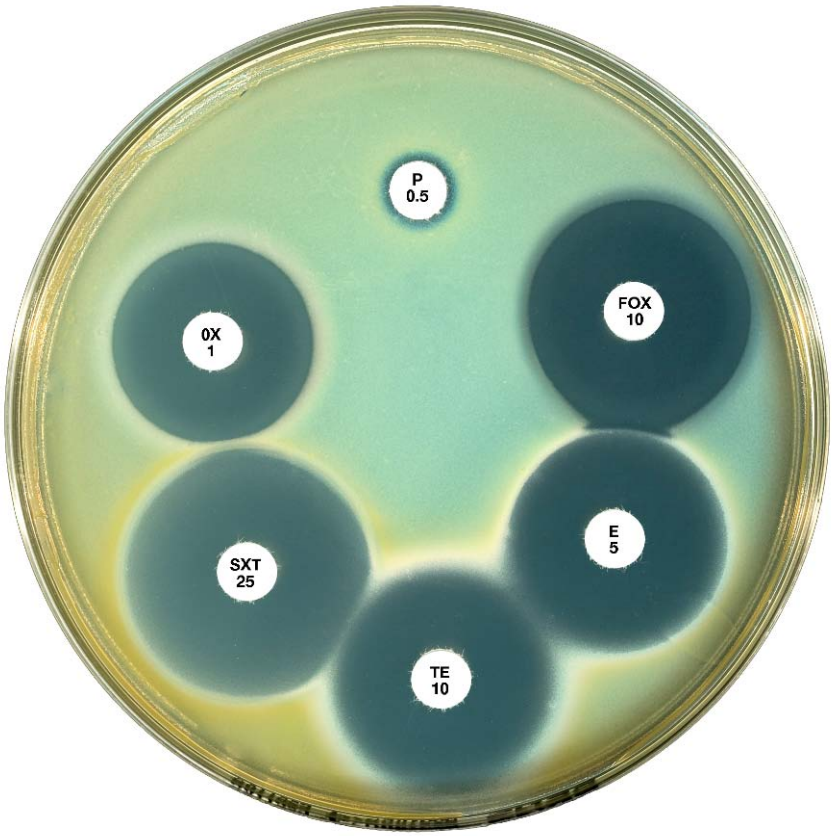
ANTIBIOTIC SUSCEPTIBILITY TESTING BY THE CDS METHOD



A MANUAL FOR MEDICAL AND VETERINARY LABORATORIES 2009

Modified Fifth Edition

8/12/2010

S. M. Bell, J. N. Pham, I. W. Carter

ANTIBIOTIC SUSCEPTIBILITY TESTING BY THE CDS METHOD

A Manual for Medical and Veterinary Laboratories

Modified Fifth Edition

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Contents

Contents 3

Preface to the Fifth Edition 6

1. Introduction 7

1.1. Unique features and basis of the CDS Test 7
1.1.1. Calibration (C) of the Test 7
1.1.2. Dichotomous (D) separation 7
1.1.3. Susceptibility (S) and break points 8
1.1.4. Interpretation of results 8
1.1.5. Performance characteristics of the CDS Test 9
References 9

2. Materials and methods 10

2.1. Materials 10
2.2. Methods 12
2.2.1. Agar plates 13
2.2.2. Preparation of the inoculum 14
2.2.3. Inoculation of plates and application of discs 16
2.2.4. Incubation of plates 16
2.2.5. Organisms with special growth requirements 16
2.2.6. Reading the zones 16
2.3. Interpretation of results 17
2.3.1. Assessment of inhibitory zone morphology 17
References 17

3. Quality assurance 18

3.1. Reference strains. 18
3.1.1. Obtaining reference strains 18
3.1.2. Handling and storage of reference strains 19
3.1.3. Testing reference strains 21
3.1.4. Measuring and recording reference strain results 21
3.2. CDS-QANTAS checklist 22
3.3. External quality assurance program 24

4. Application to Gram-positive species 25

4.1. *Corynebacterium* species 25
4.2. Enterococci 25
4.2.1. Ampicillin 25
4.2.2. Nitrofurantoin 25
4.2.3. Trimethoprim 25
4.2.4. Quinupristin/Dalfopristin 26
4.2.5. Vancomycin-resistant enterococci (VRE) 26
4.3. *Erysipelothrix* species 27
4.4. *Leuconostoc* and *Pediococcus* 27
4.5. *Nocardia* species 27
4.6. Staphylococci 28
4.6.1. Cefoxitin & oxacillin (methicillin) 28
4.6.2. Borderline oxacillin resistant *Staphylococcus aureus* (BORSA) 28
4.6.3. *Staphylococcus aureus* with low β-lactamase activity 28

4.6.4. Methicillin susceptible (mecA gene negative) *Staphylococcus aureus* (MSSA).....28
4.6.5. Methicillin resistant *Staphylococcus aureus* (MRSA).....28
4.6.6. Vancomycin resistance in *Staphylococcus aureus* 29
4.6.7. Erythromycin, clindamycin and *Staphylococcus aureus* 30
4.6.8. Coagulase negative staphylococci (CNS).....30
4.6.9. Rifampicin/sodium fusidate.....30
4.6.10. *Staphylococcus saprophyticus* from urine 30
4.7. Streptococci 31
4.7.1. Streptococcus pneumoniae 31
4.7.2. Other streptococci.....32
4.8. Erythromycin and clindamycin 32
References.....33

5. Application to Gram-negative species.....35

5.1. *Acinetobacter* species 35
5.2. *Aeromonas* species 35
5.3. *Burkholderia pseudomallei* 36
5.4. *Chryseobacterium* species (formerly *Flavobacterium* species) 36
5.5. Enterobacteriaceae.....36
5.5.1. Penicillinase β-lactamases 36
5.5.2. Inhibitor-resistant TEM β-lactamases (IRTs) 37
5.5.3. Plasmid mediated AmpC 37
5.5.4. Extended spectrum β-lactamases (ESBLs).....37
5.5.5. *Klebsiella oxytoca* and K1 β-lactamase 37
5.5.6. *Yersinia enterocolitica*.....38
5.5.7. Inducible cephalosporinases 38
5.5.8. Metallo-β-lactamases (MBLs).....39
5.5.9. *Salmonella* species and *Escherichia coli* with decreased susceptibility to ciprofloxacin 40
5.6. *Haemophilus influenzae* and *Haemophilus* species.....41
5.7. *Helicobacter pylori*.....41
5.8. *Neisseria* species 42
5.8.1. *Neisseria* meningitidis 42
5.8.2. Neisseria gonorrhoeae.....42
5.9. *Pasteurella* species 44
5.9.1. Ampicillin and benzylpenicillin susceptibility 44
5.10. *Pseudomonas aeruginosa*.....44
5.11. *Stenotrophomonas maltophilia*.....45
References.....46

6. Application to anaerobic bacteria 47

6.1. Introduction 47
6.2. Inoculum, Medium and Incubation conditions.....47
6.3. Antibiotics calibrations.....47
6.3.1. Clindamycin and anaerobes.....47

7. Application to yeasts.....48

7.1. Introduction 48
7.2. Inoculum, medium and incubation conditions 48
7.3. Interpretation of results 48
References.....48

8. Application to veterinary medicine.....49

8.1. Lincospectin 49
9. Direct antibiotic susceptibility testing 50
9.1. Urine 50
References 51
10. Tables 52
A guide to the use of the tables 52
10.1. Calibrations 53
10.2. Surrogate discs 61
10.3. Quality assurance 65
10.4. Testing and reporting β-lactam antibiotics 71
11. Plates 72
11.1. *Enterococcus faecalis, Enterococcus faecium* 72
11.2. *Enterococcus faecium, Enterococcus gallinarum & Leuconostoc* 73
11.3. *Staphylococcus aureus* 74
11.4. *MRSA* 75
11.5. *Staphylococcus saprophyticus* 76
11.6. *Acinetobacter* 77
11.7. *Aeromonas* 77
11.8. *Escherichia coli* 78
11.9. *Escherichia coli* and *Chryseobacterium indologenes* 79
11.10. *Klebsiella pneumoniae* 79
11.11. *Klebsiella oxytoca* 80
11.12. *Enterobacter and Citrobacter* 81
11.13. *Proteus penneri* 82
11.14. *Pseudomonas aeruginosa* 82
11.15. *Stenotrophomonas maltophilia* 83
11.16. Yeast 83
Acknowledgements 84

Tables

Table 3.1. Reference Strains. 18
Table 9.1. Urine sample inoculums for direct susceptibility testing. 51
Table 10.1.a. Calibrations: *Gram Positive Organisms* 53
Table 10.1.b. Calibrations: *Gram Negative Organisms* 56
Table 10.1.c. Calibrations: *Anaerobic Organisms.* 60
Table 10.1.d. Calibrations: *Yeast.* 60
Table 10.2.a. Surrogate disc testing: *Gram Positive Organisms* 61
Table 10.2.b. Surrogate disc testing: *Gram Negative Organisms* 63
Table 10.3.a. Reference strains: *Gram Positive Organisms* 65
Table 10.3.b. Reference strains: *Gram Negative Organisms* 66
Table 10.3.c. Reference strains: *Anaerobic Organisms* 70
Table 10.3.d. Reference strains: *Yeast* 70
Table 10.4.a. A guide to the testing and reporting of β-lactam antibiotics for Gram-negative organisms. 71

Preface to the Fifth Edition

At the 2008 CDS Workshop a poll was taken of the attendees as to whether there was a need for a hard copy of the Manual in view of the availability of a wealth of information on the CDS website. There was an overwhelming vote for an updated Manual and as a result we present the fifth edition here. We welcome Greg Fisher as an author of the Manual as well as the Webmaster of the CDS website.

There have been a number of changes made to this edition and these are listed on the CDS website. In summary we have attempted to make the Manual more concise and user friendly for the bench-worker. With this in mind several sections have been reformatted and those sections that are not of immediate value have been deleted. However for those who wish to view deleted portions of the Manual these can be viewed in the earlier editions that are stored in the Website archives. Susceptibility testing of more recently introduced antibiotics and for a number of new species is included in the manual. Also as our knowledge has grown of the various mechanisms of resistance we have modified some tests to ensure that the CDS method is able to detect them.

Finally, I would like to draw the reader's attention to the acknowledgements on page 83, where recognition is given to all who have made contributions to the development of the method.

Syd Bell

June, 2009.

1. Introduction

The first published description of the CDS Test appeared in “Pathology” in 1975.[1] The method was adopted quickly by diagnostic laboratories in Australasia and it is now 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, twenty one 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.

The agreed gold standard test is the agar dilution technique originally proposed by Ericsson and Sherris in 1971.[2] 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.

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, was 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. 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 strains. As far as the CDS Test is concerned, they are classed as resistant.

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 which may 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 MICs 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., 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 percent 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 percent.

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

2. Materials 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 and 10.

- "90 mm diameter" plastic Petri dishes.

- Tubes with 2.5 ml 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:

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

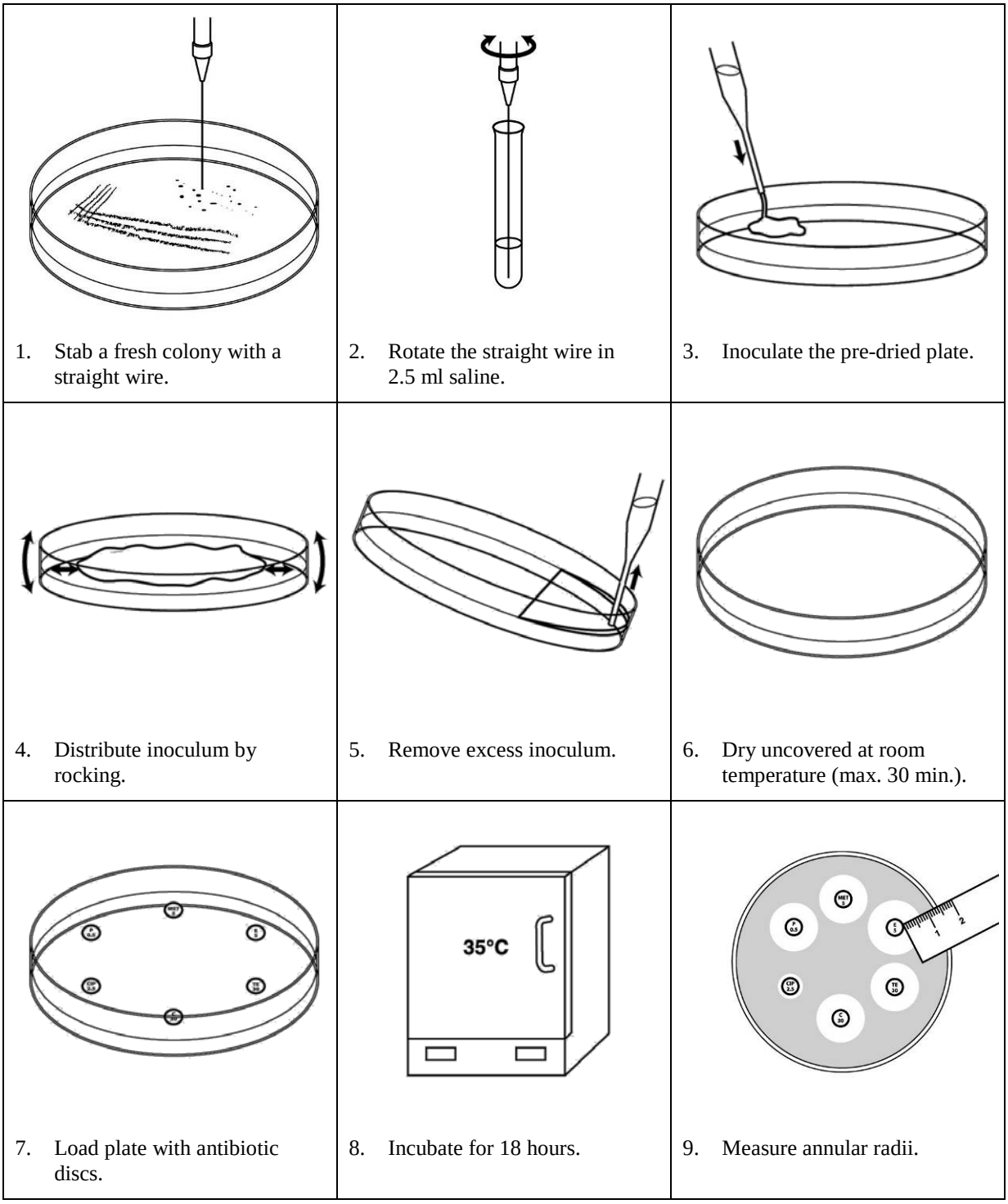
Wiltronics Research Pty Ltd	**bioMérieux Australia Pty Ltd**
Website: www.wiltronics.com.au Navigate to: Catalogue Cables and Wires (Under category) Nichrome Wire (Under Sub-category)	Pre-cut Nichrome straight wires (0.57 mm diameter) are available either unattached (MW197) or permanently attached to 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, Mast or other specified sources.
- 6 mm diameter antifungal discs supplied by Quantum Scientific Pty Ltd or Oxoid Pty Ltd.
- 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.
- 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% (eg 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.

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% (eg 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.

Casitone complex medium: To 1 L of deionised water (pH 7.2) add Bacto Casitone (Difco 225930) 9 g, yeast extract 5 g, sodium citrate 10 g, glucose 20 g, Agar Technical number 3 (Oxoid L13) 15 g, potassium di-hydrogen phosphate 0.54 g and di-sodium hydrogen phosphate 3.34 g. Mix and steam until dissolved. Autoclave and cool to 50°C before pouring.

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: C*ommercial preparations of the above media may be used provided that they comply strictly with the above descriptions.*

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, haemophili 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 up 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 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 GONORRHOEA

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

NOCARDIA SPECIES

The inoculum is prepared from a pure culture of the isolate grown for 24-48 hours on blood agar. The cells are harvested from the surface of the agar with a cotton swab 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.

YEASTS

The yeast suspension is prepared by growing yeast cultures on Sabouraud Dextrose agar at 35°C overnight in air. In sterile saline, prepare a 0.5 McFarland standard (absorbance 0.15 at 640 nm) suspension for *Candida* species or a 1.0 McFarland standard (absorbance 0.3 at 640 nm) for *Cryptococcus neoformans*.

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 10 (Tables) for correct disc potencies and Section 3.2 (CDS-QANTAS checklist) for correct storage and handling of stock and in use antibiotic discs.

2.2.4. Incubation of plates

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.

Yeasts: Casitone complex medium at 30°C in air for 24 hours. Slow growing *Candida* species or *Cryptococcus neoformans* may require 48 hours of incubation.

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. Sulphafurazole 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).

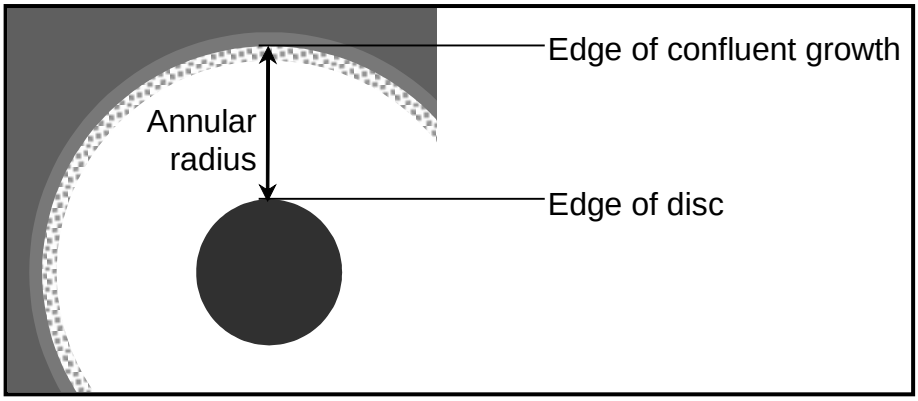


Figure 2.2 Diagram showing the annular radius of the zone of inhibition.

2.3. Interpretation of results

See Chapter 10 (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 10.1 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. 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 Accession Numbers from the University of Queensland.

Accession No.	Reference Strain	
ACM 5196	*Bacteroides fragilis*	ATCC 25285 (β-lactamase positive)
ACM 5183	*Campylobacter jejuni*	NCTC 11168
ACM 5283	*Candida parapsilosis*	ATCC 22019
ACM 5240	*Clostridium perfringens*	POW 2006
ACM 5184	*Enterococcus faecalis*	POW 1994
ACM 5185	*Escherichia coli*	NCTC 10418
ACM 5186	*Escherichia coli*	NCTC 11560 (β-lactamase positive)
ACM 5187	*Haemophilus influenzae*	NCTC 4560
ACM 5188	*Haemophilus influenzae*	NCTC 11315 (β-lactamase positive)
ACM 5239	*Neisseria gonorrhoeae*	WHO C
WHO K	*Neisseria gonorrhoeae*	WHO K
ACM 5189	*Pseudomonas aeruginosa*	NCTC 10662
ACM 5190	*Staphylococcus aureus*	NCTC 6571
ACM 5191	*Streptococcus pneumoniae*	ARL 10582

3.1.1. Obtaining reference strains

The Australian Collection of Micro-organisms (ACM) has suspended its activity for the time being.

Registered CDS users can obtain the reference strains from CDS reference laboratory:

The Antibiotic Reference Laboratory
Department of Microbiology
The Prince of Wales Hospital
Randwick NSW 2031 Australia.
Tel: (02) 9382 9046/9053
Fax: (02) 9398 4275
Email: cds@sesiahs.health.nsw.gov.au

Neisseria gonorrhoeae WHO C (ACM 5239) and WHO K may be obtained from:

The Neisseria Reference Laboratory
Department of Microbiology
The Prince of Wales Hospital
Randwick NSW 2031
Australia.
Tel: (02) 9382 9084
Fax: (02) 9398 4275
Email:
John.tapsall@sesiahs.health.nsw.gov.au
Athena.limnios@sesiahs.health.nsw.gov.au

3.1.2. Handling and storage of reference strains

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

Reference Strain	Media
Haemophilus influenzae ACM 5187 & ACM 5188	Chocolate agar
Neisseria gonorrhoeae	Chocolate agar
Candida parapsilosis ACM 5283	Sabouraud Dextrose agar
All other reference strains	Blood agar

-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 ACM 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 ACM 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 ACM 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 -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 ACM 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.

3.1.3. 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* ACM 5185, there is no need to test the same cartridge against *Pseudomonas aeruginosa* ACM 5189 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 10.3.a to d.

Bacteroides fragilis ACM 5196 and *Clostridium perfringens* ACM 5240

When performing susceptibility testing on *Helicobacter pylori* isolates but not anaerobes, *Bacteroides fragilis* ACM 5196 is the reference strain used for the quality assurance testing of metronidazole 5 μg.

When performing susceptibility testing on anaerobes, *Bacteroides fragilis* ACM 5196 (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 6 (Anaerobes), and Table 10.1.c and Table 10.3.c for more information on the testing of anaerobes.

3.1.4. 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 uncertainty of measurements for CDS testing by the laboratory.

***Staphylococcus aureus* ACM 5190**									
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.

Figure 3.1 Sample quality assurance sheet for antibiotic discs.

3.2. 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 10.3, Table 10.3.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

Organism tested……		[Y / N]
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 with an active desiccant (Bacto Lab. Ph: 02 9602 5499, Merck Microbiology. Ph: 1800 335 571).

3.3. 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 10.2.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 10.1.a to d) or is not in the 'Surrogate Disc Testing' tables (Table 10.2.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 10.4.a.

5. If requested to test an organism not calibrated for the testing by the CDS, we can extend the testing from an organism with similar growth requirement and behaviour. For example, the testing and reporting of *Chromobacterium* sp. may be extended from that of non-fastidious Gram negative bacilli such as *Pseudomonas* sp.

4. Application 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 strains is ≤ 4 mg/L (Table 10.1.a).

β-Lactamase producing *Enterococcus faecalis*

The appearance, in recent years, of rare β-lactamase producing strains 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* ACM 5184, has a zone of inhibition with a hazy edge around ampicillin 5 µg (Plate 11.1.A). β-Lactamase producing strains give a sharp edged zone of inhibition around ampicillin 5 µg (Plate 11.1.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 a β-lactamase and report the isolate resistant to ampicillin if a 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 11.1.C). The resistance to ampicillin/benzylpenicillin in *E. faecium* is associated with low affinity penicillin binding proteins.[1]

4.2.2. Nitrofurantoin

The inclusion of a nitrofurantoin 200 µg disc when performing enterococcal susceptibility testing 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 likely identification as *E. faecium* (Plate 11.1.C).

4.2.3. 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 enterococcal urinary infections but also in the development of bacteraemia.[2]

4.2.4. 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.

4.2.5. 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% are 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* ACM 5184 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 ACM 5184 (Plate 11.1.A)
    2. ***E. faecium*** have a vancomycin zone up to 4 to 6 mm with a sharp edge (Plate 11.1.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 about 2 mm around the teicoplanin 15 μg disc (Plate 11.2.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 11.2.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 11.2.C). 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 11.2.D). VRE can be distinguished from Leuconostoc and Pediococcus by its pyrrolidonyl arylamidase (PYR) activity (See section 4.4).*

4.3. *Erysipelothrix* species

Erysipelothrix species are included in the Table of Calibrations with streptococci (Table 10.1.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 11.2.D). *Leuconostoc* and *Pediococcus* species lack pyrrolidonyl arylamidase (PYR) activity and this can be used to differentiate them from VRE and other enterococci.

4.5. *Nocardia* species

Recently a limited range of commonly used antibiotics have been calibrated for susceptibility testing against *Nocardia* sp. (Table 10.1.a). Prepare the inoculum in saline broth as described in section 2.2.2 (under subheading ‘Nocardia’). Susceptibility testing is performed on Blood Sensitest Agar and incubated at 35-36°C in air for 24 to 48 hours.

Test no more than 3 antibiotic discs per plate. Discs should be distributed even around the plate and no more than 1cm from the edge of the plate. Susceptible strains of *Nocardia* sp. may 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.

With some isolates of *Nocardia* sp. the inhibitory zone around cotrimoxazole disc (SXT 25) may be hazy making the zone hard to read. Retest SXT 25 using Sensitest Agar.

For quality assurance testing, clarithromycin 5 µg, cotrimoxazole 25 µg, linezolid 10 µg and tigecycline 15 µg are tested against *S. aureus* ACM 5190 on Sensitest Agar in air at 35-37°C for 24 hours (Table 10.3.a). Amikacin 30µg and imipenem 10µg are tested against *E. coli* ACM 5185 on Sensitest Agar in air at 35-37°C for 24 hours (Table 10.3.a). Ciprofloxacin 2.5 µg and gentamicin 10µg are tested against either *S. aureus* ACM 5190 or *E. coli* ACM 5185.

4.6. Staphylococci

4.6.1. 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.

4.6.2. 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.*

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

The annular radius of the inhibitory zone around benzylpenicillin 0.5 units with β-lactamase negative *S. aureus*, such as *S. aureus* ACM 5190, is about 12 mm (Plate 11.3.A) and the edge of the inhibitory zone is hazy.

Rare strains of *S. aureus* produce low levels of β-lactamase and for these strains the annular radius of the zone of inhibition around benzylpenicillin 0.5 units may be 4 to 5 mm with a sharp edge (Plate 11.3.B). Too low an inoculum may result in semi-confluent growth with an inhibitory zone annular radius up to 7 mm. However, the edge of the zone is still sharp and these strains must be reported as resistant to penicillin.

4.6.4. 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 11.3.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 11.3.D). They are reported as susceptible to methicillin.

4.6.5. 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 11.4.A, Plate 11.4.B and Plate 11.4.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 have been 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 11.4.B). Occasionally, these strains are also 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.

4.6.6. Vancomycin resistance in *Staphylococcus aureus*

Vancomycin resistant *Staphylococcus aureus* (VRSA)

VRSA was first isolated in the USA in 2002. 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.[3] 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 11.4.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).[4] 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.

4.6.7. 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 11.4.D). The remaining 2% possess an efflux mechanism that does not confer resistance to clindamycin. See section 4.8

4.6.8. 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.

4.6.9. 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.

4.6.10. *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 benzylpenicillin 0.5 units) 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 11.5.A).

- Resistant to ampicillin but susceptible to cephalexin.

  The isolate does not have the mecA gene but does possess an inducible β-lactamase (Plate 11.5.B).

- Resistant to ampicillin and cephalexin.

  The isolate has the mecA gene (Plate 11.5.C).

4.7. Streptococci

4.7.1. 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. Isolates with an annular radius of the zone of inhibition < 6 mm are reported resistant to benzylpenicillin, amoxycillin and ampicillin. The MIC of benzylpenicillin is ≥ 0.25 mg/L

- Cefotaxime or ceftriaxone: Isolates are tested using a cefotaxime or a ceftriaxone 0.5 μg disc. Isolates with an annular radius of the zone of inhibition < 6 mm are reported resistant. The MIC is ≥ 0.5 mg/L. The MIC determination may be necessary to define adequately the susceptibility of the isolate.

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 10.1.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 10.1.a).
- Cefotaxime or ceftriaxone 0.5 μg /cefotaxime or ceftriaxone 5 μg: If the inhibitory zone is < 6 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 0.5 mg/L and 2.0 mg/L".

4.7.2. 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 10.2.a). Other streptococci including group B streptococci, *S. mitis*, *S. sanguis* etc and *S. anginosus* group showing resistance to P 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 4.7.1).

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

4.8. 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).[6,7] Today Lincomycin is rarely used in human medicine.

- 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 (lincosamide – streptogramin A) phenotype described for *Streptococcus agalactiae*.[8]

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 11.4.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.[8]

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5. Application 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. See Power points ASM 2010.

- 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 11.6.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 11.6.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. The susceptibility observed with Augmentin or Timentin is due to the combined antibacterial activities of clavulanic acid and amoxycillin or ticarcillin. 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.

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 11.7.A and Plate 11.7.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 double disc testing (Plate 11.7.A). A close examination revealed that members of *Aeromonas* sp, (except *A. sobria* which fails 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 10.4.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 10.1.b) and report as recommended in Table 10.4.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 11.9.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 trimethoprim 5 μg discs but only small zones around sulphafurazole 300 μg discs (Plate 11.9.B). This is the reverse of the pattern seen with *Stenotrophomonas maltophilia*. *Chryseobacterium* species are tested using the criteria applied to *Pseudomonas* species (Table 10.1.b) and reported using the recommendations in Table 10.4.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.

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 11.8.A).

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

Resistance to Augmentin in some strains of *E. coli* 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 but remain susceptible to cephalexin (Plate 11.8.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[5] (Plate 11.8.C, Plate 11.8.D). These enzymes are inhibited by aztreonam and boronic acid but not by clavulanic acid. Plasmid mediated AmpC producers such as *E. coli*, *K. pneumoniae* can be recognised in routine testing. They are susceptible to cefepime, a 4th generation cephalosporin and resistant to Augmentin 60 μg, cephalexin 100 μg. Those with high AmpC activity are also resistant to cefotaxime 5 μg and ceftazidime 10 μg (see Power points ASM 2010).

Susceptibilities are reported according to the standard interpretation.

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 now 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 11.10.A and Plate 11.10.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 10.4.a. Organisms of the EEC group may also have an ESBL (section 5.5.7, Plate 11.12.B and Plate 11.12.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 11.11.A). In addition to the K1 enzyme, *K. oxytoca* may also acquire a plasmid mediated ESBL (Plate 11.11.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 synergistic effect (modest keyhole) may be observed near a disc containing clavulanic acid (Augmentin or Timentin) when positioned adjacent to a cefotaxime or ceftriaxone disc (Plate 11.11.C). Greater hyper-

production of the K1 enzyme will swamp the weak inhibitory effect of clavulanic acid and the synergistic effect will no longer be visible (Plate 11.11.D).

All K1 hyper-producers show a small zone of inhibition around cephalexin 100 μg and cefotaxime 5 μg (Plate 11.11.C and Plate 11.11.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.[6] By contrast, biotypes 2 and 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 bio-serotypes 2 or 3/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 ESCHAPM Group.

In the CDS laboratory, we have been looking more closely at members of the ESCHAPM group *(Enterobacter cloacae, Enterobacter aerogenes, Serratia marcescens, Citrobacter freundii, Hafnia alvei, Aeromonas hydrophila, Aeromonas caviae, Aeromonas veronii, Providencia stuartii, Providencia rettgeri* and *Morganella morganii*) that are known to produce a chromosomal inducible cephalosporinase, inhibited by aztreonam but not by clavulanic acid (AmpC/Bush group 1). Producers of this cephalosporinase can be detected in the CDS test by a disc approximation test (Plate 11.12.A).

In addition, some members of this group can give rise, at a high frequency, 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. The species that consistently give rise to high frequency mutants (10^{-5} to 10^{-6}) are *E. cloacae, E. aerogenes* and *C. freundii* and we propose that this group be called the EEC group. We recommend that for this group, 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 group contains only one species, *S. marcescens,* which does not give rise to derepressed mutants when exposed to ceftazidime and aztreonam but does so when exposed to other β-lactams including cefotaxime, Timentin, Tazocin, cefpodoxime, cefotetan, cefoxitin, cefuroxime or cephalexin. With this species, it is considered safe only to report the results of testing of aztreonam and ceftazidime in addition to the 4th generation cephalosporins and carbapenems.

The third group consists of all members of *Aeromonas* sp except *A. sobria* which fail to produce the cephalosporinase A1. This group yielded AmpC hyper-producing 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. Furthermore, when the hyper-producing mutants that were selected by any of the eight 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. 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 group 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 group) and hyper producers of AmpC are selected only at the very low mutation rate of 10^{-8} when exposed to the β-lactams to which they are susceptible. Members of this group are tested in the usual way by the CDS and reported according to the standard interpretation.

The CDS recommendations on testing and reporting are summarised in Table 10.4. 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 double disc testing (flattened zone of CTX 5 placed near an IPM 10 disc) and the susceptibility to AMC 60 (Plate 11.13.A and Plate 11.13.B).

Inducible plasmid mediated AmpC

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.[7] Such trains 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 Bush group 3 (Ambler class B) 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^{2+}). They are not inhibited by clavulanic acid (Plate 11.9.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.

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 Power points 2010).

5.5.9. *Salmonella* species and *Escherichia coli* with decreased susceptibility to ciprofloxacin

Treatment failure has been reported with invasive *Salmonella* infections and *E. co*li septicaemia where the MIC of ciprofloxacin was ≥ 0.125 mg/L.[8] It was recommended that *Salmonella* species and blood culture *E. co*li isolates 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 and *E. coli* confer resistance to nalidixic acid with an associated decrease in the susceptibility to ciprofloxacin.[8,9] We suggest that CDS Users follow this recommendation and test all strains of *Salmonella* species causing systemic disease and blood culture *E. co*li isolates against both nalidixic acid and 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 MIC of ciprofloxacin should be determined and the isolate sent to a Reference Centre.

- 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.

5.6. *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). Do not use Sensitest Agar when preparing chocolate agar plates for susceptibility testing.

Other Haemophili

Other *Haemophilus* species such as *H. aphrophilus*, *H. paraphrophilus*, and some *H. parainfluenzae* isolated from blood culture may not grow on Haemophilus Test Medium. Susceptibility testing on these organisms is performed on chocolate Columbia Blood Agar (section 2.2.1) and incubated as described in section 2.2.4.

Haemophili with reduced susceptibility to cefotaxime

Rare isolates of *H. influenzae* or *H. parainfluenzae* may have a reduced susceptibility to cefotaxime due to changes in the PBPs. This reduced 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 with the 0.5 µg disc and≥ 6 mm with the 5 µg disc, report the susceptibility as follows: "There is reduced susceptibility to cefotaxime (or ceftriaxone) with the MIC between 0.5 mg/L and 2.0 mg/L".

5.7. *Helicobacter pylori*

A limited range of commonly used antibiotics have been calibrated for susceptibility testing against *Helicobacter pylori* (Table 10.1.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* ACM 5196 on blood Sensitest Agar in an anaerobic atmosphere at 35-37°C for 24 hours (Table 10.3.b)
- Amoxycillin 2 µg, ciprofloxacin 2.5 µg, erythromycin 5 µg, rifampicin 5 µg and tetracycline 10 µg are tested against *S. aureus* ACM 5190 on Sensitest Agar in air at 35-37°C for 24 hours (Table 10.3.a).

5.8. *Neisseria* species

5.8.1. *Neisseria* meningitidis

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.

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 and this is lower than the commonly acceptable MIC of 1 mg/L. The benzylpenicillin 0.5 u disc and the low MIC breakpoint of 0.25 mg/L may be used for epidemiological "screening" purposes.

Neisseria meningitidis and rifampicin

Rifampicin 1 μg has been calibrated for the testing of *N. meningitidis* and the annular radius of the zone of inhibition for susceptible strains is ≥ 6 mm. The MIC of rifampicin for susceptible strains is ≤ 0.5 mg/L and this is lower than the commonly acceptable MIC. The rifampicin 1 μg disc and the low MIC breakpoint may be used for epidemiological "screening" purposes.

5.8.2. Neisseria gonorrhoeae

Neisseria gonorrhoeae disc diffusion susceptibility testing is performed on chocolate Columbia Blood Agar (section 2.2.1) and incubated overnight at 35-36°C in 5% CO_2 and > 80% humidity. Benzylpenicillin, ceftriaxone, ciprofloxacin, nalidixic acid, spectinomycin and more recently cefpodoxime have been calibrated against *N. gonorrhoeae* (Table 10.1.b). Some considerations in testing and reporting susceptibility to these antibiotics follow.

Benzylpenicillin

N. gonorrhoeae may be resistant to benzylpenicillin by either of two mechanisms, β-lactamase production (penicillinase producing *N. gonorrhoeae* i.e., PPNG) and/or chromosomally mediated resistance (CMRNG). The β-lactamase can be detected using either a standard acidimetric method or a nitrocefin based method. Strains producing a β-lactamase usually give no zone of inhibition around a benzylpenicillin 0.5 u disc (P 0.5 u). Strains lacking a β-lactamase may be fully susceptible, less susceptible or resistant to benzylpenicillin[10]. These separate categories of susceptibility are defined as follows:

- Susceptible to benzylpenicillin.

  The annular radius of the inhibitory zone around a P 0.5 u disc is > 9 mm.

- Less susceptible to benzylpenicillin.

  The annular radius of the inhibitory zone around a P 0.5 u disc is between 3 mm and 9 mm.

- Resistant to benzylpenicillin (PPNG and/or CMRNG).

  The annular radius of the inhibitory zone around a P 0.5 u disc is < 3 mm.

Ceftriaxone

Resistance to ceftriaxone, i.e., properly documented treatment failure with ceftriaxone, given as a single 500 mg intramuscular dose, has not yet been recorded. "The susceptibility of *Neisseria gonorrhoeae* to the extended spectrum cephalosporins (ESCs), which include ceftriaxone, the current first line of treatment for gonorrhoea, has been decreasing worldwide. To assist in the laboratory detection of gonococci with decreased susceptibility (DS) to Ceftriaxone using the Ceftriaxone 0.5ug disc, the Cefpodoxime CPD10ug disc has recently been included to assist in the detection of gonococci with decreased susceptibility to Ceftriaxone. Thus a WHO control panel strain namely '*Neisseria gonorrhoeae* WHO K' is listed which displays decreased susceptibility to Ceftriaxone due to the presence of a penA mosaic allele, an alteration in the penicillin binding protein2 (PBP2).
Categories of susceptibility are defined as follows:

- Susceptible to ceftriaxone.

  The annular radius of the inhibitory zone around a ceftriaxone 0.5 µg disc is > 9 mm and > 12 mm around a cefpodoxime 10 µg disc.

- Decreased susceptibility to ceftriaxone.

  The annular radius of the inhibitory zone is between 5 mm and 9 mm around a ceftriaxone 0.5 µg disc and between 7 mm and 12 mm around a cefpodoxime 10 µg disc. This result should be confirmed by determining the MIC of ceftriaxone.

Ciprofloxacin

Quinolone disc testing is performed using a combination of both ciprofloxacin 1 µg (a fluoroquinolone) and nalidixic acid 30 µg (a quinolone) discs. Susceptibility to nalidixic acid is not reported but is used in determining susceptibility to ciprofloxacin. Categories of susceptibility to ciprofloxacin are defined as follows:

- Susceptible to ciprofloxacin.

  The annular radius of the inhibitory zone around both nalidixic acid 30 µg and ciprofloxacin 1 µg discs is > 6 mm.

- Less susceptible to ciprofloxacin (treatment failure rates of approximately 6% with a standard 500 mg dose).

  The annular radius of the inhibitory zone around a nalidixic acid 30 µg disc is usually 0 mm and that around a ciprofloxacin 1 µg disc is > 6 mm. These isolates are considered less susceptible to ciprofloxacin with an MIC of between 0.06 mg/L and 0.5 mg/L.

- Resistant to ciprofloxacin (treatment failure rates are approximately 40% when the strain MIC is 1 mg/L and 100% when the MIC is ≥ 4 mg/L).

  The annular radius of the inhibitory zone sizes around a ciprofloxacin 1 µg disc is ≤ 6 mm. The annular radius around a nalidixic acid 30 µg disc is usually 0 mm.

The result obtained for ciprofloxacin susceptibility can be extrapolated to other fluoroquinolones.

Spectinomycin

Categories of susceptibility are defined as follows::

- Susceptible to spectinomycin.

  The annular radius of the inhibitory zone around a spectinomycin 100 μg disc is ≥ 6 mm.

- Resistant to spectinomycin.

  The annular radius of the inhibitory zone around a spectinomycin 100 μg disc is < 6 mm (usually 0 mm).

For epidemiological and public health reasons, laboratories are advised to refer all pathogenic Neisseria isolates to the appropriate jurisdictional Neisseria Reference Laboratory for examination.

5.9. *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, gatifloxacin and tetracycline have been calibrated for *Pasteurella* species (Table 10.1.b).

5.9.1. 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.

5.10. *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).[11]

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 11.14.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 11.14.B) and Power points ASM 2010. 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.

5.11. *Stenotrophomonas maltophilia*

Most wild strains of *S. maltophilia* are usually susceptible to sulphafurazole and resistant to trimethoprim, but show a marked synergy between the two antibiotics. A pear shape or comet tail zone of inhibition between sulphafurazole and trimethoprim is typical and indicates synergy between the two antibiotics (Plate 11.15.A). However, rare strains of *S. maltophilia* may be resistant to sulphafurazole (Plate 11.15.B).

NOTE: A light/hazy growth is usually visible within the sulphafurazole inhibitory zone; this does not imply resistance to sulphafurazole. If the inhibitory zone is not easily seen, repeat the test using a 1/10 dilution of the CDS inoculum.

No zone of inhibition around an imipenem (or meropenem) disc and the marked synergy between trimethoprim and sulphafurazole suggests that the isolate is likely to be *S. maltophilia* (Plate 11.15.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 10.4.a), this can lead to a therapeutic dilemma when the isolate is resistant to sulphafurazole (Plate 11.15.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.

References

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6. Application to anaerobic bacteria

6.1. 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. For the performance of the CDS Test and quality assurance using the reference strains *C. perfringens* ACM 5240 and *B. fragilis* ACM 5196 see Chapters 2 and 3 of this Manual.

6.2. 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.

6.3. 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 10.1.c lists the antibiotics calibrated, the disc potencies, the annular radius of the inhibitory zones and the antibiotic MIC's of susceptible strains.

6.3.1. 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 4.8 for an explanation of ICR).

7. Application to yeasts

7.1. Introduction

Diagnostic laboratories are frequently requested to assess the antifungal susceptibility of yeasts. A variety of methods have been described and a standard reference procedure was adopted by the Clinical and Laboratory Standards Institute (CLSI – formerly NCCLS).[1] However, this procedure involving broth macro-dilution is too cumbersome and labour intensive for most clinical laboratories. An alternative broth micro-dilution method was developed but was not cost-effective.[2]

As a result of these deficiencies, an agar disc diffusion method was devised to test yeasts (*Candida* species and *Cryptococcus neoformans*) based on the principles of the Calibrated Dichotomous Sensitivity (CDS) Test. *C. parapsilosis* ACM 5283 (ATCC 22019) is the reference strain for antibiotic quality assurance testing.

7.2. Inoculum, medium and incubation conditions

Susceptibility testing is performed on Casitone complex medium as defined in section 2.2.1 and incubated at 30° C in air for 24 hours. Slow growing yeast may require 48 hours. The inoculum is prepared to an equivalent 0.5 McFarland standard for *Candida* species or a 1.0 McFarland standard for *Cryptococcus neoformans* as described in section 2.2.2 subsection Yeast.

7.3. Interpretation of results

Table 10.1.d lists the antifungals calibrated, the disc potencies, the annular radius of the inhibitory zones and the antifungal MIC's of susceptible strains.

The zones of inhibition of susceptibility plates incubated at 30° C are either clear or contain a very scanty fine growth. Persistent growth within the inhibitory zone is not observed with amphotericin B or caspofungin (Plate 11.16.B). *C. tropicalis* may require incubation at 35° C in the presence of 5% CO_2 to achieve clear-cut zones.

References

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8. Application to veterinary medicine

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

Ceftiofur, cefovexin, enrofloxacin, lincospectin, ofloxacin, orbifloxacin and tylosin can be extrapolated from surrogate antibiotics as described in tables Table 10.2.a and Table 10.2.b and in section 8.1.

8.1. 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 4.8). 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 4.8). 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.

8.2. Erythromycin and *Pasteurella* sp.

As requested by Veterinary Laboratories, erythromycin has been calibrated on Blood Sensitest Agar at 35oC 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

9. 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.

9.1. 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).[3] Bacterial concentrations were estimated semi-quantitatively by manual phase contrast microscopy using Kova® slides. When the bacterial concentration was estimated at $\geq 9 \times 10^{10}$/L, 25 µl of the urine sample was added to 2.5 ml of sterile saline yielding a bacterial suspension of $\geq 10^{9}$/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 smaller size of these organisms. Add 50 µl of the urine sample for bacterial concentrations of $\geq 9 \times 10^{10}$/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 13).

Table 9.1. Urine sample inoculums 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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2 **Mukerjee, C. & Reiss-Levy, E.** 1994. Evaluation of direct disc diffusion susceptibility testing for bacteriuria using diluted urine against standard CDS method. *Pathology*. 26, 201-7.

3 **Fisher, G., Simos, A. & Bell, S.M.** 2005. Direct sensitivity testing of urine samples based on the microscopic quantitation and morphology of organisms. Australian Society for Microbiology. Annual Conference, Canberra, Australia.

10. 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.

10.1. Calibrations

Table 10.1.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/Gatifloxacin	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 [d]	4 mm [d]	≤ 4
Chloramphenicol	30	4 mm	≤ 8
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
Tigecycline	15		≤ 1
Vancomycin	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] If a *Corynebacterium* species is resistant to benzylpenicillin 0.5 u, test ampicillin 5 µg.

[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/Dalfopristin.

[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 re-incubated 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. Inducible clindamycin resistance can be detected as described in section 4.8.

Table 10.1.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)
***Nocardia* species**			
(Blood Sensitest, 5% Air, 35-37°C)			
Amikacin	30		≤ 8
Ciprofloxacin	2.5		≤ 1
Clarithromycin	5		≤ 0.5
Co-trimoxazole	25		≤ 2/38
Gentamicin	10		≤ 2
Imipenem	10		≤ 4
Linezolid	10		≤ 2
Tigecycline	15		≤ 2
Staphylococci			
(Sensitest, Air 35-37°C)			
Ampicillin [h]	5		≤ 0.5
Benzylpenicillin [i]	0.5 u		≤ 0.06
Cefoxitin [j]	10		≤ 4
Cephalexin [h]	100		≤ 16
Chloramphenicol	30		≤ 8
Ciprofloxacin	2.5		≤ 1
Clindamycin [n]	2		≤ 0.5
Co-trimoxazole	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/Gatifloxacin	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
Sulphafurazole	300		≤ 64
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*).
[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 4.8.

Table 10.1.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		≤ 0.25
Cefotaxime [m]	5		≤ 2
Ceftriaxone	0.5		≤ 0.25
Ceftriaxone [m]	5		≤ 2
Chloramphenicol	30		≤ 8
Clindamycin [n]	2		≤ 0.5
Co-trimoxazole	25		≤ 0.5/9.5
Erythromycin	5		≤ 0.5
Marbofloxacin [k]	5		≤ 2
Moxifloxacin/Gatifloxacin	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 4.8.

Table 10.1.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 [c]	≤ 16[c]
Ampicillin	25		≤ 8
Apramicin [d]	15		≤ 8
Augmentin [e]	60		≤ 16/8
Aztreonam	30		≤ 8
Cefazolin	30		≤ 16
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	100		≤ 16
Chloramphenicol	30		≤ 8
Ciprofloxacin	2.5		≤ 1
Doripenem	10		≤ 4
Enoxacin	10		≤ 4
Ertapenem [f]	10		≤ 4
Gentamicin	10	4 mm	≤ 2
Imipenem	10		≤ 4
Kanamycin	50		≤ 8
Marbofloxacin [d]	5		≤ 2
Meropenem	5		≤ 2
Moxifloxacin/Gatifloxacin	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
Sulphafurazole	300		≤ 64
Tazocin [e]	55		≤ 16/2
Tetracycline	10	4°mm	≤ 4.0
Tigecycline	15		≤ 1
Timentin [e]	85		≤ 32/2
Tobramycin	10	4 mm	≤ 2
Trimethoprim	5		≤ 4

[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] Revised calibration, 2006.
[d] Antibiotic calibrated for veterinary medicine.
[e] If an ESBL is present, report Augmentin, Timentin and Tazocin for isolates from urine only.
[f] *Acinetobacter* species are considered resistant to ertapenem.
[g] For testing urinary isolates only.

Table 10.1.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/Gatifloxacin	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/Gatifloxacin	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 10.1.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[l] (mg/L)
Neisseria gonorrhoeae [k]				
(Chocolate Columbia Blood Agar, 5% CO2, 35-37°C, humidity > 80%)				
Benzylpenicillin	0.5 u			
Susceptible [k]		> 9 mm		≤ 0.03
Less susceptible [k]		3 – 9 mm		0.06 – 0.5
Resistant [k]		< 3 mm		≥ 1
Ceftriaxone/Cefpodoxime		CRO 0.5	CPD 10	Ceftriaxone
Susceptible [k]		> 9 mm	> 12 mm	< 0.03
Decreased susceptibility [k]		5 – 9 mm	≤ 12 mm	0.03 – 0.25
Resistant [k]		–		–
Ciprofloxacin/Nalidixic acid		CIP 1	NA 30	Ciprofloxacin
Susceptible [k]		> 6 mm	> 6 mm	≤ 0.03
Less susceptible [k,m]		> 6 mm	0 mm[m]	0.06 – 0.5
Resistant [k,m]		≤ 6 mm	0 mm[m]	≥ 1
Spectinomycin	100			
Susceptible [k]		≥ 6 mm		≤ 64
Less susceptible [k]		–		–
Resistant [k]		0 mm		≥ 128
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.

[k] *Neisseria gonorrhoeae* sensitivity interpretation:

Susceptible – A cure is expected following treatment with standard doses.

Less susceptible –

Quinolone agents: A cure would be expected following high oral dose regimens, e.g., 500 mg ciprofloxacin but treatment failure from lower doses.

Penicillin Group: A cure would be expected with the standard regimens used to treat male urethral gonorrhoea (e.g. a single oral dose of 3g amoxycillin) but treatment failure may occur for other infected sites, e.g., endocervix, pharynx and rectum.

Decreased susceptibility (DS) – Reduced zone size to Ceftriaxone and Cefpodoxime. Clinical significance of ceftriaxone treatment is currently uncertain.

Resistant – There is a high probability of treatment failure using current dose recommendations.

[l] MIC determined by doubling dilution agar plate incorporation method.

[m] On rare occasions (< 0.001% of isolates) a small zone to Nalidixic Acid may be observed in association with decreased ciprofloxacin zone sizes. If the findings are confirmed on repeat testing the isolate should be reported as less susceptible or resistant according to the ciprofloxacin zone size and the isolate referred for MIC testing.

Table 10.1.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 [o]	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/Gatifloxacin	2.5		≤ 1
Tetracycline	10		≤ 4
***Pseudomonas* species, *Burkholderia* species & *Chryseobacterium* species (Sensitest, air, 35-37°C)**			
Amikacin	30	4 mm	≤ 16
Augmentin [p]	60		≤ 8/4
Aztreonam	30		≤ 8
Cefepime	10		≤ 2
Cefpirome	10		≤ 2
Ceftazidime	10		≤ 4
Ciprofloxacin	2.5		≤ 1
Doripenem	10		≤ 4
Ertapenem	10		≤ 4
Gentamicin	10	4 mm	≤ 4
Imipenem	10		≤ 4
Marbofloxacin [d]	5		≤ 2
Meropenem	5		≤ 2
Moxifloxacin/Gatifloxacin	2.5		≤ 1
Norfloxacin [g]	10		≤ 4
Piperacillin	50		≤ 16
Polymyxin B	300 u	4 mm	≤ 1
Sulphafurazole	300		≤ 64
Tazocin	55		≤ 16/2
Ticarcillin	75		≤ 32
Timentin	85		≤ 32/2
Tobramycin	10	4 mm	≤ 4
Trimethoprim	5		≤ 4
***Stenotrophomonas maltophilia* [q] (Sensitest, air, 35-37°C)**			
Sulphafurazole [q]	300		≤ 64

[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
[o] *Pasteurella multocida* is tested against ampicillin 5 µg and NOT benzylpenicillin 0.5 u.
[p] *Burkholderia pseudomallei* is usually susceptible to Augmentin and can be tested against this antibiotic.
[q] See Section 5.11 for notes on testing antibiotic susceptibilities of Sulphafurazole resistant *S. maltophilia*.

Table 10.1.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	2	4 mm	≤ 0.5
Meropenem	5		≤ 2
Metronidazole	5	4 mm	≤ 2
Moxifloxacin	2.5	4 mm	≤ 2
Tazocin	55		≤ 8/2
Timentin	85		≤ 32/2

[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.

Table 10.1.d. Calibrations: *Yeast*.

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)
Yeasts			
(Casitone complex medium, air, 30°C)			
Amphotericin B	10	4 mm	≤ 0.125
Caspofungin	5	2 mm	≤ 2.0
Fluconazole	25	6 mm	≤ 16
Itraconazole	10	4 mm	≤ 1.0
Voriconazole	1	6 mm	≤ 1.0

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

10.2. Surrogate discs

Table 10.2.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]	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[d] / 10[e]
Azithromycin/Clarithromycin/Roxithromycin	Erythromycin	5
Carbapenems	Oxacillin[d]/Cefoxitin[e]	1[d] / 10[e]
Cefovecin[a]/*Ceftiofur*[a]/other Cephalosporins[b]	Oxacillin[d]/Cefoxitin[e]	1[d] / 10[e]
Cloxacillin/ Dicloxacillin/ Flucloxacillin	Oxacillin[d]/Cefoxitin[e]	1[d] / 10[e]
Enrofloxacin[a]/*Orbifloxacin*[a]	Moxifloxacin	2.5
Lincomycin	Clindamycin	2
Norfloxacin[c]/ *Ofloxacin*[a]	Ciprofloxacin	2.5
Pristinamycin	Quinupristin/Dalfopristin	15
Sulphonamides	Sulphafurazole	300
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
Sulphonamides	Sulphafurazole	300
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.
[d] Oxacillin is used for testing coagulase-negative staphylococci (except *Staphylococcus saprophyticus*) only.
[e] Cefoxitin is used for testing *Staphylococcus aureus* only.
[f] *Enterococcus faecalis* is intrinsically resistant to Pristinamycin and Quinupristin/Dalfopristin.

Table 10.2.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
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	0.5
Enrofloxacin [a]/*Orbifloxacin* [a]	Moxifloxacin	2.5
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 anginosus*, 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].

Table 10.2.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
Co-trimoxazole [c]	Sulphafurazole	300
Co-trimoxazole [c]	Trimethoprim	5
Enrofloxacin [a]/*Orbifloxacin* [a]	Moxifloxacin	2.5
Ofloxacin [a]	Ciprofloxacin	2.5
Piperacillin	Ampicillin	25
Sulphonamides	Sulphafurazole	300
Tetracyclines	Tetracycline	10
Ticarcillin	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**		
Enrofloxacin[a]/*Orbifloxacin* [a]	Moxifloxacin	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.
[c] Resistance to co-trimoxazole is indicated by resistance to both sulphafurazole and trimethoprim.

Table 10.2.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	Benzylpenicillin[d]	0.5 u
Cefovecin [a]/*Ceftiofur* [a]	Ampicillin	5
Enrofloxacin[a]/*Orbifloxacin* [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
Cotrimoxazole [c]	Trimethoprim	5
Cotrimoxazole [c]	Sulphafurazole	300
Enrofloxacin [a]/*Orbifloxacin* [a]	Moxifloxacin	2.5
Norfloxacin / *Ofloxacin* [a]	Ciprofloxacin	2.5
Stenotrophomonas maltophilia		
Cotrimoxazole	Sulphafurazole	300

[a] Antibiotic used in veterinary medicine only.
[c] Resistance to cotrimoxazole is indicated by resistance to both sulphafurazole and trimethoprim.
[d] *Pasteurella multocida* is tested against ampicillin 5 μg and NOT benzylpenicillin 0.5 u.

10.3. Quality assurance

Table 10.3.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* ACM 5184 (Blood Sensitest, CO_2 35°C)**		
Ampicillin	5	5.9 – 9.2
Chloramphenicol	30	6.3 – 8.7
Gentamicin	200	6.6 – 9.9
Linezolid	10	6.6 – 9.0
Nitrofurantoin	200	6.1 – 8.7
Streptomycin	300	5.4 – 7.9
Tigecycline	15	6.6 – 9.5
Teicoplanin	15	3.1 – 5.3
Vancomycin	5	2.0 – 3.7
***Staphylococcus aureus* ACM 5190 (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
Cephalexin	100	10.7 – 15.5
Chloramphenicol	30	7.8 – 11.4
Ciprofloxacin	2.5	9.2 – 12.4
Clarithromycin [e]	5	9.4 – 11.8
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
Gatifloxacin	2.5	10.1 – 14.9
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
Sulphafurazole	300	9.3 – 13.7
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.

[e] Clarithromycin is calibrated only for *Nocardia* species.

Table 10.3.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* ACM 5191**		
(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
Co-trimoxazole	25	7.0 – 9.2
Erythromycin	5	7.1 – 12.9
Gatifloxacin	2.5	5.6 – 8.4
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 10.3.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* ACM 5196** [c]		
(Blood Sensitest, anaerobic, 35-37°C)		
Metronidazole	5	7.1 – 13.5
***Campylobacter jejuni* ACM 5183**		
(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* (ACM 5196) may be used as the reference strain when testing *H. pylori* against metronidazole. When testing anaerobic organisms, *Clostridium perfringens* (ACM 5240) should be used as the reference organism.

Table 10.3.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* ACM 5185 [d]**		
(Sensitest, air, 35–37°C)		
Amikacin	30	6.7 – 10.3
Ampicillin	25	7.5 – 10.7
Apramicin [d]	15	5.3 – 7.9
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
Doripenem	10	11.4 – 14.7
Gatifloxacin	2.5	11.2 – 14.8
Enoxacin	10	9.7 – 15.7
Ertapenem	10	12.1 – 16.1
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 [d]	25	5.0 – 7.8
Streptomycin [e]	25	6.2 – 7.8
Sulphafurazole	300	6.7 – 10.7
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* ACM 5186**		
(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* ACM 5185, there is no need to test these against *Pseudomonas aeruginosa* ACM 5189 as well and vice versa.

[e] Antibiotic used in veterinary medicine only

Table 10.3.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* ACM 5187 (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
Cefpodoxime	10	10.9 – 14.1
Ceftriaxone	0.5	9.1 – 12.9
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
Gatifloxacin	2.5	13.5 – 17.1
Moxifloxacin	2.5	10.6 – 15.2
Tetracycline	10	8.2 – 13.0
***Haemophilus influenzae* ACM 5188 (HTM [f] agar, 5% CO_2, 35-37°C)**		
Augmentin	15	7.7 – 10.1
***Neisseria gonorrhoeae* ACM 5239 (WHO C) (Chocolate Columbia Blood Agar, 5% CO_2, 35-37°C, humidity > 80%)**		
Benzylpenicillin	0. 5 u	2.1 – 4.1
Cefpodoxime	10	10.9 – 14.5
Ceftriaxone	0.5	8.2 – 11.0
Ciprofloxacin	1	12.7 – 16.3
Nalidixic acid	30	11.3 – 14.5
Spectinomycin	100	6.9 – 8.9
***Neisseria gonorrhoeae* WHO K (Chocolate Columbia Blood Agar, 5% CO_2, 35-37°C, humidity > 80%)**		
Cefpodoxime	10	7.6 – 11.1
Ceftriaxone	0.5	7.3 – 9.3

[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* ACM 5185, there is no need to test these against *Pseudomonas aeruginosa* ACM 5189 as well and vice versa.

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

Table 10.3.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* ACM 5189** [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]
Gatifloxacin	2.5	7.8 – 11.4
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* ACM 5185, there is no need to test these against *Pseudomonas aeruginosa* ACM 5189 as well and vice versa.

[g] Ertapenem and Moxifloxacin should be tested against *E. coli* ACM 5185.

Table 10.3.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* ACM 5240**		
(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* ACM 5196**		
(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.

Table 10.3.d. Reference strains: *Yeast*

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

Antibiotic reported	Disc potency (µg)	Annular radii (mm) [b]
***Candida parapsilosis* ACM 5283**		
(Casitone Complex medium, aerobic, 30°C)		
Amphotericin B	10	8.1 – 9.7
Caspofungin	5	3.9 – 5.5
Fluconazole	25	12.5 – 15.7
Itraconazole	10	6.2 – 7.4
Voriconazole	1	15.7 – 17.7

[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.

10.4. Testing and reporting β-lactam antibiotics

Table 10.4.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	R
Aeromonas with A1 cephalosporinase & A2 carbapenemase	R	R	**T***	**T***	R	**T**	R	R	R [e]	N[a]	R	R
Aeromonas sobria with A2 carbapenemase only	R	R	**T**	**T**	**T**	**T**	**T**	**T**	R	N[a]	**T**	**T**
Proteus vulgaris / penneri [f]	R	**U**	R	**T** [f]	R [f]	R	**T**	**T**	**T**	N[a]	**U**	**U**
Enterobacteriaceae with ESBL	R	**U**	R	R	R	R	**T**	R	**T**	N[a]	**U**	**U**
Isolate with a metallo-β-lactamase	R	R	**T**	R	R	R	R	R	R	N[a]	R	R
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**
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*= Can be tested and reported (See section 5.2 on *Aeromonas*).
U = Test isolates from urine 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, Citrobacter freundii.*

[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 5.11 for notes on testing *S. maltophilia* against these and other antibiotics.

11. Plates

11.1. *Enterococcus faecalis, Enterococcus faecium*

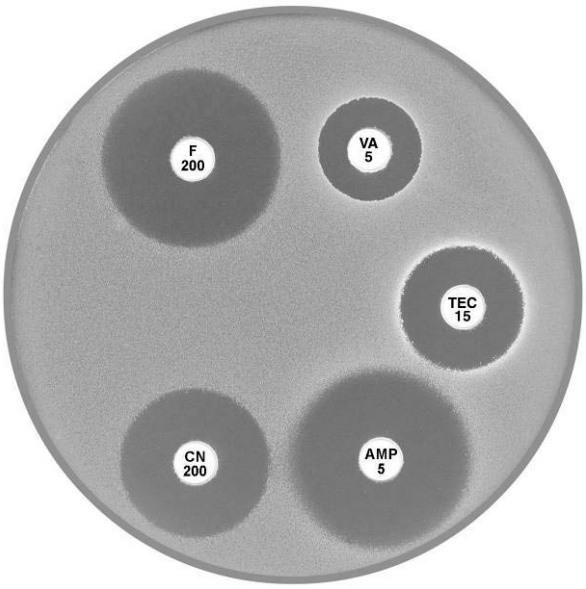

Plate 11.1.A *Enterococcus faecalis* and ampicillin
The diffuse edge to the zone of inhibition > 4 mm around ampicillin (AMP 5) indicates susceptibility.

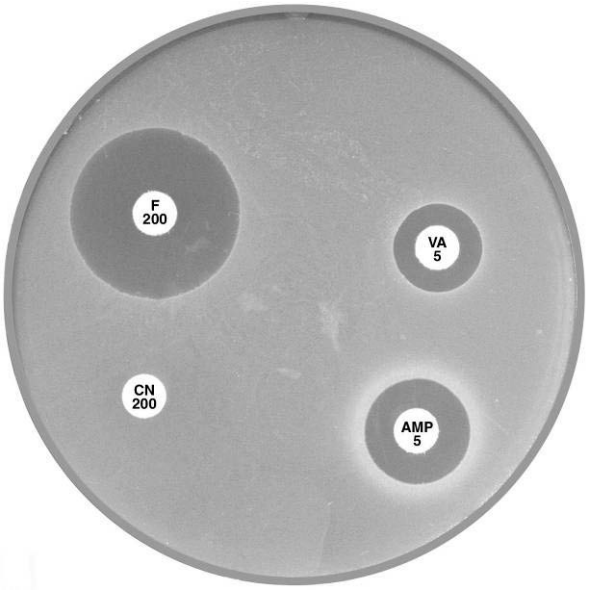

Plate 11.1.B β-Lactamase producing *Enterococcus faecalis*
The sharp edge and the reduced inhibitory zone around ampicillin (AMP 5) indicate β-lactamase production and resistance.

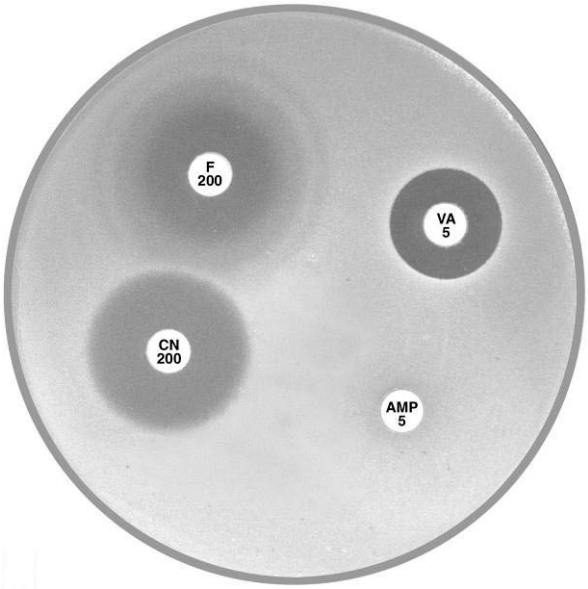

Plate 11.1.C *Enterococcus faecium* and nitrofurantoin
The hazy edged zone of inhibition around nitrofurantoin and no zone of inhibition around ampicillin is typical of *E. faecium*.

11.2. *Enterococcus faecium, Enterococcus gallinarum & Leuconostoc*

Plate 11.2.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.

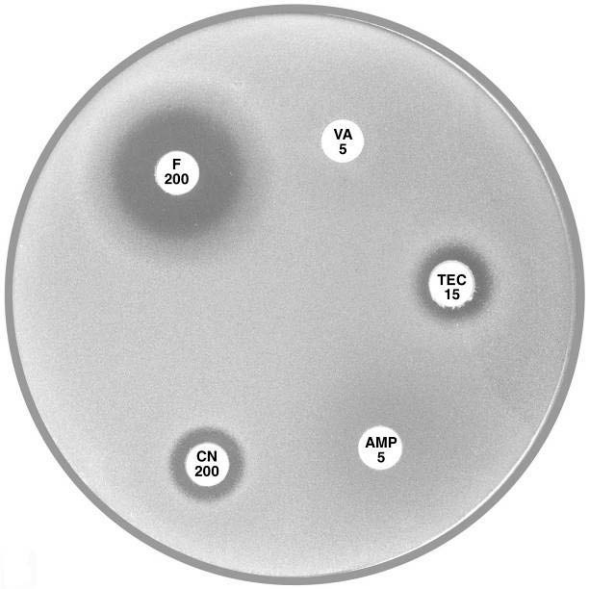

Plate 11.2.B *Enterococcus faecium* with VanA type vancomycin resistance
Resistance to both vancomycin (VA 5) and teicoplanin (TEC 15) is typical of the vanA phenotype.

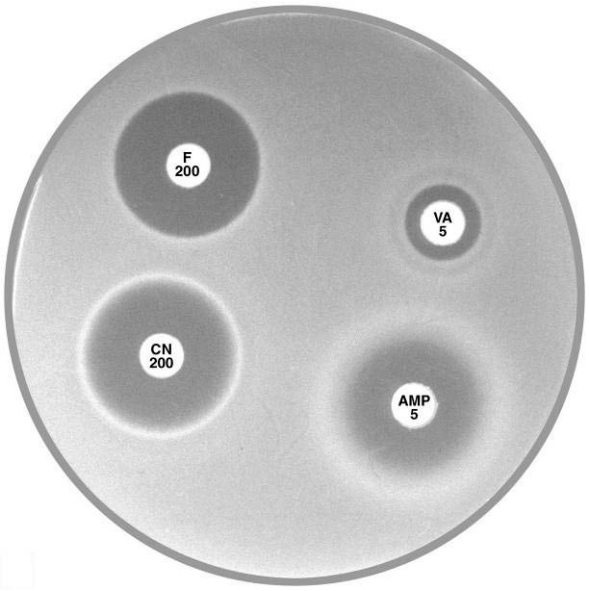

Plate 11.2.C *Enterococcus gallinarum* with intrinsic VanC type vancomycin resistance
Note the sharp edge of the reduced inhibitory zone around vancomycin (VA 5).

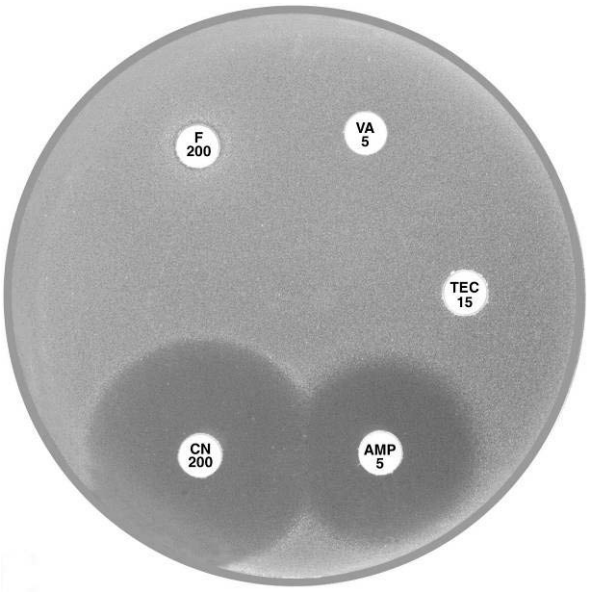

Plate 11.2.D *Leuconostoc* versus vancomycin and teicoplanin
This organism has high inherent resistance to vancomycin (VA 5) and teicoplanin (TEC 15).

11.3. *Staphylococcus aureus*

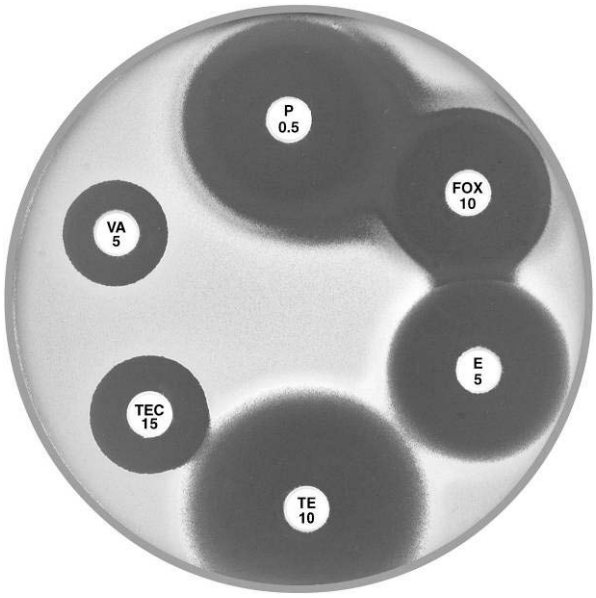

Plate 11.3.A *Staphylococcus aureus* ACM 5190

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.

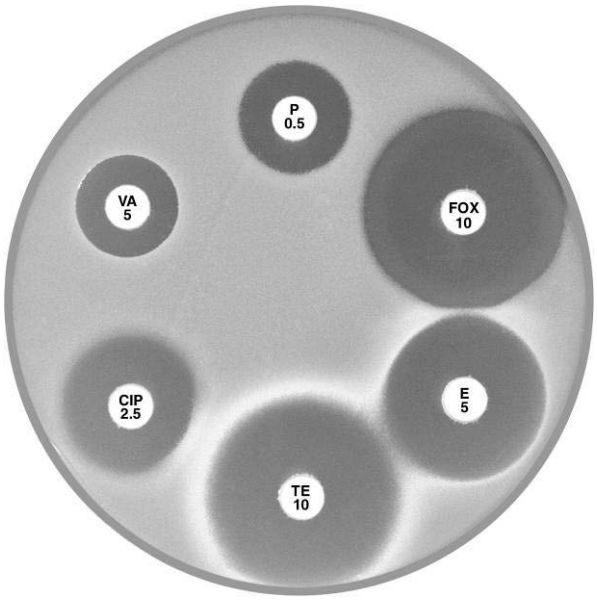

Plate 11.3.B *Staphylococcus aureus* with low β-lactamase activity

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 inhibition zone indicates β lactamase production.

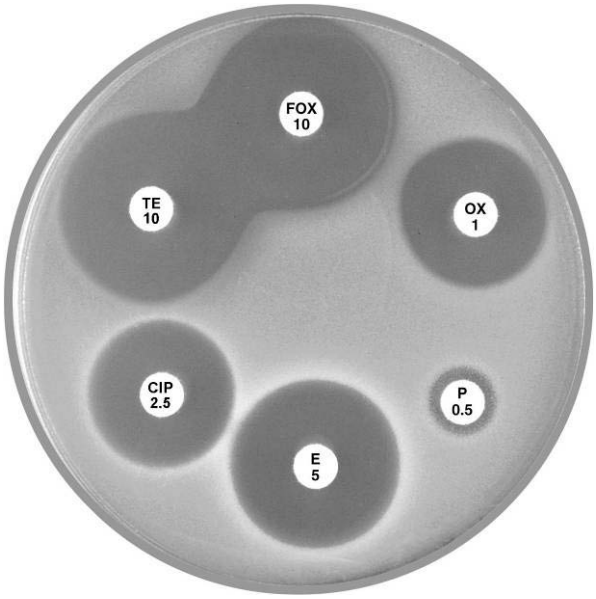

Plate 11.3.C *Staphylococcus aureus* resistant to penicillin only

Note the 9 mm annular radius of the zone of inhibition around cefoxitin (FOX 10).

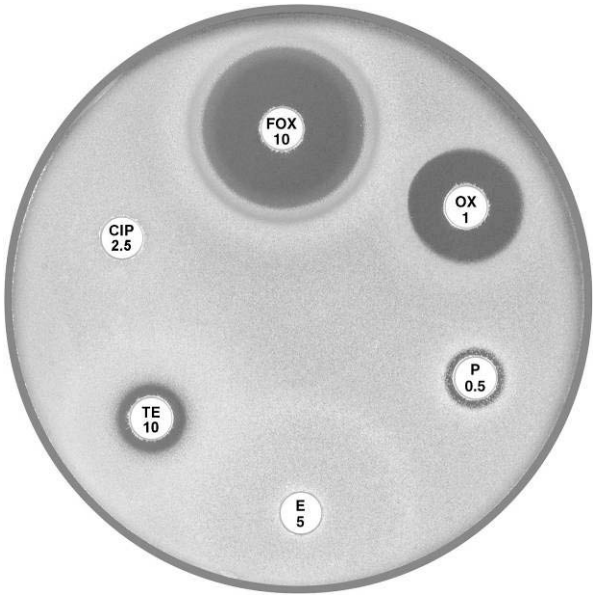

Plate 11.3.D mecA gene negative multiresistant *Staphylococcus aureus* (MSSA)

This isolate shows susceptibility to cefoxitin (FOX 10) and resistance to the other antibiotics tested.

11.4. *MRSA*

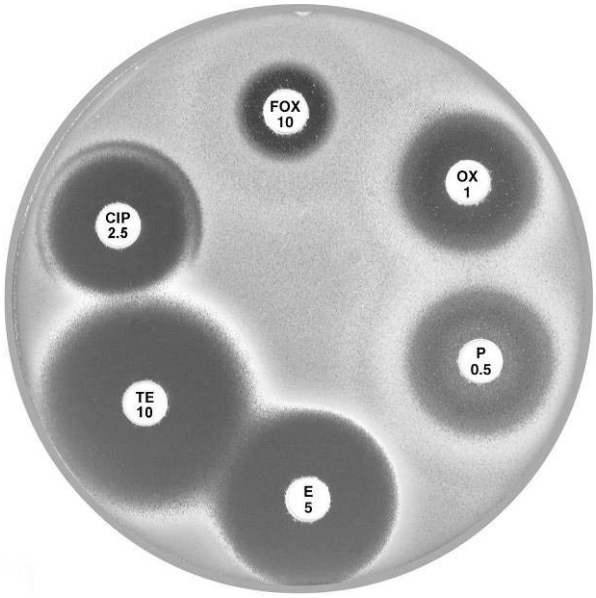

Plate 11.4.A Non multiresistant *mecA* gene positive *Staphylococcus aureus* (MRSA) lacking β –lactamase activity
This isolate is reported as resistant to methicillin and all other β-lactams.

Plate 11.4.B Non multiresistant MRSA
This isolate is susceptibility to erythromycin (E 5), tetracycline (TE 10), cotrimoxazole (SXT 25) and ciprofloxacin (CIP 2.5).

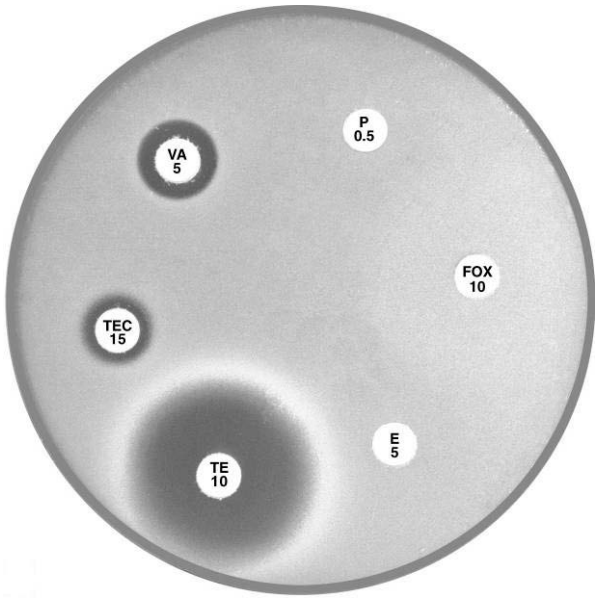

Plate 11.4.C MRSA with reduced susceptibility to vancomycin (VISA/GISA)
Note the reduced inhibitory zone around vancomycin (VA 5) and teicoplanin (TEC 15).

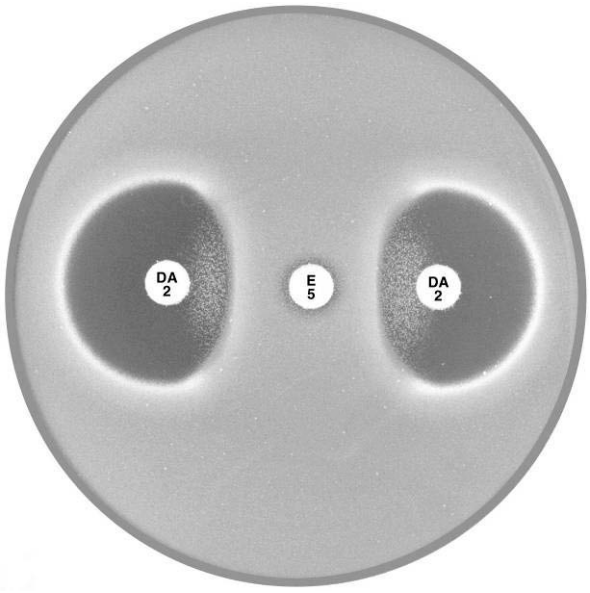

Plate 11.4.D MRSA with inducible clindamycin resistance (ICR)
Inducible clindamycin resistance is indicated by the absence of an inhibitory zone around erythromycin (E 5) and a flattening of the inhibitory zone around clindamycin (DA 2) adjacent to the erythromycin disc.

11.5. *Staphylococcus saprophyticus*

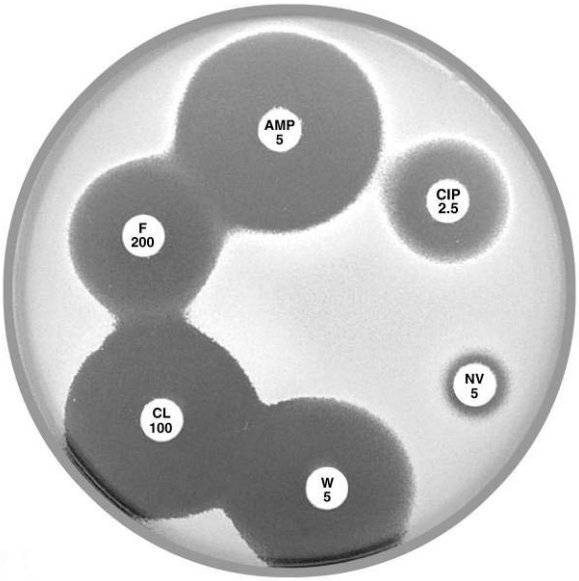

Plate 11.5.A *mecA* gene negative *Staphylococcus saprophyticus*
Typical of urinary isolates. Resistant to novobiocin (NV 5) but susceptible to ampicillin (AMP 5) and cephalexin (CL 100).

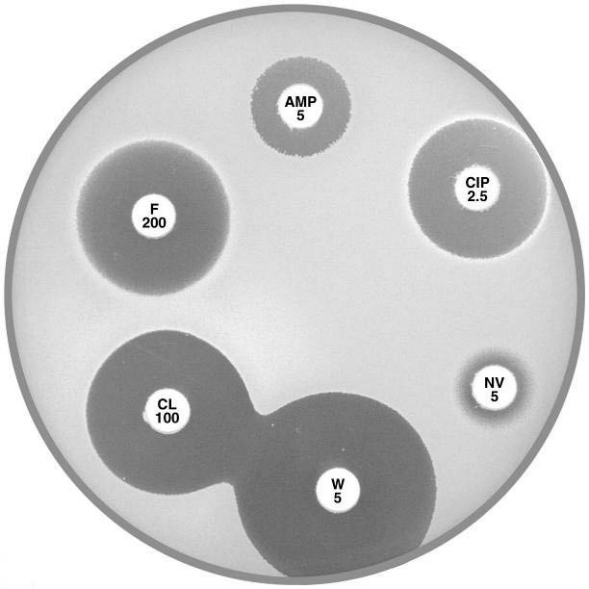

Plate 11.5.B *mecA* gene negative *Staphylococcus saprophyticus w*ith an inducible β-lactamase
Urinary isolate *showing* resistance to ampicillin (AMP 5) but susceptibility to cephalexin (CL 100).

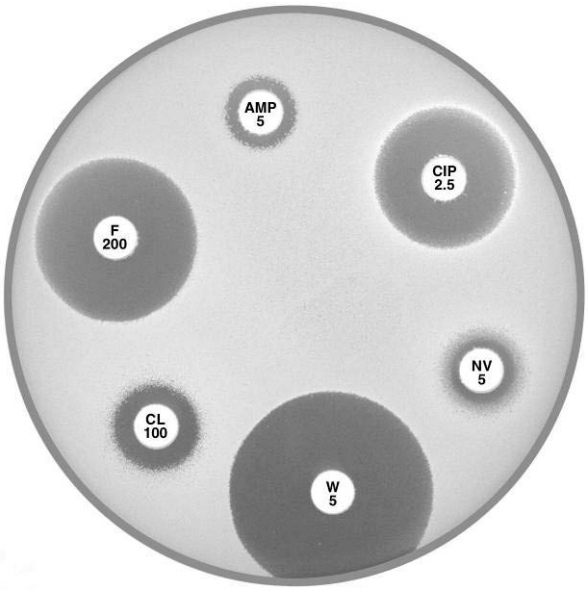

Plate 11.5.C *mecA* gene positive *Staphylococcus saprophyticus*
Urinary isolate showing resistance to ampicillin (AMP 5) and cephalexin (CL 100).

11.6. *Acinetobacter*

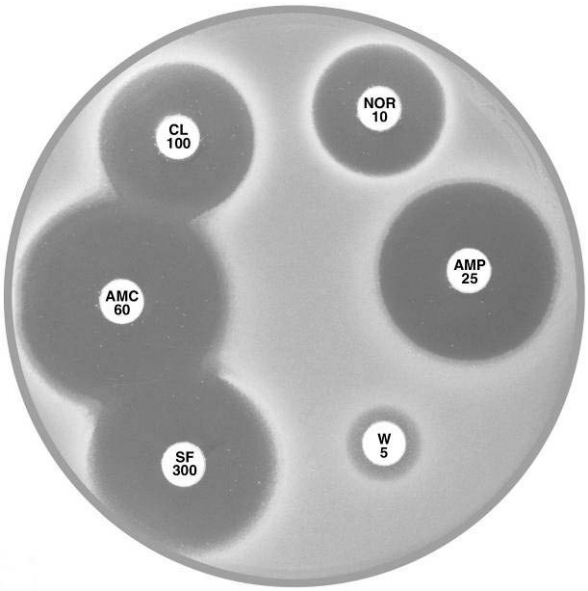

Plate 11.6.A A*cinetobacter lwoffi*
A large inhibitory zone around ampicillin (AMP 25), a smaller one around cephalexin (CL 100), resistance to trimethoprim (W 5) and a lack of synergy with sulphafurazole (SF 300) are typical of A. *lwoffi*

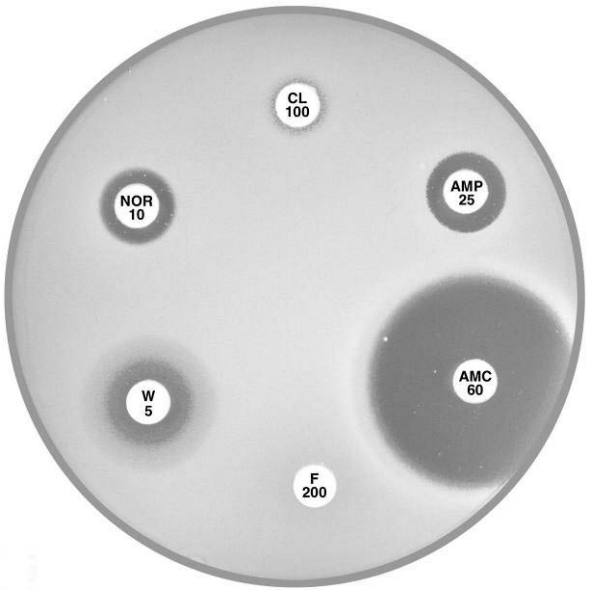

Plate 11.6.B *Acinetobacter baumannii*
Note the large inhibitory zone around Augmentin (AMC 60), a small one around ampicillin (AMP 25) and none around cephalexin (CL 100).

11.7. *Aeromonas*

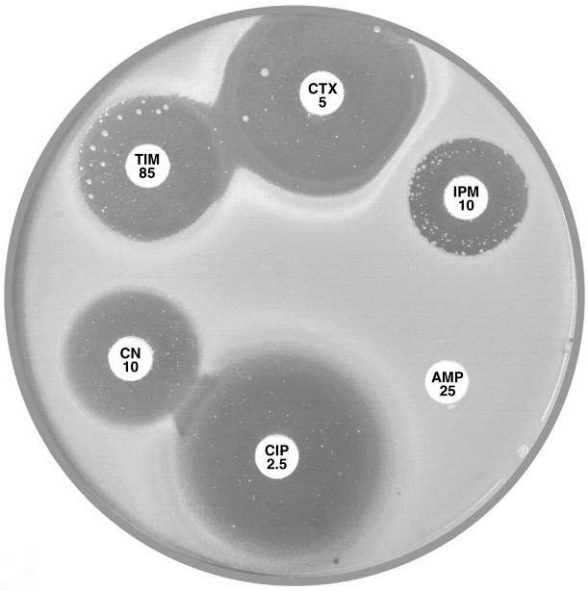

Plate 11.7.A *Aeromonas hydrophilia* with cephalosporinase A1 & carbapenemase A2
The flattening of the inhibitory zone around cefotaxime (CTX 5) indicates an inducible A1 cephalosporinase. The reduced inhibitory zone around imipenem (IPM 10) with resistant colonies indicates an A2 carbapenemase.

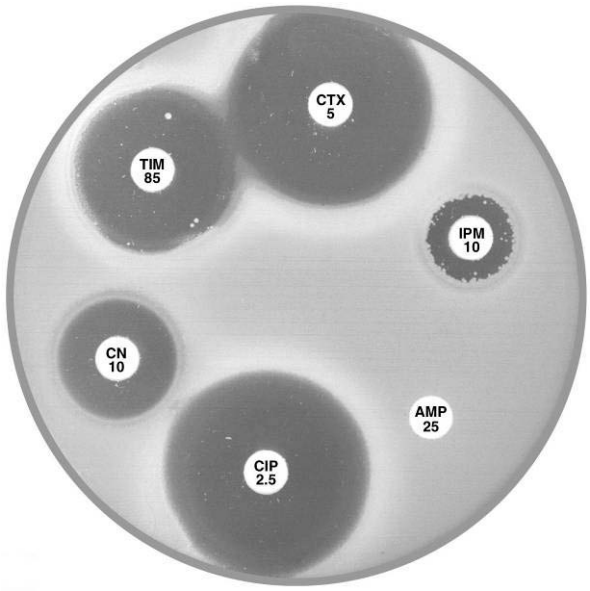

Plate 11.7.B *Aeromonas sobria* with carbapenemase A2 but lacking cephalosporinase A1
The A2 carbapenemase is indicated by the small inhibitory zone around imipenem (IPM 10) and resistant colonies within this zone.

11.8. *Escherichia coli*

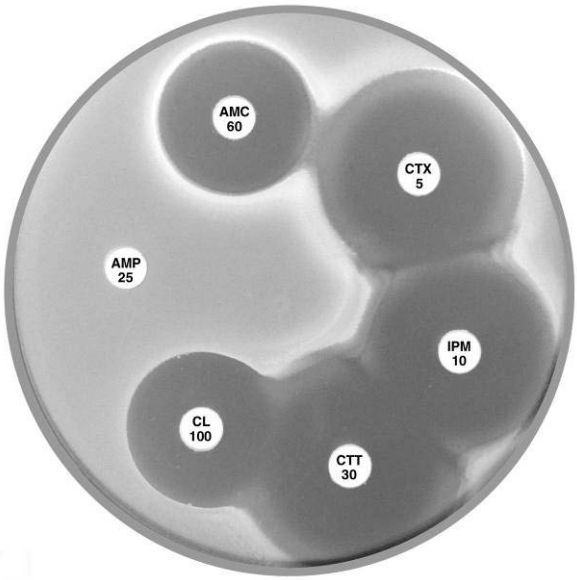

Plate 11.8.A *Escherichia. coli* ACM 5186 producing TEM-1 β-lactamase
This strain is resistant to ampicillin (AMP 25), but susceptible to Augmentin (AMC 60), cefotaxime (CTX 5), imipenem (IPM 10), cefotetan (CTT 30) & cephalexin (CL 100).

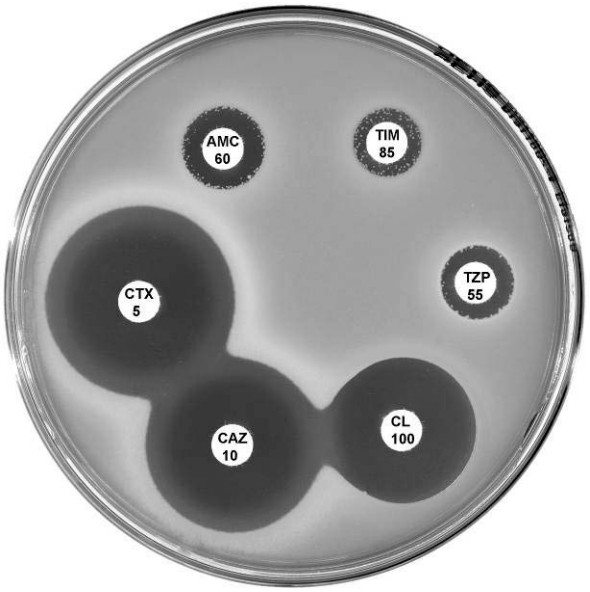

Plate 11.8.B *Escherichia. coli* with an Inhibitor Resistant TEM (IRT)
This isolate is resistant to the inhibiting effect of both clavulanic acid and tazobactam in Augmentin, Timentin and Tazocin. The isolate remains susceptible to the cephalosporins.

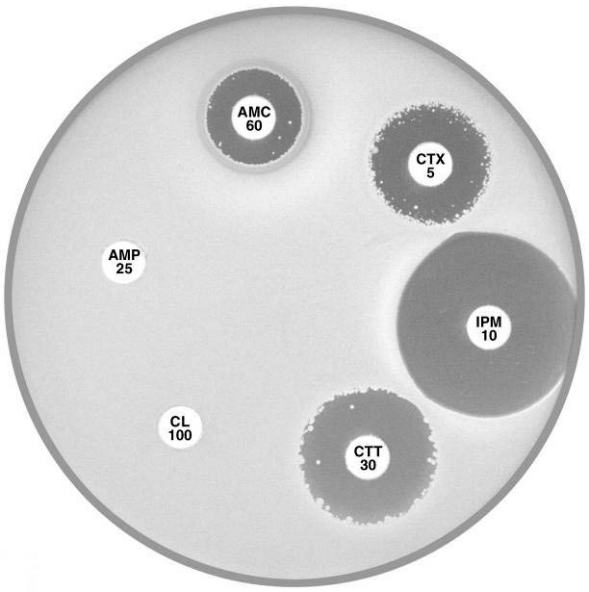

Plate 11.8.C *Escherichia coli* with low activity of the AmpC β-lactamase
Showing a reduced zone of inhibition around cefotaxime (CTX 5) and no zone around cephalexin (CL 100).

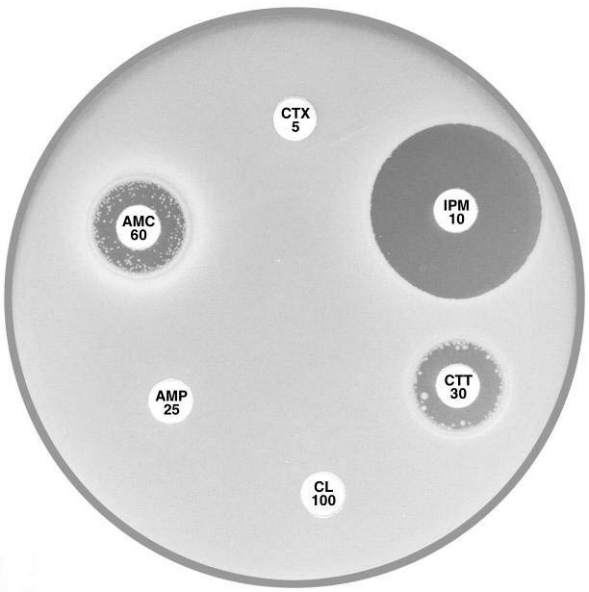

Plate 11.8.D *Escherichia coli* with high activity of the AmpC β-lactamase
Note the absence of an inhibitory zone around both cefotaxime (CTX 5) and cephalexin (CL 100).

11.9. *Escherichia coli* and *Chryseobacterium indologenes*

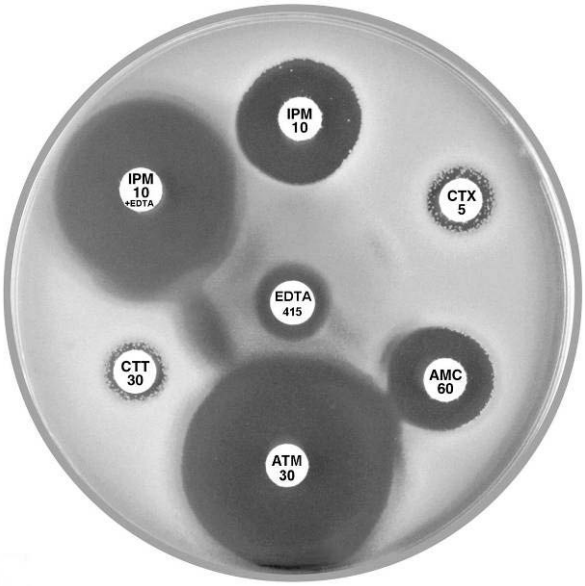

Plate 11.9.A *Escherichia coli* producing a zinc metallo β-lactamase
Showing resistance to cefotaxime, cefotetan and Augmentin but susceptible to aztreonam. Note the borderline resistance to imipenem and the inhibition of β-lactamase activity by EDTA.

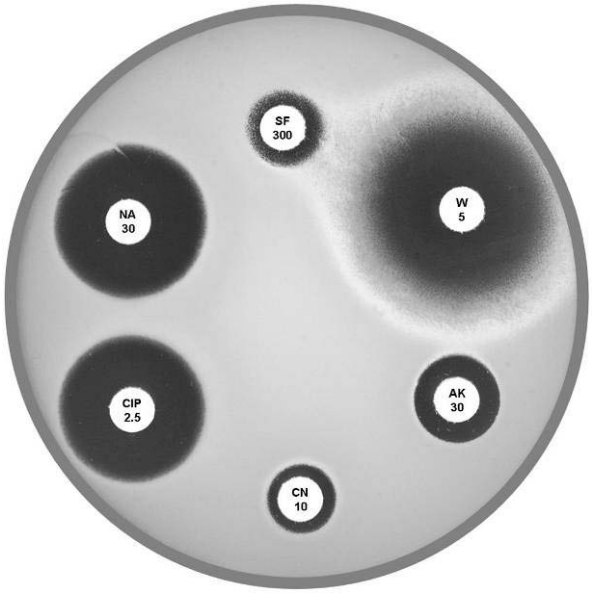

Plate 11.9.B *Chryseobacterium indologenes*
Chryseobacterium showing susceptibility to trimethoprim (W 5) and resistance to sulphafurazole (SF 300) and resistance to aminoglycosides (AK 30 and CN 10). This strain is susceptible to the tested quinolones.

11.10. *Klebsiella pneumoniae*

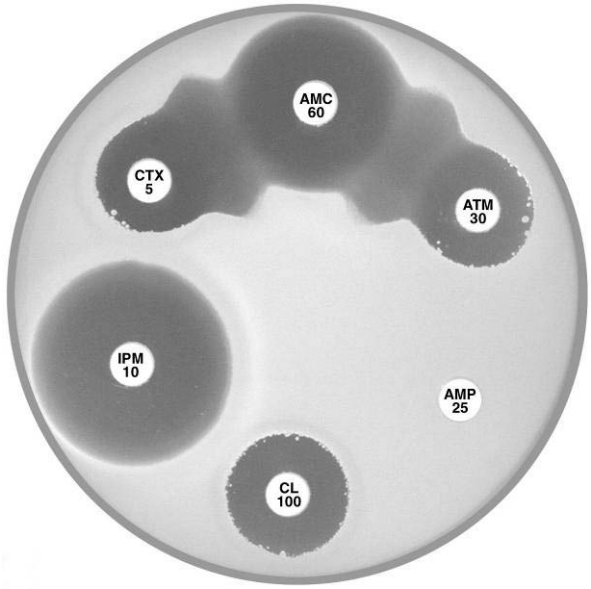

Plate 11.10.A *Klebsiella pneumoniae* producing an ESBL
ESBL production is indicated by the inhibition of the β-lactamase by clavulanic acid and the resulting "keyhole" inhibition pattern between cefotaxime, aztreonam and Augmentin.

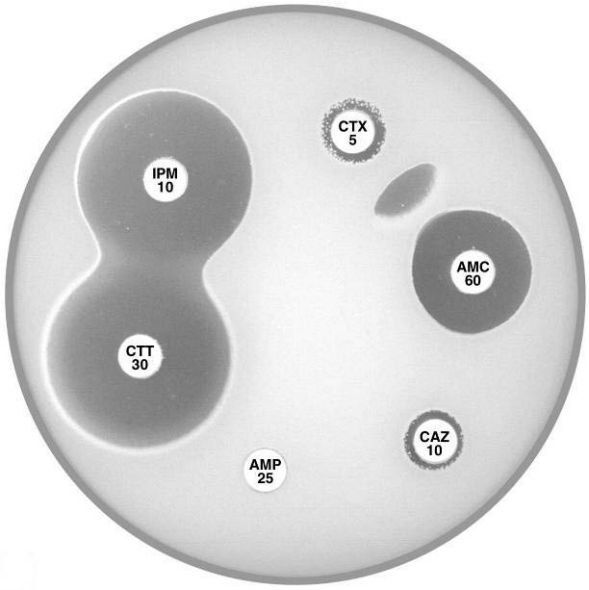

Plate 11.10.B *Klebsiella pneumoniae* producing a higher activity ESBL
The higher level of ESBL production is indicated by the inhibition of the β-lactamase by clavulanic acid and the resulting elliptical inhibitory zone between cefotaxime and Augmentin.

11.11. *Klebsiella oxytoca*

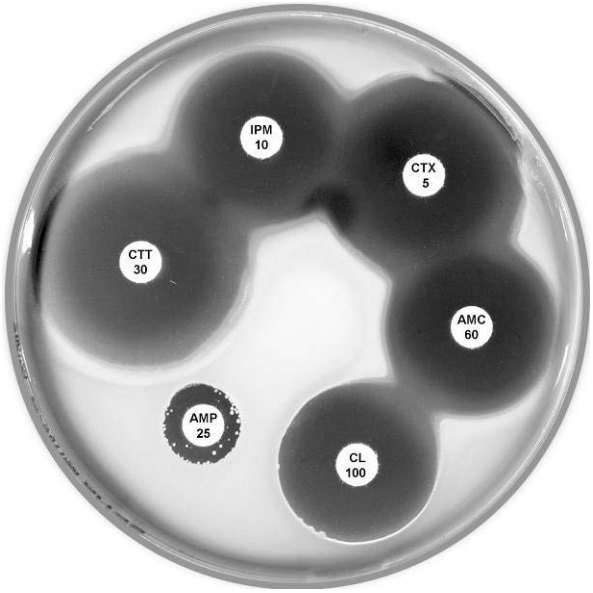

Plate 11.11.A *Klebsiella oxytoca* with a low basal level of K1 β-lactamase
This isolate is resistant to ampicillin only.

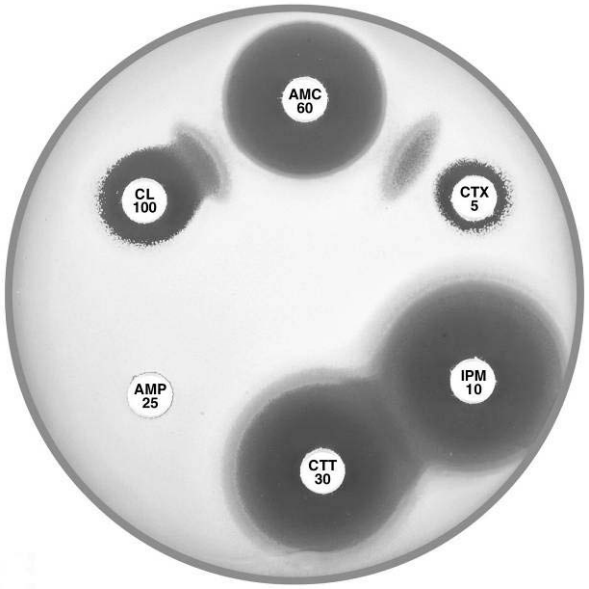

Plate 11.11.B *Klebsiella oxytoca* with a low basal level of K1 β-lactamase and an ESBL
ESBL production is demonstrated by the "keyhole" and elliptical inhibitory zones between cephalexin (CL 100) and cefotaxime (CTX 5) respectively and Augmentin (AMC 60).

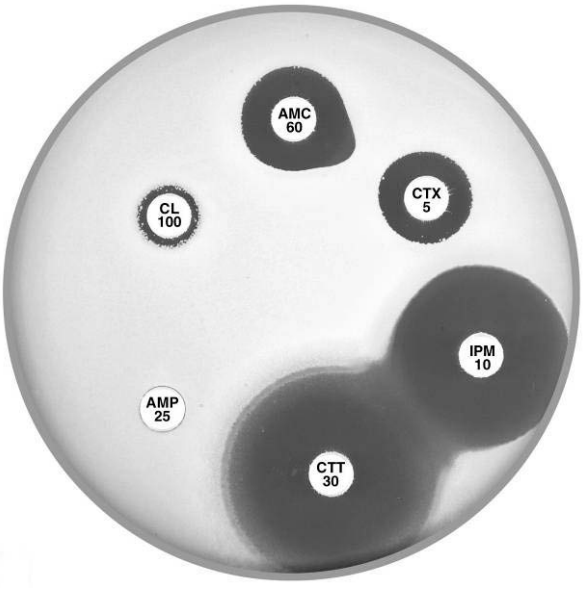

Plate 11.11.C *Klebsiella oxytoca* with a high level of the K1 β-lactamase
This isolate shows the tiny "keyhole" effect between cefotaxime (CTX 5) and Augmentin (AMC 60) and the small inhibitory zone around cephalexin (CL 100).

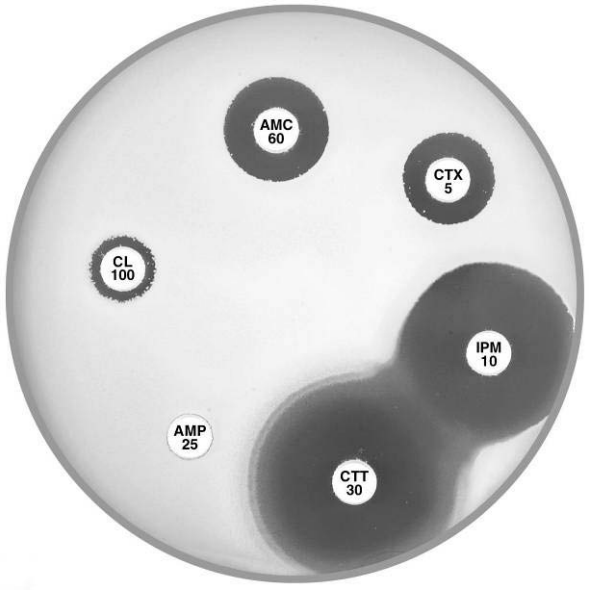

Plate 11.11.D *Klebsiella oxytoca* with a higher level of the K1 β-lactamase
There is no obvious synergy between cephalexin (CL 100) or cefotaxime (CTX 5) and Augmentin (AMC 60).

11.12. *Enterobacter and Citrobacter*

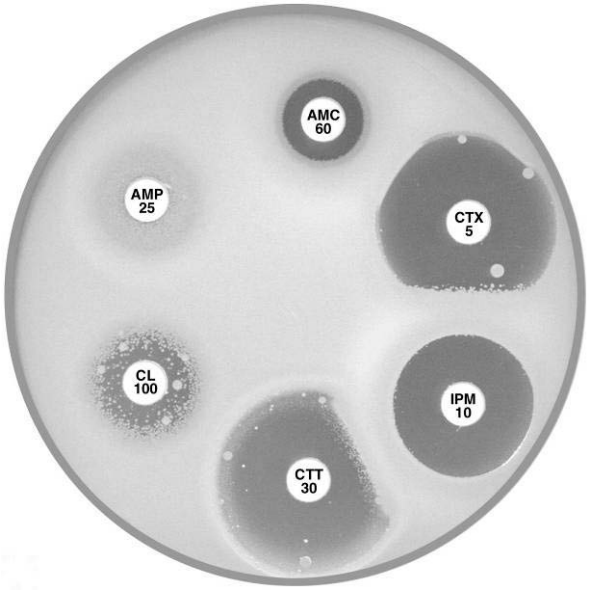

Plate 11.12.A *Enterobacter cloacae* with AmpC β-lactamase
The flattened inhibitory zone around imipenem (IPM 10) adjacent to cefotaxime/cefotetan (CTX 5, CTT 30) demonstrates the presence of a basal inducible cephalosporinase**.**

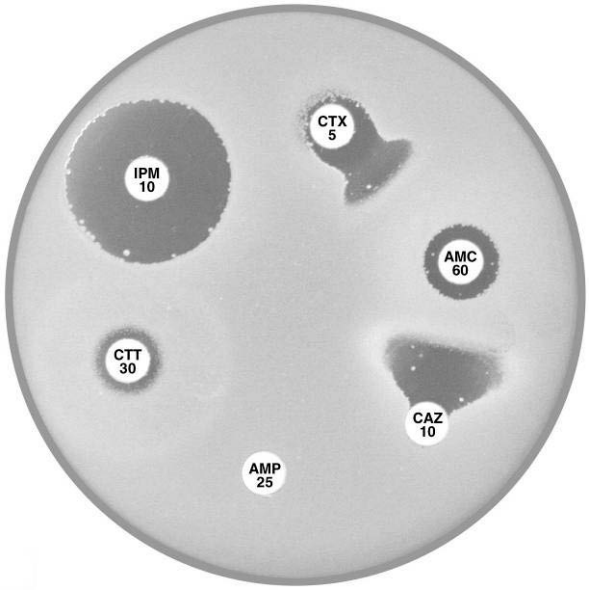

Plate 11.12.B *Enterobacter cloacae* producing higher levels of the AmpC β-lactamase and also an ESBL
Note the resistance to cefotaxime (CTX 5) and cefotetan (CTT 30).

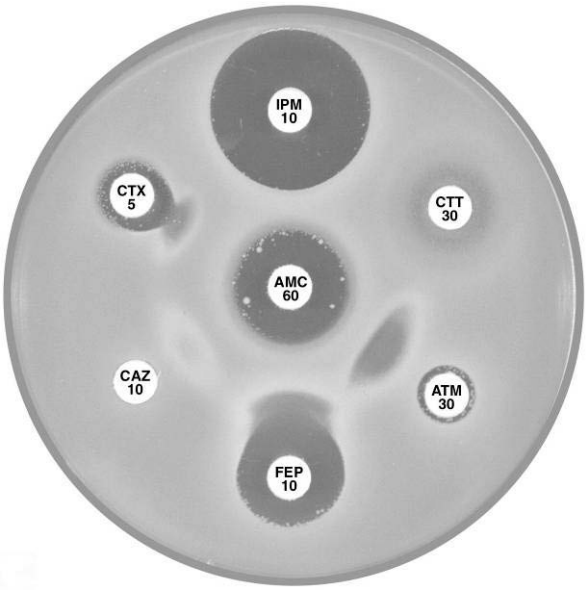

Plate 11.12.C *Citrobacter freundii* with derepressed AmpC β-lactamase and ESBL
The ESBL is indicated by the keyhole pattern between cefepime (FEP 10), aztreonam and augmentin (AMC 60) (ATM 30). AmpC derepression is indicated by the resistance to cefotetan (CTT 30).

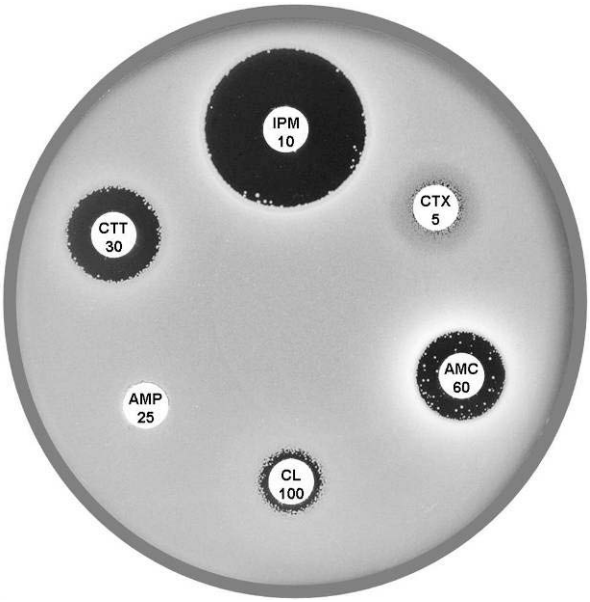

Plate 11.12.D *Citrobacter freundii* with derepressed AmpC β-lactamase
Note the resistance to cefotaxime (CTX 5) and cefotetan (CTT 30).

11.13. *Proteus penneri*

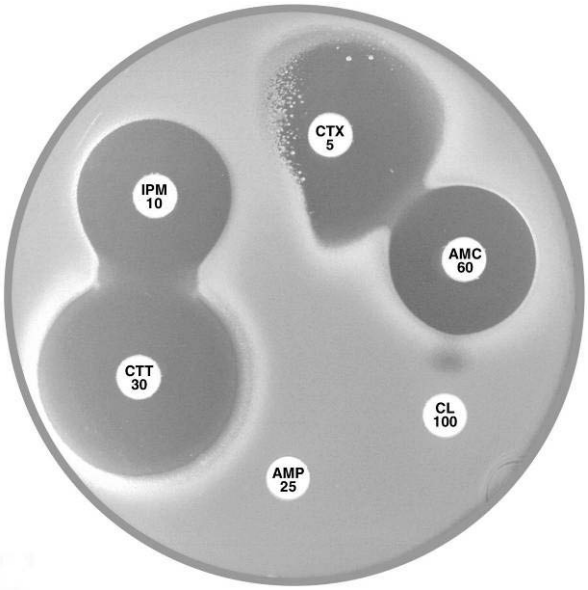

Plate 11.13.A *Proteus penneri* with an inducible group 2e β-lactamase.
The large inhibitory zone around Augmentin (AMC 60) and the flattening of the cefotaxime (CTX 5) zone adjacent to imipenem (IPM 10) indicate the presence of an inducible group 2e β-lactamase.

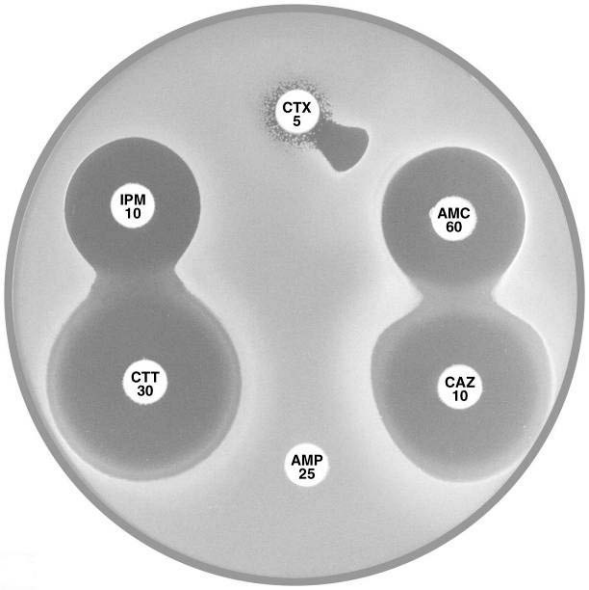

Plate 11.13.B *Proteus penneri* with a derepressed group 2e β-lactamase.
The susceptibility to ceftazidime and cefotetan and the synergy between Augmentin and cefotaxime suggests a group 2e β-lactamase. The resistance to cefotaxime indicates hyper-production of the β-lactamase.

11.14. *Pseudomonas aeruginosa*

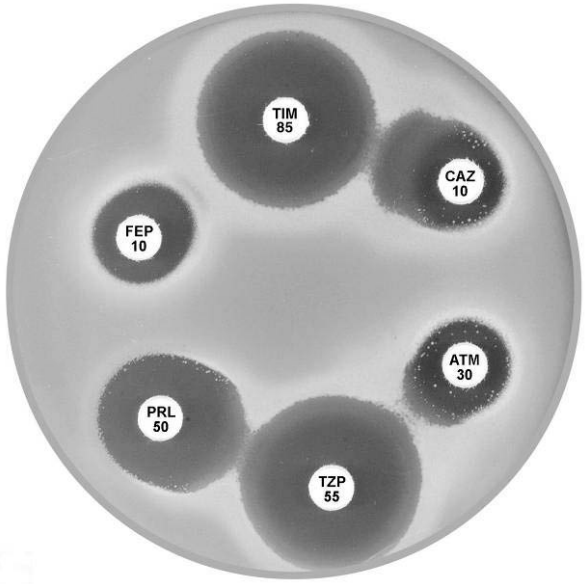

Plate 11.14.A *Pseudomonas aeruginosa* producing an ESBL
Showing synergy between Timentin (TIM 85) & ceftazidime (CAZ 10) and between Tazocin (TZP 55) & aztreonam (ATM 30).

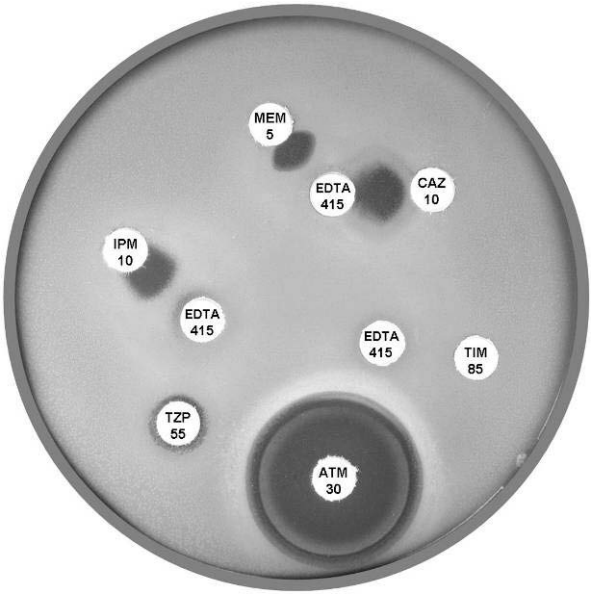

Plate 11.14.B *Pseudomonas aeruginosa* with a zinc-metallo-carbapenemase
Showing the inhibition of the carbapenemase by EDTA.

11.15. *Stenotrophomonas maltophilia*

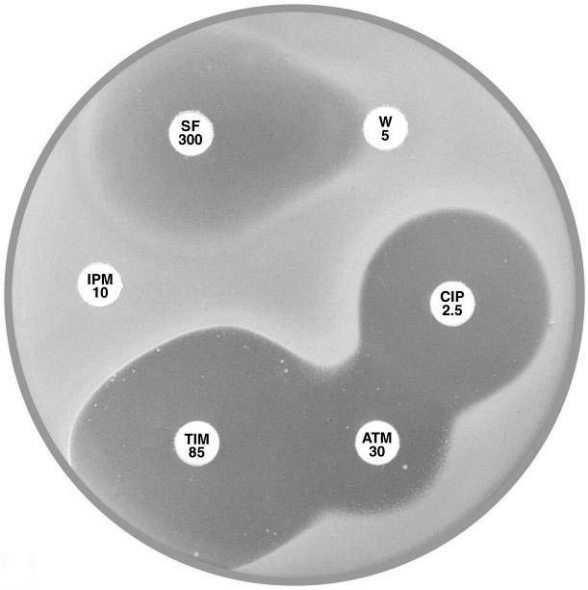

Plate 11.15.A *Stenotrophomonas maltophilia*
Note the lack of an inhibitory zone around imipenem, the pear shaped zone of inhibition between sulphafurazole and trimethoprim and the resistant colonies within the Timentin and aztreonam inhibitory zones.

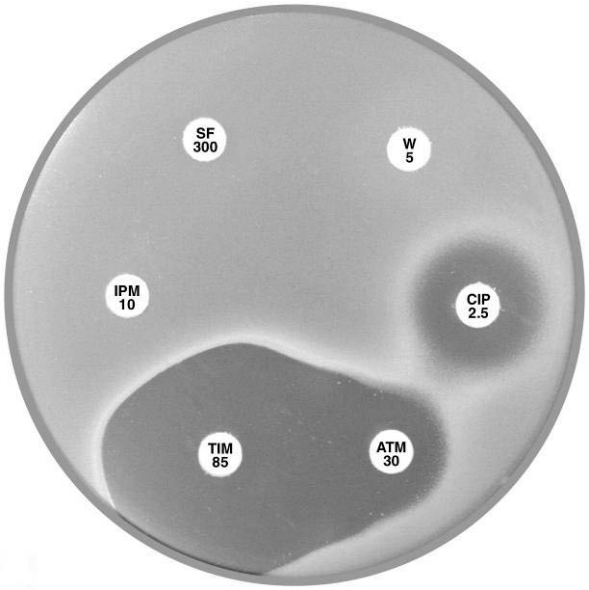

Plate 11.15.B Sulphafurazole resistant *Stenotrophomonas maltophilia*
No zone around sulphafurazole (SF 300).

11.16. Yeast

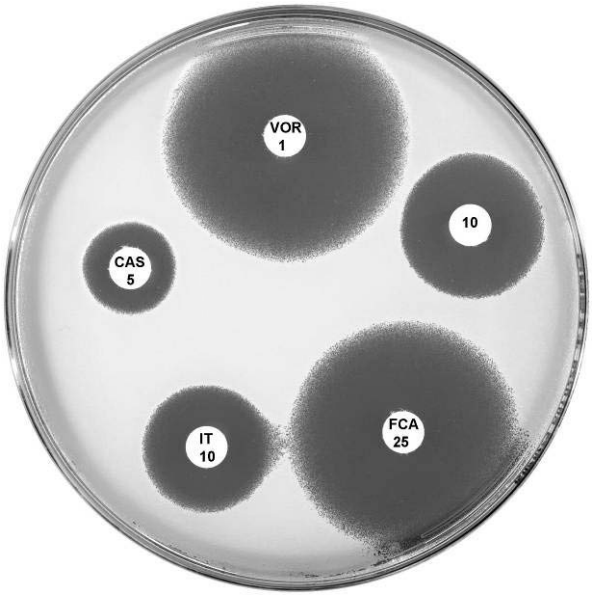

Plate 11.16.A *Candida parapsilosis* ACM 5283
Showing susceptibility to voriconazole (VOR 1), amphotericin B (10), fluconazole (FCA 25), itraconazole (IT 10) and caspofungin (CAS 5).

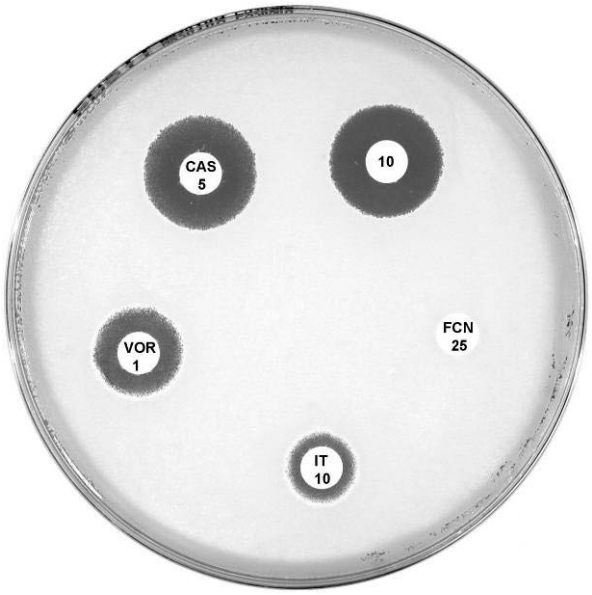

Plate 11.16.B *Candida albicans* isolate
Showing resistance to voriconazole (VOR 1), itraconazole (IT 10) and fluconazole (FCA 25), and susceptibility to caspofungin (CAS 5) and amphotericin B (10).

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ANTIBIOTIC SUSCEPTIBILITY TESTING BY THE CDS METHOD

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The Antibiotic Reference Laboratory

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