THE PRINCE OF WALES HOSPITAL

The Prince of Wales Hospital,

Cnr. High & Avoca Streets,

Randwick. N.S.W. 2031.

SMB/BG 20th August, 1991.

Dear Colleague,

C.D.S. USERS GROUP

NEWSLETTER No. 3

Report of the CDS Users Group Workshop held at the

ASM Gold Coast Meeting 1991

The CDS Users Group Workshop at this years ASM was attended by over 70 participants. Unfortunately, because of the restricted size of the venue, there was insufficient space to accommodate all those who wished to attend. We apologise to those people and shall ensure that won't occur at future meetings.

The meeting was organised to promote maximum audience participation and this opportunity was taken up by the participants. This resulted in a very active discussion and valuable feedback on practical aspects of the CDS method. For those who were unable to attend the meeting we have enclosed a very brief summary of each of the presentations and/or the overheads and slides used by the speakers.

Kind regards.

Yours sincerely,

S. M. BELL.

INTRODUCTION

The meeting was organised by four members of the Antibiotic Laboratory of the Prince of Wales Hospital who each presented some aspect of the CDS method.Prof. Sydney M. Bell (SMB) chaired the meeting and introduced each of the other speakers: Mrs Jeanette N. Pham (JNP), Mr. Alex S. Jimenez (ASJ) and Dr. Barrie J. Gatus (BJG).The second part of the workshop was dedicated to Questions and Answers.

PART 1

CONCEPTS IN DISC SUSCEPTIBILITY TESTING(SMB)

Summary

The messages in this presentation are:

1.A properly calibrated disc test is in fact a highly accurate, reproducible method of determining an MIC.

2.The test predicts a successful outcome to antibiotic therapy by defining a SUSCEPTIBLE isolate as one where, ideally, there has been a prior correlation of the MIC with a favourable clinical response.

3.It encourages the view that in any test of antibiotic susceptibility, the absolute specificity of the test (ie. NO FALSE susceptible isolates) should be preserved even at the expense of missing some strains that could be susceptible.

Overheads

The Aim: to determine antibiotic susceptibility.

The Measurement: the estimation of an MIC which is expressed as equal to or less than a defined value.

The Purpose: to assist in the choice of most appropriate antibiotic.

The Meaning: a prediction of a successful antibiotic treatment.

The Basis of the Prediction: a prior correlation of MIC with a positive clinical response.

The Sensitivity of the Test: the percentage of susceptible isolates correctly determined to be susceptible.

The Specificity of the Test: the percentage of those isolates, determined to be susceptible, which are genuinely susceptible.

The Predictive Requirements: the absolute specificity of the test must be preserved even at the expense of a diminished sensitivity.

STAPHYLOCOCCI AND THE PENICILLINS(JNP)

A. *Staphylococcus aureus* v/s benzylpenicillin

We are now using **penicillin 0.5 u** discs.Staphylococci are incubated at **35^{o}C**, not at 37 ^{o}C.

Susceptible strains: strains without a plasmid coding for the penicillinase enzyme (eg. *Staphylococcus aureus* NCTC). Susceptible strains give a zone of inhibition of approximately 12 mm of annular radius with a Pen 0.5 u disc.

Resistant strains: strains producing a plasmid mediated **penicillinase**. Resistant strains give no zone of inhibition or a zone up to 2 mm of annular radius with a Pen 0.5 u disc.

Note: Watch out for rare strains of *Staph. aureus* producing low levels of penicillinase with a **reduced zone** of 4-6 mm and a typical **sharp edge**. These strains with a benzylpenicillin MIC of 0.06 mg/L are not picked up by other methods including MIC determination.

B. *Staphylococcus aureus* v/s methicillin

We are now using **methicillin 5 µg** discs. Plates are incubated at **35^{o}C.**There is no need to incubate at 30 ^{o}C.

- Resistance to methicillin is due to the presence of PBP 2a with poor affinity for methicillin and all other β-lactams including imipenem and cephalothin.

- **Resistance to methicillin = resistance to all β-lactams**.
- *Staph. aureus* coding for PBP 2a are **Methicillin-resistant *Staph. aureus*** or **MRSA**.
- **Multiple resistance** to various antibiotics is a marker for the majority of MRSA, not for all MRSA.
- **There are 3 phenotypes of MRSA**

Homogenous resistance: the majority of cells appear to be resistant to high levels of methicillin, with no zone around Met 5 disc, at 35°C or 37°C**.**

Thermosensitive-heterogenous resistance:

At **37°C**, only a low number of cells are resistant to methicillin. These strains have a large methicillin zone with resistant colonies inside the zone.

At **35°C**, high number of cells are resistant to methicillin. These strains have no zone (majority of clinical isolates of MRSA belong to this group) or a zone of light growth up to the disc (rare).

Heterogenous resistance: Rare strains of MRSA with a very low number of cells resistant to methicillin. These strains have a methicillin zone with an annular radius of 6 to 7 mm with resistant colonies seen in the zone of inhibition.If in doubt, incubate the plate for another 24 h.

C. Coagulase-negative staphylococci v/s benzylpenicillin

Susceptible strains are strains with an annular radius > 6 mm with a Pen 0.5 u disc.

Resistant strains are strains with an annular radius < 6 mm with a Pen 0.5 u disc.

EXCEPTION: Novobiocin-resistant coagulase-negative staphylococci (*Staph. saprophyticus*) from urine. These organisms produce low levels of non-inducible penicillinase (ie. susceptible to penicillin) and give inhibitory zones of 5-6 mm in annular radius with a Pen 0.5 u disc. Investigation is in progress.

D. Resistance to methicillin and coagulase-negative staphylococci

*Homogenous resistance***:** All cells appear to be resistant to high levels of methicillin, with zone around Met 5 disc < 6 mm**.**

*Heterogenous resistance***:** Unlike *Staph. aureus*, **a large number** of coagulase-negative staphylococci express this type of resistance. These strains have a **large methicillin zone > 6 mm with resistant colonies inside the zone**. In order not to miss the resistance, make sure that a proper CDS inoculum of 10^7 cfu/ml is used to flood the plate and incubate the plate for further 24 h if in doubt.

CEPHALOSPORINS(BJG)

CEPHALOTHIN

Intravenous only, NOT for urinary tract infections

1.What is it used for?

- **For Gram-positive organisms**
- **Patients allergic to penicillin, systemic infections with *Staph. aureus,* streptococci groups A, C, G.**
- For Gram-negative, superseded by cefotaxime, ceftriaxone , ceftazidime.

2.Role of β-lactamase in resistance to cephalothin?

- ***Staph. aureus:*** **Susceptible to methicillin = Susceptible to cephalothin**
- *Enterobacteriaceae:* Cephalothin is not stable against TEM, SHV β-lactamases: appear susceptible at 10^4 inoculum but resistant at 10^6 inoculum. Susceptibilty to cephalothin of members of the *Enterobacteriaceae* (except those producing a class I chromosomal β-lactamase) can be **inferred from ampicillin**.

CEPHALEXIN

Calibrated for urinary infections ONLY (100 µg disc).

Test only *E. coli, Klebsiella, Proteus mirabilis* and *Citrobacter diversus*. Do not test organisms producing inducible cephalosporinases and Extended Beta Lactamases (ESBL).These organisms are considered resistant to cephalexin.

INDUCTION OF BETA-LACTAMASES IN *ENTEROBACTERIACEAE* and *PSEUDOMONAS AERUGINOSA* (BJG)

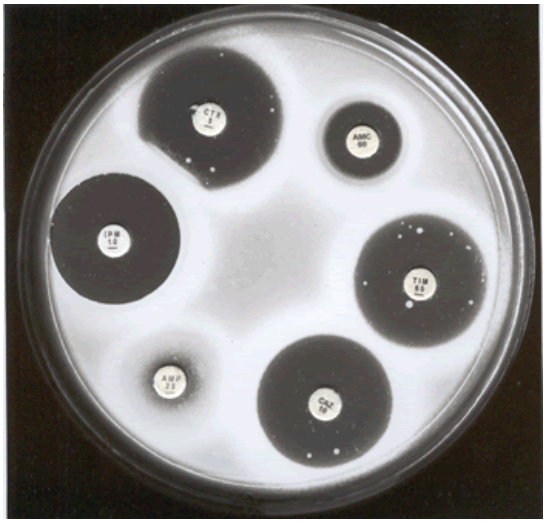
Summary

Enterobacter cloacae, Citrobacter freundii, Serratia marcescens, Providencia sp., *Morganella morganii* possess a Class I inducible β-lactamase. Induction of this enzyme by a β-lactam antibiotic is an *in-vitro* phenomenon and does not confer resistance in-vivo.Mutants that hyperproduce β-lactamase are selected at a high frequency (10^{-5} to 10^{-6}) by β-lactams (except imipenem).The selection of these mutants *in-vivo* confers resistance.Imipenem is not affected by Class I β-lactamases and is active against hyperproducing mutants.The species mentioned above are considered resistant to ALL β-lactam antibiotics, except imipenem, which nevertheless must be tested.*Pseudomonas aeruginosa* also possess an inducible Class I β-lactamase but the rate of mutation to resistance is low (10^{-9}).Therefore, agents such as piperacillin and ticarcillin can be used for therapy provided the organism is susceptible when tested.

1. A. ENTEROBACTERIACEAE

- *Enterobacter cloacae, Citrobacter freundii, Serratia marcescens, Providencia* sp., *Morganella morganii* possess a **Class I inducible β-lactamase** (cephalosporinases).
- Induction of this enzyme by a β-lactam antibiotic is an *in-vitro* phenomenon and **does not confer resistance** *in-vivo*.
- Can be demonstrated *in-vitro* using a **disc approximation** test with **IMIPENEM 10** and **CEFOTAXIME 5** discs.

In the diagram below, imipenem has induced Class I inducible β-lactamase in a strain of *Enterobacter cloacae* resulting in a flattened zone of inhibition around cefotaxime disc.Note the presence of β-lactamase hyperproducing resistant mutant colony in cefotaxime zone.



2. *B. PSEUDOMONAS AERUGINOSA*

Pseudomonas aeruginosa also possess an inducible Class I β-lactamase but the rate of mutation to resistance is low (10^{-9}). *Ps. aeruginosa* are not likely to give rise to β-lactamase hyperproducing mutants except in sequestered sites (cystic fibrosis, osteomyelitis). Therefore, agents such as piperacillin and ticarcillin can be used for therapy provided the organism is susceptible when tested.

DETAILS OF SUSCEPTIBILITY TESTING BY THE CDS METHOD(ASJ)

Materials

1. Dehydrated media
- Sensitest Agar (Oxoid CM409)
- Columbia Agar Base (Oxoid CM331, BBL or Lab M)
2. Defibrinated horse blood.
3. 90 mm diameter plastic Petri dishes.
4. 2.5 ml of sterile isotonic saline in 13 mm x 100 mm test tubes.
5. 10 cm of 0.56 mm diameter nichrome wire in a loop holder.

Available from: Australia Electrical Electronics, 342-350 Parramatta Road, Petersham, NSW 2049. Tel: (02) 568 3888, Fax: (02) 568 3144

6. Pasteur pipettes.
7. 6 mm diameter antibiotic discs supplied only by Oxoid, BBL or Mast.
8. Disc dispenser (maximum of 6 discs) available from Oxoid and BBL suppliers.
9. Max/min thermometers.
10. Clear plastic mm ruler.

Preparation of agar plates

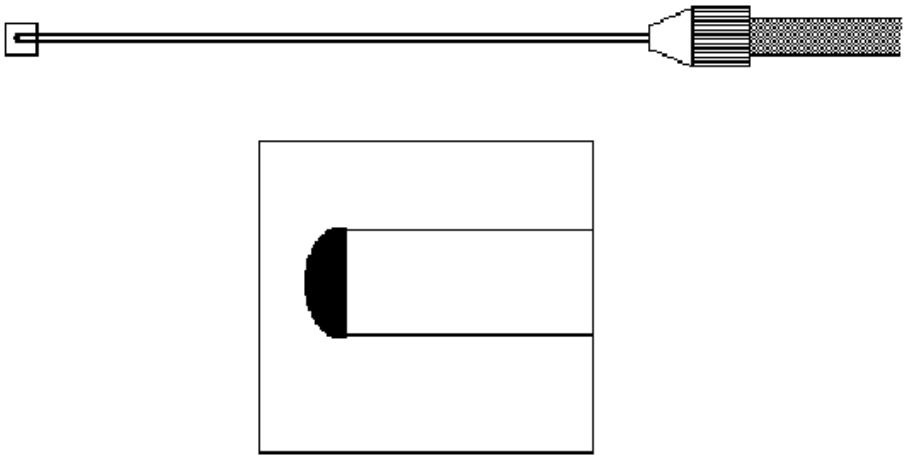
1. Dehydrated media must be prepared strictly according to the manufacturer's instructions.
- 5% Horse blood is added to Sensitest Agar to prepare blood Sensitest Agar.
- 8% Horse blood is added to Columbia Agar Base and heated to 80°C until chocolated (approximately 30 min. for 2 litres) to prepare "chocolate agar".
2. 20 ml of agar is dispensed into Petri dishes.
3. Agar plates are stored at 4°C for a maximum of 14 days.
4. Plates are dried face down with the lid removed in an incubator at 35°C. This will usually take 1 hour in a fan-forced incubator or 2 hours in an ordinary incubator.
5. Dried plates may be stored in the refrigerator for a maximum of 2 days.

The inoculum

1. The desired inoculumis 10^7 cfu/ml

HEAVY INOCULUM = SLIGHT DECREASE IN ZONE SIZES
LIGHT INOCULUM = MARKED INCREASE IN ZONE SIZES
May miss β-lactamase producing staphylococci
May not detect resistant colonies in zone

2. Use an overnight culture preferably on blood agar to prepare the inoculum.
3. The standard method to obtain the inoculum is to stab 1 colony (1-2 mm of diameter).That should result in bacterial material being **visible** on the tip of the straight wire as shown in the diagram below.



4. If bacterial material is not visible using the standard method then one of the following methods may be used:

- Stab 3-5 colonies (suitable for small colonies).
- Tease the colony apart and pick up bacterial material (suitable for sticky colonies).
- Stab the edge of confluent growth (this may be necessary with *Strep. milleri* and *Strep. pneumoniae*). This is the least preferable method since there is a possibility that the inoculum may not be pure.

5. Inoculate the saline by rotating the straight wire at least 10 times with the tip in contact with the bottom of the tube.
6. Mix up and down at least 10 times using a Pasteur pipette.

Inoculation of plates

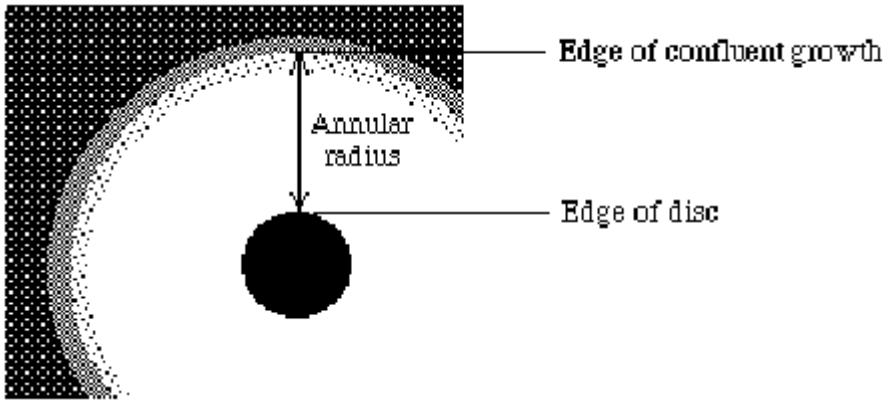
1. Flood agar plate and remove excess. If 2 plates are to be flooded then half the suspension can be used for each plate.
2. Remove the lid and place the plate next to a Bunsen burner to dry. This will usually take 5 - 10 min. Plates must **NOT** be left longer than 45 min.
3. Apply antibiotic discs.

Incubation of plates

Most organisms are incubated at 35°C overnight in air.
Y. enterocolitica: 30°C in air.

Reading the zones

1. Measure the zones from the back of the plate where possible.
2. Measure the annular radius which is the shortest distance from the edge of the disc to the edge of confluent growth. The edge of confluent growth usually corresponds to the sharpest edge of the zone.



Interpretation

Annular radius ≥ 6 mm = SUSCEPTIBLE

< 6 mm = RESISTANT

There are some exceptions (see Table 1) eg *Pseudomonas aeruginosa* with aminoglycosides where the interpretation is:

Annular radius ≥ 4 mm = SUSCEPTIBLE

< 4 mm = RESISTANT

Quality Control

1.Media

Check the approximate depth and weight of agar.Test the control strains and check that zone sizes are within the acceptable ranges and make sure that a good confluent lawn of growth is obtained.

2. Inoculum

Cells materials seen on the tip of the straight wire

A lawn of confluent growth on the sensitivity plate after overnight incubation

3. Antibiotic discs

- Check that correct disc potency is used eg. ampicillin 2μg for *H. influenzae*, not ampicillin 25 μg
- Stock must be stored at -20^{o}C
- Disc in use are stored in an air tight container (dispenser) with a desiccator at 4^{o}C.Augmentin and Timentin are preferably kept at -20^{o}C.
- Discs must be allowed to reach room temperature before opening the container to avoid condensation of moisture on the disc as this will inactivate antibiotics such as benzylpenicillin.
- Do not use disc past the expiry date.

4. Incubation temperature is easily monitored using a max/min thermometer.

5. Reference strains used in quality control

The following reference strains are used with the CDS method:

Staphylococcus aureus	NCTC 6571.
Escherichia coli	NCTC 10418.
Escherichia coli	NCTC 11560.
Pseudomonas aeruginosa	NCTC 10662.
Haemophilus influenzae	NCTC 4560.
Yersinia enterocolitica	IP 22273 (biotype 4, serotype O:3)

These strains may be obtained from:

Antibiotic Laboratory,
Department of Microbiology,
The Prince of Wales Hospital,
Randwick, NSW 2031,
Australia.
Tel: (02) 399-4053.
Fax: (02) 399-1120.

PART 2

Members who attended the meeting were divided into six groups and each was asked one of the following questions.

Q1. Gentamicin gave a 5.5 mm zone of inhibition with an isolate of *Escherichia coli.* The organism was tested again by the registrar and the gentamicin zone was found to be 6.0 mm. The MIC of gentamicin was determined by the scientific officer and the registrar. The results were compared as follows. The MIC for susceptible strains is ≤ 1 mg/L.

	Zone size (mm)	MIC(mg/L)
Scientific officer results	5.5 (R)	1 (S)
Registrar results	6.0 (S)	2 (R)

Who is correct?Who is wrong?What do these results mean?How would you interpret these results?

Q2. You are asked to test sulphafurazole and trimethoprim against:

Staphylococcus aureus	Coagulase (-) staphylococci
Streptococci	Enterococci
Haemophilus influenzae	*Enterobacteriaceae*
Xanthomonas maltophilia	

What will you do and why?

How will you report the results?

Q3. Anaerobic streptococci, *Corynebacteria* spp., *Listeria* spp. and *Neisseria meningitidis* are not yet calibrated using the CDS method. You are asked to report the antibiotic susceptibility of these strains. What would you do and why?

Q4. You are asked to test chloramphenicol and bacitracin susceptibilities for a strain of *Staphylococcus aureus* which was isolated from the ear of a patient with otitis externa. What will you do and why? Do you have any comments on reporting the susceptibility of antibiotics for topical use?

Q5. A *Klebsiella* spp. sent by the Q.A.P. was tested using the CDS method. Cefotaxime gave a zone of inhibition of 3mm. The Q.A.P. result stated that the organism was SUSCEPTIBLE to cefotaxime. Discuss these results?

Q6. How are MIC breakpoints derived?

Answers to the questions asked of members.

Q1. The answer is that no one was wrong. There are errors associated with both MIC determination and disc testing. The difference of 1 dilution is within the acceptable error of an MIC determination and the difference of 0.5 mm is within the acceptable error of the CDS method. The reason why this strain was a problem is that it had an MIC which lies on the breakpoint and therefore will sometimes be called susceptible and sometimes resistant.

According to the CDS method if a strain is found to be susceptible you can be confident that it is susceptible. On the other hand, some strains which are found to be resistant may, in fact, be susceptible. However, these strains have an MIC which is around the "breakpoint" and are not to be confused with highly resistant strains. In the case described above it would be acceptable to call this strain susceptible.

Q2. Testing of sulphafurazole and trimethoprim:

Staphylococcus aureus	Does not work clinically. DO NOT test.
Coagulase (-) staphylococci	Both antibiotics calibrated for urinary infections only.
Streptococci	These require blood in agar for growth;thus cannot test because of the presence of sulphonamide and trimethoprim inhibitors.
Enterococci	These are resistant to sulphafurazole (and other sulphonamides) but susceptible *in vitro* to trimethoprim. The combination of sulphamethoxazole and trimethoprim has been used for urinary tract infections with enterococci but there have been reports of "breakthrough" bacteraemia in patients with urinary infections who were treated with this combination. The combination, sulphamethoxazole and trimethoprim, should not be used for the therapy of systemic infections with enterococci.
Haemophilus influenzae	These grow only on chocolate agar containing sulphonamide and trimethoprim inhibitors;thus cannot test.
Enterobacteriaceae	Both calibrated for use with the CDS method.
Xanthomonas maltophilia	This species is resistant to all aminoglycosides, all β-lactam antibiotics and the quinolones;mutants which are selected at a high frequency (10^{-4} to 10^{-5}) are resistant to these agents. Most strains of *X. maltophilia* are resistant to trimethoprim but susceptible to sulphafurazole and there is synergy between both antibiotics. The combination cotrimoxazole is the treatment of choice for serious infections caused by *X. maltophilia.*

Q3. Anaerobic streptococci Anaerobes have not been calibrated for the CDS test. All should be susceptible to penicillin.

Corynebacteria Grow poorly, thus cannot test accurately with disc diffusion method. If growth is good then may use disc diffusion test but not yet calibrated.

Listeria spp. At present ALL susceptible to penicillin, ampicillin.

Neisseria meningitidis At present ALL susceptible to penicillin, cefotaxime and chloramphenicol. Not calibrated to date.

Haemophilus influenzae All tested so far are susceptible to rifampicin.

As a result of the discussion related to this question, we tentatively approached members to enquire whether one laboratory per state would act as a reference centre to collect and examine the susceptibility of *Listeria* spp., *Neisseria meningitidis* and *Haemophilus influenzae* to the antibiotics mentioned above. Some members volunteered their services and we shall investigate the process whereby the creation of these reference centres can come into being.

Q4. The susceptibility of antibiotics for topical use should not be reported. There is no correlation between the *in vitro* susceptibility of organisms and the *in vivo* response using topical antibiotics. If there is intense pressure from the clinician to report a result for topical antibiotic use, then it is reasonable to give a result for those antibiotics which have been calibrated for systemic use. However, the significance of any report is uncertain.

Q5. The strain of *Klebsiella* was reported as susceptible according to the Q.A.P. using the NCCLS breakpoint (MIC ≤ 8 mg/L = susceptible). However, using the CDS method the isolate was resistant (MIC ≤ 1 mg/L = susceptible). Using the CDS method with a cefotaxime 5 μg disc, there was a zone of inhibition of 3 mm which corresponds to an MIC of 4 mg/L. We have a collection of *Klebsiella* strains producing novel β-lactamases (cefotaximases) that hydrolyse cefotaxime, ceftazidime and other cephalosporins. The MIC of cefotaxime for these strains ranges from 2 to 32 mg/L and mutants with MIC of 32 mg/L can be selected at a high frequency (10^{-5}). Wild strains of *Klebsiella* normally have a cefotaxime MIC of 0.03 mg/L. The cefotaximase β-lactamase is inhibited by clavulanic acid and an interesting phenomenon which we term "the key hole effect" can be observed when a cefotaxime disc is placed near a Timentin or Augmentin disc. Try it!

The diagram shows an easy way to recognise cefotaximases. Place a CTX 5 disc and an AMC 60 or TIM 85 disc with the adjacent edges 1.5 to 2 cm apart on a CDS plate. Inhibition of the cefotaximase β-lactamase by clavulanic acid results in a zone of inhibition with the shape of a "key hole" (the key hole effect).

Q6. MIC "breakpoints" are derived from multiple sources.

DIRECT CORRELATIONS OF MIC AND CLINICAL RESPONSE

Life versus Death

Modifies the course of the disease

Shortens the duration of illness

Prevents the disease

INDIRECT CORRELATIONS OF MIC AND CLINICAL RESPONSE

Animal experiments:By extrapolation from animal studies but caution with the results, eg., penicillin can kill guinea pigs. *Salmonella typhi* is susceptible *in vitro* to the aminoglycoside antibiotics and in mice these agents are therapeutically effective. However, in humans, the aminoglycosides are ineffective for the treatment of typhoid.

By analogy to similar successful antibiotics, eg., the β-lactam group and the various aminoglycosides.

IN VITRO SUGGESTIONS OF LIKELY SUCCESS

If there is variation in susceptibility it distributes in a bimodal fashion.

If antibiotic unaffected by the mechanism of resistance associated with treatment failures of related antibiotics, eg., methicillin resistant to staphylococcal β-lactamase.

A FALSE AND MISLEADING BASIS OF PREDICTION

Assessing a susceptible MIC by its relationship to achievable antibiotic blood levels.

Class Representatives

The following table is a guide which shows the class representative antibiotic which can be used to extrapolate the susceptibility of other antibiotics for testing against groups/species of bacteria.

Representative antibiotic (Disc content)	Group/species	Antibiotics Represented
Ampicillin (25 μg)	Enterococci	Amoxycillin, benzylpenicillin
	Enterobacteriaceae[1]	Cephalothin, piperacillin, ticarcillin
	Acinetobacter spp.	Piperacillin
Augmentin (3 μg)	*Y. enterocolitica*	Cephalosporins
Benzylpenicillin (0.5 μg)	Gram (+) cocci	Penicillin V, ampicillin, amoxycillin
Cefaclor (30 μg)	*H. influenzae*	Augmentin
Cefotaxime (5 μg)	*Enterobacteriaceae*[1]	Ceftazidime, ceftriaxone, aztreonam
Erythromycin (5 μg)	Gram (+) cocci	Lincomycin, clindamycin
Methicillin (5 μg)	*Staph. aureus*	Cloxacillin, flucloxacillin, cephalothin, Augmentin
Piperacillin (50 μg)	*Ps. aeruginosa*	Azlocillin, mezlocillin
Polymyxin (300 u)	*Ps. aeruginosa*	Colistin
Sulphafurazole (300 μg)	Enterococci	ALL other sulphonamides
	Enterobacteriaceae	ALL other sulphonamides
Tetracycline (30 μg)	Gram (+) cocci	ALL other tetracyclines
	Enterobacteriaceae	ALL other tetracyclines

1Certain members of the Enterobacteriaceae, i.e., *Enterobacter cloacae, Citrobacter freundii, Serratia marcescens, Providencia* spp., *Morganella morganii*and indole (+) *Proteus* spp., should be considered resistant to ALL cephalosporins, penicillins, cephamycins and aztreonam but may be susceptible to imipenem.