which are β-lactamase negative) produce a low level cephalosporinase of AmpC type. These organisms are resistant to cephalexin 100 µg (inhibitory zone annular radius 2- 3 mm) but may appear susceptible to ampicillin 25 µg with a zone marginally > 6 mm. They should be reported as resistant to ampicillin.

Note: We recommend the testing in parallel of ampicillin 25 µg and cephalexin 100 µg discs with all *Acinetobacter* species. See Power points ASM 2011 (on the CDS website).

5.2. *Aeromonas* species

Two chromosomal β-lactamases A1 and A2 have been described in *Aeromonas* species.

A2, a carbapenemase, hydrolyses carbapenems but not cephalosporins or cephamycins. It may show heterogeneous expression of resistance and consequently, resistance to imipenem and meropenem may not be detectable by any conventional method, including MIC determination, and false reporting of susceptibility to carbapenems may occur. (See Plate 13.7.A and Plate 13.7.B). *A. caviae* strains do not possess the A2 carbapenemase and can be tested against carbapenems[3]. All other *Aeromonas* species should be reported as resistant to carbapenems.

A1, an inducible cephalosporinase inhibited by aztreonam but not by clavulanic acid, hydrolyses cephalosporins and cephamycins but not carbapenems (AmpC/Bush group 1) and can be detected by adjacent disc testing (Plate 13.7.A). A close examination revealed that members of *Aeromonas* sp, (except *A. sobria/veronii* both of which failed to produce the cephalosporinase A1), yielded hyper-producing of AmpC mutants at an extremely variable rate when exposed to cefotaxime, Timentin, Tazocin, cefpodoxime, cefotetan, cefoxitin, cefuroxime or cephalexin. Within each species the rate varied from 10^{-5} to 10^{-8}. On the other hand, we were unsuccessful in our attempts to obtain any hyper-producing mutants from the same strains by exposure to ceftazidime or aztreonam. Also, when the hyper-producing mutants that were selected by any of the β-lactams listed above were tested against ceftazidime or aztreonam, all were susceptible to these two antibiotics. It appears that the substrate specificity of AmpC of *Aeromonas* sp. is different to that of the AmpC of the EEC group (see section 5.5.7). Based on these observations we recommend that it is only safe to report ceftazidime, aztreonam susceptibility as well that of the 4th generation cephalosporins and carbapenems, the latter only in the case of *A. caviae*. (See Table 12.4.a guide to the reporting of β-lactam antibiotics).

5.3. *Burkholderia pseudomallei*

Isolates of this species possess an inducible chromosomal cephalosporinase that is inhibited by clavulanic acid. They are usually susceptible to Augmentin and ceftazidime. These antibiotics together with cotrimoxazole are used for treatment. Test *B. pseudomallei* using the criteria applied to *Pseudomonas* species with the exception of Augmentin 60 μg (Table 12.1.b) and report as recommended in Table 12.4.a.

5.4. *Chryseobacterium* species (formerly *Flavobacterium* species)

*C. meningosepticum (*formerly *Flavobacterium meningosepticum*) and *C. indologenes* (formerly *Flavobacterium indologenes*) are the most commonly encountered species of this genus. These species are often resistant to β-lactams and may possess a zinc-metallo-carbapenemase. Both species are also resistant to aminoglycosides (Plate 13.9.B) and can be resistant to quinolones[4].

Cotrimoxazole is the antibiotic of choice for the treatment of infections with *Chryseobacterium* species. Isolates typically show a large zone of inhibition around the cotrimoxazole 25 μg discs (Plate 13.9.B).. *Chryseobacterium* species are tested using the criteria applied to *Pseudomonas* species (Table 12.1.b) and reported using the recommendations in Table 12.4.a.

5.5. Enterobacteriaceae

The predominant mechanism of resistance to β-lactam antibiotics in Enterobacteriaceae and Vibrionaceae is the production of β-lactamases. The number of identified significant β-lactamases has increased considerably with a consequential increase in the complexity of

their classification. The use of a heavier inoculum and the careful selection of appropriate disc potencies in the CDS method facilitate the *in vitro* demonstration of β-lactamase mediated resistance. Adjacent disc testing and inhibitory zone morphology, both integral parts of the CDS method, assist in the identification of these β-lactamases.

A note about Meropenem and Imipenem: Meropenem has replaced Imipenem as a therapeutic agent in Australia so laboratories should report the susceptibilities to Meropenem and not Imipenem. The CDS recommendation is to continue to load the susceptibility plate with both Meropenem 5 µg and Imipenem 10 µg but not to use Imipenem as a surrogate for Meropenem because some strains of Proteus that are resistant to Imipenem are susceptible to Meropenem. The Imipenem 10 µg disc is useful in assessment of zone morphology (an essential part of the CDS), for example, inducible cephalosporins are most readily detected by the placement of an Imipenem 10µg disc adjacent to the Cefotaxime 5 µg disc.

5.5.1. Penicillinase (broad spectrum) β-lactamases

TEM-1, TEM-2 (*E. coli*, *P. mirabilis*, *C. koseri*) and SHV-1 (*K. pneumoniae*) are penicillinases of Bush group 2b (Ambler class A) commonly found in the specified organisms. They are inhibited by clavulanic acid and confer resistance to ampicillin and cephalothin but not to cephalexin or the extended spectrum cephalosporins (Plate 13.8.A).

5.5.2. Inhibitor-resistant TEM β-lactamases (IRTs)

Resistance to Augmentin in some isolates of *Escherichia coli* and rare isolates of *Klebsiella pneumonia* might be due to the production of mutant forms of TEM-1 β-lactamase. These TEM β-lactamases are far less susceptible to inhibition by clavulanic acid than the original TEM enzyme and are called inhibitor-resistant TEM β-lactamases or IRTs. IRT producing *E. coli* are resistant to Augmentin, Timentin, Tazocin but remain susceptible to cephalexin (Plate 13.8.B).

5.5.3. Plasmid mediated AmpC

Non-inducible plasmid mediated cephalosporinases of Bush group 1 (Ambler class C or AmpC) with varying activity have been found in *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella* species (Plate 13.8.C, Plate 13.8.D[5]. These enzymes are inhibited by aztreonam and boronic acid but not by clavulanic acid.

Screening for AmpCs

Plasmid mediated AmpC producers such as *E.coli*, and *K.pneumoniae* can be recognised in routine testing. They are susceptible to cefepime (a 4th generation cephalosporin), and resistant to Augmentin 60 µg and cephalexin 100 µg. Those with high AmpC activity are also resistant to cefotaxime 5 µg and ceftazidime 10 µg.
The susceptibilities are reported according to the standard interpretation.

Confirmation of AmpC (optional)

The use of boronic acids has long been recognised as a specific inhibitor of AmpC β-lactamases[6,7]. The expression of an AmpC can be confirmed by demonstrating synergy between 200 µg boronic acid disc and an adjacent cefotaxime 5 µg, Augmentin 60 µg, cephalexin 100 µg and ceftazidime 10 µg (Plate 13.9.A, Plate 13.9.B; see Power points ASM 2010 on the CDS website).

Note:
To prepare a solution containing 200 µg in each disc (i.e. 8 mg/ml), weigh 400 mg boronic acid (Thianaphthene-2-boronic acid from Sigma - Product number: 499978) and dissolve the powder in 50 ml 70% ethanol in a volumetric flask. Sterilise by filtering through a membrane filter 0.45 µm. Dispense in 10 ml aliquots. The solution is stable in the dark at room temperature.
Boronic acid discs used in phenotypic detection of AmpC are prepared by lining up 6mm blank paper discs in a Petri dish. Drop 25 µL boronic acid onto each disc. Let dry for 24-48 hours (until dry) with lid on. Keep Petri dish protected from light. Transfer dry discs to a jar and store in a drawer. This solution cannot be applied to antibiotic discs because of the presence of ethanol.

5.5.4. Extended spectrum β-lactamases (ESBLs)

The abbreviation 'ESBL' is used to refer to plasmid mediated extended spectrum β-lactamases, though there are reports of these integrating into the bacterial chromosome. These enzymes can hydrolyse extended spectrum cephalosporins and aztreonam but do not hydrolyse the cephamycins (cefoxitin and cefotetan). They are inhibited by clavulanic acid and can be acquired by all members of the Enterobacteriaceae. ESBL production can be detected by adjacent disc testing: position a disc containing clavulanic acid (Augmentin 60 µg or Timentin 85 µg) adjacent to a cephalosporin disc. An elliptical clearing between the two discs indicates the inhibition of the β-lactamase by clavulanic acid (Plate 13.10.A and Plate 13.10.B). This synergistic effect may be subtle and harder to detect in isolates with a high activity ESBL (no zone around a CTX 5). In these cases, repositioning the discs slightly closer together can intensify the effect and confirm the presence of an ESBL. Report susceptibilities for ESBL producing isolates as recommended in Table 12.4.a. Organisms of the EEC group may also have an ESBL (section 5.5.7, Plate 13.12.B and Plate 13.12.C)

5.5.5. *Klebsiella oxytoca* and K1 β-lactamase

K. oxytoca produces a chromosomal β-lactamase known as K1 β-lactamase. Like ESBLs, K1 is inhibited by clavulanic acid but to a lesser degree. Typically, K1 is produced at low (basal) level conferring resistance to ampicillin only with no detectable synergy between clavulanic acid and cephalosporin discs (Plate 13.11.A). In addition to the K1 enzyme, *K. oxytoca* may also acquire a plasmid mediated ESBL (Plate 13.11.B).

Hyper production of the K1 β-lactamase

Some strains of *K. oxytoca* hyper-produce the K1 enzyme and have a reduced zone of inhibition of < 6 mm around an Augmentin 60 µg disc. A mild synergy may be observed near a disc containing clavulanic acid (Augmentin or Timentin) when positioned adjacent to a cefotaxime or ceftriaxone disc (Plate 13.11.C). Greater hyper-production of the K1 enzyme will swamp the weak inhibitory effect of clavulanic acid and the synergy will no longer be visible (Plate 13.11.D).

K1 hyper-producers show a small zone of inhibition around cephalexin 100 µg and cefotaxime 5 µg (Plate 13.11.D). They also have a zone < 6mm in annular radius around Timentin 85 µg and Tazocin 55 µg discs.

Susceptibilities are reported according to the standard interpretation.

5.5.6. *Yersinia enterocolitica*

Yersinia enterocolitica susceptibility testing is performed at 30°C on Sensitest Agar using all the criteria applied to members of the Enterobacteriaceae. Although the presence of an inducible cephalosporinase enzyme B (Ambler class C, Bush group 1 β-lactamase) was described in this species, it was shown this β-lactamase is not highly inducible in the predominant virulent biotype 4, serotype O:3[8] By contrast, biotype 2 or 3, serotype O:5,27, the second most commonly bio-serotypes found in Australia, produce a highly inducible cephalosporinase enzyme B. Induction of β-lactamase may be demonstrated by the flattening of the zone of inhibition around a cefotaxime 5 µg disc adjacent to an imipenem 10 µg disc. It is advisable to report biotype 2 or 3, serotype O:5,27 resistant to penicillins, penicillin/inhibitor combinations and all cephalosporins except cefpirome and cefepime that can be tested.

5.5.7. Inducible cephalosporinases

Further studies on the ESCHAPPM Group.

ESCHAPPM (or ESCAPPM) is a mnemonic for a group of Enterobacteriaceae *(Enterobacter cloacae, Enterobacter aerogenes*, *Serratia marcescens*, *Citrobacter freundii, Hafnia alvei, Aeromonas hydrophila, Aeromonas caviae, Aeromonas veronii, Providencia stuartii, Providencia rettgeri* a*nd Morganella morganii*) that are known to produce chromosomal inducible cephalosporinases, inhibited by aztreonam but not by clavulanic acid (AmpC/Bush group 1). Producers of these types of cephalosporinase can be detected in the CDS test by an adjacent disc test (Plate 13.12.A). In the CDS laboratory, we have been looking more closely at members of this group and we have found that as far as AmpC production is concerned ESCHAPPM is not a homogeneous group but consists of 4 distinct subgroups.

The first subgroup comprises *E. cloacae* complex[9]*, E. aerogenes* and *C. freundii* complex[10] and we propose that this subgroup be called the EEC subgroup. This subgroup gives rise, at a high frequency (10^{-5} to 10^{-6}), to derepressed mutants that are AmpC enzyme hyper-producers. This may not always be obvious on disc testing, so that where this enzyme is detected, the strain is reported as resistant to all cephalosporins except the 4th generation irrespective of the zone size. We recommend that for this subgroup, only the results with 4th generation cephalosporins (cefepime and cefpirome) and carbapenems be reported. We consider it unsafe to attempt to interpret the results of testing with other β-lactams.

The second subgroup contains only one species, *S. marcescens,* which does not give rise to derepressed mutants when exposed to ceftazidime, Tazocin and aztreonam but does so when exposed to other β-lactams including cefotaxime, Timentin, cefpodoxime, cefotetan, cefoxitin, cefuroxime or cephalexin. With this species, it is considered safe only to report the results of testing of aztreonam, Tazocin and ceftazidime in addition to the 4th generation cephalosporins and carbapenems.

The third subgroup consists of all members of *Aeromonas* sp. with the exception of *A. sobria* and *A.veronii* both of which do not produce the cephalosporinase A1. When exposed to cefotaxime, Timentin, Tazocin, cefpodoxime, cefotetan, cefoxitin, cefuroxime or cephalexin this subgroup yields AmpC hyper-producing mutants at an extremely variable rate. Within each species, the rate varies from 10^{-5} to 10^{-8}. On the other hand, we have been unsuccessful in our attempts to obtain any hyper-producing mutants from the same strains by exposure to ceftazidime or aztreonam. Furthermore, when the hyper-producing mutants that are selected by any of the eight β-lactams listed above are tested against ceftazidime or aztreonam, all are susceptible to these two antibiotics. It appears that the substrate specificity of AmpC of

Aeromonas sp. is different to that of the AmpC of the EEC subgroup. Based on these observations, we recommend that it is only safe to report ceftazidime and aztreonam susceptibility as well that of the 4th generation cephalosporins and carbapenems, the latter only in the case of *A. caviae* (see section 5.2).

The fourth subgroup consists of *H. alvei*, *P. stuartii, P. rettgeri* and *M. morganii.* Wild strains are resistant only to ampicillin, Augmentin and cephalexin (a characteristic pattern of this subgroup) and hyper producers of AmpC are selected only at a very low mutation rate of 10^{-8} when exposed to the β-lactams to which they are susceptible. Members of this subgroup are tested in the usual way by the CDS and reported according to the standard interpretation.

Note: As with *Acinetobacter* species, the testing in parallel of ampicillin 25 µg and cephalexin 100 µg discs is strongly recommended with all members of Enterobacteriaceae.

The CDS recommendations on testing and reporting are summarised in Table 12.4. (A guide to the reporting of β-lactam antibiotics).

P. vulgaris* and *P. penneri

P vulgaris and *P. penneri* possess a chromosomal inducible cephalosporinase of class A or Bush group 2e, inhibited by clavulanic acid but not by aztreonam. In the absence of some other resistant mechanism, these organisms are susceptible to Augmentin and derepressed mutants remain susceptible to ceftazidime, cefoxitin and cefotetan. Detection is possible using an adjacent disc testing (flattened zone of CTX 5 placed near an IPM 10 disc) and the susceptibility to AMC 60 (Plate 13.13.A and Plate 13.13.B).

Citrobacter amalonaticus

Citrobacter koseri and *Citrobacter amalonaticus* are two species within the genus Citrobacter that have similar biochemical patterns and cannot be differentiated by usual laboratory techniques such as API or Vitek. However, *C. amalonaticus* produces a chromosomally mediated inducible cephalosporinase of class A that in many respects resembles that of *Proteus vulgaris* and *Proteus penneri* (Plate 13.13.A) while *C. koseri* lacks this enzyme.[11] Although *C. amalonaticus*, is grouped with *P. vulgaris/P. penneri* in Table 12.4. its β-lactam antibiotic susceptibility profile is slightly different. On the CDS plate, a typical pattern of *C. amalonaticus* is flattening of the zone of CTX 5 adjacent to the IPM 10 disc, a zone > 6 mm around cefepime 10 µg and a borderline AMC 60 zone**.**

Inducible plasmid mediated Amp C

Rare strains of Enterobacteriaceae other than those in the EEC group may acquire a plasmid mediated inducible cephalosporinase equivalent to that possessed by the EEC group.[12] Such strains show an inhibitory zone < 6 mm around a CTX 5 disc with resistant colonies in the zone. The susceptibilities are reported as for the EEC group.

5.5.8. Metallo-β-lactamases (MBLs)

These Ambler class B (Bush group 3) plasmid mediated β-lactamases hydrolyse penicillins, cephalosporins and carbapenems but not aztreonam and are plasmid mediated. They require zinc ions for enzymic catalysis and so are inhibited by EDTA (through chelation of Zn^{++}). They are not inhibited by clavulanic acid (Plate 13.9.A).

Screening for MBLs

The MBLs of the Enterobacteriaceae hydrolyse carbapenems less efficiently than they hydrolyse other β-lactams, consequently isolates that express an MBL may still appear susceptible to both imipenem and meropenem. However, their more efficient hydrolysis of other β-lactams and their non-inhibition by clavulanic acid can be used to screen for their presence.

Resistance observed with a cefepime 10 µg disc and the absence of a synergistic zone of inhibition between this disc and an adjacent Augmentin 60 µg disc (containing clavulanic acid) is suggestive of the presence of an MBL. Note: Cefepime is not hydrolysed by AmpC β-lactamases.

Confirmation of an MBL

The expression of an MBL can be confirmed by demonstrating the loss of enzymic activity following chelation of zinc ions. Position a disc loaded with EDTA 415 µg, 10 mm, edge to edge, from a cefepime 10 µg or imipenem 10 µg disc. A zone of growth inhibition between the two discs indicates the presence of an MBL. Confirmation can also be achieved by parallel testing of a carbapenem disc and an EDTA supplemented carbapenem disc (see plate 13.14.B, plate 13.15.A and plate 13.15.B).

When using EDTA discs to perform MBL confirmatory testing it is necessary to include an aztreonam 30 µg disc (ATM 30). As aztreonam is not affected by MBL an MBL producer will be susceptible to ATM 30 unless it also expresses an ESBL. The co-presence of an MBL and an ESBL can be clearly demonstrated in members of the Enterobacteriaceae by the resistance to ATM 30 and the synergy observed between ATM 30 and an adjacent AMC 60 disc.
With *Pseudomonas aeruginosa*, some isolates may show a non-specific synergy between EDTA and a beta-lactam disc including ATM 30 that does not indicate the presence of an MBL.
Note: EDTA discs used in phenotypic detection of MBL are prepared by lining up 6 mm paper discs in a Petri dish. Drop 25 µL EDTA 0.05M (Ethylenediaminetetraacetic acid, Sigma, 431788) onto each disc. Let dry for 24 - 48 hour (until dry) with the lid on in 35^{o}C incubator. Transfer discs to a jar and store in a draw.

Co-expression of MBL and ESBL

It is not uncommon for Enterobacteriaceae isolates to express both an ESBL and an MBL. In these cases, expression of an ESBL cannot be detected in the usual way. An aztreonam 30 µg disc placed in the centre of the plate will show the typical "key hole" with an Augmentin 60 µg disc. The presence of the MBL should be confirmed as described above see (plate 13.15.B).

5.5.9. *Klebsiella pneumoniae* carbapenemases (KPCs)

Although KPC producing *K. pneumoniae* have been reported in recent years in Europe and USA, the first strain isolated in Australia was reported in September 2010. KPCs are essentially "super" ESBLs of Ambler class A (Bush group 2) plasmid mediated β-lactamases that hydrolyses all β-lactam antibiotics including the carbapenems. Although inhibited by clavulanic acid and tazobactam, the enzyme is very efficient and affects all β-lactams including Timentin, Augmentin and Tazobactam (see plates 13.16.A & B).

Due to the low potency discs and the relatively high inoculum used in the CDS test, KPC producers are readily recognised as resistant to all β-lactam antibiotics tested including the carbapenems. The confirmation can be performed phenotypically or by PCR testing.

5.5.10. *Salmonella* species with decreased susceptibility to ciprofloxacin in systemic infection

Treatment failure has been reported with invasive *Salmonella* infections where the MIC of ciprofloxacin was ≥ 0.125 mg/L[13]. On this basis it was recommended that *Salmonella* species isolated from blood culture should be tested against both nalidixic acid and ciprofloxacin. A single point mutation in the quinolone resistance-determining region (QRDR) of the topoisomerase gene *gyrA* in *Salmonella* species confers resistance to nalidixic acid with an associated decrease in the susceptibility to ciprofloxacin[13,14]. Although similar mutations have been described in *E. coli* and more recently in other members of the Enterobacteriaceae it is not known if the increase in MIC up to 0.5 mg/L that may result from the mutation has an adverse effect on the clinical response to ciprofloxacin in Gram negative septicaemia. Until more clinical evidence is available we recommend that CDS Users only test *Salmonella* species causing systemic disease against nalidixic acid as well as ciprofloxacin. The susceptibility to ciprofloxacin is reported as follows:

- The annular radius of the zone of inhibition is ≥ 6 mm around nalidixic acid 30 µg and ≥ 6 mm around ciprofloxacin 2.5 µg

  The MIC of ciprofloxacin is < 0.125 mg/L.

  Report as susceptible to ciprofloxacin.

- The annular radius of the zone of inhibition is < 6 mm (usually 0 mm) around nalidixic acid 30 µg and ≥ 6 mm around ciprofloxacin 2.5 µg

  There is a decreased susceptibility to ciprofloxacin with an MIC of ≥ 0.125 mg/L.

  Report the susceptibility as follows: “There is decreased susceptibility to ciprofloxacin with the MIC between 0.125 mg/L and 1 mg/L. Treatment failure with ciprofloxacin has been reported with these strains”.

- The annular radius of the zone of inhibition is < 6 mm (usually 0 mm) around nalidixic acid 30 µg and < 6 mm around ciprofloxacin 2.5 µg

  The MIC of ciprofloxacin is > 1 mg/L. Report as resistant to ciprofloxacin

5.5.11. Azithromycin 15 µg disc for the testing of *Salmonella typhi* and other *Salmonella* species isolated from blood culture.

Salmonella typhi and other *Salmonella* species isolated from blood culture have been calibrated in the CDS using an azithromycin 15 µg disc. The Australian Antibiotic Guidelines recommend azithromycin as the treatment of choice for *Salmonella typhi*. Although it is surprisingly high we have tentatively accepted a susceptible breakpoint of 16 mg/L as this is based on clinical outcome and *in vitro* testing reported in the literature[15,16]. The cut off of the annular radius for susceptible strains is 4 mm. The acceptable range obtained with *E. coli* NCTC 10418 is 5.4 – 7.0 mm.

5.5.12. Salmonella and gentamicin.

Salmonella are facultative intracellular pathogens. Gentamicin has been shown to be ineffective in killing intracellular Salmonella[17]. Do not report gentamicin for Salmonella.

5.5.13. Fosfomycin 200 µg disc for the testing of Enterobacteriaceae from uncomplicated UTI.

This antibiotic has been calibrated for the CDS for use in uncomplicated urinary tract infections using a fosfomycin/trometamol 200µg disc (Oxoid, FOT 200, CT0758). It is emphasised that this antibiotic has not been calibrated for systemic use and it is best regarded as a urinary antiseptic. Enterobacteriaceae are tested on Sensitest agar. When there is a double zone of confluent growth, the measurement of the annular radius is performed on the inner zone.

MIC of susceptible strains	≤ 32 mg/L
Annular radius of susceptible strains	≥ 6 mm

Note: With this antibiotic *in vitro* mutation to resistance is high with the majority of strains tested and this is demonstrated by the presence of resistant colonies within the inhibitory zones on disc testing. However, it is claimed that the reason for the clinical efficacy is that a fosfomycin urine level of > 128 mg/L is maintained for over 24h after a single 3g oral dose[18]. *Acinetobater* species are considered inherently resistant to fosfomycin.

5.6. *Haemophilus influenzae* and *Haemophilus* species

Haemophilus influenzae

Susceptibility testing of *H. influenzae* is performed on Haemophilus Test Medium (section 2.2.1) and incubated as described in section 2.2.4. Some rare strains of *H. influenzae* grow poorly or not at all on Haemophilus Test Medium - Susceptibility testing of these strains can be performed on chocolate Columbia Blood Agar (section 2.2.1) and incubated as described in 2.2.4.

It has been described with *H.influenzae* or *H. parainfluenzae* that increases in MIC to ampicillin and other β-lactam antibiotics is most likely due to the production of a β-lactamase (TEM type or ROB-1), altered PBPs or in some rare cases a combination of both. These isolates are referred to as β-lactamase positive amoxicillin-clavulanate resistant (BLPACR) strains. Both TEM type and ROB-1 β-lactamases are class A serine β-lactamases which confer resistance to ampicillin and are inhibited by clavulanic acid. Their presence can be confirmed using Nitrocefin hydrolysis.

Haemophili with decreased susceptibility to cefotaxime

Isolates of *H. influenzae* or *H. parainfluenzae* may have altered PBPs with a lowered affinity for β-lactam antibiotics resulting in resistance to ampicillin and decreased susceptibility to other β-lactam antibiotics especially cephalosporins. These isolates are referred to as β-lactamase negative ampicillin-resistant (BLNAR) strains[19]. This decreased susceptibility can be confirmed by susceptibility testing with a cefotaxime or a ceftriaxone 5 µg disc in parallel with the cefotaxime or ceftriaxone 0.5 µg disc. If the inhibitory zone is < 6 mm with

the 0.5 µg disc and ≥ 6 mm with the 5 µg disc, report the susceptibility as follows: "There is decreased susceptibility to cefotaxime (or ceftriaxone) with the MIC between 0.5 mg/L and 2.0 mg/L".

Other Haemophili

Other *Haemophilus* species such as *H. aphrophilus* and *H. paraphrophilus* are now included in *Aggregatibacter sp*. and are described in Section 9.

5.7. *Helicobacter pylori*

A limited range of commonly used antibiotics has been calibrated for susceptibility testing against *Helicobacter pylori* (Table 12.1.b). The inoculum is prepared in Brain Heart Infusion broth as described in section 2.2.2, subsection '*Helicobacter pylori*'. Susceptibility testing is performed on chocolate Columbia Blood Agar (section 2.2.1) and incubated at 35-37°C under microaerophilic conditions for 72 hours.

Test no more than 3 antibiotic discs per plate. Discs should be distributed evenly around the plate and no more than 1cm from the edge of the plate. Susceptible strains of *H. pylori* have large zones of inhibition and these may overlap if discs are positioned any closer than instructed above. Susceptibilities are reported as per the standard interpretation (6 mm).

H. pylori is a difficult organism to work with and dies readily. For this reason, it is not suitable for use as a reference strain for quality assurance testing.

- Metronidazole 5 µg is tested against *B. fragilis ATCC 25285* on blood Sensitest Agar in an anaerobic atmosphere at 35-37°C for 24 hours (Table 12.3.)
- Amoxycillin 2 µg, ciprofloxacin 2.5 µg, erythromycin 5 µg, rifampicin 5 µg and tetracycline 10 µg are tested against *S. aureus* NCTC 7561 on Sensitest Agar in air at 35-37°C for 24 hours (Table 12.3.a).

5.8. *Pasteurella* species

The CDS method has now been extended to include other *Pasteurella* species (*P. gallinarum*, *P. pneumotropica* and *Mannheimia haemolytica* (previously *Pasteurella haemolytica*) in addition to *P. multocida*). Some strains require CO_2 to grow and it is, therefore, now recommended that susceptibility testing of *Pasteurella* species be performed on blood Sensitest Agar (Section 2.2.1) at 35-37°C, in an atmosphere of 5% CO_2. Ampicillin, benzylpenicillin, ciprofloxacin, moxifloxacin and tetracycline have been calibrated for *Pasteurella* species (Table 12.1.b).

5.8.1. Ampicillin and benzylpenicillin susceptibility

Pasteurella multocida

P. multocida should be tested against ampicillin 5 µg. Do not use benzylpenicillin 0.5 u. An inhibitory zone with an annular radius of ≥ 6 mm around the ampicillin 5 µg disc indicates susceptibility to benzylpenicillin.

***Pasteurella* species (other than *P. multocida*)**

Two antibiotic discs, benzylpenicillin 0.5 u and ampicillin 5 µg are used for testing and reporting the susceptibility to benzylpenicillin, ampicillin, amoxycillin and ceftiofur. Categories of susceptibility are defined as follows:

- Susceptible to benzylpenicillin, ampicillin, amoxycillin and ceftiofur.

  The annular radius of the inhibitory zone around benzylpenicillin 0.5 u is ≥ 4 mm.

- Reduced susceptibility to benzylpenicillin, ampicillin, amoxycillin and ceftiofur.

  The annular radius of the inhibitory zone around benzylpenicillin 0.5 u is < 4 mm but ≥ 6 mm around ampicillin 5 µg.

  Report as: "There is decreased susceptibility to benzylpenicillin, ampicillin, amoxycillin and ceftiofur with the MIC between 0.25 and 2.0 mg/L."

- Resistant to benzylpenicillin, ampicillin, amoxycillin and ceftiofur.

  The annular radius of the inhibitory zone around ampicillin 5 µg is < 6 mm.

5.9. *Pseudomonas aeruginosa*

P. aeruginosa possesses an inducible chromosomal Bush group 1, AmpC β-lactamase. However, the rate of mutation to resistance is low (approximately 10^{-9}), unlike members of the Enterobacteriaceae. *In vivo*, *P. aeruginosa* is unlikely to give rise to hyperproducing mutants except in sequestered sites (cystic fibrosis, osteomyelitis[19]).

P. aeruginosa may also acquire an ESBL. Double disc testing with a Timentin disc (contains clavulanic acid) adjacent to a ceftazidime disc, facilitates detection of these ESBLs (Plate 13.14.A).

A zinc-metallo-carbapenemase (or MBL, Ambler class B or Bush group 3) has been found in some strains of *Pseudomonas aeruginosa*. Pigmented MBL producing *P. aeruginosa* isolates are highly resistant to all β-lactams with no zone of inhibition except aztreonam. See section 5.5.8. for additional information on the nature and detection of these enzymes (Plate 13.14.) and Power Points ASM 2011. When performing the detection of MBL, be aware that with some *P. aeruginosa,* EDTA may show non-specific synergy with all antibiotics discs including aztreonam and non β-lactam antibiotic discs. Therefore, it is necessary to always include an ATM 30 disc in MBL confirmatory test.

GES β-lactamases: Multidrug resistant *P.aeruginosa* has been associated with GES-2. Although rare, GES enzymes have been identified worldwide. The GES enzyme (Guiana extended spectrum) is an Ambler class A extended spectrum β lactamase, first described in 2000[21]. They are plasmid encoded and have a broad hydrolysis spectrum including penicillin, extended spectrum cephalosporins and may extend to cephamycins as well as imipenem. Isolates displaying carbapenem resistance and that have a negative disc approximation test with EDTA (see 5.5.8) may possess a GES enzyme.

5.9.1. Fosfomycin 200 µg disc for the testing of *Pseudomonas* species from uncomplicated UTI.

This antibiotic has been calibrated for the CDS for use in uncomplicated urinary tract infections using a fosfomycin/trometamol 200µg disc (Oxoid, FOT 200, CT0758). It is emphasised that this antibiotic has not been calibrated for systemic use and it is best regarded as a urinary antiseptic. *Pseudomonas sp.* are tested on Sensitest agar.

MIC of susceptible strains	≤ 32 mg/L
Annular radius of susceptible strains	≥ 6 mm

Note: With this antibiotic *in vitro* mutation to resistance is high with the majority of strains tested and this is demonstrated by the presence of resistant colonies within the inhibitory zones on disc testing. However, it is claimed that the reason for the clinical efficacy is that a fosfomycin urine level of > 128 mg/L is maintained for over 24h after a single 3g oral dose. When there is a double zone of confluent growth, the measurement of the annular radius is performed on the inner zone.

5.10. *Stenotrophomonas maltophilia*

Most wild strains of *S. maltophilia* are usually susceptible to sulphonamide and resistant to trimethoprim, but show a marked synergy between the two antibiotics. A pear shape or comet tail zone of inhibition between cotrimoxazole (sulphonamide component) and trimethoprim is typical and indicates synergy between the two antibiotics (Plate 13.17.A). However, rare strains of *S. maltophilia* may be resistant to sulphonamide as well as trimethoprim and therefore appear resistant to cotrimoxaxole (SXT 25) (Plate 13.17.B).

NOTE: S. maltophilia usually have a hazy or light growth visible within the SXT 25 inhibitory zone. This does not imply resistance to co-trimoxazole or sulphamethoxazole. Repeat the test using a 1/10 dilution of the CDS inoculum. The inhibitory zone will then be more distinctive and easier to read.

No zone of inhibition around an imipenem (or meropenem) disc and the marked synergy between trimethoprim and cotrimoxazole suggests that the isolate is likely to be *S. maltophilia* (Plate 13.17.A).

Although some isolates of *S. maltophilia* appear to be susceptible to aminoglycosides at 35°C, the MICs of aminoglycosides recorded at 30°C with these strains were 32 or 64 fold higher than those recorded at 35°C. Therefore, in the CDS test *S. maltophilia* is considered to be resistant to all aminoglycosides

S. maltophilia possesses two chromosomal β-lactamases – L1, a penicillinase/carbapenemase inhibited by EDTA and L2, a cephalosporinase inhibited by clavulanic acid. There is a high rate of mutation (10^{-4} to 10^{-6}) to resistance to β-lactams, aminoglycosides and quinolones and should be considered resistant to all antibiotics intended for use as monotherapy.

The drug of choice for the treatment of infections caused by S. maltophilia is cotrimoxazole.

Although we recommend that *S. maltophilia* be reported resistant to all β-lactam antibiotics (Table 12.4), this can lead to a therapeutic dilemma when the isolate is resistant to sulphonamides (Plate 13.17.B) or the patient is allergic to sulphonamides. In these difficult, uncommon situations, test aztreonam, ceftazidime, piperacillin, Timentin, Tazocin, ciprofloxacin and moxifloxacin using the criteria set out for *Pseudomonas* species (susceptibility equals an annular radius of ≥ 6 mm). A warning such as "A combination of antibiotics is necessary for successful therapy" should then be issued with the susceptibility report.

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6. Application of the CDS to *Neisseria Sp.*

6.1 Preliminary Testing by the Routine Laboratory

Please note: This section is intended to encompass the preliminary testing of *Neisseria* species by routine diagnostic laboratories. Those laboratories that need to carry out more detailed examinations on either *Neisseria meningitidis* or *Neisseria gonorrhoeae* should consult section 6.2 on *Neisseria* provided by Prof Lahra. The majority of countries where the CDS is used have highly specialised public health laboratories that carry out detailed antibiotic susceptibility and other studies on both *Neisseria meningitidis* and *Neisseria gonorrhoeae.* It is important that isolates of both these species are sent without delay to the appropriate reference centre.

Neisseria meningitides

If the routine laboratory has ready access to a reference laboratory who can provide a rapid turnaround time they may elect to forgo testing. Otherwise, *Neisseria meningitidis* susceptibility testing is performed on blood Sensitest Agar (Section 2.2.1.) and incubated at 35-36°C, in 5% CO_2. Invasive isolates of *N. meningitidis* should be sent to a reference centre for serotyping and confirmation of identity and antibiotic susceptibilities. The antibiotics calibrated for *Neisseria meningitis* are shown in Table 12.1.b.

Neisseria meningitidis and benzylpenicillin

Benzylpenicillin 0.5 u (P 0.5 u) has been calibrated for the testing of *N. meningitidis* and the annular radius of the zone of inhibition for susceptible strains is ≥ 4 mm. The MIC of benzylpenicillin for susceptible strains is ≤ 0.25 mg/L.

Note 1: Some authorities recommend a susceptible breakpoint of ≤ 1 mg/L, however the "susceptible" breakpoint of ≤ 0.25 mg/L for penicillin was established with wild type isolates of *Neisseria meningitidis* and correlated well with clinical response. Strains with a diminished susceptibility to penicillin appeared after the initial calibrations and there is a dearth of strong clinical evidence to indicate that infections with these strains would respond to treatment with penicillin. This should be kept in mind when interpreting susceptibility test reports of other methods where the susceptible breakpoint is higher than 0.25 mg/L.

Note 2: More recently the Neisseria Reference laboratory has raised an issue regarding the accuracy of the CDS Test in Testing *N. meningitis* to penicillin. This is contrary to the strong evidence of the CDS Laboratory which demonstrates a highly satisfactory separation of *N. meningitis* into Resistant and Susceptible. Until this is resolved laboratories may chose to either perform and report the results of the test or advise the clinician to continue with empirical treatment.

Neisseria gonorrhoeae

Disc susceptibility testing by the routine laboratory is inappropriate for two reasons. First susceptibility testing of *Neisseria gonorrhoeae* is a complex procedure and more appropriately performed by a specialised laboratory. Secondly, clinical practice is not to await

results of antibiotic susceptibility testing before initiating treatment. The choice of antibiotic is made empirically and based on the epidemiological data gathered by the reference centre.

Note 1 Section 6.2 below is supplied in its entirety by Prof. Monica Lahra of the Neisseria Reference Laboratory, Microbiology Department, Randwick. Comments or questions about the contents of this section should be addressed to the Neisseria Reference Laboratory.

Note 2 In section 6.2 the results of "CDS testing" are reported in at least 3 categories based on zone sizes. Strictly speaking these tests are not CDS Tests where, by definition, it only reports zones sizes dichotomously as susceptible or resistant. For the sake of expediency some licence has been granted to the reference laboratory to modify the CDS in this way but this does not indicate support for the wider application of this modification or any other modifications outside the reference laboratory.

6.2 Testing by the Reference Laboratory

Application of the CDS to Neisseria gonorrhoeae and Neisseria meningitidis

This section is intended to provide a guide for testing and interpreting antimicrobial susceptibility testing in a diagnostic setting for antibiotics clinically relevant to the treatment of gonococcal and meningococcal disease.

Laboratory surveillance to monitor antimicrobial resistance in *N. gonorrhoeae* and *N. meningitidis* from invasive meningococcal disease cases is performed by National Neisseria Network (NNN): the Australian Gonococcal Surveillance Programme (AGSP)[1], and the Australian Meningococcal Surveillance Programme (AMSP)[2], The NNN is a collaboration of jurisdictional reference laboratories, in co-operation with private and public sector laboratories[3]. For epidemiological and public health reasons, laboratories are requested to refer all *N. gonorrhoeae* isolates to the appropriate jurisdictional Neisseria Reference Laboratory.

METHODS

Minimum Inhibitory Concentration

The determination of Minimum Inhibitory Concentration (MIC) by the agar plate dilution method is the gold standard for determining the category of susceptibility of *N. gonorrhoeae* and *N. meningitidis*[3,4].

The CDS-applied Etest® MIC method[5] has been validated by the WHO Collaborating Centre for STD, and Neisseria Reference Laboratory, Sydney. For a link to this method see http://www.sciencedirect.com/science/article/pii/S0732889316300955.

CDS Disc Diffusion Method

CDS testing of *N. gonorrhoeae* and *N. meningitidis* using the AGSP and AMSP method and interpretive criteria is performed as described in section 2.2, with the modification of Chocolate Columbia Blood Agar as the testing media and incubated 18-24 hours at 36°C ±

1°C in 5% CO_2 in air with > 80% humidity. The annular radius (AR) of the zone of inhibition of growth around the antibiotic disc is then measured and reported according to the AGSP and AMSP interpretive criteria.

INTERPRETATIVE CRITERIA FOR *N. GONORRHOEAE*

Table 1: AGSP Interpretative Criteria[3,6,7] for *N. gonorrhoeae* MIC values.

Antibiotic MIC (mg/L)	Sensitive	Less sensitive [a]	Resistant
Ceftriaxone	<0.06	0.06-0.25	Not defined [b]
Azithromycin	<1.0	Not defined	≥ 1.0 [c]
Penicillin	<0.06	0.06 – 0.5	>0.5
Ciprofloxacin	<0.06	0.06-0.5	>0.5
Gentamicin	<8	8-16	>16
Spectinomycin	≤64	Not defined	>64

Table 1 Notes:

[a] The term used to describe intermediate susceptibility in ceftriaxone is "decreased susceptibility".

[b] The absence or rare occurrence of an adequate number of evidence-based correlates between the MIC of isolates and treatment outcome means the breakpoint for resistance cannot yet be determined. [8, 9]

[c] The Centre for Disease Control (CDC), Atlanta, USA states that in the absence of established criteria, the use of critical MICs ≥1.0mg/L to interpret the susceptibility of *N. gonorrhoeae* to this agent is recommended until more extensive assessments of clinical treatment outcome to this agent is available. [4]

The annular radius parameters used for testing *N. gonorrhoeae* susceptibility and their indicative MIC and category of susceptibility are shown in tables 2 and 3:

Table 2: Antibiotic annular radius and indicative susceptibility and for *N.gonorrhoeae*

Antibiotic & Disc Potency	Annular Radius	Indicative MIC (mg/L)	Indicative Category
Ceftriaxone 0.5µg	≥10mm	<0.06	**Susceptible**
	≤9mm[d]	0.06 – 0.25	**Decreased Susceptibility** [d]
Azithromycin 15µg	>6mm[e]	<1.0	**Susceptible**
	≤6mm[e]	≥1.0	**Resistant** [f]
Penicillin 0.5U	> 9mm	<0.06	**Susceptible**
	3 – 9mm[g]	0.06 – 0.50[g]	**Less Susceptible**
	<3mm[g]	>0.5 [g]	**Resistant**
Gentamicin 30 µg	≥ 6 mm	≤ 4	**Susceptible**
	<6 mm [h]	>4[h]	**See comment** [h]
Spectinomycin 100µg	≥ 6 mm	<128	**Susceptible**
	<6 mm	≥128	**Resistant** [i]

Table 3: Ciprofloxacin annular radius and indicative susceptibility and for *N.gonorrhoeae*

Antibiotic	Annular radius Ciprofloxacin 1 µg	Annular radius Nalidixic acid 30 µg	Indicative MIC (mg/L)	Susceptibility Category
Ciprofloxacin [j]	> 11mm	> 6mm	<0.06	Susceptible
	6 –11mm	≤ 6 mm	0.06 – 0.5	Less Susceptible
	≤ 6mm	≤ 6mm	>0.5	Resistant

Tables 2 and 3 Notes:

[d] Internal validations have shown that using the ceftriaxone 0.5µg disc, *N. gonorrhoeae* isolates with AR of 5-9mm have an MIC value in the range of 0.016-0.125mg/L. Determination of ceftriaxone MIC is required to definitively assign the category of susceptibility. Isolates with suspected decreased susceptibility or resistance to ceftriaxone should be referred to the appropriate jurisdictional Neisseria Reference Laboratory for MIC testing.

[e] Internal validations have shown that a small proportion of isolates (5-10%) that have an AR of the inhibitory zone to azithromycin 15 µg disc of 6.0-7.0mm have an azithromycin MIC ≥ 1.0mg/L. It is therefore recommended that strains with AR of the inhibitory zone to azithromycin 15 µg disc of ≤7.0mm should have the azithromycin MIC value determined and the susceptibility category confirmed.

[f] The Centre for Disease Control (CDC), Atlanta, USA state that in the absence established criteria, the use of critical MICs ≥1.0mg/L to interpret the susceptibility of *N.gonorrhoeae* to this agent is recommended until more extensive assessments of clinical treatment outcome to this agent is available. [4]

[g] Isolates with AR close to the cut off for less sensitive/resistant category (i.e. 2-4mm) may have an MIC that is 1 standard two-fold dilution below the MIC resistant breakpoint. The susceptibility category of such isolates should be confirmed by MIC testing and referred to the appropriate jurisdictional Neisseria Reference Laboratory.

[h] Internal validations have shown that using the gentamicin 30µg disc, *N. gonorrhoeae* isolates with AR of 2-5mm have an MIC value in the range of 4-16 mg/L. The absence or rare occurrence of an adequate number of evidence-based correlates between the AR of isolates with an MIC value of ≥ 8 mg/L and treatment outcome means the AR breakpoint and indicative category of susceptibility cannot yet be determined with certainty. Determination of gentamicin MIC is required to definitively assign the category of susceptibility. Isolates with suspected intermediate susceptibility or resistance to gentamicin should be confirmed by MIC testing and referred to the appropriate jurisdictional Neisseria Reference Laboratory.

[i] Resistance observed to spectinomycin is rare. Any isolates suspected to have spectinomycin resistance should be confirmed by MIC testing and referred to the appropriate jurisdictional Neisseria Reference Laboratory.

[j] Testing is performed using a combination of both ciprofloxacin 1 µg and nalidixic acid 30 µg discs. The category of susceptibility for ciprofloxacin is derived by considering the annular radius measurements obtained with both antibiotic discs.

INTERPRETATIVE CRITERIA FOR *N. MENINGITIDIS*

Table 4: AMSP interpretative criteria[2,3] of MIC for *N.meningitidis*

Antibiotic MIC (mg/L)	Sensitive	Less Sensitive	Resistant
Ceftriaxone	≤0.25	Not defined[k]	Not defined[k]
Penicillin	<0.06	0.06 – 0.5	>0.5
Ciprofloxacin	<0.06	0.06-0.5	>0.5
Rifampicin	≤0.5	Not defined	>0.5

Table 4 Notes:

[k] The absence or rare occurrence of isolates with MIC values above the susceptible breakpoint are very rare or not yet reported. The absence or rare occurrence of resistant strains precludes defining any result categories other than sensitive. [10]

The annular radius parameters for the various antibiotics used for testing *N. meningitidis* susceptibility and their indicative MIC and category of susceptibility are shown in tables 5 and 6:

Table 5: Antibiotic annular radius and indicative susceptibility and for *N.meningitidis*

Antibiotic	Annular Radius	Indicative MIC (mg/L)	Susceptibility Category
Ceftriaxone 0.5µg	≥9 mm	≤0.25	Susceptible
	≤8 mm	Refer to reference lab for MIC testing[l]	
Penicillin	See notes[m]	See notes[m]	See notes[m]
Rifampicin 1µg	≥ 6 mm	<1	Susceptible
	<6 mm	≥1	Resistant[n]

Table 6: Ciprofloxacin annular radius and indicative susceptibility and for *N.meningitidis*

Antibiotic	Annular radius Ciprofloxacin 1 µg	Annular radius Nalidixic acid 30 µg	Indicative MIC (mg/L)	Susceptibility Category
Ciprofloxacin o	> 11 mm	> 6 mm	<0.06	Susceptible
	6 –11 mm	≤ 6 mm	0.06 – 0.5	Less susceptible
	≤ 6 mm	≤ 6 mm	>0.5	Resistant

Tables 5 and 6 Notes:

[l] The absence or rare occurrence of an adequate number of evidence-based correlates between the MIC of isolates and treatment outcome means the indicative MIC cannot be stated with any degree of certainty. The susceptibility category of such isolates should be confirmed by MIC testing and referred to the appropriate jurisdictional Neisseria Reference Laboratory[10].

[m] Penicillin disc diffusion testing, using the CDS method and chocolate agar, is not recommended to determine the antimicrobial susceptibility category of *N. meningitidis* to penicillin. Internal validation testing of *N. meningitidis* against penicillin 0.5U disc produced results with a high percentage interpretive susceptibility category error, particularly in strains that had an MIC equivalent at close to the various category breakpoints. This has been reported elsewhere[2,17]. MIC determination by Etest® or agar dilution is required for antimicrobial susceptibility categorisation.

[n] Isolates with a rifampicin annular radius measurement of <6mm should be referred for MIC testing. Isolates with a rifampicin annular radius measurement of >2mm but <6 mm should be referred for MIC testing. Resistance to rifampicin is uncommon. The number of isolates available for testing with a rifampicin MIC ≥ 0.5 in internal validation studies is small. The significance of rifampicin MIC ≥ 1mg/L but <100mg/L is not certain, however, the breakpoint for resistance is considered by the AMSP to be ≥1mg/L[2,3,12].

[o] Testing is performed using a combination of both ciprofloxacin 1 µg and nalidixic acid 30 µg discs. The category of susceptibility for ciprofloxacin is derived by considering the annular radius measurements obtained with both antibiotic discs.

INTENDED USE OF REFERENCE STRAINS AND QUALITY CONTROL

The World Health Organisation (WHO) reference strains are those recommended for antimicrobial susceptibility testing of *N. gonorrhoeae*[8,13] and *N. meningitidis*. To reduce the risk of exposure, the use of *N. meningitidis* control strains is not recommended.
The WHO reference strains are available from the WHO Collaborating Centre for STD, and Neisseria Reference Laboratory, Sydney.

Tel: +61 293829084; Email: NSWPATH-WHOCCSYDNEY@health.nsw.gov.au

Table 7: WHO *N. gonorrhoeae* reference strains recommended for QC and acceptable annular radius measurement ranges.

Antibiotic & Disc Potency	Reference Strain	Mean A.R. (Range)	Expected MIC range (mg/L)	Susceptibility Category
Ceftriaxone 0.5µg	WHO C	9.5mm (8.3 – 10.6)	0.008-0.032	Susceptible
	WHO L	5.5mm (4.4 – 6.7)	0.064-0.25	Decreased Susceptible
Azithromycin 15µg	WHO C	10mm (8.3 – 11.3)	0.064-0.25	Susceptible
	WHO P	5.5mm (4.2 – 7.0)	1.0-4.0	Resistant
Penicillin 0.5U	WHO C	3mm (2.1 - 4.7)	0.25-1.0	Less Susceptible
	WHO L	0mm	1.0-4.0	Resistant
Spectinomycin 100µg	WHO C	8mm (6.7-9.1)	<64	Susceptible
	WHO O	0mm	>1024	Resistant
Ciprofloxacin 1µg	WHO C	14mm (12.5 – 16.0)	0.004-0.016	Susceptible
	WHO L	0mm	≥32	Resistant
Nalidixic Acid 30µg	WHO C	13mm (11.0 – 14.0)	-	Susceptible
	WHO L	0mm	-	Resistant

References

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7. Application of the CDS to anaerobic bacteria

7.1. Introduction

Susceptibility testing of anaerobic bacteria is not performed routinely unless infections such as osteomyelitis, infected prosthetic devices and brain abscesses are present and the knowledge of the antimicrobial susceptibility may be crucial for treatment. The CDS has been calibrated for *Propionebacterium sp., Peptostreptococcus sp., Clostridium sp., Bacteroides sp., Prevotella sp., Fusobacterium sp.* For the performance of the CDS Test and quality assurance using the reference strains *C. perfringens* and *B. fragilis*, see Chapters 2 and 3 of this Manual.

7.2. Inoculum, Medium and Incubation conditions

Anaerobes are tested on supplemented Brucella Medium Base (Oxoid CM0169), (Section 2.2.1) and incubated anaerobically at 35-37°C for 24 hours (slow growing organisms require 48 hours). The inoculum is prepared to an equivalent 0.5 McFarland standard as described in section 2.2.2 subsection Anaerobes.

7.3. Antibiotics calibrations

Augmentin 3 µg, benzylpenicillin 0.5 units, cefoxitin 30 µg, clindamycin 2 µg, meropenem 5 µg, metronidazole 5 µg, moxifloxacin 2.5 µg, Tazocin 55 µg and Timentin 85 µg were calibrated for testing anaerobic bacteria. Table 12.1.c lists the antibiotics calibrated, the disc potencies, the annular radius of the inhibitory zones and the antibiotic MIC's of susceptible strains.

7.3.1. Clindamycin and anaerobes.

When clindamycin is tested against anaerobes, an erythromycin 5 µg disc must be placed 13 mm edge to edge from the clindamycin 2 µg disc. This is to detect inducible clindamycin resistance (ICR) (See Section 4.7 for an explanation of ICR).

8. Application to yeast

Please note: This section has been deleted.

9. Application to Unusual organisms

It is simply not possible to calibrate formally the CDS for every organism that could be associated with an infective process. In the first place gathering a sufficient number of unusual isolates can be extremely difficult and secondly we do not have the resources to carry out the procedure on endless number of species. The advice given below is based on less formal testing in the CDS laboratory of these species. When requested, the interpretation of results of testing of organisms not calibrated by the CDS may be extrapolated, with caution, from that of organisms with similar characteristics and growth requirements. The report should include a comment to indicate it is a provisional result and if necessary the susceptibilities may need to be confirmed by a quantitative technique. The species where this applies are grouped below.

Aerococcus sp., *Gemella* sp., *Granulicatella* sp., *Abiotrophia*: For these catalase negative, Gram positive cocci, the interpretation of AST results is extrapolated from those of streptococci. As with streptococci they are tested on Blood Sensitest Agar in an atmosphere of 5% CO_2 and with the antibiotic discs calibrated for *Streptococcus* sp. If the organism has an inhibitory zone < 6mm with P 0.5 u and an inhibitory zone ≥ 6mm with AMP 5 µg, the organism is reported as having a decreased susceptibility to penicillin, amoxycillin, ampicillin with an MIC between 0.25 and 2mg/L. *Aerococcus* sp. are generally susceptible to penicillin and resistant to aminoglycosides. *Aerococcus* sp. can also be tested against ciprofloxacin 2.5 µg disc calibrated for *Staphylococcus* sp.

Pediococcus, Leuconostoc (catalase negative, Gram positive cocci): These species are inherently resistant to vancomycin and teicoplanin. Interpretation of testing of these species is the same as that for streptococci. They are tested on Blood Sensitest Agar, in an atmosphere of 5% CO_2 and with the antibiotic discs calibrated for *Streptococcus* sp. As with streptococci, if the organism has an inhibitory zone < 6mm with P 0.5 u and an inhibitory zone ≥ 6mm with AMP 5 µg, the organism is reported as having a decreased susceptibility to penicillin, amoxycillin, ampicillin with an MIC between 0.25 and 2mg/L.

Coryneform group *(Arcanobacterium, Dermabacter, Rothia* sp*)*: These species are tested on Blood Sensitest Agar , in an atmosphere of 5% CO_2 and with the antibiotic discs calibrated for *Corynebacterium* sp. and interpretation of the results is the same as that with *Corynebacterium* sp. Interpretation of AST results is extrapolated from those of *Corynebacterium* sp. If the organism has an inhibitory zone of < 6mm with P 0.5u and an inhibitory zone of ≥ 6mm with AMP 5 µg, the organism is reported as having a decreased susceptibility to penicillin, amoxycillin, ampicillin with an MIC between 0.25 and 2mg/L.

Bacillus sp*:* The interpretation of results may be extrapolated from those of *Corynebacterium* sp. However, unlike *Corynebacterium* sp., some members of *Bacillus* species may produce a β-lactamase. Therefore, we recommend only the use of a P 0.5 u disc for the testing and reporting of the susceptibility to penicillin, amoxycillin and ampicillin. Do not test *Bacillus* sp. against AMP 5 µg disc as this disc may give false susceptibility with β-lactamase positive isolates.

Non-fastidious, non-Enterobacteriaceae Gram Negative Group: Including *Achromobacter* sp., *Chromobacter* sp. and *B. bronchoseptica* can be tested as outlined in the *Pseudomonas, Burkholderia & Chryseobacterium* species (Table 12.1.b).

Actinobacillus sp., *Aggregatibacter sp., Cardiobacterium, Capnocytophaga*, *Eikenella, Kingella* and other fastidious Gram negative organisms: The interpretation of AST results is extrapolated from those of *Pasteurella* sp. They are tested on Blood Sensitest Agar, in an atmosphere of 5% CO_2 using the discs calibrated for *Pasteurella* sp. Slow growing organisms may require a suspension adjusted to an equivalent 0.5 McFarland standard instead of the standard inoculum of 10^7 cfu/mL.

10. Application to veterinary medicine

Apramycin, Marbofloxacin, Neomycin and Spectinomycin have been calibrated by the CDS methods for veterinary use.

Ceftiofur, cefovexin, enrofloxacin, framycetin, lincospectin, ofloxacin, orbifloxacin and tylosin can be extrapolated from surrogate antibiotics as described in tables Table 12.2.a and Table 12.2.b and in section 10.1.

10.1. Lincospectin

Lincospectin is a combination of lincomycin and spectinomycin. When susceptibility testing is requested for this combination: Gram positive isolates are initially tested against erythromycin 5 μg and, if required, against clindamycin 2 μg; Gram negative isolates are tested against spectinomycin 25 μg. Susceptibilities are reported as follows.

Gram positive organisms

- Isolates susceptible to erythromycin are susceptible to clindamycin and lincomycin and therefore lincospectin.
- Staphylococci resistant to erythromycin are usually (98%) resistant to clindamycin and lincomycin and therefore lincospectin. There is no need to perform adjacent disc testing with erythromycin and clindamycin.
- $iMLS_B$ phenotypes (ICR positive) of streptococci and corynebacteria show a flattening of the clindamycin inhibitory zone adjacent to erythromycin (see section 4.7). These isolates are resistant to clindamycin and lincomycin and therefore to lincospectin.
- M phenotypes (ICR negative) of streptococci and corynebacteria show a zone of inhibition ≥ 6 mm in annular radius around clindamycin with no flattening of the zone adjacent to erythromycin (see section 4.7). These isolates are susceptible to clindamycin and lincomycin and therefore to lincospectin.

Gram negative organisms

Isolates susceptible to spectinomycin are reported as susceptible to lincospectin. Isolates resistant to spectinomycin are reported as resistant to lincospectin.

10.2. Erythromycin and *Pasteurella* sp.

As requested by Veterinary Laboratories, erythromycin has been calibrated on Blood Sensitest Agar at 35°C in 5% CO2 atmosphere for the testing of *Pasteurella* sp. isolated from respiratory infection specimens of dogs and cats.

Annular radius of susceptible strains	≥ 4 mm
Susceptible MIC	≤ 2 mg/L

10.3. Oxacillin and Staphyloccus intermedius group SIG).

The *Staphylococcus intermedius* group comprises three separate species – *Staphylococcus intermedius*, *Staphylococcus pseudintermedius* and *Staphylococcus delphini*. Methicillin susceptible (*mec A* gene negative) SIG have a zone of inhibition to oxacillin 1μg discs >

6mm and should be reported as susceptible to methicillin. Methicillin resistant (*mec A* gene positive) SIG have a zone of inhibition to oxacillin 1µg disc <6mm and should be reported as resistant to methicillin.
When the identification of the isolate is not available at the time of susceptibility testing then both cefoxitin 10µg disc and oxacillin 1µg discs should be tested. If both have a zone >6mm then the isolate should be reported as susceptible to methicillin. See Power point ASM 2015.

11. Direct antibiotic susceptibility testing

The temptation to perform a susceptibility test directly on the specimen isolated from a patient is great as it has the potential to save at least 24 hours in the provision of the result. It is argued that this may be of critical value in the management of the patient. However, this is only the case if the result can be guaranteed to be accurate and this is not always the case. A premature inaccurate result may do more harm than good and in the vast majority of cases it is of little advantage to hasten the result because the patient can be maintained on a broad antibiotic cover until an accurate assessment is made. One area where we are often tempted to provide direct susceptibility testing is in blood cultures. However, in an extensive retrospective analysis Mahrer showed that while there is a reasonable agreement between direct and formal susceptibility testing there were a number of serious discrepancies between the two examinations[1]. Consequently, we do not recommend direct CDS susceptibility testing of blood cultures; the only acceptable direct susceptibility testing is on urine and this is described below.

11.1. Urine

Direct antibiotic susceptibility testing of urine facilitates the early reporting of antimicrobial susceptibilities. The usefulness of early reporting was established previously[2].

In a recent study, the volume of the urine sample used for direct susceptibility testing was adjusted based upon the sample bacterial concentration, to achieve a suspension equivalent to the standard CDS suspension (10^{10}/L)[2]. Bacterial concentrations were estimated semi-quantitatively by manual phase contrast microscopy using Kova® slides. When the bacterial concentration was estimated at ≥ 9 x 10^{10}/L, 25 µl of the urine sample was added to 2.5 ml of sterile saline yielding a bacterial suspension of ≥ 10^9/L. A 250 µl inoculum was used for all lower bacterial concentrations giving a bacterial suspension of < 10^{10}/L. In this study an acceptable lawn of bacterial growth was achieved for 95% of urine samples having bacterial concentration ranging from < 10^9/L to > 10^{11}/L, thereby reducing the number of susceptibilities that had to be repeated.

Bacterial concentrations reported by automated urine analysers may not be accurate enough to permit reliable adjustment of the inoculum. We recommend that laboratories using these analysers use a 250 µl inoculum for all samples.

When manual microscopic morphology suggests enterococci, streptococci or staphylococci, a larger inoculum is required to compensate for the lower viable count of these organisms. Add 50 µl of the urine sample for bacterial concentrations of ≥ 9 x 10^{10}/L and 250 µl for all lower concentrations.

After incubation, the lawn of growth should be confluent, i.e., similar to that obtained with the standard CDS inoculum. If the lawn is semi-confluent or worse, too heavy or mixed, the result should be withheld and susceptibility testing repeated from a plate culture of the organism using a conventional prepared CDS inoculum (section 2.2.2, page 16).

Table 11.1. Urine sample inocula for direct susceptibility testing.

Bacterial Morphology	Quantitation*	Urine Sample Inoculum†
Rods	$< 9 \times 10^{10}$/L	250 µl
	$\geq 9 \times 10^{10}$/L	25 µl
Cocci	$< 9 \times 10^{10}$/L	500 µl
	$\geq 9 \times 10^{10}$/L	50 µl
Automated Urine Analysers		250 µl

* A sample bacterial concentration of 9×10^{10}/L is equivalent to 1000 bacteria per small square of the Kova® chamber.

† Add the inoculum to 2.5 ml sterile saline and use this suspension to flood the susceptibility plate.

References

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12. Tables

A guide to the use of the tables

There are 4 sets of tables containing information essential for the performance of the CDS Test. These tables are intended for both Clinical and Veterinary laboratories. Antibiotics used in veterinary medicine only are flagged.

Calibration tables 1a, 1b, 1c & 1d collectively summarise much of the basic information used in the CDS Test. They list all the organisms and antimicrobials tested, the media used, conditions of incubation, disc potencies, cut off sizes for unusual annular radii and the MICs for susceptible strains (breakpoints). The tables are updated regularly and operators should ensure that they are using the latest versions of these tables. It is most important to pay particular attention to the footnotes included with each table as these highlight exceptions, restrictions and some specific directions. For organisms not included in the Table of Calibrations, extrapolate the testing from that established for similar organisms**.**

Surrogate disc testing tables 2a and 2b list those antimicrobials where the susceptibility can be inferred from the results obtained with a closely related agent, the "surrogate disc". The table is arranged according to bacterial species and the relationship between the antimicrobials is valid only for the species indicated. This table is also updated regularly as data are accumulated which either invalidate the relationship or enable us to add agents to the list. Laboratories may find it useful to include a comment on the susceptibility report that the result reported with a particular antimicrobial indicates the susceptibility to another.

Quality assurance tables 3a, 3b, 3c & 3d list the 13 reference strains, the media, the incubation conditions of testing and the expected range of zone sizes observed with each disc of a stated potency. The footnotes explain how the acceptable ranges of zone sizes were derived and recommend the indications for and the frequency of testing the reference strains.

Table 4 is a guide through the maze of testing and reporting the susceptibility of Gram negative aerobic species to β-lactam antibiotics. The production of one or more β-lactamases is an important and common mechanism of resistance in these species but, for several reasons, resistance may be difficult or impossible to demonstrate by the usual methods of antibiotic susceptibility testing. The table sets out, in some detail, those species where resistance should be assumed on the basis of previous documentation of the presence of a stable mechanism of resistance and those where susceptibility can be reliably demonstrated by the disc test. This table also relies heavily on the footnotes to draw attention to exceptions and special circumstances.

12.1. Calibrations

Table 12.1.a. Calibrations: *Gram Positive Organisms*

Antibiotics, disc potencies, annular radii and MIC for susceptible strains, media and incubation conditions.

Antibiotic	Disc potency (µg)	Exception to the standard interpretation [a]	MIC (mg/L)
***Corynebacterium* species (Blood Sensitest, CO_2, 35-37°C)[b]**			
Ampicillin [c]	5		≤ 2
Benzylpenicillin	0.5 u		≤ 0.125
Chloramphenicol	30		≤ 8
Ciprofloxacin	2.5		≤ 1
Clindamycin [n]	2		≤ 0.5
Erythromycin	5		≤ 0.5
Fusidic acid	2.5		≤ 0.5
Marbofloxacin [k]	5		≤ 2
Moxifloxacin	2.5		≤ 1
Quinupristin/Dalfopristin	15		≤ 2
Rifampicin	1		≤ 0.5
Teicoplanin	15	2 mm	≤ 8
Tetracycline	10		≤ 4
Tigecycline	15		≤ 1
Vancomycin	5	2 mm	≤ 4
Enterococci (Blood Sensitest, CO_2, 35-37°C)			
Ampicillin [d]	5	4 mm[d]	≤ 4
Chloramphenicol	30	4 mm	≤ 8
Doxycycline [e]	30	4 mm	≤ 16
Fosfomycin [e]	200		≤ 64
Gentamicin	200		≤ 256
Linezolid	10		≤ 4
Marbofloxacin [k]	5		≤ 2
Nitrofurantoin [e]	200	4 mm	≤ 64
Quinupristin/Dalfopristin [f]	15		≤ 2
Streptomycin	300	4 mm	≤ 512
Teicoplanin	15	2 mm	≤ 8
Tetracycline	10		≤ 4
Tigecycline	15		≤ 1
Vancomycin [g]	5	(See foot note)[g]	≤ 4
***Listeria* species (Blood Sensitest, CO_2, 35-37°C)**			
Ampicillin	5		≤ 1
Cotrimoxazole	25		≤ 0.5/9.5
Gentamicin	10		≤ 1

[a] The standard 6 mm cut-off applies where no exception has been specified.

[b] Slow growers are incubated for 48h.

[c] *Corynebacterium* species: benzylpenicillin 0.5 u/ampicillin 5 µg: If the inhibitory zone is < 6 mm with a benzylpenicillin 0.5 u disc and ≥ 4 mm with an ampicillin 5 µg disc, report the susceptibility as follows: "There is decreased susceptibility to penicillin, ampicillin and amoxycillin with the MIC between 0.25 mg/L and 2.0 mg/L"

[d] Perform a nitrocefin based test to detect β–lactamase activity if the zone of inhibition has a sharp edge and an annular radius > 4 mm. β–Lactamase–positive isolates are reported as resistant.

[e] For testing urine isolates only.

[f] *Enterococcus faecalis* is intrinsically resistant to pristinamycin and quinupristin/qalfopristin.

[g] A zone of inhibition with a hazy edge and a light growth towards the disc indicates a low level resistance to vancomycin (VanB type), irrespective of the size of the inhibitory zone. The light growth will be more evident if the plate is reincubated for 48 h.

[k] Antibiotic calibrated for veterinary medicine.

[n] Isolates with inducible clindamycin resistance (iMLS$_B$ phenotype) will have a large zone to clindamycin, but should be reported as resistant (see section 4.7).

Table 12.1.a. Calibrations: *Gram Positive Organisms* cont.

Antibiotics, disc potencies, annular radii and MIC for susceptible strains, media and incubation conditions.

Antibiotic	Disc potency (µg)	Exception to the standard interpretation [a]	MIC (mg/L)
Staphylococci (Sensitest, Air 35-37°C)			
Ampicillin [h]	5		≤ 0.5
Benzylpenicillin [i]	0.5 u		≤ 0.06
Cefoxitin [j]	10		≤ 4
Ceftaroline [m]	5		≤ 1
Cephalexin [h]	100		≤ 16
Chloramphenicol	30		≤ 8
Ciprofloxacin	2.5		≤ 1
Clindamycin [n]	2		≤ 0.5
Cotrimoxazole	25		≤ 1/19
Erythromycin	5		≤ 0.5
Fusidic acid	2.5		≤ 0.5
Gentamicin	10		≤ 1
Kanamycin	50		≤ 8
Linezolid	10		≤ 4
Marbofloxacin [k]	5		≤ 2
Moxifloxacin	2.5		≤ 1
Mupirocin	5		≤ 2
Neomycin [k]	30	4 mm	≤ 8
Nitrofurantoin [e]	200		≤ 32
Novobiocin [k]	5		≤ 1
Oxacillin [l]	1		≤ 0.25
Quinupristin/Dalfopristin	15		≤ 2
Rifampicin	1		≤ 0.5
Teicoplanin	15	2 mm	≤ 8
Tetracycline	10		≤ 4
Tigecycline	15		≤ 1
Trimethoprim	5		≤ 4
Vancomycin	5	2 mm	≤ 4

[a] The standard 6 mm cut-off applies where no exception has been specified.
[e] For testing urine isolates only.
[h] For testing *Staphylococcus saprophyticus* only.
[i] Not for testing *Staphylococcus saprophyticus*.
[j] For testing Staphylococcus aureus only.
[k] Antibiotic calibrated for veterinary medicine.
[l] For testing coagulase-negative staphylococci (except *Staphylococcus saprophyticus*).
[m] For testing MRSA only.
[n] Isolates with inducible clindamycin resistance (iMLS$_B$ phenotype) will have a large zone to clindamycin, but should be reported as resistant. Inducible clindamycin resistance can be detected as described in section 4.7.

Table 12.1.a. Calibrations: *Gram Positive Organisms* cont.

Antibiotics, disc potencies, annular radii and MIC for susceptible strains, media and incubation conditions.

Antibiotic	Disc potency (µg)	Exception to the standard interpretation [a]	MIC (mg/L)
Streptococci & *Erysipelothrix* species (Blood Sensitest, CO_2, 35-37°C)[m]			
Ampicillin [m]	5	4 mm	≤ 2
Benzylpenicillin	0.5 u		≤ 0.125
Cefotaxime	0.5	4mm	≤ 0.5
Cefotaxime [m]	5		≤ 2
Ceftriaxone	0.5	4mm	≤ 0.5
Ceftriaxone [m]	5		≤ 2
Chloramphenicol	30		≤ 8
Clindamycin [n]	2		≤ 0.5
Cotrimoxazole	25		≤ 0.5/9.5
Erythromycin	5		≤ 0.5
Marbofloxacin [k]	5		≤ 2
Moxifloxacin	2.5	4 mm	≤ 1
Nitrofurantoin [e]	200	4 mm	≤ 32
Quinupristin/Dalfopristin	15		≤ 2
Rifampicin	1		≤ 0.5
Teicoplanin	15	2 mm	≤ 8
Tetracycline	10		≤ 4
Tigecycline	15		≤ 1
Vancomycin	5	2 mm	≤ 4

[a] The standard 6 mm cut-off applies where no exception has been specified.

[e] For testing urine isolates only

[k] Antibiotic calibrated for veterinary medicine.

[m] NOT for testing *Streptococcus pneumoniae* from CSF. If *Streptococcus pneumoniae* or any other *Streptococcus* species from a site other than CSF is resistant to benzylpenicillin 0.5 u, cefotaxime 0.5 µg or ceftriaxone 0.5 µg then test ampicillin 5 µg, cefotaxime 5 µg and ceftriaxone 5 µg.

[n] Isolates with inducible clindamycin resistance (iMLS$_B$ phenotype) will have a large zone to clindamycin, but should be reported as resistant. Inducible clindamycin resistance can be detected as described in section 4.7.

Table 12.1.b. Calibrations: *Gram Negative Organisms*

Antibiotics, disc potencies, annular radii and MIC for susceptible strains, media and incubation conditions.

Antibiotic	Disc potency (µg)	Exception to the standard interpretation [a]	MIC (mg/L)
***Acinetobacter* species, Enterobacteriaceae & Vibrionaceae (Sensitest, air, 35-37°C)** [b]			
Amikacin	30	4 mm	≤ 16
Ampicillin [c]	25		≤ 8
Apramicin [d]	15	4 mm	≤ 8
Augmentin [e]	60		≤ 8/4
Azithromycin*	15	4 mm	≤ 16
Aztreonam	30		≤ 8
Cefazolin	30		≤ 4
Cefepime	10		≤ 2
Cefotaxime	5		≤ 1
Cefotetan	30		≤ 8
Cefoxitin	30		≤ 8
Cefpirome	10		≤ 2
Cefpodoxime	10		≤ 2
Ceftazidime	10		≤ 4
Ceftriaxone	5		≤ 1
Cefuroxime	30		≤ 8
Cephalexin[c]	100		≤ 16
Chloramphenicol	30		≤ 8
Ciprofloxacin	2.5		≤ 1
Cotrimoxazole	25		≤ 1/19
Doripenem	10		≤ 4
Enoxacin	10		≤ 4
Ertapenem[f]	10		≤ 4
Fosfomycin[g]	200		≤ 32
Gentamicin	10	4 mm	≤ 2
Imipenem	10		≤ 4
Kanamycin	50		≤ 8
Marbofloxacin [d]	5		≤ 2
Meropenem	5		≤ 2
Moxifloxacin	2.5		≤ 1
Nalidixic acid [g]	30		≤ 4
Neomycin [d]	30	4 mm	≤ 8
Nitrofurantoin [g]	200		≤ 32
Norfloxacin [g]	10		≤ 4
Polymyxin B	300 u	4 mm	≤ 1
Spectinomycin [d]	25		≤ 32
Streptomycin [d]	25		≤ 16
Tazocin [e, @]	55		≤ 16/2
Tetracycline	10	4 mm	≤ 4
Tigecycline	15		≤ 1
Timentin [e, &]	85		≤ 16/2
Tobramycin	10	4 mm	≤ 2
Trimethoprim	5		≤ 4

* For the testing of *Salmonella typhi* and other *Salmonella* species isolated from blood culture.

[a] The standard 6 mm cut-off applies where no exception has been specified.

[b] *Yersinia enterocolitica* is incubated in air at 30° C.

[c] Test ampicillin 25 and cephalexin 100 in parallel. Strains resistant to cephalexin but appear susceptible to ampicillin (presence of chromosomal inducible AmpC β-lactamase) are reported as resistant to ampicillin/amoxicillin.

[d] Antibiotic calibrated for veterinary medicine.

[@] Piperacillin in the presence of 2mg/L tazobactam.

[&] Ticarcillin in the presence of 2mg/L clavulanic acid.

[e] If an ESBL is present, report Augmentin, Timentin and Tazocin for isolates from uncomplicated UTI only.

[f] *Acinetobacter* species are considered resistant to ertapenem.

[g] For testing urinary isolates only.

Table 12.1.b. Calibrations: *Gram Negative Organisms* cont.

Antibiotics, disc potencies, annular radii and MIC for susceptible strains, media and incubation conditions.

Antibiotic	Disc potency (µg)	Exception to the standard interpretation[a]	MIC (mg/L)
Branhamella catarrhalis (Moraxella catarrhalis)			
(Blood Sensitest, 5% CO_2, 35-37°C)			
Benzylpenicillin	0.5 u		≤ 0.25
Cefaclor	30		≤ 4
Cefpodoxime	10		≤ 2
Cefuroxime	30		≤ 4
Chloramphenicol	10		≤ 4
Ciprofloxacin	2.5		≤ 1
Cotrimoxazole	25		≤ 1/19
Erythromycin	5		≤ 0.5
Marbofloxacin [d]	5		≤ 2
Moxifloxacin	2.5		≤ 1
Tetracycline	10		≤ 4
***Campylobacter* species**			
(Blood Sensitest, microaerophilic, 42°C)			
Ciprofloxacin	2.5		≤ 1
Erythromycin	5	4 mm	≤ 0.5
Gentamicin	10		≤ 1
Tetracycline	10		≤ 4
***Haemophilus* species**			
(HTM agar [h], 5% CO_2, 35-37°C)			
Ampicillin	5		≤ 1
Augmentin	15		≤ 2/1
Cefaclor	30		≤ 4
Cefotaxime	0.5		≤ 0.25
Cefotaxime	5		≤ 1
Cefpodoxime	10		≤ 2
Ceftriaxone	0.5		≤ 0.25
Ceftriaxone	5		≤ 1
Cefuroxime	30		≤ 4
Chloramphenicol	10		≤ 2
Ciprofloxacin	2.5		≤ 1
Cotrimoxazole	25		≤ 1/19
Marbofloxacin [d]	5		≤ 2
Moxifloxacin	2.5		≤ 1
Tetracycline	10		≤ 4
Helicobacter pylori			
(Chocolate Columbia Blood Agar, microaerophilic, 35-37°C)			
Amoxycillin	2		≤ 1
Ciprofloxacin	2.5		≤ 1
Erythromycin [i]	5		≤ 0.5
Metronidazole	5		≤ 4
Rifampicin [j]	5		≤ 2
Tetracycline	10		≤ 4

[a] The standard 6 mm cut-off applies where no exception has been specified.

[d] Antibiotic calibrated for veterinary medicine.

[h] Haemophilus Test Medium Base containing 15 mg/L of freshly prepared haematin and NAD.

[i] Erythromycin 5 µg is the surrogate disc for reporting the susceptibility to clarithromycin. The MIC of clarithromycin for susceptible strains is ≤ 0.5 mg/L.

[j] Rifampicin 5 µg is the surrogate disc for reporting the susceptibility to rifabutin.

Table 12.1.b. Calibrations: *Gram Negative Organisms* cont.

Antibiotics, disc potencies, annular radii and MIC for susceptible strains, media and incubation conditions.

Antibiotic	Disc potency (µg)	Exception to the standard interpretation[a]	MIC[k] (mg/L)
Neisseria meningitidis			
(Blood Sensitest, 5% CO2, 35-37°C)			
Benzylpenicillin	0.5 u	4 mm	≤ 0.25
Cefotaxime	0.5		≤ 0.25
Ceftriaxone	0.5		≤ 0.25
Chloramphenicol	10		≤ 2
Ciprofloxacin	2.5		≤ 1
Rifampicin	1		≤ 0.5

[a] The standard 6 mm cut-off applies where no exception has been specified.

Table 12.1.b. Calibrations: *Gram Negative Organisms* cont.

Antibiotics, disc potencies, annular radii and MIC for susceptible strains, media and incubation conditions.

Antibiotic	Disc potency (µg)	Exception to the standard interpretation[a]	MIC (mg/L)
***Pasteurella* species (Blood Sensitest, 5% CO2, 35-37°C)**			
Ampicillin [m]	5		≤ 2
Benzylpenicillin	0.5 u	4 mm	≤ 0.25
Ciprofloxacin	2.5		≤ 1
Erythromycin [d]	5	4 mm	≤ 2
Marbofloxacin [d]	5		≤ 2
Moxifloxacin	2.5		≤ 1
Tetracycline	10		≤ 4
***Pseudomonas* species, *Burkholderia* species & *Chryseobacterium* species (Sensitest, air, 35-37°C)**			
Amikacin	30	4 mm	≤ 16
Augmentin [n]	60		≤ 8/4
Aztreonam	30		≤ 8
Cefepime	10		≤ 2
Cefpirome	10		≤ 2
Ceftazidime	10		≤ 4
Ciprofloxacin	2.5		≤ 1
Cotrimoxazole	25		≤ 2/38
Doripenem	10		≤ 4
Ertapenem	10		≤4
Fosfomycin [g]	200		≤ 32
Gentamicin	10	4 mm	≤ 4
Imipenem	10		≤ 4
Marbofloxacin [d]	5		≤ 2
Meropenem	5		≤ 2
Moxifloxacin	2.5		≤ 1
Norfloxacin [g]	10		≤ 4
Piperacillin	50		≤ 16
Polymyxin B	300 u	4 mm	≤ 1
Tazocin[@]	55		≤ 16/2
Ticarcillin[d]	75		≤ 32
Timentin[&]	85		≤ 16/2
Tobramycin	10	4 mm	≤ 4
Trimethoprim	5		≤ 4
***Stenotrophomonas maltophilia* [o] (Sensitest, air, 35-37°C)**			
Cotrimoxazole [o]	25		≤ 2/38

[a] The standard 6 mm cut-off applies where no exception has been specified.
[d] Antibiotic calibrated for veterinary medicine.
[g] For testing urine isolates only
[m] *Pasteurella multocida* is tested against ampicillin 5 µg and NOT benzylpenicillin 0.5 u.
[n] *Burkholderia pseudomallei* is usually susceptible to Augmentin and can be tested against this antibiotic.
[@] Piperacillin in the presence of 2mg/L tazobactam.
[&] Ticarcillin in the presence of 2mg/L clavulanic acid.
[o] See Section 5.11 for notes on testing antibiotic susceptibilities of Sulphonamide resistant *maltophilia*.

Table 12.1.c. Calibrations: *Anaerobic Organisms*.

Antibiotics, disc potencies, annular radii and MIC for susceptible strains, media and incubation conditions.

Antibiotic	Disc potency (µg)	Exception to the standard interpretation [a]	MIC (mg/L)
Anaerobic organisms [b]			
(Supplemented Brucella Medium Base, anaerobic, 35-37°C) [c]			
Augmentin*	3	4 mm	≤ 1/2
Benzylpenicillin	0.5 u		≤ 0.25
Cefoxitin	10		≤ 4
Clindamycin[d]	2	4 mm	≤ 0.5
Meropenem	5		≤ 2
Metronidazole	5	4 mm	≤ 2
Moxifloxacin	2.5	4 mm	≤ 2
Tazocin	55		≤ 8/2

* Amoxycillin in the presence of 2mg/L clavulanic acid.

[a] The standard 6 mm cut-off applies where no exception has been specified.

[b] Slow growers should be incubated for 48 h.

[c] Brucella Medium Base containing 5% defibrinated horse blood, haemin 5 mg/L and vitamin K 1 mg/L.

[d] An erythromycin 5µg disc must be placed 13mm edge to edge to detect inducible clindamycin resistance as described in section 4.7.

12.2. Surrogate discs

Table 12.2.a. Surrogate disc testing: *Gram Positive Organisms*

Antibiotics that can be reported based on susceptibility results obtained with a surrogate disc.

Antibiotic reported	Surrogate disc used	Disc potency (µg)
***Corynebacterium* species**		
Amoxycillin/Ampicillin/Penicillin V	Benzylpenicillin	0.5 u
Azithromycin/Clarithromycin/Roxithromycin	Erythromycin	5
Carbapenems	Benzylpenicillin	0.5 u
Cefovecin [a] */Ceftiofur* [a] /other Cephalosporins [b]	Benzylpenicillin	0.5 u
Enrofloxacin [a] */Orbifloxacin* [a]	Moxifloxacin	2.5
Lincomycin	Clindamycin	2
Norfloxacin [c]	Ciprofloxacin	2.5
Pristinamycin	Quinupristin/Dalfopristin	15
Tetracyclines	Tetracycline	10
Tylosin [a]	Erythromycin	5
Enterococci		
Amoxycillin/Benzylpenicillin	Ampicillin	5
Enrofloxacin [a] */Orbifloxacin* [a] */Pradofloxacin* [a]	Moxifloxacin	2.5
Pristinamycin [f]	Quinupristin/Dalfopristin	15
***Listeria* species**		
Amoxycillin/Benzylpenicillin	Ampicillin	5
Staphylococci (except *S. saprophyticus* from urine)		
Amoxycillin/Ampicillin/Penicillin V	Benzylpenicillin	0.5 u
Augmentin	Oxacillin [d]/Cefoxitin [e]	1 / 10
Azithromycin/Clarithromycin/Roxithromycin	Erythromycin	5
Carbapenems	Oxacillin [d]/Cefoxitin [e]	1 / 10
Cefovecin [a] */Ceftiofur* [a] /other Cephalosporins [b]	Oxacillin [d]/Cefoxitin [e]	1 / 10
Cloxacillin/ Dicloxacillin/ Flucloxacillin	Oxacillin [d]/Cefoxitin [e]	1 / 10
Enrofloxacin [a] */Orbifloxacin* [a] */Pradofloxacin* [a]	Moxifloxacin	2.5
Framycetin [a]	Neomycin	30
Lincomycin	Clindamycin	2
Norfloxacin [c]/ *Ofloxacin* [a]	Ciprofloxacin	2.5
Pristinamycin	Quinupristin/Dalfopristin	15
Tetracyclines	Tetracycline	10
Tylosin [a]	Erythromycin	5
***Staphylococcus saprophyticus* from urine**		
Amoxycillin/Benzylpenicillin/Penicillin V	Ampicillin	5
Augmentin	Cephalexin	100
Cefovecin [a] */Ceftiofur* [a] /other Cephalosporins [b]	Cephalexin	100
Cloxacillin/Dicloxacillin/Flucloxacillin	Cephalexin	100
Enrofloxacin [a] */Orbifloxacin* [a]	Moxifloxacin	2.5
Norfloxacin [c]/ *Ofloxacin* [a]	Ciprofloxacin	2.5
Tetracyclines	Tetracycline	10

[a] Antibiotic used in veterinary medicine only.
[b] Ceftazidime is inactive against Gram positive organisms.
[c] Reporting of norfloxacin is for urine isolates only.
[f] *Enterococcus faecalis* is intrinsically resistant to pristinamycin and quinupristin/Ddalfopristin.
[d] Oxacillin is used for testing coagulase-negative staphylococci (except *Staphylococcus saprophyticus*) only.
[e] Cefoxitin is used for testing *Staphylococcus aureus* only.

Table 12.2.a. Surrogate disc testing: *Gram Positive Organisms* cont.

Antibiotics that can be reported based on susceptibility results obtained with a surrogate disc.

Antibiotic reported	Surrogate disc used	Disc potency (µg)
Streptococci [g]		
Amoxycillin/Ampicillin/Augmentin /Penicillin V	Benzylpenicillin	0.5 u
Amoxycillin/Augmentin/ Benzylpenicillin	Ampicillin [h]	5
Augmentin [j]	Benzylpenicillin	0.5 u
Augmentin [j]	Ampicillin	5
Azithromycin/Clarithromycin/Roxithromycin	Erythromycin	5
Carbapenems	Benzylpenicillin	0.5 u
Cefovecin [a]/*Ceftiofur* [a]	Benzylpenicillin	0.5 u
Cefovecin [a]/*Ceftiofur* [a]	Cefotaxime [i]	5
Cephalosporins (*except ceftiofur*) [a]	Cefotaxime/Ceftriaxone[k]	0.5/5
Enrofloxacin [a] */Orbifloxacin* [a]	Moxifloxacin	2.5
Flucloxacillin	Benzylpenicillin	0.5 u
Lincomycin	Clindamycin	2
Pristinamycin	Quinupristin/Dalfopristin	15
Tetracyclines	Tetracycline	10
Tylosin [a]	Erythromycin	5

[a] Antibiotic used in veterinary medicine only.

[g] For streptococci groups A, B, C, G and *Streptococcus milleri group*, the susceptibility to benzylpenicillin, ampicillin, amoxycillin and cephalosporin antibiotics (except ceftazidime) is extrapolated from the testing of benzylpenicillin 0.5 u.

[h] NOT for testing *Streptococcus pneumoniae* from CSF. Test if isolate is resistant to benzylpenicillin 0.5 u, cefotaxime 0.5 mg or ceftriaxone 0.5 mg and reported as having a decreased susceptibility.

[i] If the organism is resistant to benzylpenicillin 0.5 u and susceptible to cefotaxime 5 µg, report as having a decreased susceptibility to *Cefovecin* [a]/*Ceftiofur* [a].

[j] For the reporting of all *Streptococcus* species including *S. pneumoniae* from sites other than CSF. If the organism is resistant to benzylpenicillin 0.5 u and susceptible to Ampicillin 5 µg, report as having a decreased susceptibility.

[k] If the organism is resistant to cefotaxime/ceftriaxone 0.5 and susceptible to cefotaxime/ceftriaxone 5 µg, report as having a decreased susceptibility.

Table 12.2.b. Surrogate disc testing: *Gram Negative Organisms*

Antibiotics that can be reported based on susceptibility results obtained with a surrogate disc.

Antibiotic reported	Surrogate disc used	Disc potency (μg)
Acinetobacter, Enterobacteriaceae & *Vibrionaceae* species		
Amoxycillin	Ampicillin	25
Cefovecin [a] /*Ceftiofur* [a]	Cefotaxime	5
Cephalothin [b]	Ampicillin	25
Ceftriaxone	Cefotaxime	5
Cefotaxime	Ceftriaxone	5
Enrofloxacin [a] /*Orbifloxacin* [a] /*Pradofloxacin* [a]	Moxifloxacin	2.5
Framycetin [a]	Neomycin	30
Ofloxacin [a]	Ciprofloxacin	2.5
Piperacillin	Ampicillin	25
Tetracyclines	Tetracycline	10
Ticarcillin [a]	Ampicillin	25
Branhamella catarrhalis (Moraxella catarrhalis)		
Azithromycin/Clarithromycin/Roxithromycin	Erythromycin	5
Amoxycillin/Ampicillin/Penicillin V	Benzylpenicillin	0.5 u
Augmentin	Cefuroxime/Cefaclor	30
Cefovecin [a] /*Ceftiofur* [a]	Cefuroxime/Cefaclor	30
Cephalosporins	Cefuroxime/Cefaclor	30
Enrofloxacin [a] /*Orbifloxacin* [a]	Moxifloxacin	2.5
Ofloxacin [a]	Ciprofloxacin	2.5
Tetracyclines	Tetracycline	10
***Campylobacter* species**		
Azithromycin /*Clarithromycin* [a] /*Roxithromycin* [a]	Erythromycin	5
Enrofloxacin [a] /*Orbifloxacin* [a] / *Ofloxacin* [a]	Ciprofloxacin	2.5
Tetracyclines	Tetracycline	10
***Haemophilus influenzae/Haemophilus* species**		
Amoxycillin	Ampicillin	5
Cefepime	Cefotaxime/Ceftriaxone	0.5
Cefotaxime	Ceftriaxone	0.5
Cefpirome	Cefotaxime/Ceftriaxone	0.5
Ceftazidime	Cefotaxime/Ceftriaxone	0.5
Cefovecin [a] /*Ceftiofur* [a]	Cefuroxime/Cefaclor	30
Ceftriaxone	Cefotaxime	0.5
Cephalexin	Cefuroxime/Cefaclor	30
Enrofloxacin [a] /*Orbifloxacin* [a]	Moxifloxacin	2.5
Ofloxacin [a]	Ciprofloxacin	2.5
Tetracyclines	Tetracycline	10
Helicobacter pylori		
Clarithromycin	Erythromycin	5
Rifabutin	Rifampicin	5

[a] Antibiotic used in veterinary medicine only.
[b] Not for *Acinetobacter* species.

Table 12.2.b. Surrogate disc testing: *Gram Negative Organisms* cont.

Antibiotics that can be reported based on susceptibility results obtained with a surrogate disc.

Antibiotic reported	Surrogate disc used	Disc potency (μg)
Neisseria meningitidis		
Ampicillin/Amoxycillin	Benzylpenicillin	0.5 u
Cefotaxime	Ceftriaxone	0.5
Cefovecin [a]/*Ceftiofur* [a]	Benzylpenicillin	0.5 u
Ceftriaxone	Cefotaxime	0.5
Enrofloxacin [a] */Orbifloxacin* [a]	Moxifloxacin	2.5
***Pasteurella* species**		
Amoxycillin/Benzylpenicillin	Ampicillin	5
Ampicillin/amoxycillin[c]	Benzylpenicillin	0.5 u
Augmentin	Ampicillin	5
Cephalexin	Ampicillin	5
Cefovecin [a]/*Ceftiofur* [a]	Ampicillin	5
Enrofloxacin [a] */Orbifloxacin* [a] /*Pradofloxacin* [a]	Moxifloxacin	2.5
Norfloxacin / *Ofloxacin* [a]	Ciprofloxacin	2.5
Tetracyclines	Tetracycline	10
***Pseudomonas* species, *Burkholderia* species & *Chryseobacterium* species**		
Azlocillin	Piperacillin	50
Colistin	Polymyxin B	300 u
Enrofloxacin [a] */Orbifloxacin* [a]	Moxifloxacin	2.5
Norfloxacin / *Ofloxacin* [a]	Ciprofloxacin	2.5

[a] Antibiotic used in veterinary medicine only.

[c] *Pasteurella multocida* is tested against ampicillin 5 μg and NOT benzylpenicillin 0.5 u.

12.3. Quality assurance

Table 12.3.a. Reference strains: *Gram Positive Organisms*

Antibiotics, disc potencies and acceptable zones of inhibition for reference strains[a]

Antibiotic reported	Disc potency (µg)	Annular radii (mm)[b]
***Enterococcus faecalis* POW 1994 (Blood Sensitest, CO_2 35°C)**		
Ampicillin	5	5.9 – 9.2
Chloramphenicol	30	6.3 – 8.7
Doxycycline	30	7.0 – 11.0
Fosfomycin	200	7.1 – 10.7
Gentamicin	200	6.6 – 9.9
Linezolid	10	6.6 – 9.0
Nitrofurantoin	200	6.1 – 8.7
Streptomycin	300	5.4 – 7.9
Tetracycline	10	7.0 – 10.2
Tigecycline	15	6.6 – 9.5
Teicoplanin	15	3.1 – 5.3
Vancomycin	5	2.0 – 3.7

[a] Reference strain testing must be performed: (i) In conjunction with the clinical isolate, or at least once weekly; (ii) When a new batch of medium is used; (iii) When a new batch of discs is used.

[b] The acceptable range (95% confidence limits) is the mean ± 2 standard deviations. The mean was derived from > 120 measurements with different operators using different batches of both agar and discs. It is statistically acceptable to use one hundred measurements to represent the "normal distribution" and this gives a confidence limit of 95%, meaning an in-built MU of 5% for the test.

Table 12.3.a. Reference strains: *Gram Positive Organisms* cont.

Antibiotics, disc potencies and acceptable zones of inhibition for reference strains[a]

Antibiotic reported	Disc potency (µg)	Annular radii (mm) [b]
***Staphylococcus aureus* NCTC 6571 (Sensitest, air 35°C)**		
Amoxycillin [c]	2	9.1 – 11.9
Ampicillin	5	12.1 – 18.1
Benzylpenicillin	0.5 u	8.7 – 13.5
Cefoxitin	10	7.1 – 10.1
Ceftaroline	5	10.5 – 14.1
Cephalexin	100	10.7 – 15.5
Chloramphenicol	30	7.8 – 11.4
Ciprofloxacin	2.5	9.2 – 12.4
Clindamycin	2	8.5 – 12.9
Cotrimoxazole	25	10.1 – 13.3
Erythromycin	5	8.0 – 10.8
Fusidic acid	2.5	8.6 – 12.6
Gentamicin	10	6.6 – 9.4
Kanamycin	50	7.8 – 9.6
Linezolid	10	7.9 – 13.1
Marbofloxacin [d]	5	8.9 – 11
Moxifloxacin	2.5	10.9 – 14.5
Mupirocin	5	7.4 – 12.2
Neomycin [d]	30	8.1 – 12.9
Nitrofurantoin	200	6.7 – 10.3
Novobiocin [d]	5	6.1 – 12.5
Oxacillin	1	7.4 – 10.4
Quinupristin/Dalfopristin	15	9.2 – 12.4
Rifampicin	1	9.3 – 12.5
Rifampicin [c]	5	12.7 – 15
Teicoplanin	15	3.4 – 6.1
Tetracycline	10	11.3 – 14.4
Tigecycline	15	10.3 – 13.2
Trimethoprim	5	8.5 – 11.3
Vancomycin	5	2.8 – 4.9

[a] Reference strain testing must be performed: (i) In conjunction with the clinical isolate, or at least once weekly; (ii) When a new batch of medium is used; (iii) When a new batch of discs is used.

[b] The acceptable range (95% confidence limits) is the mean ± 2 standard deviations. The mean was derived from > 120 measurements with different operators using different batches of both agar and discs. It is statistically acceptable to use one hundred measurements to represent the "normal distribution" and this gives a confidence limit of 95%, meaning an in-built MU of 5% for the test.

[c] Amoxycillin 2 µg and rifampicin 5 µg are used for the susceptibility testing of *H. pylori* only.

[d] Antibiotic used in veterinary medicine only.

Table 12.3.a. Reference strains: *Gram Positive Organisms* cont.

Antibiotics, disc potencies and acceptable zones of inhibition for reference strains[a]

Antibiotic reported	Disc potency (µg)	Annular radii (mm)[b]
***Streptococcus pneumoniae* ARL 10582**		
(Blood Sensitest, 5% CO_2, 35–37°C)		
Ampicillin	5	10.8 – 15.2
Benzylpenicillin	0.5 u	8.3 – 14.8
Cefotaxime	0.5	9.3 – 14.8
Cefotaxime	5	10.9 – 15.3
Ceftriaxone	0.5	9.1 – 14.3
Ceftriaxone	5	11.5 – 15.2
Chloramphenicol	30	8.0 – 13.2
Clindamycin	2	7.1 – 9.9
Cotrimoxazole	25	7.0 – 9.2
Erythromycin	5	7.1 – 12.9
Moxifloxacin	2.5	5.6 – 8.6
Quinupristin/Dalfopristin	15	6.4 – 9.2
Rifampicin	1	7.5 – 10.8
Teicoplanin	15	5.1 – 8.0
Tetracycline	10	9.5 – 11.5
Tigecycline	15	9.7 – 12.6
Vancomycin	5	5.1 – 8.6

[a] Reference strain testing must be performed: (i) In conjunction with the clinical isolate, or at least once weekly; (ii) When a new batch of medium is used; (iii) When a new batch of discs is used.

[b] The acceptable range (95% confidence limits) is the mean ± 2 standard deviations. The mean was derived from > 120 measurements with different operators using different batches of both agar and discs. It is statistically acceptable to use one hundred measurements to represent the "normal distribution" and this gives a confidence limit of 95%, meaning an in-built MU of 5% for the test.

Table 12.3.b. Reference strains: *Gram Negative Organisms*

Antibiotics, disc potencies and acceptable zones of inhibition for reference strains[a]

Antibiotic reported	Disc potency (µg)	Annular radii (mm)[b]
***Bacteroides fragilis* ATCC 25825**		
(Blood Sensitest, anaerobic, 35-37°C)		
Metronidazole	5	7.1 – 13.5
***Campylobacter jejuni* NCTC 11168**		
(Blood Sensitest, microaerophilic, 42°C)		
Ciprofloxacin	2.5	9.2 – 16.9
Erythromycin	5	6.4 – 12.4
Gentamicin	10	7.0 – 11.0
Tetracycline	10	10.8 – 16.8

[a] Reference strain testing must be performed: (i) In conjunction with the clinical isolate, or at least once weekly; (ii) When a new batch of medium is used; (iii) When a new batch of discs is used.

[b] The acceptable range (95% confidence limits) is the mean ± 2 standard deviations. The mean was derived from > 120 measurements with different operators using different batches of both agar and discs. It is statistically acceptable to use one hundred measurements to represent the "normal distribution" and this gives a confidence limit of 95%, meaning an in-built MU of 5% for the test.

[c] *Bacteroides fragilis* (ATCC 25285) may be used as the reference strain when testing *H. pylori* against metronidazole. When testing anaerobic organisms, *Clostridium perfringens* (POW 2006) should be used as the reference organism.

Table 12.3.b. Reference strains: *Gram Negative Organisms* cont.

Antibiotics, disc potencies and acceptable zones of inhibition for reference strains[a]

Antibiotic reported	Disc potency (µg)	Annular radii (mm) [b]
***Escherichia coli* NCTC 10418 [d]**		
(Sensitest, air, 35–37°C)		
Amikacin	30	6.7 – 10.3
Ampicillin	25	7.5 – 10.7
Apramicin [e]	15	5.3 – 7.9
Azithromycin	15	5.4 – 7.0
Aztreonam	30	13.7 – 15.9
Cefazolin	30	6.7 – 12.7
Cefepime	10	11.9 – 15.3
Cefotaxime	5	9.7 – 13.7
Cefotetan	30	11.9 – 14.8
Cefoxitin	30	9.8 – 13.0
Cefpirome	10	11.9 – 14.6
Cefpodoxime	10	10.3 – 12.7
Ceftazidime	10	9.3 – 14.1
Ceftriaxone	5	10.5 – 14.3
Cefuroxime	30	8.3 – 11.1
Cephalexin	100	6.9 – 10.9
Chloramphenicol	30	8.7 – 11.9
Ciprofloxacin	2.5	12.4 – 15.8
Cotrimoxazole	25	10.2 – 13.0
Doripenem	10	11.4 – 14.7
Enoxacin[e]	10	9.7 – 15.7
Ertapenem	10	12.1 – 16.1
Fosfomycin	200	5.7 – 9.7
Gentamicin	10	6.2 – 9.4
Imipenem	10	10.3 – 13.5
Kanamycin	50	6.2 – 11.8
Marbofloxacin [e]	5	13.2 – 15.3
Meropenem	5	11.0 – 14.4
Moxifloxacin	2.5	10.0 – 13.4
Nalidixic acid	30	8.9 – 12.1
Neomycin [e]	30	6.0 – 8.6
Nitrofurantoin	200	6.3 – 9.5
Norfloxacin	10	10.4 – 16.4
Polymyxin B	300 u	5.1 – 7.5
Spectinomycin	25	5.0 – 7.8
Streptomycin [e]	25	6.2 – 7.8
Tetracycline	10	4.5 – 8.6
Tigecycline	15	9.7 – 12.6
Tobramycin	10	6.4 – 8.4
Trimethoprim	5	8.8 – 13.6
***Escherichia coli* NCTC 11560**		
(Sensitest, air, 35-37°C)		
Augmentin	60	6.4 – 9.6
Timentin	85	6.0 – 8.4
Tazocin	55	7.4 – 9.2

[a] Reference strain testing must be performed: (i) In conjunction with the clinical isolate, or at least once weekly; (ii) When a new batch of medium is used; (iii) When a new batch of discs is used.

[b] The acceptable range (95% confidence limits) is the mean ± 2 standard deviations. The mean was derived from > 120 measurements with different operators using different batches of both agar and discs. It is statistically acceptable to use one hundred measurements to represent the "normal distribution" and this gives a confidence limit of 95%, meaning an in-built MU of 5% for the test.

[d] If antibiotic discs are tested with *Escherichia coli*, NCTC 11560 there is no need to test these against *Pseudomonas aeruginosa* NCTC 10662 as well and vice versa.

[e] Antibiotic used in veterinary medicine only

Table 12.3.b. Reference strains: *Gram Negative Organisms* cont.

Antibiotics, disc potencies and acceptable zones of inhibition for reference strains[a]

Antibiotic reported	Disc potency (µg)	Annular radii (mm)[b]
***Haemophilus influenzae* NCTC 4560 (HTM[f] agar, 5% CO_2, 35-37°C)**		
Ampicillin	5	7.0 – 11.1
Cefaclor	30	8.1 – 12.1
Cefotaxime	0.5	9.2 – 12.8
Cefotaxime	5	12.6 – 16.1
Cefpodoxime	10	10.9 – 14.1
Ceftriaxone	0.5	9.1 – 12.9
Ceftriaxone	5	13.3 – 16.3
Cefuroxime	30	8.3 – 12.8
Chloramphenicol	10	11.1 – 14.3
Ciprofloxacin	2.5	11.1 – 15.9
Cotrimoxazole	25	9.0 – 12.5
Moxifloxacin	2.5	10.6 – 15.2
Tetracycline	10	8.2 – 13.0
***Haemophilus influenzae* NCTC 11315 (HTM[f] agar, 5% CO_2, 35-37°C)**		
Augmentin	15	7.7 – 10.1

[a] Reference strain testing must be performed: (i) In conjunction with the clinical isolate, or at least once weekly; (ii) When a new batch of medium is used; (iii) When a new batch of discs is used.

[b] The acceptable range (95% confidence limits) is the mean ± 2 standard deviations. The mean was derived from > 120 measurements with different operators using different batches of both agar and discs. It is statistically acceptable to use one hundred measurements to represent the "normal distribution" and this gives a confidence limit of 95%, meaning an in-built MU of 5% for the test.

[f] Haemophilus Test Medium Base containing 15 mg/L freshly prepared Haematin and NAD.

Table 12.3.b. Reference strains: *Gram Negative Organisms* cont.

Antibiotics, disc potencies and acceptable zones of inhibition for reference strains[a]

Antibiotic reported	Disc potency (µg)	Annular radii (mm) [b]
***Pseudomonas aeruginosa* NCTC 10662** [d] **(Sensitest, air, 35-37°C)**		
Amikacin	30	7.4 – 10.6
Aztreonam	30	8.3 – 13.1
Cefepime	10	8.1 – 11.3
Cefpirome	10	8.1 – 10.6
Ceftazidime	10	7.5 – 11.9
Ciprofloxacin	2.5	8.9 – 14.5
Doripenem	10	12.6 – 16.6
Ertapenem [g]	10	– [g]
Fosfomycin	200	8.0 – 10.8
Gentamicin	10	5.5 – 9.5
Imipenem	10	7.9 – 10.3
Meropenem	5	9.7 – 14.8
Moxifloxacin [g]	2.5	– [g]
Norfloxacin	10	10.1 – 12.6
Piperacillin	50	8.1 – 12.9
Polymyxin B	300 u	5.2 – 7.2
Ticarcillin	75	7.3 – 12.1
Tobramycin	10	7.0 – 10.6

[a] Reference strain testing must be performed: (i) In conjunction with the clinical isolate, or at least once weekly; (ii) When a new batch of medium is used; (iii) When a new batch of discs is used.

[b] The acceptable range (95% confidence limits) is the mean ± 2 standard deviations. The mean was derived from > 120 measurements with different operators using different batches of both agar and discs. It is statistically acceptable to use one hundred measurements to represent the "normal distribution" and this gives a confidence limit of 95%, meaning an in-built MU of 5% for the test.

[d] If antibiotic discs are tested with *Escherichia coli NCTC 11580*, there is no need to test these against *Pseudomonas aeruginosa* NCTC 10662as well and vice versa.

[g] Ertapenem and moxifloxacin should be tested against *E. coli* NCTC 11560

Table 12.3.c. Reference strains: *Anaerobic Organisms*

Antibiotics, disc potencies and acceptable zones of inhibition for reference strains[a]

Antibiotic reported	Disc potency (µg)	Annular radii (mm) [b]
***Clostridium perfringens* POW 2006**		
(Supplemented Brucella Medium Base, anaerobic, 35-37°C) [c]		
Benzylpenicillin	0. 5 u	6.3 – 7.9
Cefoxitin	10	7.3 – 9.7
Clindamycin	2	6.1 – 8.1
Meropenem	5	10.1 – 12.9
Metronidazole	5	4.3 – 6.7
Moxifloxacin	2.5	4.7 – 6.3
***Bacteroides fragilis* ATCC 25285**		
(Supplemented Brucella medium Base, anaerobic, 35-37°C) [c]		
Augmentin	3	5.1 – 8.3
Tazocin	55	9.0 – 12.2
Timentin	85	11.6 – 16.0

[a] Reference strain testing must be performed: (i) In conjunction with the clinical isolate, or at least once weekly; (ii) When a new batch of medium is used; (iii) When a new batch of discs is used.

[b] The acceptable range (95% confidence limits) is the mean ± 2 standard deviations. The mean was derived from > 120 measurements with different operators using different batches of both agar and discs. It is statistically acceptable to use one hundred measurements to represent the "normal distribution" and this gives a confidence limit of 95%, meaning an in-built MU of 5% for the test.

[c] Brucella Medium Base containing 5% defibrinated horse blood, haemin 5 mg/L and vitamin K 1 mg/L.

12.4. Testing and reporting β-lactam antibiotics

Table 12.4.a. A guide to the testing and reporting of β-lactam antibiotics for Gram negative organisms.

Organism	Antibiotic [b,c] AMP	AMC	ATM	CAZ	CXM,CL,CPD CRO,CTX,KZ	CPO FEP	CTT	FOX	IPM,MEM ETP, DOR	PRL	TIM	TZP
Enterobacteriaceae of the EEC group [d]	R	R	R	R	R	**T**	R	R	**T**	N[a]	R	R
Serratia marcescens	R	R	**T**	**T**	R	**T**	R	R	**T**	N[a]	R	**T**
Aeromonas with A1 cephalosporinase & A2 carbapenemase	R	R	**T***	**T***	R	**T**	R	R	R [e]	N[a]	R	R
Aeromonas sobria/veronii with A2 carbapenemase only	R	R	**T**	**T**	**T**	**T**	**T**	**T**	R	N[a]	**T**	**T**
Morganella morganii	R	**T***	**T***	**T***	**T***	**T***	**T***	**T***	**T***	N[a]	**T***	**T***
Proteus vulgaris / penneri [f]	R	**U**	**T**	**T**	R	**T**	**T**	**T**	**T**	N[a]	**U**	**U**
C. amalonaticus	R	**U**	R	**T**	R	**T**	**T**	**T**	**T**	N[a]	**U**	**U**
Enterobacteriaceae with ESBL	R	**U**	R	R	R	R	**T**	R	**T**	N[a]	**U**	**U**
Enterobacteriacae with an MBL	R	R	**T**	R	R	R	R	R	R	N[a]	R	R
Enterobacteriaceae with an AMP C[I]	R	R	**T**	R	R	T	T	T	T	N[a]	T	T
Pseudomonas species / *Burkholderia* species [g] / *Chryseobacterium* species	R	R [g]	**T**	**T**	R	**T**	R	R	**T**	**T**	**T**	**T**
P. aeruginosa with ESBL	R	R	R	R	R	R	R	R	**T**	R	**U**	**U**
P. aeruginosa with an MBL	R	R	**T**	R	R	R	R	R	R	R	R	R
Stenotrophomonas maltophilia [h]	R	R	R [h]	R [h]	R	R	R	R	R	R [h]	R [h]	R [h]

[a] N = Not calibrated for this species.

[b] R = The isolate is resistant to the antibiotic because it possesses a mechanism of resistance that may not be demonstrated by disc testing.
T = Can be tested and reported. **T*** See (section 5.2 on *Aeromonas* & Morganella in sect 5.5.7)
U = Test isolates from uncomplicated UTI only. Isolates from other sites are considered RESISTANT.

[c]

AMP	ampicillin	CL	cephalexin	DOR	doripenem	KZ	cefazolin
AMC	Augmentin	CPD	cefpodoxime	ETP	ertapenem	MEM	meropenem
ATM	aztreonam	CPO	cefpirome	FEP	cefepime	PRL	piperacillin
CAZ	ceftazidime	CRO	ceftriaxone	FOX	cefoxitin	TIM	Timentin
CXM	cefuroxime	CTT	cefotetan	IPM	imipenem	TZP	Tazocin
CTX	cefotaxime						

[d] *Enterobacter aerogenes, Enterobacter cloacae* complex*, Citrobacter freundii* complex.

[e] *A. caviae* does not possess a carbapenemase (A2) and can be tested against imipenem, meropenem, ertapenem and doripenem.

[f] Isolates with high β-lactamase activity may give no zone around CTX 5 µg but show a "key-hole" effect that may be mistaken as an indication of the presence of an ESBL. However, unlike ESBL producers, they may be susceptible to ceftazidime.

[g] *B. pseudomallei* is usually susceptible to Augmentin and can be tested against this antibiotic.

[h] See Section 5.10 for notes on testing *S. maltophilia* against these and other antibiotics.

[I] This group varies in the susceptibility to betalactams- see sections 5.5.3 & 5.5.7.

13. Plates

13.1. *Enterococcus faecalis*, *Enterococcus faecium*

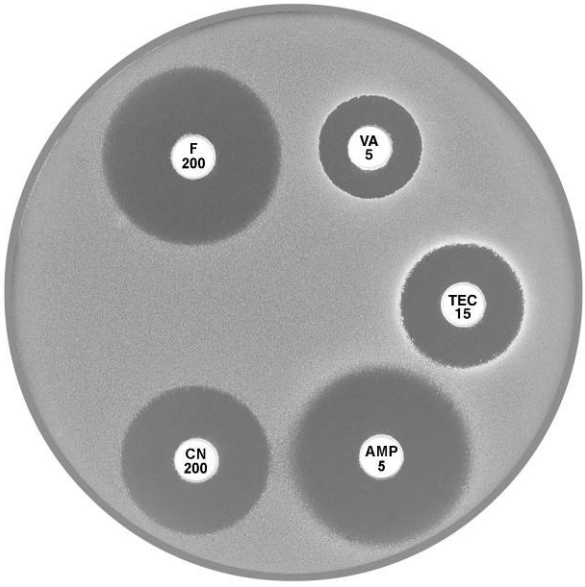

Plate 13.1.A *Enterococcus faecalis* and ampicillin
The diffuse edge to the zone of inhibition > 4 mm around ampicillin (AMP 5) indicates susceptibility.

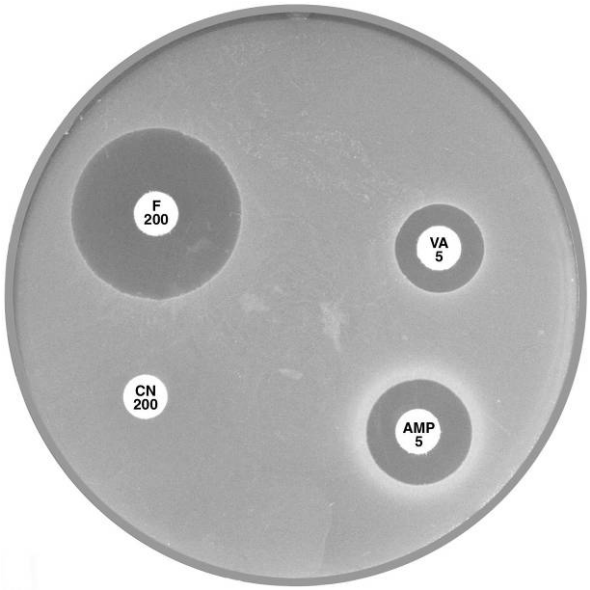

Plate 13.1.B *Enterococcus faecalis* producing β-Lactamase
The sharp edge and the reduced inhibitory zone around ampicillin (AMP 5) indicate β-lactamase production and resistance.

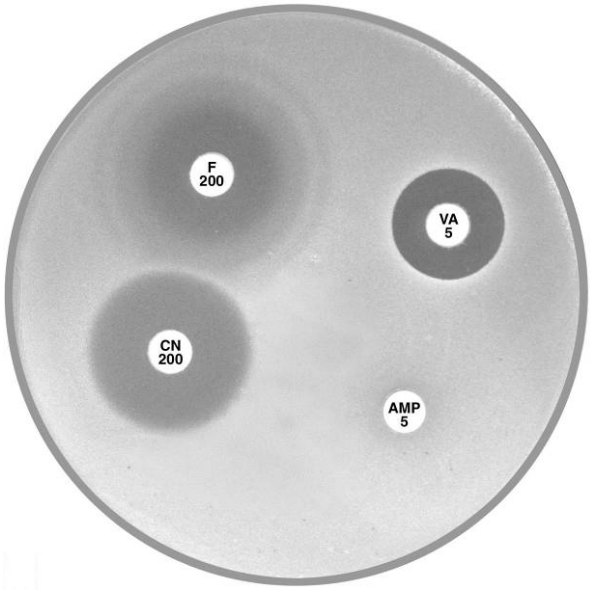

Plate 13.1.C *Enterococcus faecium* and nitrofurantoin
The hazy edged zone of inhibition around nitrofurantoin and no zone of inhibition around ampicillin is characteristic of *E. faecium*.

13.2. *Enterococcus faecium, Enterococcus gallinarum & Leuconostoc*

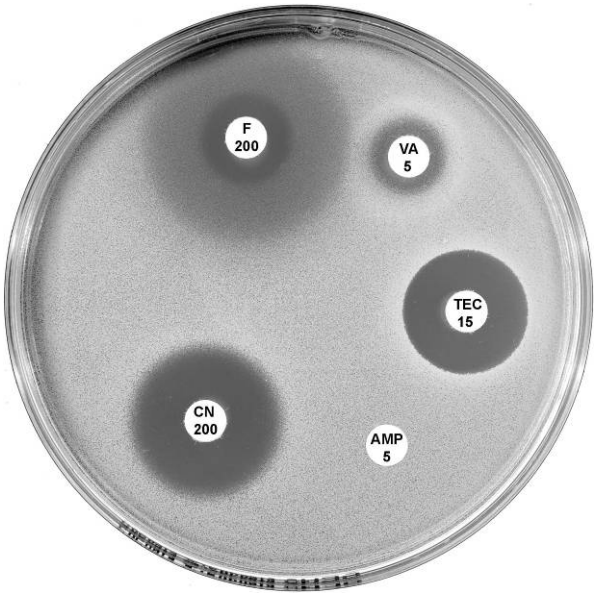

Plate 13.2.A *Enterococcus faecium* with VanB type vancomycin resistance
The hazy edged inhibitory zone around vancomycin (VA 5) and the diffuse growth towards the disc is frequently observed with this phenotype.

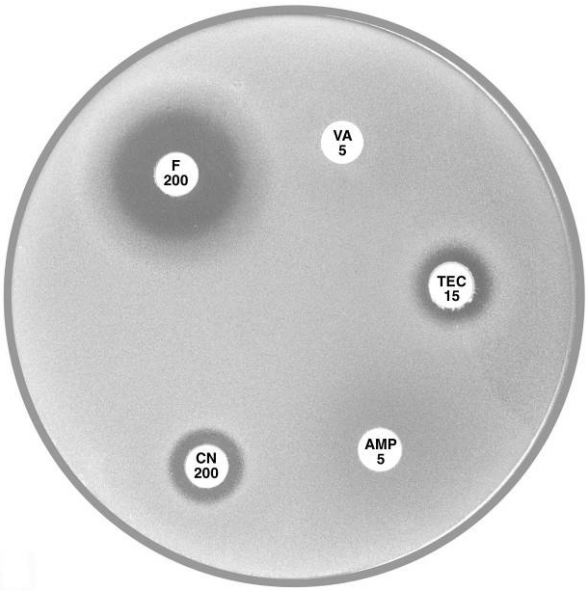

Plate 13.2.B *Enterococcus faecium* with VanA type vancomycin resistance
Resistance to both vancomycin (VA 5) and teicoplanin (TEC 15) is typical of the vanA phenotype.

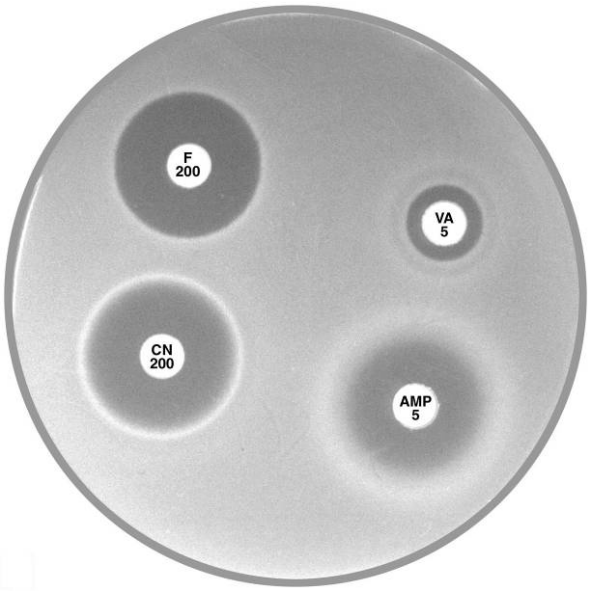

Plate 13.2.C *Enterococcus gallinarum* with intrinsic VanC type vancomycin resistance
Note the sharp edge of the reduced inhibitory zone around vancomycin (VA 5).

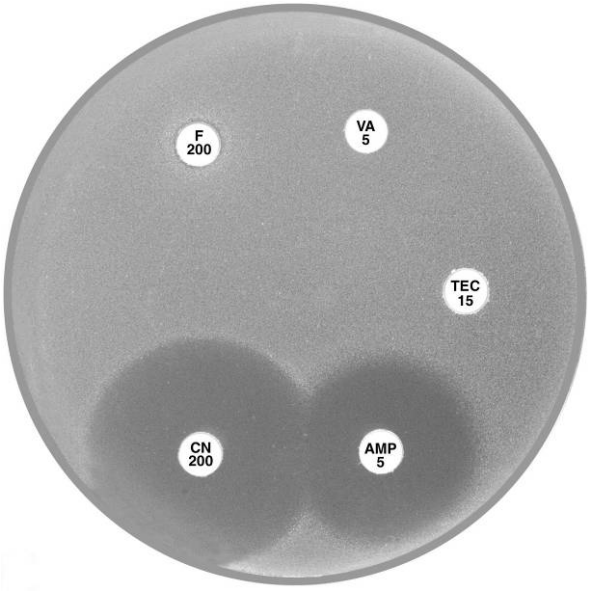

Plate 13.2.D *Leuconostoc* versus vancomycin and teicoplanin
This organism has high inherent resistance to vancomycin (VA 5) and teicoplanin (TEC 15).

13.3. *Staphylococcus aureus*

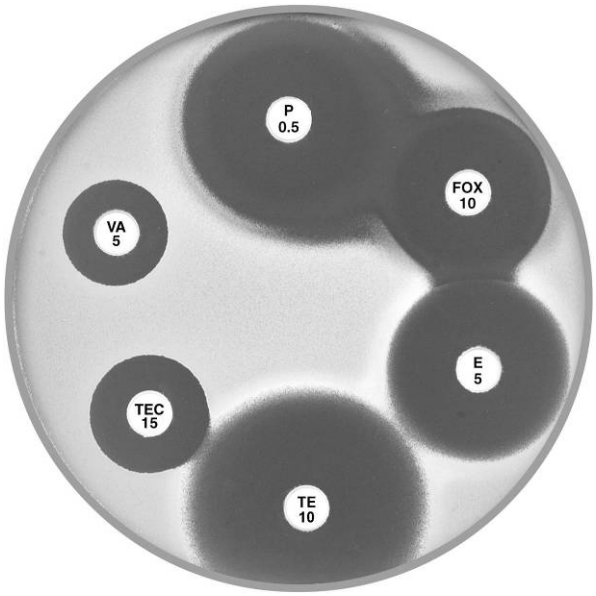

Plate 13.3.A *Staphylococcus aureus NCTC* 6571
This organism is fully susceptible and has large inhibitory zones around penicillin (P 0.5), cefoxitin (FOX 10), vancomycin (VA 5) and teicoplanin (TEC 15); 12 mm, 10 mm, 3 mm and 5 mm respectively.

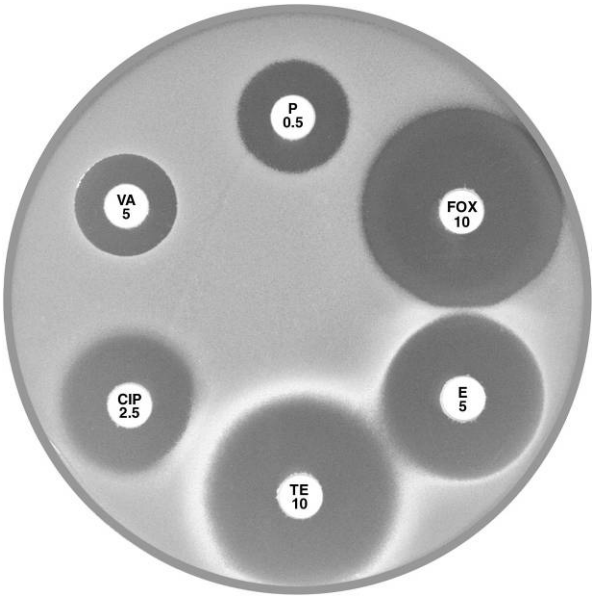

Plate 13.3.B *Staphylococcus aureus* with low β-lactamase activity
The reduced inhibitory zone around penicillin (P 0.5) with an annular radius of 4 to 5 mm indicates resistance to penicillin. The sharp edge of the inhibitory zone confirms β lactamase production.

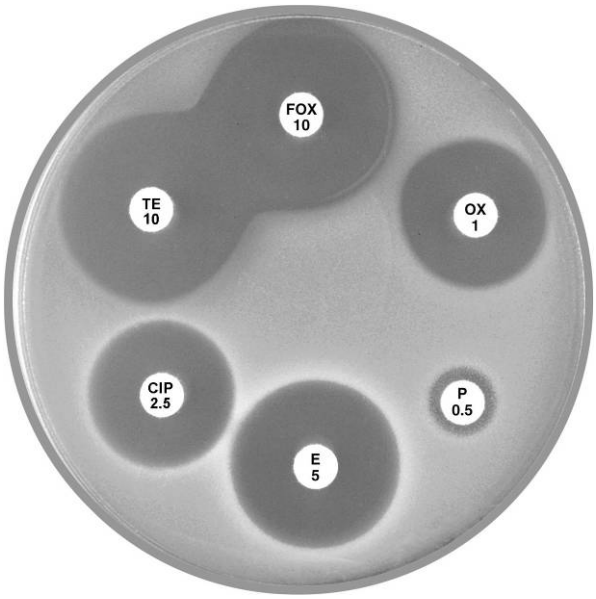

Plate 13.3.C *Staphylococcus aureus* resistant to penicillin only
Note the 9 mm annular radius of the zone of inhibition around cefoxitin (FOX 10).

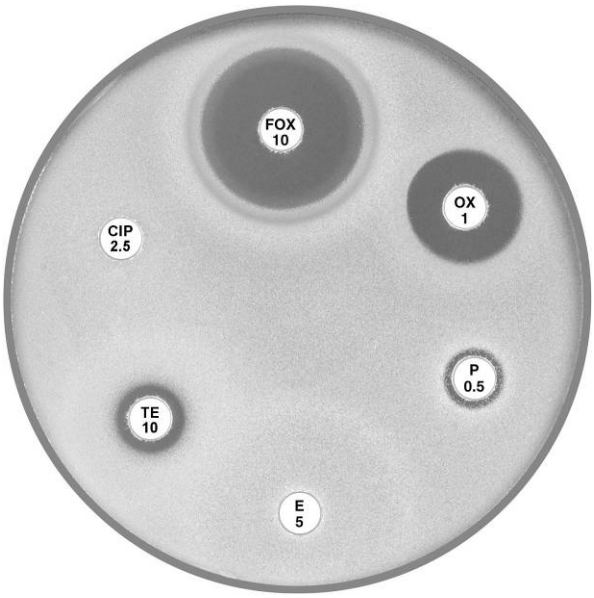

Plate 13.3.D *Staphylococcus aureus* (MSSA) *mecA* gene negative multiresistant
This isolate shows susceptibility to cefoxitin (FOX 10) and resistance to the other antibiotics tested.

13.4. *MRSA*

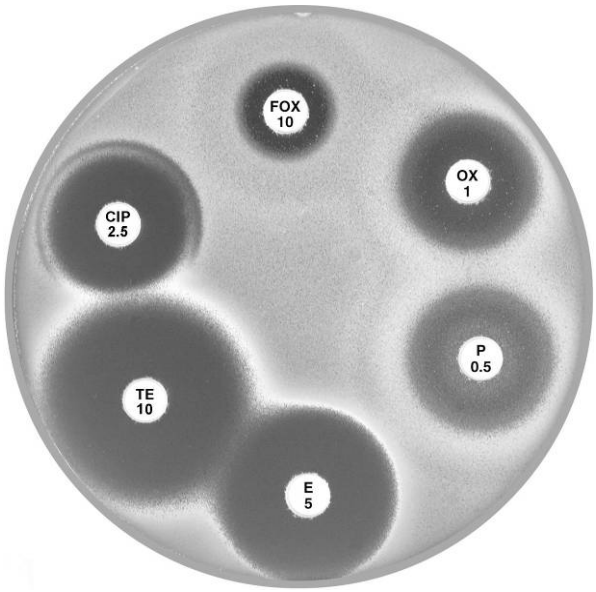

Plate 13.4.A *Staphylococcus aureus* (NMRMRSA) Non multiresistant, *mecA* gene positive but lacks β –lactamase activity
This isolate is reported as resistant to methicillin and all other β-lactams.

Plate 13.4.B *Staphylococcus aureus* (NMRMRSA)
This isolate is susceptible to erythromycin (E 5), tetracycline (TE 10), cotrimoxazole (SXT 25) and ciprofloxacin (CIP 2.5) but is reported as resistant to methicillin.

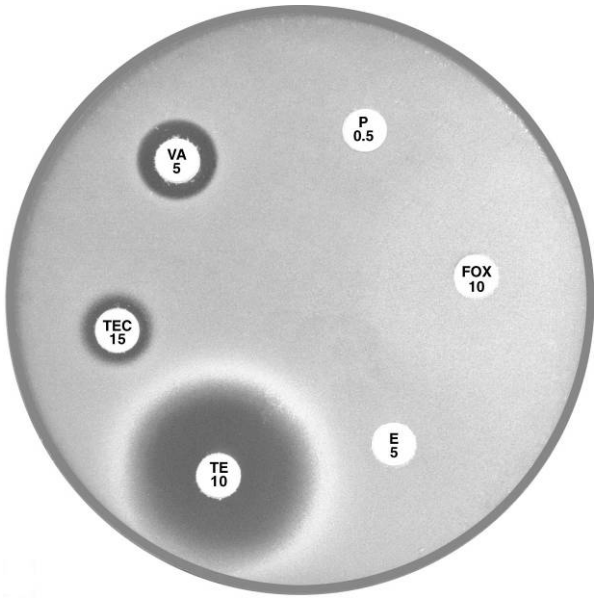

Plate 13.4.C MRSA with reduced susceptibility to vancomycin (VISA/GISA)
Note the reduced inhibitory zone around vancomycin (VA 5) and teicoplanin (TEC 15).

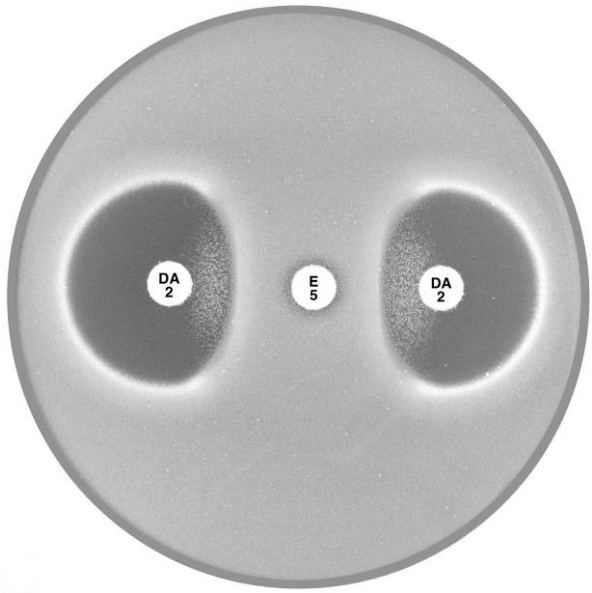

Plate 13.4.D MRSA with inducible clindamycin resistance (ICR)
Inducible clindamycin resistance (ICR) is indicated by the absence of an inhibitory zone around erythromycin (E 5) and a flattening of the clindamycin (DA 2) zone adjacent to the erythromycin disc.

13.5. *Staphylococcus saprophyticus*

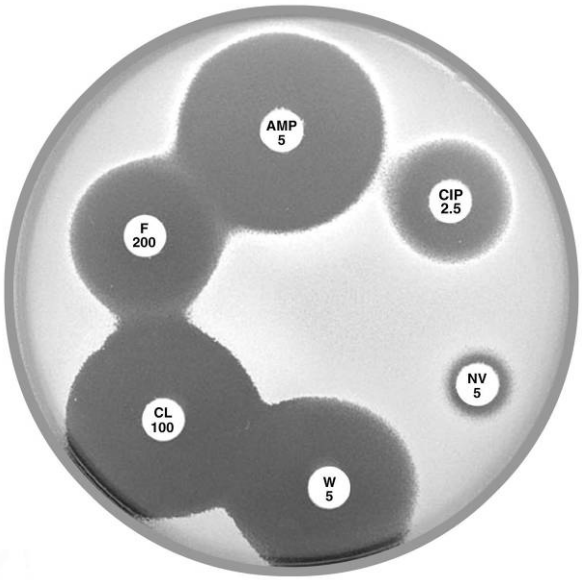

Plate 13.5.A *Staphylococcus saprophyticus mecA* gene negative
Typical of urinary isolates. Resistant to novobiocin (NV 5) but susceptible to ampicillin (AMP 5) and cephalexin (CL 100).

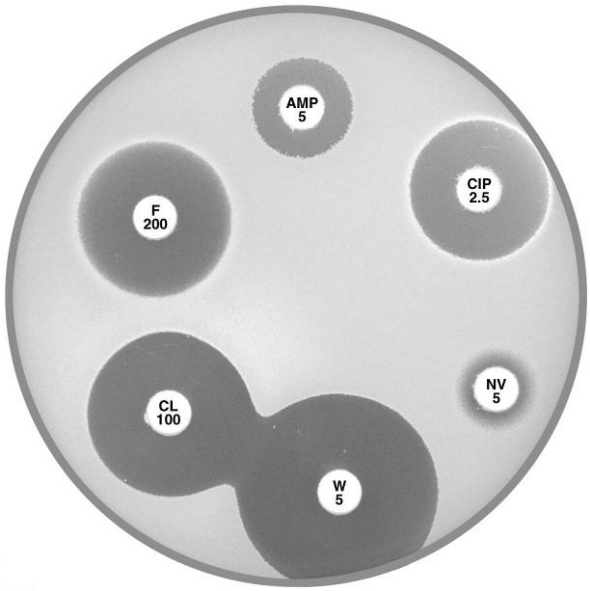

Plate 13.5.B *Staphylococcus saprophyticus mecA* gene negative *wi*th an inducible β-lactamase
Urinary isolate resistant to ampicillin (AMP 5) but susceptible to cephalexin (CL 100).

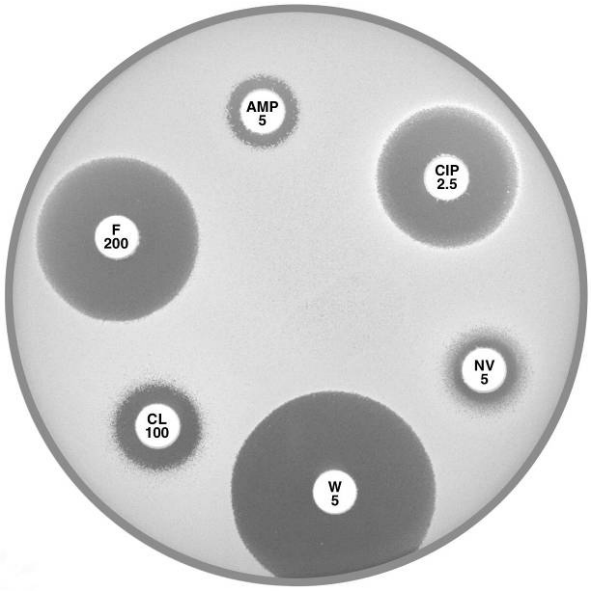

Plate 13.5.C *Staphylococcus saprophyticus mecA* gene positive
Urinary isolate showing resistance to ampicillin (AMP 5) and cephalexin (CL 100).

13.6. *Acinetobacter*

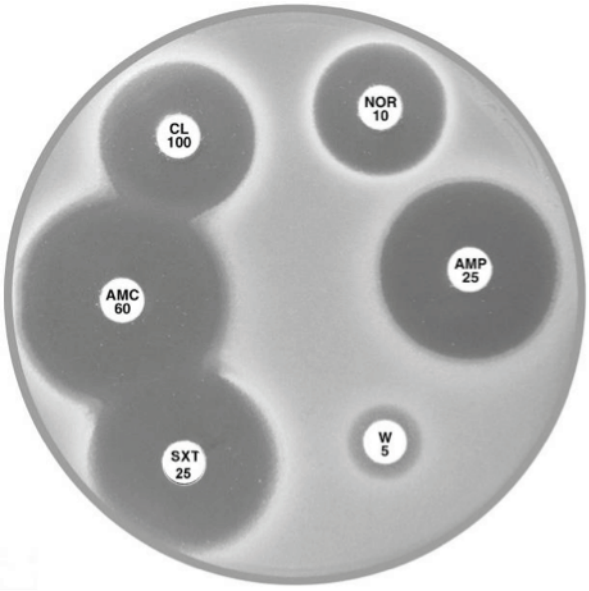

Plate 13.6.A A*cinetobacter lwoffi*
A large inhibitory zone around ampicillin (AMP 25), a smaller one around cephalexin (CL 100), resistance to trimethoprim (W 5) and a lack of synergy with sulphonamide (SXT 25) are typical of A. *lwoffi*

Plate 13.6.B *Acinetobacter baumannii*
Note the large inhibitory zone around Augmentin (AMC 60), a small one around ampicillin (AMP 25) and none around cephalexin (CL 100).

13.7. *Aeromonas*

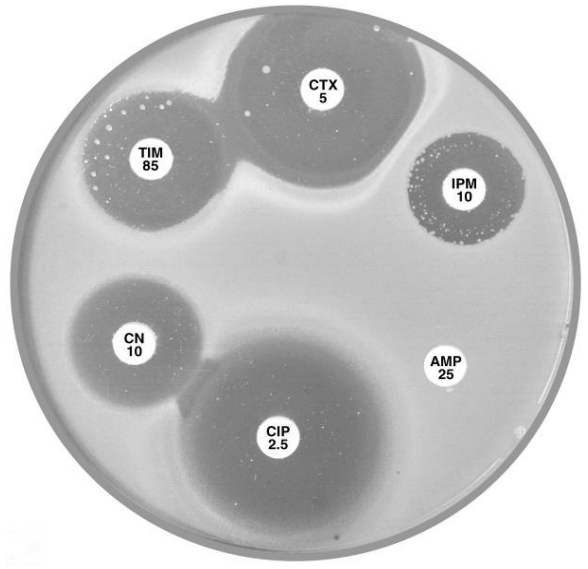

Plate 13.7.A *Aeromonas hydrophilia* with cephalosporinase A1 & carbapenemase A2
The flattening of the inhibitory zone around cefotaxime (CTX 5) indicates an inducible A1 cephalosporinase. The reduced inhibitory zone around imipenem (IPM 10) with resistant colonies indicates an A2 carbapenemase.

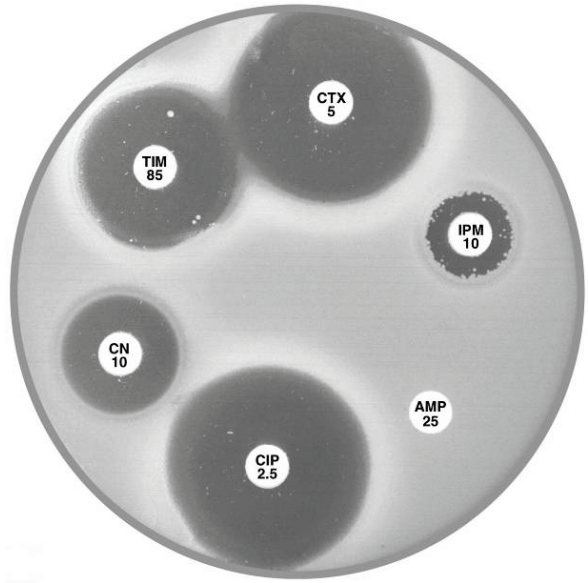

Plate 13.7.B *Aeromonas sobria* with carbapenemase A2 but lacking cephalosporinase A1
The A2 carbapenemase is indicated by the small inhibitory zone around imipenem (IPM 10) and resistant colonies within this zone.

13.8. *Escherichia coli*

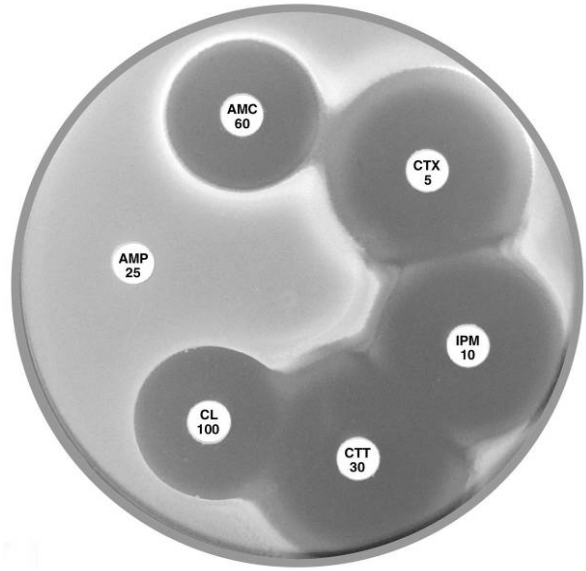

Plate 13.8.A *Escherichia. coli* NCTC 11560 producing TEM-1 β-lactamase
This strain is resistant to ampicillin (AMP 25), but susceptible to Augmentin (AMC 60), cefotaxime (CTX 5), imipenem (IPM 10), cefotetan (CTT 30) & cephalexin (CL 100).

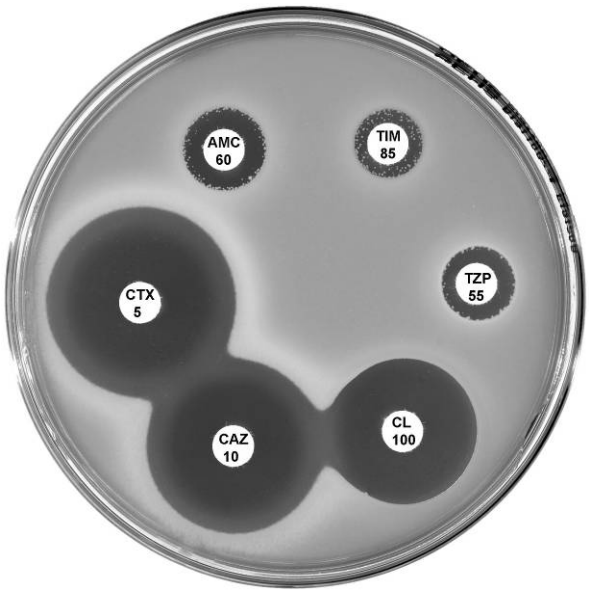

Plate 13.8.B *Escherichia. coli* with an Inhibitor Resistant TEM (IRT)
This isolate is resistant to the inhibiting effect of both clavulanic acid and tazobactam in Augmentin, Timentin and Tazocin. The isolate remains susceptible to the cephalosporins.

Plate 13.8.C *Escherichia coli* with low activity of the AmpC β-lactamase
Showing a zone inhibition >6mm around cefotaxime (CTX 5) and no zone around cephalexin (CL 100).

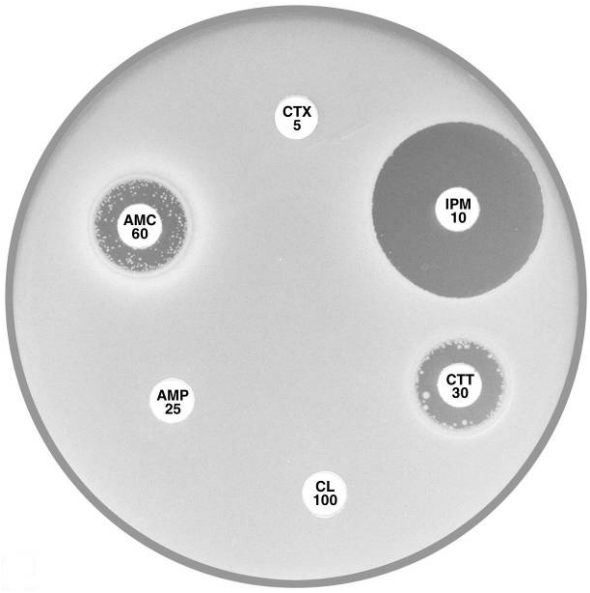

Plate 13.8.D *Escherichia coli* with high activity of the AmpC β-lactamase
Note the absence of an inhibitory zone around both cefotaxime (CTX 5) and cephalexin (CL 100).

13.9. *Escherichia coli* and *Chryseobacterium indologenes*

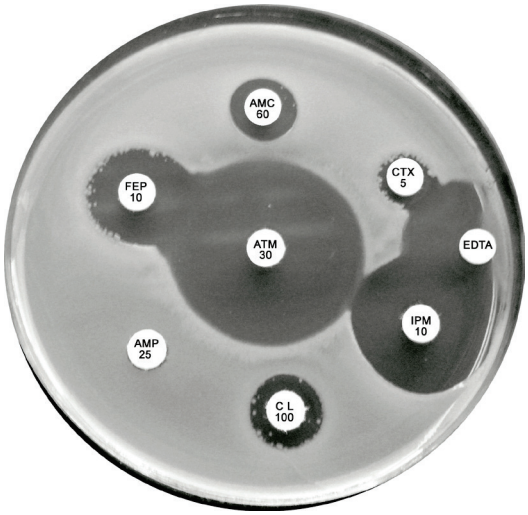

Plate 13.9.A *Escherichia coli* producing a zinc metallo β-lactamase
Showing resistance to cefotaxime (CTX), cefepime (FEP) and Augmentin (AMC) but susceptible to aztreonam. The lack of synergy between AMC and CTX/FEP suggesting the absence of an ESBL. Note the inhitory zone > 6mm around imipenem and the inhibition of β-lactamase activity by EDTA.

Plate 13.9.B *Chryseobacterium indologenes*
Chryseobacterium showing susceptibility to cotrimoxazole (SXT 25) and resistance to ceftazidime (CAZ 10) and aminoglycosides (AK 30 and CN 10). This strain is susceptible to the tested quinolones.

Plate 13.9.C *Escherichia coli* Plasmid mediated AmpC β-lactamase (low activity)
Synergy observed between a BA 200 ug (BA, blank) disc and the β-lactam discs, CL 100 (4 mm), AMC 60 (reduced) and CTX 5 (slightly reduced).

Plate 13.9.D *Escherichia coli* Plasmid mediated AmpC β-lactamase (higher activity). Synergy observed between a BA 200µg disc and the β-lactam discs, CL 100 (no mm), AMC 60 (4mm), CAZ (4mm) and CTX 5 (numerous R. col)

13.10. *Klebsiella pneumoniae*

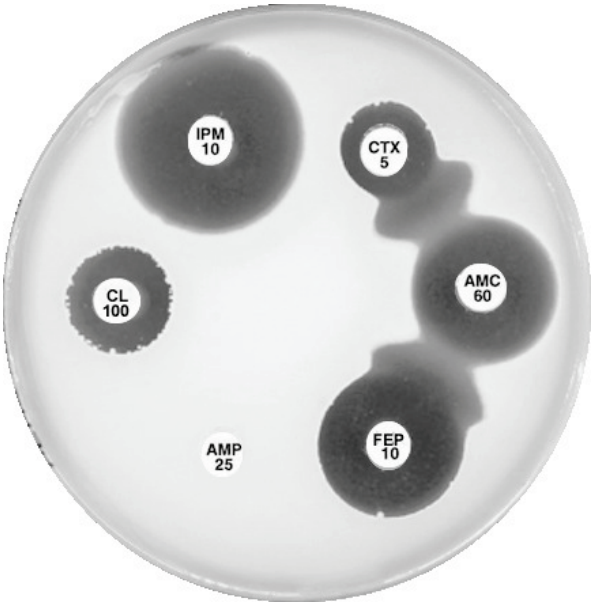

Plate 13.10.A *Klebsiella pneumoniae* producing an ESBL
ESBL production is indicated by the inhibition of the β-lactamase by clavulanic acid and the resulting "keyhole" inhibition pattern between cefotaxime, cefepime and Augmentin.

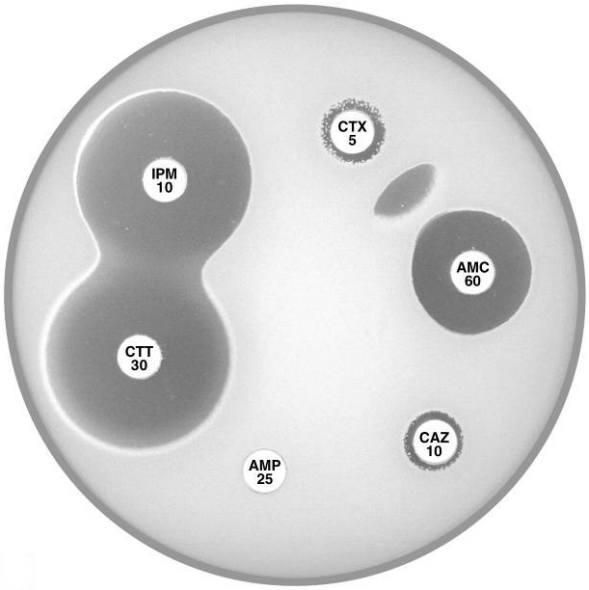

Plate 13.10.B *Klebsiella pneumoniae* producing a higher activity ESBL
The higher level of ESBL production is indicated by the inhibition of the β-lactamase by clavulanic acid and the resulting elliptical inhibitory zone between cefotaxime and Augmentin.

13.11. *Klebsiella oxytoca*

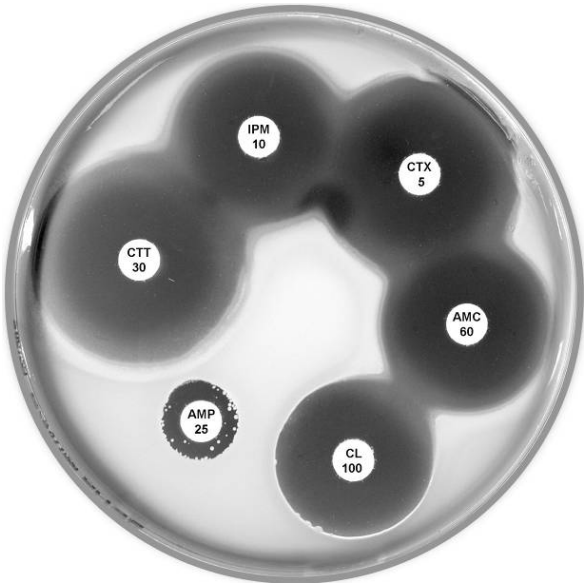

Plate 13.11.A *Klebsiella oxytoca* with a low basal level of K1 β-lactamase
This isolate is resistant to ampicillin only.

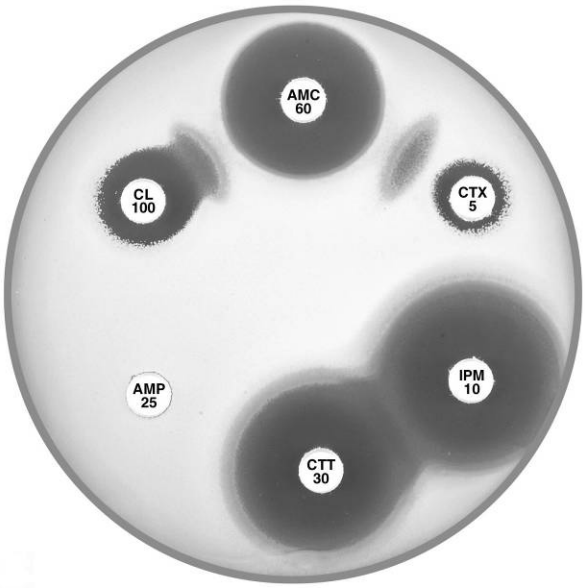

Plate 13.11.B *Klebsiella oxytoca* with a low basal level of K1 β-lactamase and an ESBL
ESBL production is demonstrated by the "keyhole" and elliptical inhibitory zones between cephalexin (CL 100) and cefotaxime (CTX 5) respectively and Augmentin (AMC 60).

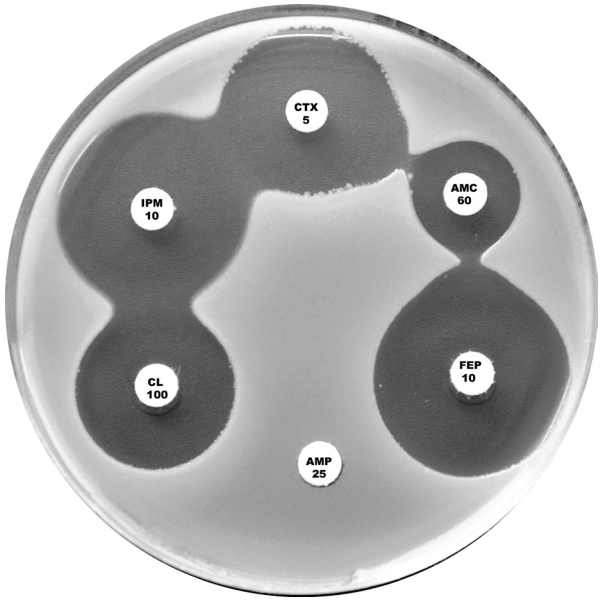

Plate 13.11.C *Klebsiella oxytoca* with a moderate level of the K1 β-lactamase
This isolate shows a mild synergy between cefotaxime (CTX 5)/cefepime (FEP 10) and Augmentin (AMC 60).

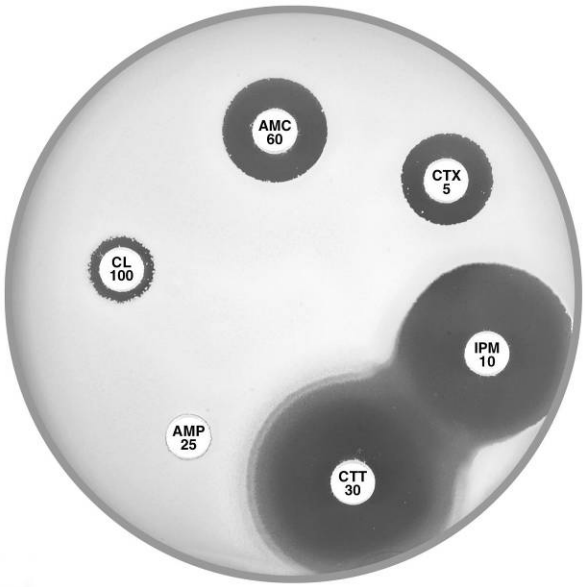

Plate 13.11.D *Klebsiella oxytoca* with a higher level of the K1 β-lactamase
There is no obvious synergy between cephalexin (CL 100) or cefotaxime (CTX 5) and Augmentin (AMC 60).

13.12. *Enterobacter and Citrobacter*

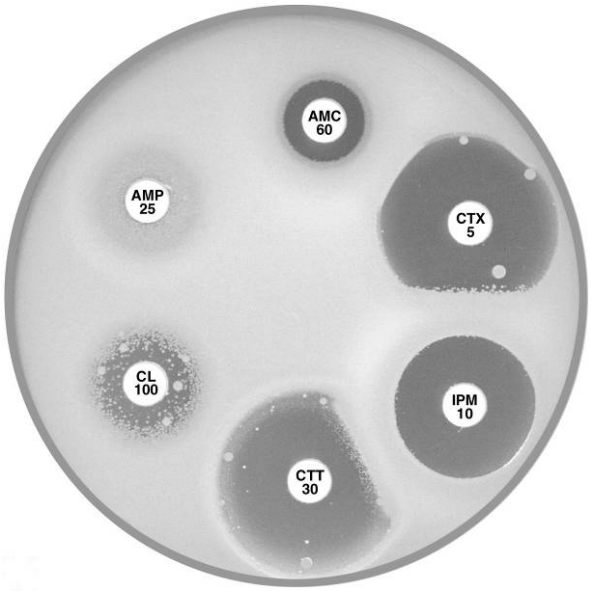

Plate 13.12.A *Enterobacter cloacae* with AmpC β-lactamase
The flattened inhibitory zone around imipenem (IPM 10) adjacent to cefotaxime/cefotetan (CTX 5, CTT 30) demonstrates the presence of a basal inducible cephalosporinase**.**

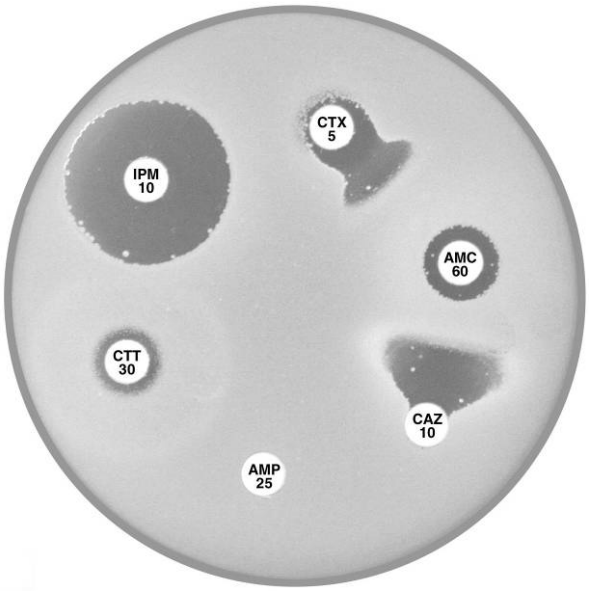

Plate 13.12.B *Enterobacter cloacae* producing higher levels of the AmpC β-lactamase and also an ESBL
Note the resistance to cefotaxime (CTX 5) and cefotetan (CTT 30).

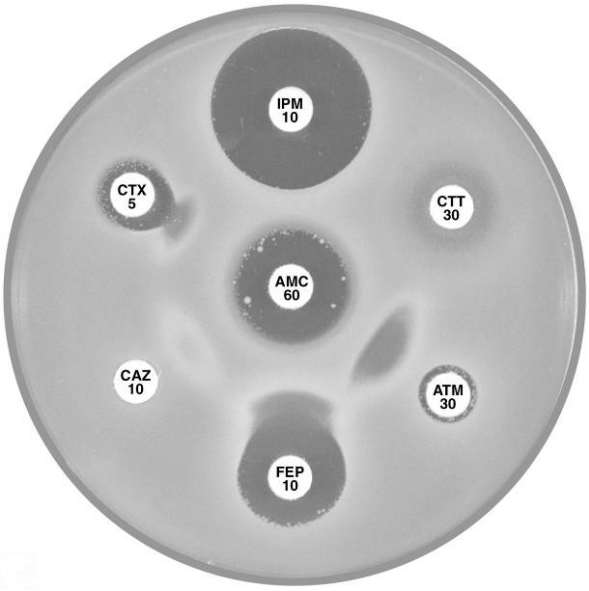

Plate 13.12.C *Citrobacter freundii* with derepressed AmpC β-lactamase and ESBL
The ESBL is indicated by the keyhole pattern between cefepime (FEP 10), aztreonam and Augmentin (AMC 60) (ATM 30). AmpC derepression is indicated by the resistance to cefotetan (CTT 30).

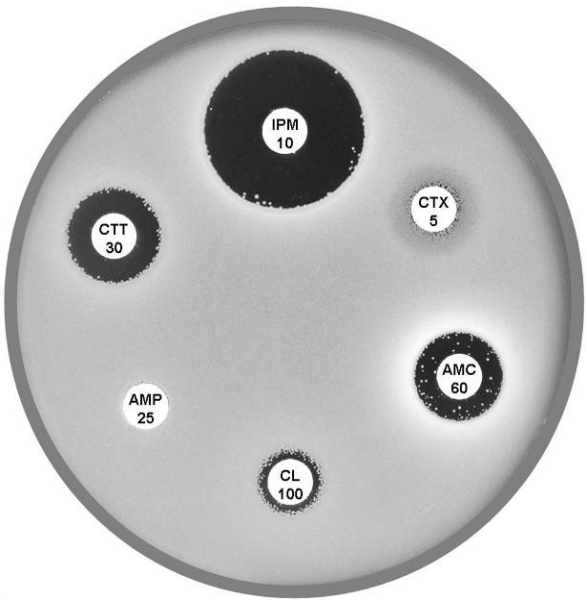

Plate 13.12.C *Citrobacter freundii* with derepressed AmpC β-lactamase
Note the resistance to cefotaxime (CTX 5) and cefotetan (CTT 30).

13.13. *Proteus penneri*

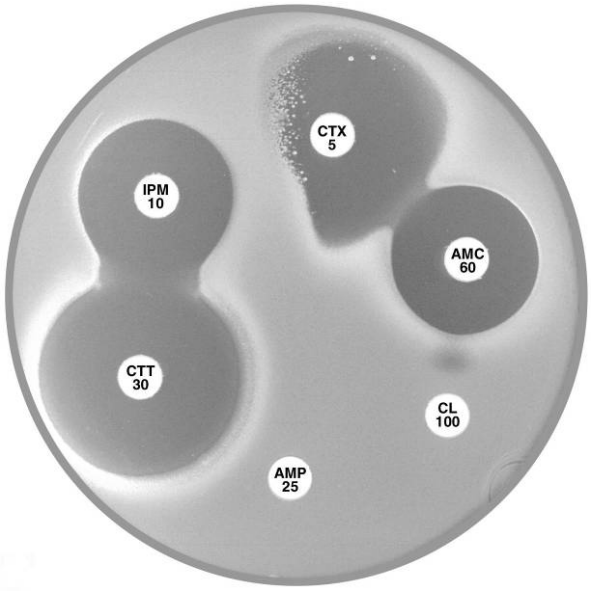

Plate 13.13.A *Proteus penneri* with an inducible group 2e β-lactamase.
The large inhibitory zone around Augmentin (AMC 60) and the flattening of the cefotaxime (CTX 5) zone adjacent to imipenem (IPM 10) indicate the presence of an inducible group 2e β-lactamase.

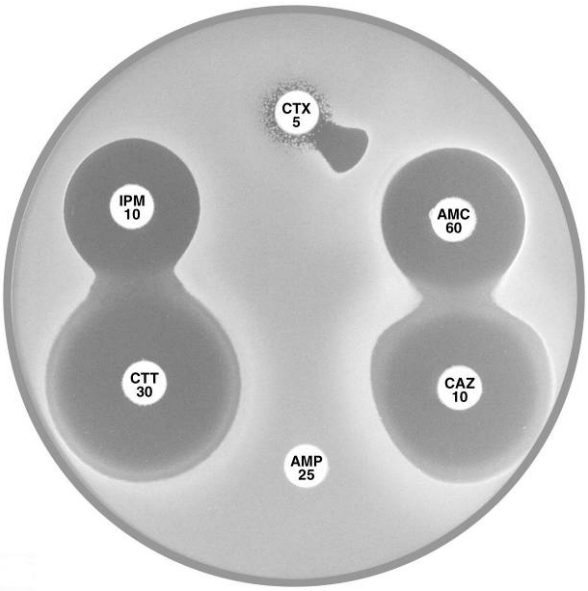

Plate 13.13.B *Proteus penneri* with a derepressed group 2e β-lactamase.
The susceptibility to ceftazidime and cefotetan and the synergy between Augmentin and cefotaxime suggests a group 2e β-lactamase. The resistance to cefotaxime indicates hyper-production of the β-lactamase.

13.14. *Pseudomonas aeruginosa*

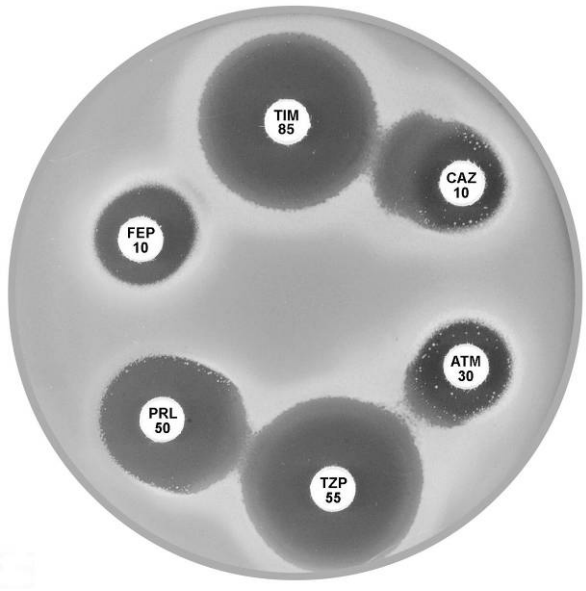

Plate 13.14.A *Pseudomonas aeruginosa* producing an ESBL
Showing synergy between Timentin (TIM 85) & ceftazidime (CAZ 10) and between Tazocin (TZP 55) & aztreonam (ATM 30).

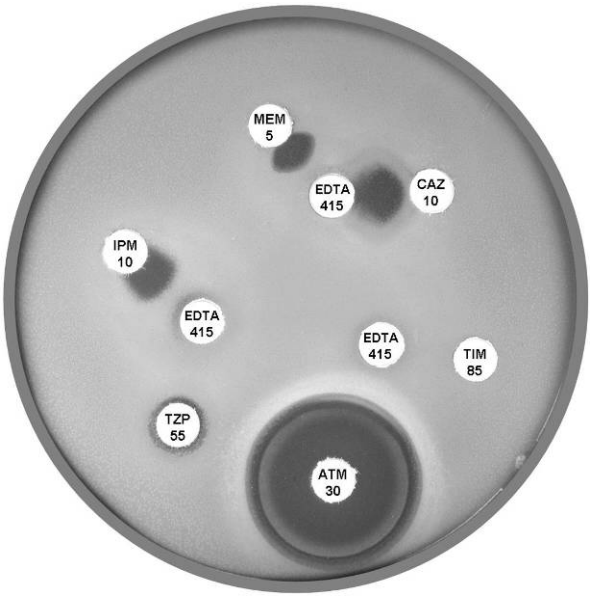

Plate 13.14.B *Pseudomonas aeruginosa* with a zinc-metallo-carbapenemase
Showing the inhibition of the carbapenemase by EDTA.

13.15. Metallo-β-lactamases (MBLs)

Plate 13.15.A *K. pneumoniae* producing an MBL on routine CDS test.
Resistant to AMP 25, AMC 60, CTX 5, CL100 and FEP 10, colonies at the edge of imipenem (IPM 10) zone ≥ 6 mm, see sect. 5.5.8

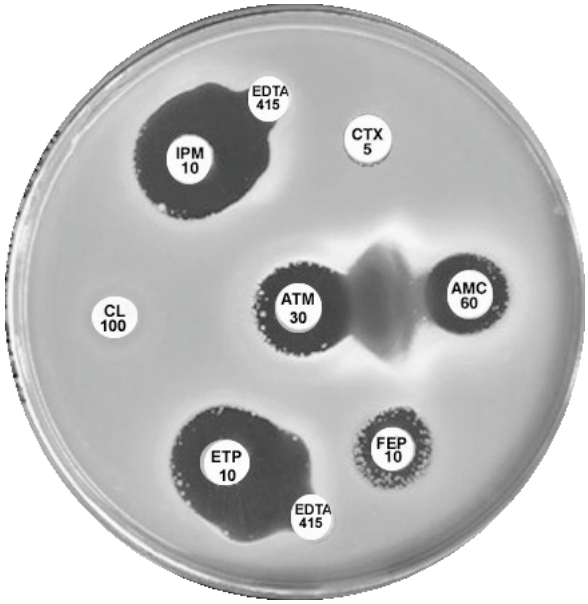

Plate 13.15.B. *K. pneumoniae* producing an MBL and an ESBL
MBL because EDTA confirmatory test positive, ESBL because small ATM (aztreonam) with synergy between ATM and AMC (Augmentin).

13.16. K. pneumoniae carbapenemase (KPC)

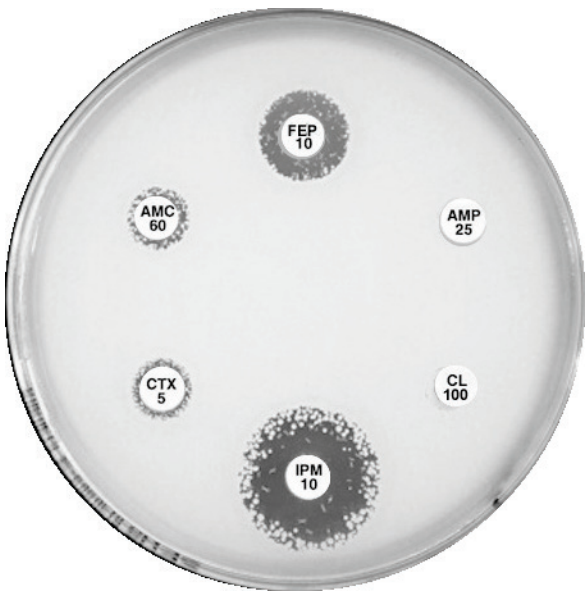

Plate 13.16.A *K. pneumoniae* producing a KPC in routine CDS test.
Resistant to all, numerous resistant colonies in imipenem (IPM) zone. Negative for ESBL and MBL. Perform confirmatory test for KPC

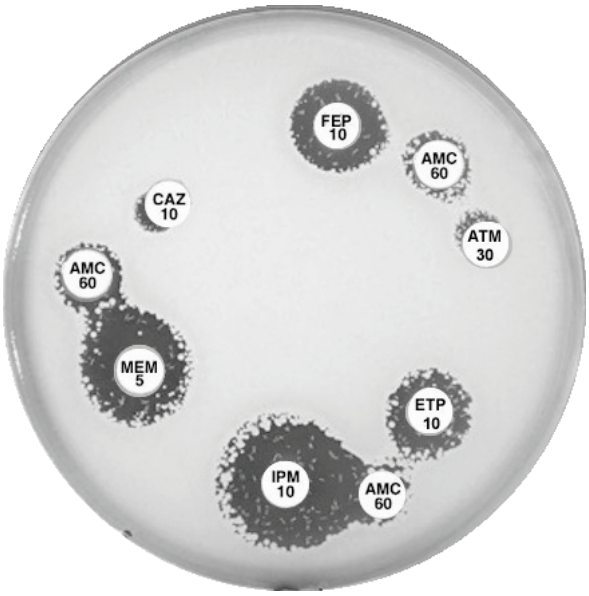

Plate 13.16.B *K. pneumoniae from Greece* producing an KPC-2
Confirmatory test: Synergy between AMC 60 and IPM 10 hydrolyses carbapenem, a "super" ESBL. See Sect. 5.5.9

13.17. *Stenotrophomonas maltophilia*

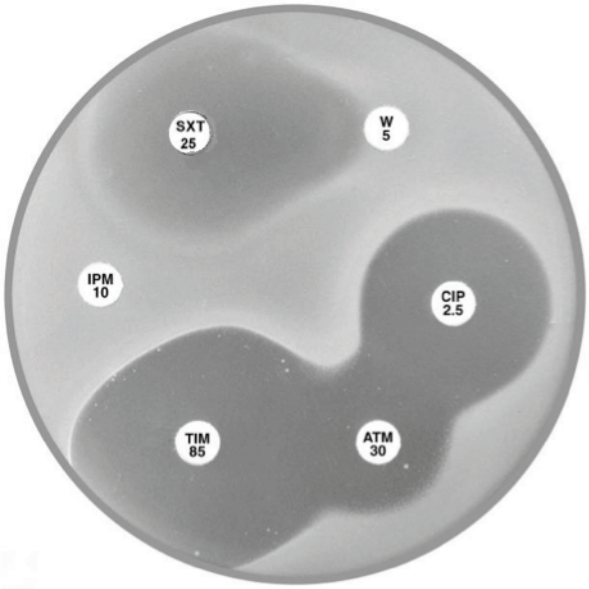

Plate 13.17.A *Stenotrophomonas maltophilia*
Note the lack of an inhibitory zone around imipenem, the pear shaped zone of inhibition between cotrimoxazole and trimethoprim and the resistant colonies within the Timentin and aztreonam inhibitory zones.

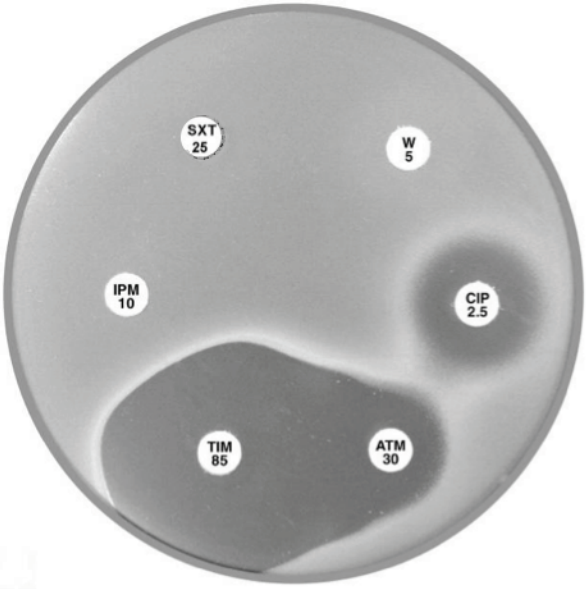

Plate 13.17.B Sulphonamide resistant *Stenotrophomonas maltophilia*
No zone around cotrimoxazole (SXT 25).

Acknowledgements

The CDS antibiotic susceptibility test has been developed over a period in excess of 40 years and during that time a large number of people have had an involvement one way or another in the development of the method. In some cases those who have made specific contributions have been acknowledged in earlier editions of the Manual and on the CDS website. Here we would like to acknowledge and thank all of those who contributed and supported us in our endeavours. They are:

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Syd Bell

October 2016

National Association of Testing Authorities, Australia

This communiqué is designed to provide laboratories performing in vitro diagnostic medical device (IVD) assays with information about NATA's approach to accreditation and relationship with the Therapeutic Goods Administration (TGA).

NATA's Role in Meeting Regulatory Requirements for In-house IVDs

In-house in vitro diagnostic medical devices (IVDs) are in general, pathology tests that have been developed or modified by a medical laboratory to carry out testing on human samples, where the results are intended to assist in clinical diagnosis or in making decisions concerning clinical management.

Under the Therapeutic Goods Administration's (TGA)[1] new regulatory framework for IVDs that commenced on 1 July 2010, laboratories manufacturing in-house IVDs are required to meet specific regulatory requirements to legally perform their in-house IVDs.

Should a facility supply their IVDs to outside clients they become a commercial IVD manufacturer and will require a different IVD approval process as defined by the regulations.

The National Pathology Accreditation Advisory Council (NPAAC) standard *Requirements for the Development and Use of In-house In Vitro Diagnostic Devices (IVDs)* is one of the key components of the regulatory framework for Class 1-3 in-house IVDs.

This standard sets out the requirements for quality, safety and performance that manufacturers of in-house IVDs must meet to supply their IVDs in Australia.

Compliance and NATA accreditation

Laboratories performing Class 1 -3 IVDs are able to demonstrate compliance with the NPAAC standard and meet TGA regulatory requirements by maintaining NATA/RCPA accreditation under ISO 15189 as a Medical Testing laboratory.

Laboratory organisations with appropriate NATA corporate accreditation can be considered as a laboratory network, and can manufacture and distribute in-house tests within their network.

NATA's role is to assess laboratories for compliance with the NPAAC standards and ISO 15189, and where they are found to meet the requirements, to accredit laboratories which manufacture Class 1-3 in-house IVDs as Medical Testing laboratories.

To ensure the regulatory requirements of in-house IVDs are met NATA will:

- assess compliance with NPAAC standards and ISO 15189;
- review technical documentation for a sample of in-house IVDs (e.g. in-house IVDs implemented or changed since the last assessment);
- conduct an on-site assessment of the laboratory Quality Management System;
- collaborate with the TGA in the accreditation of laboratories and laboratory networks that manufacture in-house IVDs; and
- notify the TGA of any severe compliance issues or deficiencies found in relation to in-house IVD's, where non-conformances are considered not to have been appropriately addressed;

[1] The TGA administers the *Therapeutic Goods Act 1989* and subordinate legislation, such as the Therapeutic Goods (Medical Devices) Regulations 2002, which establish the IVD regulatory framework.

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Any non-conformances identified by NATA for IVDs will be handled via the normal accreditation processes which are in place for other non-conformances identified in NATA assessments.

Transitional period for in-house IVDs

The Regulations include a transitional period to allow time for laboratories that manufacture in-house IVD to comply with the new system. The transition period has been set at four years from the commencement of the new regulations on 1 July 2010 until 30 June 2014. By the end of this period all in-house IVDs supplied in Australia must comply with the new regulatory requirements. From 1 July 2010 the new in-house IVD framework is being applied progressively to laboratories undergoing accreditation to ensure that they will comply with the requirements by 30 June 2014. In addition to NATA accreditation, by this date laboratories must have notified the TGA of all the in-house IVDs that they manufacture.

The NATA assessment team will be responsible for ensuring a laboratory's in-house IVDs have been appropriately assessed.

Technical requirements for an In-house IVD include:

- Maintaining a comprehensive list of in-house IVDs stating the name of each IVD and its "Class". It is the laboratory's responsibility to assign the Class, according to the classification rules specified in the regulations[2]. This information will then be reviewed by the TGA upon notification to the in-house IVD database.
- There must be a documented procedure in place detailing the design, development and release of an in-house IVD;
- The laboratory must have an established procedure for the monitoring, analysis and improvement of in-house IVDs, including the reporting of any adverse events.
- Documentation relating to each individual in-house IVD must be maintained so that the minimum requirements outlined in the NPAAC standard are shown to have been met. In particular, the following points should be addressed:
    - An executive summary describing the introduction and general requirements of the assay;
    - A review of the design and technology used;
    - Summary of the production process;
    - Details of technical validation performed;
    - A summary of specimen types used;
    - Minimum requirements for specimen types and numbers;
    - Source of reference material;
    - Acceptance /rejection criteria;
    - Formal evaluation of the data collected, including any statistical analysis
    - Final summary of results with a conclusion or recommendations;
    - For established in-house IVDs, a review of the technical and clinical relevance and its ongoing satisfactory performance should be conducted at least annually
    - Formal sign off by senior staff members

[2] Classification rules, Schedule 2A of the *Therapeutic Goods (Medical Devices) Regulations 2002*

Pre-assessment
As part of the pre-assessment process, NATA will request

Applicant laboratories
(also includes accredited facilities proposing assays not previously assessed prior to the last visit :

- the list of in-house IVDs being put forward for accreditation including approximate date of introduction
- a copy of the corporate validation procedure;
- A full validation report for each assay type introduced since 2007;
- Validation **summary** reports for each in-house IVD, including modifications requiring validation, introduced since 2007

For facilities with **all** IVDs assessed at last visit
- the list of in-house IVDs currently in use, including approximate date of introduction
- a copy of the corporate validation procedure;
- A full validation report for each assay type (not each individual test) introduced since the time of the last visit;
- Validation summary reports for each new in-house IVD implemented since the time of last visit
- Validation summary reports for any existing (previously accredited) in-house assays that has undergone changes requiring validation since the time of the last visit.

Assessment
The assessment team will review a sample of in-house IVDs to ensure the availability of appropriate supporting documentation and ongoing monitoring of performance.

The assessment team will also confirm by sampling, the completeness of the list of in-house IVDs notified to the TGA.

There will not be a systematic review of all in-house IVDs previously accredited by NATA, beyond a general review of the laboratory's practices to ensure ongoing compliance with requirements of the standard.

Validation data for assays introduced prior to 2007 which are being assessed for the first time should be made available at assessment. These will be reviewed in conjunction with assay performance data. Facilities will generally not be expect to "re-validate" assays prior to 2007, however, should there be issues identified with the assay in terms of fitness for purpose or poor performance, corrective action will be required.

Should you require any further information please contact Andrew Griffin, Deputy Sector Manager – Life Sciences on (03) 9274 8212 or Andrew.griffin@nata.com.au

ANTIBIOTIC SUSCEPTIBILITY TESTING BY THE CDS METHOD

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The Antibiotic Reference Laboratory

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