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3rd August, 1998.

Dear Colleague,

C.D.S. USERS GROUP

Newsletter No. 9

This newsletter comprises three parts:

A. The proceedings of the CDS Users Group Workshop conducted at the Australian Society for Microbiology Annual Meeting in Adelaide in September, 1997.

B. Suggestions to CDS Users on how to improve their performance in the Quality Assurance Programme.

C. Synopsis of the ASIG Workshop.

There is some confusion about the handling of reference strains used for quality control of the CDS method of antibiotic susceptibility testing and, therefore, advice on the matter is included in this issue of the newsletter.

An updated list of CDS Reference Laboratories is enclosed with a new member from Singapore. Finally, as usual, a CDS Users Group Workshop will take place at this year's ASM meeting in Hobart. Please find the abstract and, also, the questionnaire which will assist us in planning the workshop.

Yours faithfully,

S. M. BELL,
For the Antibiotics Laboratory.
S. M.Bell, B. J. Gatus & J. N. Pham

CDS USERS GROUP NEWSLETTER No. 9

PROCEEDINGS OF THE ASM MEETING

ADELAIDE 1997

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The Antibiotics Laboratory,
S.M. Bell, B.J. Gatus & J.N. Pham.

A. THE CDS USERS GROUP WORKSHOP.

1. Preparation of fresh solutions of haematin and NAD for incorporation in Haemophilus Test Medium.

HAEMIN 10 mg/ml (1%)
Haemin chloride (Equine) from Sigma, Code No H 7903

- In a 10 ml volumetric flask, dissolve 100 mg haemin in 7 ml NaOH 0.2 M.
- Make up to 10 ml by adding distilled water.
- Sterilise by filtering through a millipore filter size 0.45 µm.

Note: When haemin is added to NaOH a chemical change occurs and haemin is converted to HAEMATIN.

NAD 10 mg/ml (1%)
β-Nicotinamide adenine dinucleotide from ICN, Code No 100319

- Dissolve 100 mg in 10 ml distilled water in a beaker using a stirrer.
- Sterilise by filtering through a millipore filter size 0.45 µm.

Add 1.5 ml of each of the haematin and NAD solutions freshly prepared to 1L of HTM agar base which has been autoclaved and allowed to cool to 50^0C.
Note: Aliquots (1.5 ml or 3 ml) of solutions of haematin and NAD may be kept at -70^0C or -20^0C for up to 6 months.

2. Co-trimoxazole 25µg.

Co-trimoxazole 25 µg discs contain 1.25 µg trimethoprim and 23.75 µg sulphamethoxazole.
The MIC of co-trimoxazole was determined using 1 part of trimethoprim in the presence of 19 parts of sulphamethoxazole. The MIC of co-trimoxazole for susceptible strains is that of trimethoprim in the presence of sulphamethoxazole:

Haemophilus influenzae	S ≤ 1.0 / 19.0 mg/L
Streptococcus pneumoniae	S ≤ 0.5 / 9.5 mg/L

3. Macrolides and *Haemophilus influenzae.*

Haemophilus influenzae is considered resistant to erythromycin and roxithromycin. The range of MICs of erythromycin observed with wild strains of this species is greater than that observed with susceptible streptococci and staphylococci (Table 1). This applies similarly for roxithromycin.

Note: *Erythromycin and roxithromycin are not calibrated for use with either the CDS or the NCCLS methods for testing H. influenzae.*

Table 1. The MIC of erythromycin for wild susceptible strains of streptococci, staphylococci and haemophili related to the CDS and NCCLS MIC breakpoints. The MIC was determined on appropriate media for these genera.

		Erythromycin MIC (mg/L)			
Species	MIC (mg/L)	CDS	NCCLS		
Streptococci	0.03 - 0.06	S ≤ 0.5	S ≤ 0.25	I = 0.5	R ≥ 1.0
Staphylococci	0.12 - 0.25	S ≤ 0.5	S ≤ 0.25	I = 0.5	R ≥ 1.0
Haemophili	1.0 - 4.0	Nil	Nil	Nil	Nil

4. Performance of Haemophilus Test Medium supplemented with fresh haematin and NAD.

Overall, CDS Users stated that they were satisfied with the new HTM medium. One person found that some strains of *H. influenzae* grew very poorly on the medium but this is a problem which at present cannot be overcome. We would be grateful to receive such strains to investigate their growth requirements.

- HTM plates can be stored at 4^0C for up to 2 weeks without being placed in plastic bags and up to 4 weeks within plastic bags.

5. Augmentin 3 μg and cefaclor 30 μg for testing *H. influenzae.*

We received enquiries concerning the zones around Augmentin 3 μg (amoxycillin 2 μg and clavulanate 1 μg) discs.

I. It was reported that with 2 non-encapsulated strains of *H. influenzae,* the annular radius of the zone of inhibition around Augmentin was 2 to 3 mm whilst those around ampicillin 5 μg and cefaclor 30 μg were approximately 6.5 mm. These observations were confirmed by us. The most likely explanation for the findings is that these isolates are less susceptible to β-lactam antibiotics because of an "intrinsic" mechanism which is possibly due to altered penicillin-binding-proteins (PBP's) (Bell *et al*. 1980: *Lancet*; 270-80). **Note**: Usually, the annular radius of the zone of inhibition around ampicillin 5 μg and cefaclor 30 μg observed with wild, susceptible non-encapsulated strains ranges from 9 to 10 mm and with Augmentin 3 μg ranges from 6 to 7 mm. With the two unusual isolates mentioned above, report them as susceptible to ampicillin, cefaclor and Augmentin. We are investigating the use of a different Augmentin disc that contains amoxycillin at a potency which has equivalent antibacterial activity compared with the ampicillin 5 μg disc.

ii. Two other non-encapsulated strains of *H. influenzae* were found to be resistant to cefaclor with the annular radius of the zone of inhibition 3 mm around a cefaclor 30 μg disc but 6.5 mm around an ampicillin 5 μg disc and 3 mm around an Augmentin 3 μg disc. The explanation for the reduced zone around Augmentin 3 μg was discussed above. On the other hand, the selective resistance to cefaclor but not to ampicillin can be explained by a change in one or more PBP's which influence the susceptibility to second generation cephalosporins (cefaclor, cephalexin) more than to ampicillin and amoxycillin. This is analogous to the changes of pneumococcal PBP's which confer "differential resistance" to penicillin and third generation cephalosporins. In the instances cited above, report the isolate resistant to cefaclor but susceptible to ampicillin and Augmentin.
Note: An Augmentin 15 μg disc is being investigated for testing *H. influenzae*.

6. Enterococci and vancomycin 5 μg.

Since publication of Newsletter No 8, we obtained a clinical isolate of *Enterococcus faecium* with a vancomycin MIC of 8 mg/L and there was hazy growth up to the edge of a vancomycin 5 μg disc. In the interim, the MIC of vancomycin for susceptible strains of enterococci has been changed to ≤ 4.0 mg/L (previously ≤ 8.0 mg/L). **This MIC "breakpoint" applies to streptococcci and staphylococcci also.**

Note: There are modifications to Newsletter No 8 under the heading "4. Vancomycin 5 μg for the testing of *Enterococcus species, Staphylococcus* species and *Streptococcus* species" (page 1).

a. **Enterococci.**
Four patterns of the zone of inhibition around a vancomycin 5 μg disc were observed:

1. A clear zone of inhibition with an annular radius ≥ 2 mm and a sharp edge: MIC of vancomycin ≤ 4 mg/L.
2. Hazy growth within the zone of inhibition with an annular radius of approximately 2 mm and a hazy edge: MIC of vancomycin 8 to 32 mg/L.
3. No zone of inhibition: MIC of vancomycin ≥ 128 mg/L.
   Note: We have no enterococci where the MIC of vancomycin is 64 mg/L. If anyone has such a strain we would be most grateful to receive it.
4. An annular radius of the zone of inhibition of approximately 1.5 mm to 2.0 mm with a clear zone was observed around a vancomycin 5 μg disc with strains of *E. gallinarum* and *E. casseliflavus* (we have no strains of *E. flavescens* to investigate). The reduced but clear zone of inhibition which corresponds with an MIC of 8.0 mg/L of vancomycin applied consistently with strains of *E. gallinarum* and *E. casseliflavus.*
   Note: *E. gallinarum, E. casseliflavus & E. flavescens* are inherently resistant to vancomycin (MIC of 8 mg/L).

7. Nitrofurantoin and enterococci.

With all strains of *Enterococcus faecium* which we tested, the MIC of nitrofurantoin was 64 mg/L and there was a corresponding annular radius of the zone of inhibition approximately 5 mm around a nitrofurantoin 200 μg disc. In contrast, the MIC of nitrofurantoin for susceptible strains of *Enterococcus faecalis* was found to be 16 mg/L with the corresponding annular radius of the zone of inhibition in the range 7 to 9 mm. For enterococci only, the MIC of nitrofurantoin for susceptible strains has been changed to ≤ 64 mg/L and the annular radius of inhibition for susceptible strains has been reduced to ≥ 4 mm (Revised Table 1 Calibrations, 1997, page 26 of this Newsletter). The MIC "breakpoint" of nitrofurantoin and the annular radius of inhibition around a nitrofurantoin 200 μg disc for susceptible strains remains unchanged for other bacterial species.

8. Testing of organisms NOT YET calibrated for the CDS method.

It is possible to perform antimicrobial disc susceptibility tests against organisms which are not included in the table of calibrations. However, some caution needs to be exercised.

- The organisms must be fast growers ie. produce a reasonable lawn culture after overnight incubation. If organisms grow slowly, disc diffusion testing may not be possible.
- Determine the appropriate medium for testing eg. Sensitest Agar or blood Sensitest Agar.

- Choose a panel of antibiotics which seems appropriate for the species to be tested. For example it is reasonable to use those antibiotics which are calibrated for streptococci to test *Corynebacterium* species under similar conditions of media, temperature and atmosphere of incubation which would be used for testing streptococci.
- When testing species which are not commonly encountered, beware that there may be some resistant mechanism present which you are unaware of. If this is so, there may be a spurious interpretation of the results.
- Issue a guarded susceptibility report.

For example when *Corynebacterium* species are tested, use the antibiotics calibrated for streptococci. With a penicillin 0.5 u disc an annular radius ≥ 6 mm indicates that the MIC of benzylpenicillin is ≤ 0.25 mg/L (Table 1 Calibrations, 1997). Conversely, an annular radius < 6 mm corresponds with an MIC of penicillin > 0.25 mg/L. Whether the antibiotic tested is appropriate for therapy is another issue.

9. Testing of *Streptococcus pneumoniae* isolated from sites other than CSF.

The MIC of benzylpenicillin, cefotaxime and ceftriaxone for susceptible strains of pneumococci isolated from CSF is ≤ 0.25 mg/L with a corresponding annular radius of the zone of inhibition ≥ 6 mm when penicillin 0.5 u, cefotaxime 0.5 µg and ceftriaxone 0.5 µg discs are used. For *Strep. pneumoniae* isolated from sites other than CSF eg. sputum or blood culture, there is a general consensus that a susceptible MIC breakpoint of ≤ 2.0 mg/L for penicillin, cefotaxime and ceftriaxone is acceptable. The isolation of *Strep. pneumoniae* from sputum and blood culture where the MIC of benzylpenicillin, cefotaxime and ceftriaxone is > 0.25 mg/L justifies the introduction of "differential disc testing". We are investigating the use of higher disc potencies of penicillin, cefotaxime and ceftriaxone for testing not only *Streptococcus pneumoniae* but other species of streptococci which may be relatively resistant to these antibiotics eg. *Streptococcus mitis* and *sanguis.* The results will be announced later.

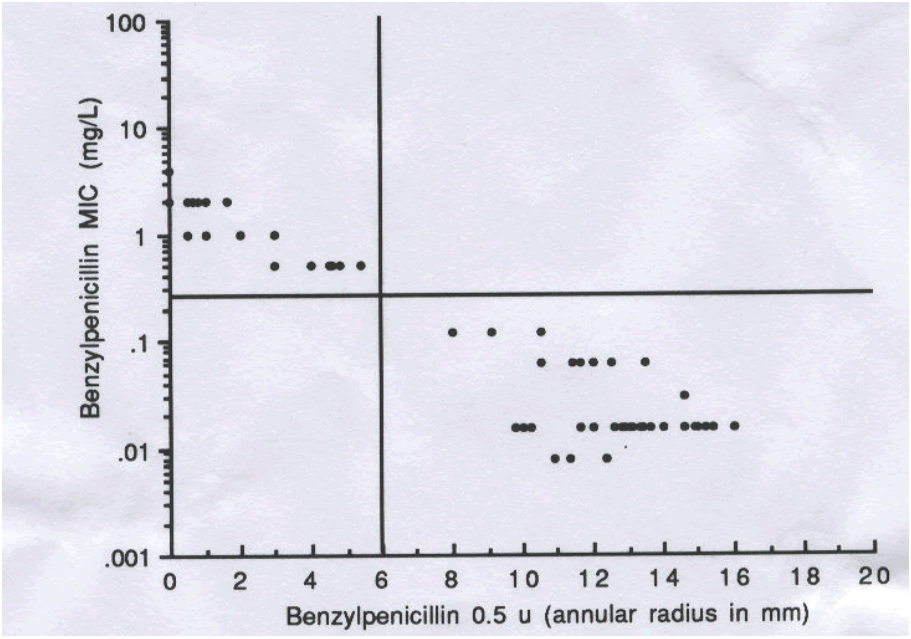
NOTES:

- **A conventional penicillin, cefotaxime and ceftriaxone MIC should be performed on ALL PNEUMOCOCCI WHICH ARE ISOLATED FROM CSF IF THEY ARE FOUND TO BE RESISTANT WHEN PENICILLIN 0.5 u, CEFOTAXIME 0.5µg and CEFTRIAXONE 0.5 µg are tested. Laboratories which are unable to perform a conventional MIC or an E-test should send the isolate to one where these procedures can be undertaken. However, take care when performing an E-test since there are problems with the test. These were detailed by both the QAP and the ASIG Workshop held in Adelaide.**

• Calibration graph.

Penicillin 0.5 u.

Figure 1. A calibration graph of penicillin which relates the Log MIC of penicillin to the annular radius of the zone of inhibition around a penicillin 0.5 u disc for clinical isolates of *Streptococcus pneumoniae* and other streptococcal species The MIC was determined on blood Sensitest Agar using a standard 10^4 cfu spot inoculum after overnight incubation in an atmosphere of 5 % CO_2.



CDS QUIZ ASM 1997
RECOGNITION OF RESISTANT MECHANISMS AND PATTERNS OF SUSCEPTIBILITY ASSIST IN IDENTIFICATION OF ORGANISMS

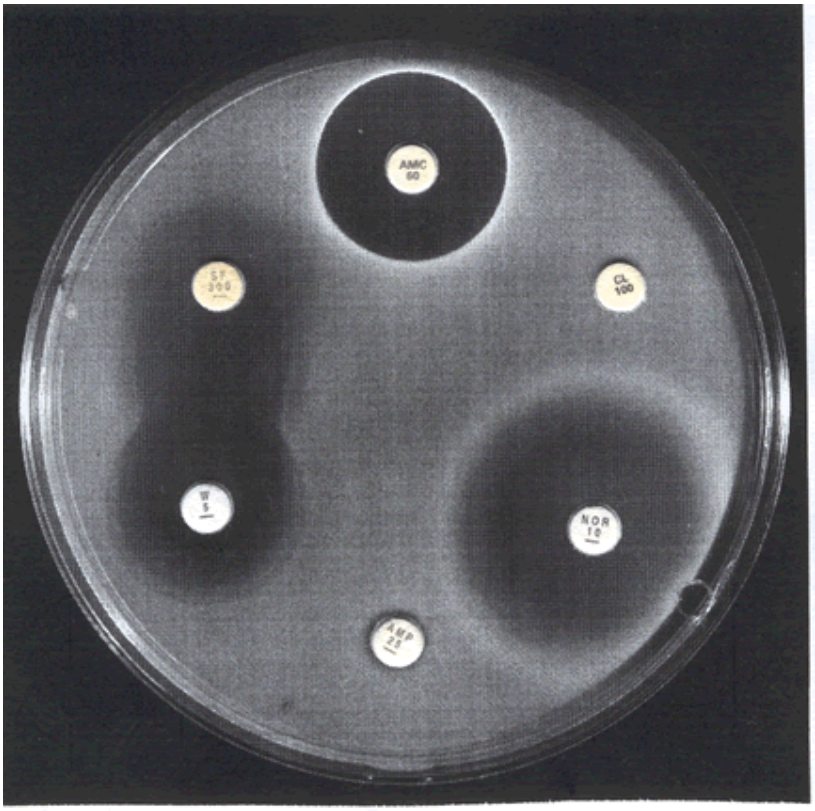
INTRODUCTION.

With the CDS method not only can the susceptibility of individual antibiotics be determined but by thoughtful placement of discs, the presumptive identification of bacterial genera and even species is possible also. Of critical significance is an understanding of β-lactamases, particularly those of Gram-negative bacteria. If the reader takes time to read and digest the contents of Table 4, Newsletter No. 7, "Simplified Classification of Bacterial β-lactamases", the patterns of interaction of β-lactam antibiotics with these enzymes which are shown in most of the accompanying slides should enable the observer to identify the isolate presumptively. REMEMBER THE CLUES FOR THE INTERPRETATION OF THE PATTERNS OBSERVED RELATE TO THE INTERACTION OF β-LACTAM AGENTS WITH β-LACTAMASES.

In the following photographs the scale is slightly bigger than normal. Please read the questions, look at the photographs and determine what you think is the reply before referring to the answers later in the Newsletter. This is meant to be a teaching exercise and is designed to illustrate that the selection and placement of antibiotic discs can reveal a great deal of information regarding resistance mechanisms and allow the presumptive identification of organisms.

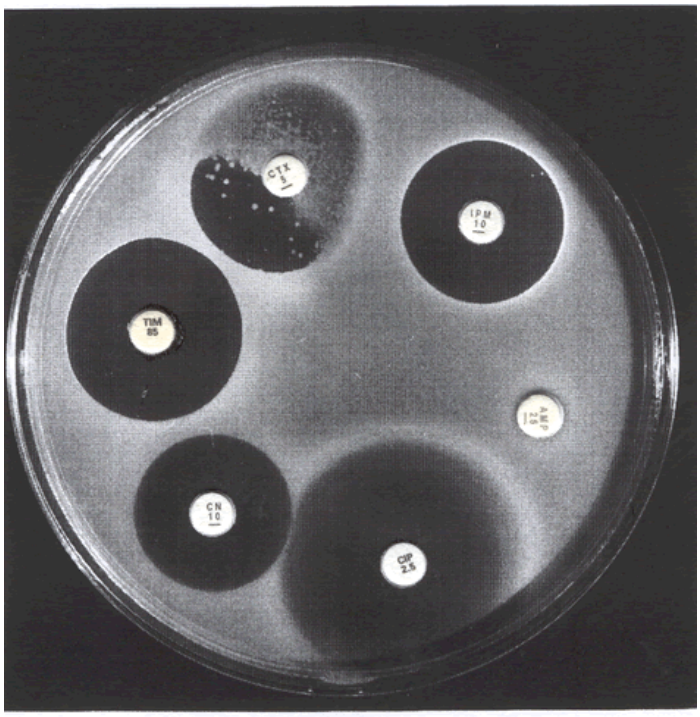
1. This organism was isolated from the urine of a patient who was febrile.

It was a non-lactose fermenter, indole negative and phenylalanine positive. How would you report the susceptibilities of the antibiotics ? What does the pattern of the susceptibility to β-lactams indicate ? What might this organism be ?



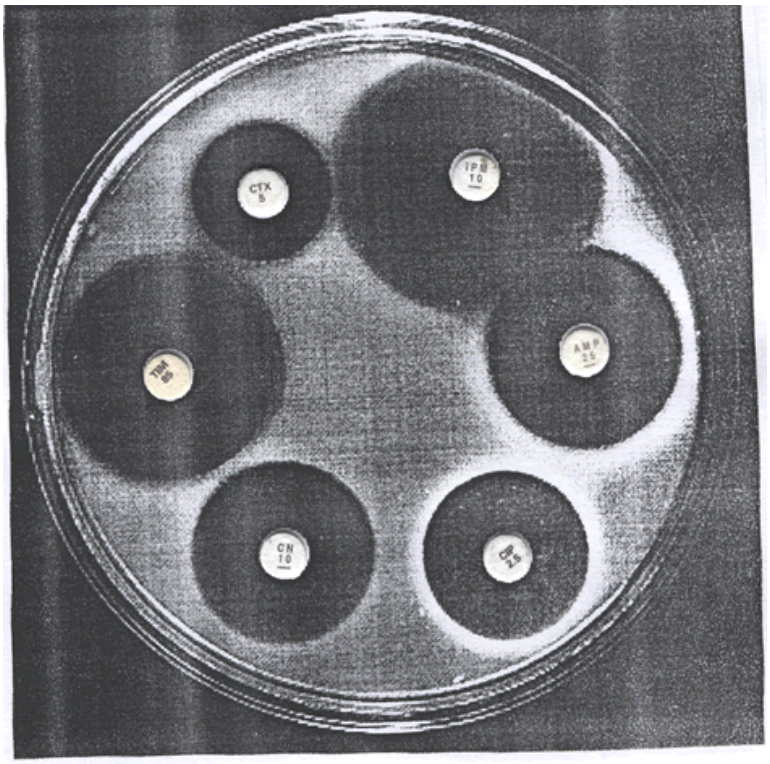
2. This organism was isolated from blood culture of a patient with symptoms of a urinary tract infection.

It was a non-lactose fermenter, indole negative and phenylalanine positive. How would you report the susceptibilities of the antibiotics ? What does the pattern of susceptibility to β-lactams indicate ? Could this be confused with another bacterial species ? If so why ? What other biochemical tests may assist in identifying the isolate ?



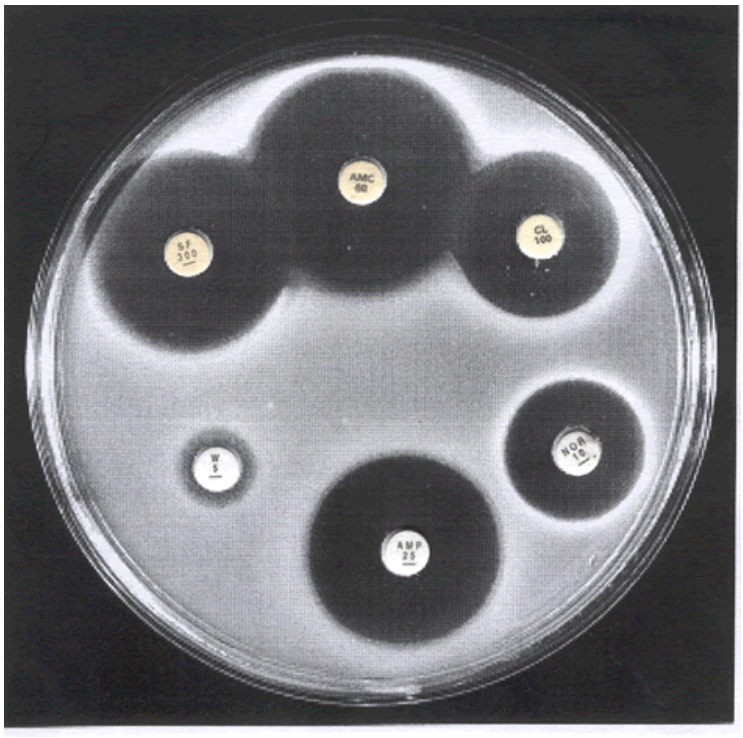
3. This organism was isolated from blood culture of a patient with a central line in situ.

It was a non-lactose fermenter and indole negative. How would you report the susceptibilities of the antibiotics ? Is there anything unusual about the susceptibility to β-lactams ? What do you think the species is ?



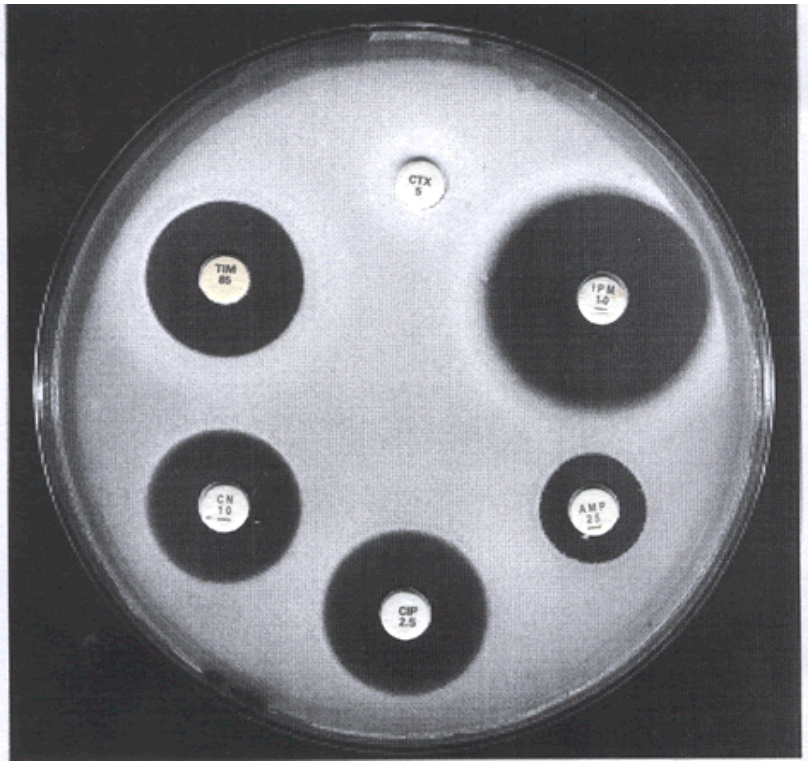
4. This organism was isolated from the urine of a patient with an indwelling urinary catheter.

It was a non-lactose fermenter and indole negative. How would you report the susceptibilities of the antibiotics ? Is there anything unusual about the susceptibility to antibiotics on this slide ? What do you think the species is ?



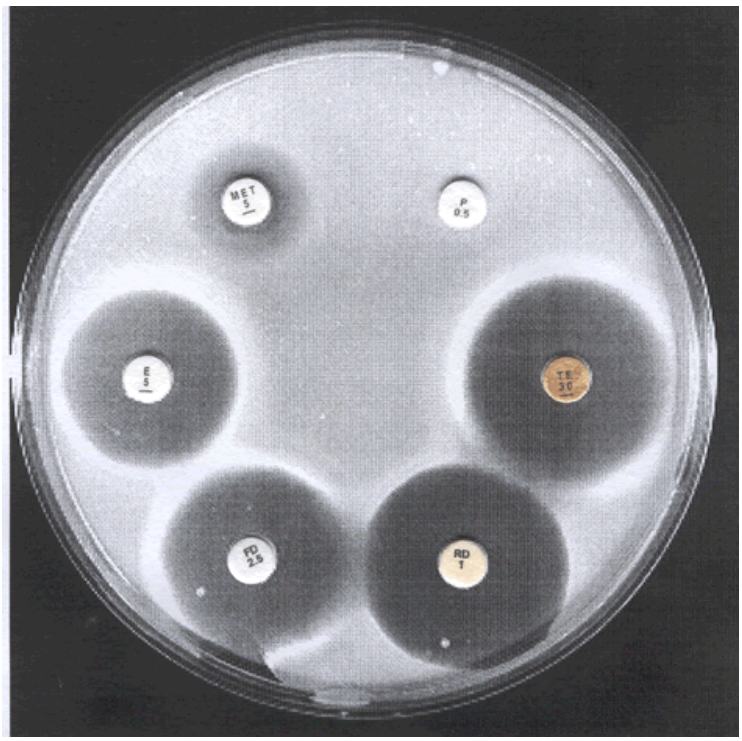
5. This organism was isolated from blood culture of a patient with an indwelling urinary catheter.

It was a non-lactose fermenter and indole negative. How would you report the susceptibilities of the antibiotics ? Is there anything unusual about the susceptibility to β-lactam antibiotics on this slide ? What do you think the species is ?



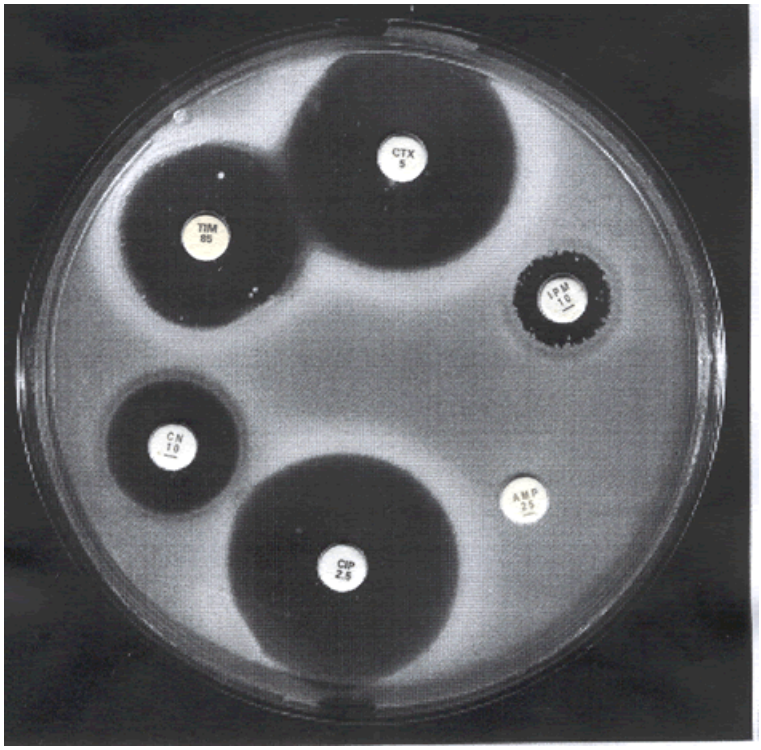
6. A heavy growth of *Staphylococcus aureus* was isolated from a post-operative wound swab.

How would you report the susceptibilities of antibiotics shown ? Is there anything unusual about the susceptibility of this organism to the antibiotics tested ?



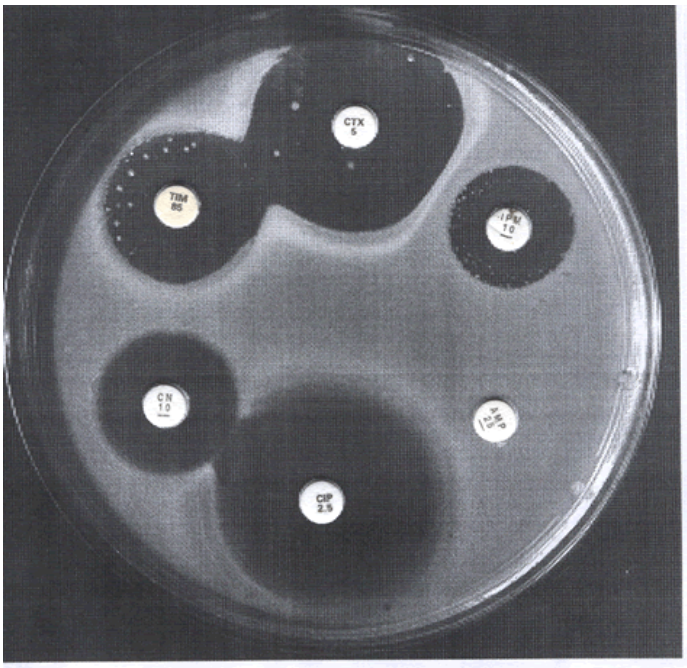
7. This organism was isolated from a wound swab of a footballer who sustained a cut to the leg during a rugby match.

There were β-haemolytic zones around coliform-like colonies growing on blood agar. How would you report the susceptibilities of the antibiotics on this slide ? What mechanism of resistance to β-lactam antibiotics is present ? What bacterial species do you think this is ? What one biochemical test would in all probability allow you to identify the species presumptively ?



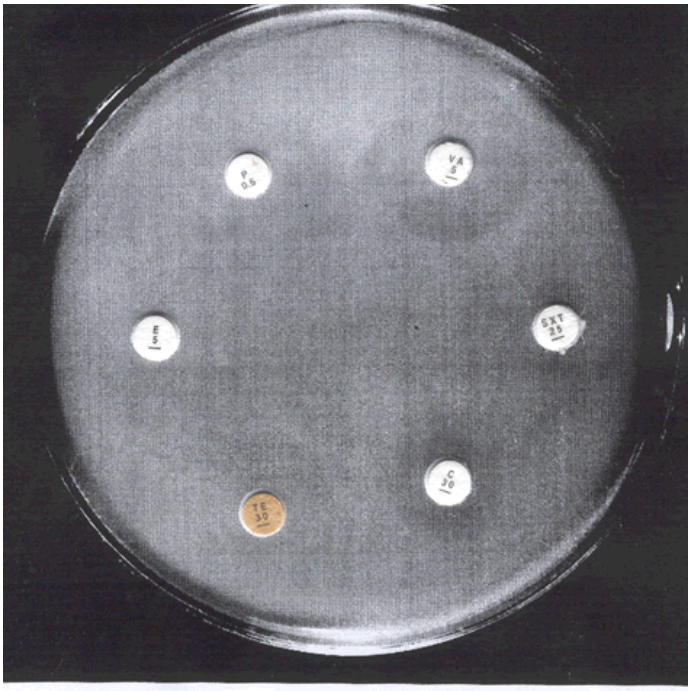
8. This organism was isolated from blood culture of a male with alcoholic liver cirrhosis. He ate a dozen oysters 24 hours before he became ill with a high fever and rigors.

There were β-haemolytic zones around coliform-like colonies growing on blood agar. How would you report the susceptibilities of the antibiotics ? What mechanism of resistance to β-lactam antibiotics is present ? What bacterial species do you think this is ?



9. A heavy growth of *Streptococcus pneumoniae* was isolated from the sputum of an 18 year old female who presented to her General Practitioner with shortness of breath, a fever and a cough which had been present for 4 days.

The susceptibility to antibiotics which may be prescribed commonly in General Practice is shown on the slide. How would you report the susceptibility of the antibiotics shown ? Is there any thing else you would like to perform which at present, you are not capable of doing so ? If there is, what is it and why would you perform it ?



ANSWERS TO QUIZ.

1. This organism was isolated from the urine of a patient who was febrile.
It was a non-lactose fermenter, indole negative and phenylalanine positive. How would you report the susceptibilities of the antibiotics ? What does the pattern of the susceptibility to β-lactams indicate ? What might this organism be ?

• *Reporting the susceptibilities.*
The antibiotics are those which might be tested commonly against "coliform-like" organisms isolated from the urine.

Ampicillin	**R ---** β-lactamase present
Trimethoprim	**S**
Sulphafurazole	**S**
Augmentin	**S** --- clavulanate has inhibited β-lactamase
Cephalexin	**R** --- β-lactamase probably active against cephalosporins
Norfloxacin	**S**

• *Pattern of susceptibility to β-lactams.*
The organism possesses a β-lactamase which hydrolyses not only ampicillin but cephalexin. However clavulanic acid inhibits this β-lactamase.

• *What might the organism be* ?
The deamination of phenylalanine and the inability to ferment lactose indicate that the organism is likely to be a *Proteus* species. In addition, the inability to produce indole indicates that the isolate may be a *Proteus mirabilis* which produces an **ESBL** (no zone around cephalexin). **Note**: If there is no zone around cephalexin and the isolate does produce an ESBL a "keyhole effect" or an elliptical area of clearing may not be observed. Cefotaxime is superior to cephalexin for demonstrating the presence of an ESBL using disc approximation tests. Alternatively, the isolate may be *Proteus penneri* which produces an **inducible Class 2e β-lactamase** that is inhibited by clavulanic acid. Induction of β-lactamase cannot be demonstrated on this slide because of the types of antibiotics tested.

• *Most likely identification.*
Could be *Proteus mirabilis* with an ESBL or *Proteus penneri.*

2. This organism was isolated from blood culture of a patient with symptoms of a urinary tract infection.

It was a non-lactose fermenter, indole negative and phenylalanine positive. How would you report the susceptibilities of the antibiotics ? What does the pattern of susceptibility to β-lactams indicate ? Could this be confused with another bacterial species ? If so why ? What other biochemical tests may assist in identifying the isolate ?

• *Reporting the susceptibilities.*

The antibiotics tested are those from a panel of 12 which are used for "coliform-like" organisms isolated from blood culture at The Prince of Wales Hospital.

Gentamicin	**S**
Ciprofloxacin	**S**
Ampicillin	**R ---** β-lactamase present
Imipenem	**S**
Cefotaxime	**R** --- induction of β-lactamase (some type of cephalosporinase) and resistant mutants present
Timentin	**S** --- cephalosporinase inhibited by clavulanic acid

• *Pattern of susceptibility to β-lactams.*

The phenomenon of induction of β-lactamase is demonstrated by flattening of the zone of inhibition around the cefotaxime disc adjacent to the imipenem disc. Also, there are resistant mutant colonies present within the zone of inhibition around cefotaxime. "Swarming" (a phenomenon characteristic of some *Proteus* spp.) is evident within the zone of inhibition around cefotaxime. The **inducible β-lactamase** is a **Class 2e cephalosporinase** and is inhibited by clavulanic acid which is evident from the large zone of inhibition around Timentin. **Note**: There are no resistant mutant colonies within the zone of inhibition around Timentin.

• *Confusion with other bacterial species.*

The demonstration of induction of a cephalosporinase together with the deamination of phenylalanine and the inability to ferment lactose indicate that the organism is either a *Providencia* spp., *Morganella* spp., *Proteus vulgaris* or *Proteus penneri*. However, the inability to produce indole excludes *Proteus vulgaris*, *Providencia* and *Morganella* spp.. Although, *Proteus mirabilis* and *Proteus penneri* are both indole-negative, only *Proteus penneri* produces an **inducible Class 2e cephalosporinase**. Clavulanic acid contained in Timentin has inhibited the β-lactamase produced by this organism. This is similar to that observed with Augmentin in the photograph for question 1. **Note**: *Proteus vulgaris* and *Proteus penneri* produce an **inducible Class 2e β-lactamase** and are susceptible to Augmentin and Timentin whereas *Providencia* and *Morganella* spp. produce an inducible **Class 1' β-lactamase** and are resistant to Augmentin but susceptible to Timentin.

• *Most likely identification.*

Proteus penneri ---This is the same organism as in question 1 ! Note the difference in the pattern of susceptibility to β-lactams between the two pictures.

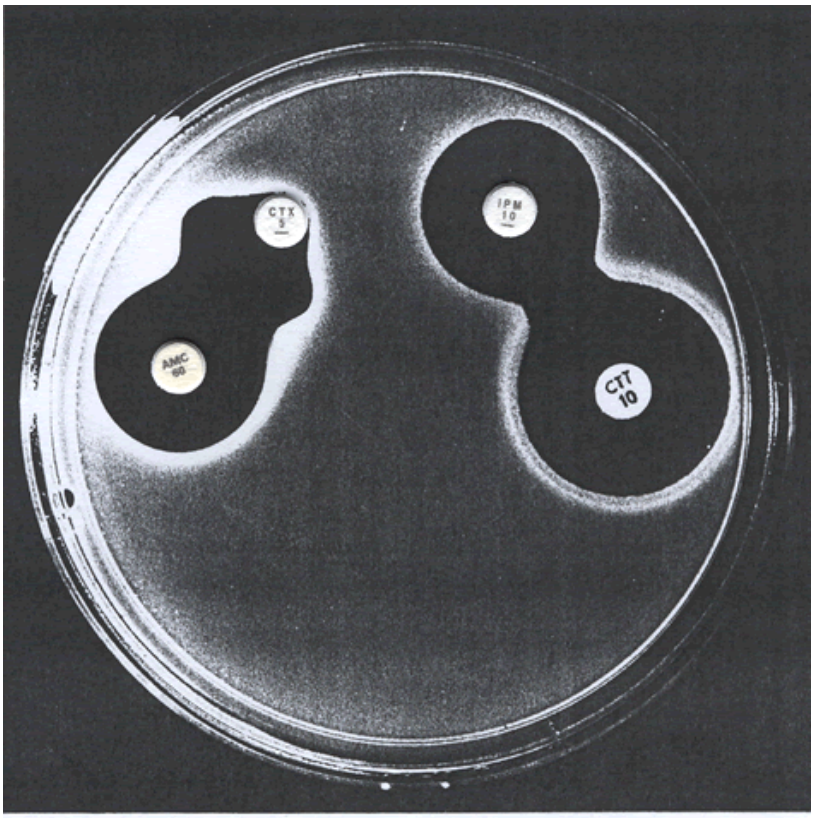
• *Ancillary biochemical tests.*

The utilisation of maltose assists in differentiating *Proteus penneri* from *Proteus mirabilis*.

Proteus penneri	MALTOSE	+	(100 %)
Proteus mirabilis	MALTOSE	-	(0 %)

INFORMATION PHOTOGRAPH.

This is another strain of *Proteus penneri*. There is high level resistance to cefotaxime indicating that the organism is a derepressed mutant which hyperproduces **Class 2 e β-lactamase**. The organism is susceptible to Augmentin and clavulanic acid has inhibited the β-lactamase. One might think that an ESBL is present since there is a "keyhole type" effect because of the interaction of clavulanic acid contained in Augmentin with the zone of inhibition around cefotaxime. Note also, the susceptibility to the cephamycin antibiotic cefotetan (CTT 10). This together with the "keyhole type" effect may make one think even moreso that an ESBL is present. However, this strain does NOT produce an ESBL.



3. This organism was isolated from blood culture of a patient with a central line in situ.

It was a non-lactose fermenter and indole negative. How would you report the susceptibilities of the antibiotics ? Is there anything unusual about the susceptibility to β-lactams ? What do you think the species is ?

• *Reporting the susceptibilities.*

The antibiotics tested are those from a panel of 12 which are used for "coliform-like" organisms isolated from blood culture at The Prince of Wales Hospital.

Gentamicin	**S**
Ciprofloxacin	**S**
Ampicillin	**S --- β**-lactamase absent
Imipenem	**S**
Cefotaxime	**R** --- annular radius of the zone 5.8 mm
Timentin	**S**

• *Unusual features of susceptibilities to β-lactams.*

It is odd to see a larger zone of inhibition around ampicillin compared with a smaller zone of inhibition around cefotaxime. β-Lactamase is not present since there would be either no zone or a reduced one around ampicillin. Note the very large zone of inhibition around Timentin (the explanation for this will appear in the answer to question 5).

• *Most likely identification.*

The pattern of susceptibility to β-lactam antibiotics is typical of *Acinetobacter lwoffii*. Generally, *Acinetobacter lwoffii* is less susceptible to most cephalosporins compared with ampicillin. The reasons for this are not entirely clear but may be explained on poorer permeability of some types of cephalosporins into the cell compared with the penicillins.

4. This organism was isolated from the urine of a patient with an indwelling urinary catheter.

It was a non-lactose fermenter and indole negative. How would you report the susceptibilities of the antibiotics ? Is there anything unusual about the susceptibility to antibiotics on this slide ? What do you think the species is ?

• *Reporting the susceptibilities.*

The antibiotics are those which might be tested commonly against "coliform-like" organisms isolated from the urine.

Ampicillin	**S**
Trimethoprim	**R**
Sulphafurazole	**S**
Augmentin	**S**
Cephalexin	**S** --- smaller zone than around ampicillin. An Unusual observation
Norfloxacin	**S**

- Note: Although antibiotic susceptibilities were performed we would not issue a result because the isolate was from a catheter specimen of urine.

• *Unusual features of the antibiotic susceptibilities.*

This is the same organism as in question 3 !! Note the difference in the pattern of susceptibility to β-lactams between the two pictures. Also note the very large zone of inhibition around Augmentin (the explanation for this will appear in the answer to question 5).

There is a larger zone of inhibition around ampicillin compared with that around cephalexin. In addition, the isolate is susceptible to sulphafurazole but resistant to trimethoprim. This pattern is typical of that seen with *Acinetobacter lwoffii*. In our experience most *Acinetobacter* species are resistant to trimethoprim and this appears to be a characteristic feature of this genus.

• *Most likely identification. Acinetobacter lwoffii*.

5. This organism was isolated from blood culture of a patient with an indwelling urinary catheter.

It was a non-lactose fermenter and indole negative. How would you report the susceptibilities of the antibiotics ? Is there anything unusual about the susceptibility to β-lactam antibiotics on this slide ? What do you think the species is ?

• *Reporting the susceptibilities.*

The antibiotics tested are those from a panel of 12 which are used for "coliform-like" organisms isolated from blood culture at The Prince of Wales Hospital.

Gentamicin	**S**
Ciprofloxacin	**S**
Ampicillin	**R ---**β-lactamase present
Imipenem	**S**
Cefotaxime	**R** --- no zone of inhibition; cephalosporinase present ?
Timentin	**S**

• *Unusual features of the susceptibilities to β-lactams.*

There is a reduced zone of inhibition around ampicillin BUT no zone at all around cefotaxime. Note the relatively large zone of inhibition around Timentin. One might think that the resistance to cefotaxime is due to hyperproduction of a cephalosporinase from a derepressed mutant possessing a Class 1 β-lactamase. However, if this were the case there should be no zone around ampicillin and in all probability no zone around Timentin. Alternatively, one might think that an ESBL is present but there is neither a "key hole" effect nor an elliptical area of clearing between cefotaxime and Timentin. Neither of these interpretations is correct.

• *Most likely identification.*

The pattern of susceptibility to β-lactam antibiotics is that seen typically with *Acinetobacter* "*baumannii like" species.*

• Special note.

1. *Acinetobacter* "*baumannii* like" species possess a **chromosomal cephalosporinase** which unlike that of *Enterobacter cloacae* etc. IS NOT INDUCIBLE.

2. **Clavulanic acid has inherent antibacterial activity against all *Acinetobacter* spp!** The MIC of clavulanic acid with isolates of *Acinetobacter lwoffii* which were tested ranged from 1 to 2 mg/L whilst with *Acinetobacter* "*baumannii* like" species the MIC of clavulanic acid ranged from 4 to 32 mg/L. The susceptibility of *Acinetobacter* species to both Augmentin and Timentin is in part due to the presence of clavulanic acid.
Note: When β-lactamase was extracted from *Acinetobacter* "*baumannii* like" species and incubated with clavulanic acid there was no inhibition of enzyme activity by this β-lactam agent. Therefore, the large zones of inhibition around Augmentin and Timentin observed with *Acinetobacter* "*baumannii* like" species are not because of inhibition of the cephalosporinase by clavulanic acid.

6. A heavy growth of *Staphylococcus aureus* was isolated from a post-operative wound swab.

How would you report the susceptibilities of antibiotics shown ? Is there anything unusual about the susceptibility of this organism to the antibiotics tested ?

• *Reporting the susceptibilities*
The antibiotics tested are those from a panel of 12 which are used for staphylococci isolated from wound swabs at The Prince of Wales Hospital.

Erythromycin	**S**
Tetracycline	**S**
Fusidate	**S** --- note mutants BUT ZONE > 6 mm
Rifampicin	**S** --- note mutants BUT ZONE > 6 mm
Penicillin	**R**
Methicillin	**R** --- zone < 6 mm and resistant mutant colonies

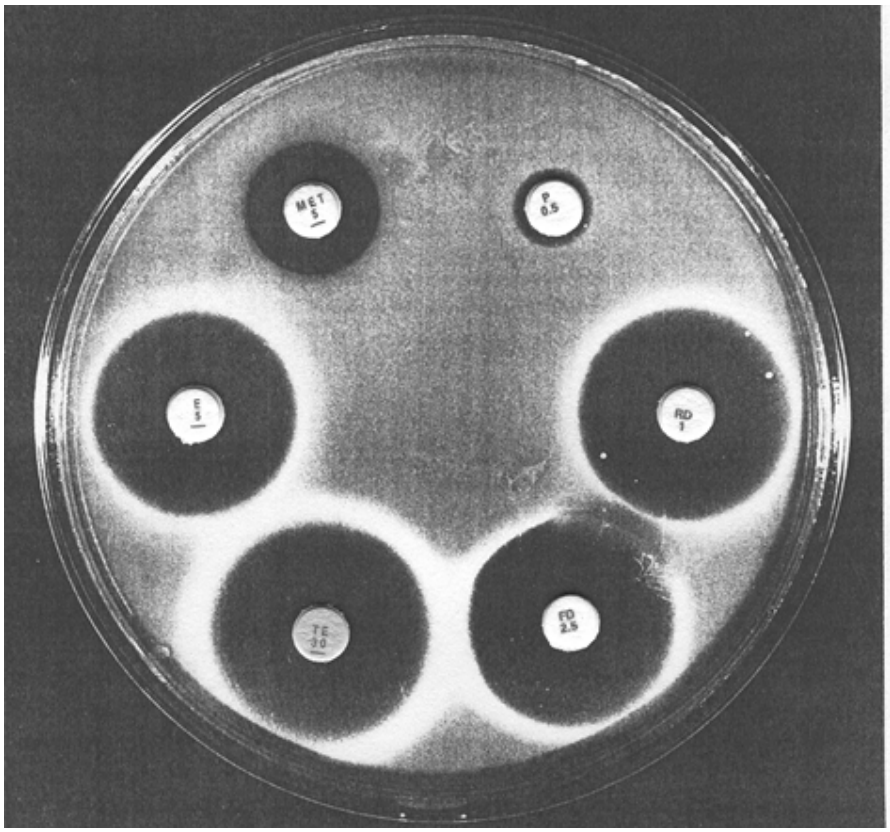
• *Unusual features of the antibiotic susceptibilities.*
The antibiotic susceptibilities are unusual insofar that stains of MRSA are resistant usually to erythromycin, tetracycline (also ciprofloxacin). MRSA means methicillin-resistant *Staphylococcus aureus* NOT MULTIPLY resistant *Staphylococcus aureus*.

• **Note:** Although there are colonies within the zones of inhibition around both rifampicin and fusidate, the annular radius of the zone of inhibition around both antibiotic discs is > 6 mm. These colonies are mutants which are resistant to rifampicin and fusidate and may be present at a high frequency (10^{-5}) in strains of staphylococci (not only MRSA). As a consequence, rifampicin and fusidate should not be used alone for the treatment of staphylococcal infections since these mutants will be selected during therapy. Comments such as "Both agents should be used in combination to prevent the emergence of resistant mutants" should accompany the susceptibility report.

INFORMATION PHOTOGRAPH.

This is an unusual strain of MRSA provided by Nichalas Nuttall where the annular radius of the zone of inhibition around methicillin is 5 mm. If the inoculum is too light the annular radius of the zone of inhibition may be > 6 mm and the isolate might be reported as susceptible to methicillin whereas it is in fact resistant to this antibiotic. The mec A gene was present and there are resistant mutant colonies inside the zone of inhibition around methicillin. If you are in doubt about the susceptibility to methicillin reincubate the plate for a further 24 hours and observe if resistant mutant colonies are present within the zone of inhibition around methicillin.

• **Note**: The pattern of susceptibility observed with non-methicillin-resistant strains of *Staphylococcus aureus* is that there is no zone around a penicillin 0.5 u disc but an annular radius of the zone of inhibition approximately 8 mm around a methicillin 5 μg disc. Such strains produce β-lactamase which hydrolyses penicillin but not methicillin. If the zone of inhibition around methicillin is reduced to approximately 6 mm, be aware that the isolate may be a MRSA. In this case, look for colonies within the zone of inhibition and if in doubt reincubate the plate for a further 24 hours. Also, repeat the test if you think that the original inoculum was too light.



7. This organism was isolated from a wound swab of a footballer who sustained a cut to the leg during a rugby match.

There were β-haemolytic zones around coliform-like colonies growing on blood agar. How would you report the susceptibilities of the antibiotics on this slide ? What mechanism of resistance to β-lactam antibiotics is present ? What bacterial species do you think this is ? What one biochemical test would in all probability allow you to identify the species presumptively ?

• *Reporting the susceptibilities.*
The antibiotics tested are those from a panel of 12 which are used for "coliform-like" organisms which may be significant clinically and are isolated from wound swabs at The Prince of Wales Hospital.

Gentamicin	**S**
Ciprofloxacin	**S**
Ampicillin	**R ---** β-lactamase present
Imipenem	**R ---** β-lactamase present and resistant mutants within zone
Cefotaxime	**S**
Timentin	**R** --- annular radius of the zone > 6 mm with resistant mutants present

• *Mechanism of resistance to β-lactams*
Resistant to ampicillin indicates that some type of β-lactamase is present but the isolate is susceptible to cefotaxime and the phenomenon of induction of a cephalosporinase is not present (observe the zone around cefotaxime adjacent to the imipenem disc). An unusual feature is that the organism is resistant to imipenem (annular radius of the zone of inhibition < 6 mm) and resistant mutant colonies are present within the zone of inhibition. **Note**: Although the annular radius of the zone of inhibition around Timentin is > 6 mm there are resistant mutant colonies present inside the zone. Overall, the pattern of susceptibility to β-lactam antibiotics indicates that the isolate produces a carbapenemase/penicillinase **Class 3 metallo-enzyme type β-lactamase** which is found in species of *Aeromonas sobria*. Although not apparent on this slide, this enzyme is INDUCIBLE and confers resistance to penicillin and carbapenem antibiotics (imipenem and meropenem) but not to cefotaxime or ceftriaxone.

• *What is the species ?*

Most likely *Aeromonas sobria.* The pattern of susceptibility to β-lactams observed in this photo is typical of this species.

• *Biochemical test*

Perform a test for OXIDASE --- should be POSITIVE.

Note: Any "coliform like" colonies growing on blood agar which produce zones of β-haemolysis should be subjected to a test for oxidase production. In our laboratory at The Prince of Wales Hospital, we have noticed that during periods of wet weather there is an increase in the incidence of isolation of *Aeromonas* species from wound swabs from patients who sustained cuts whilst working in the garden or playing sport on grassy fields. *Aeromonas* species are ubiquitous in the environment, particularly where water is present.

8. This organism was isolated from blood culture of a male with alcoholic liver cirrhosis. He ate a dozen oysters 24 hours before he became ill with a high fever and rigors.

There were β-haemolytic zones around coliform-like colonies growing on blood agar. How would you report the susceptibilities of the antibiotics ? What mechanism of resistance to β-lactam antibiotics is present ? What bacterial species do you think this is ?

• *Reporting the susceptibilities*

The antibiotics tested are those from a panel of 12 which are used for "coliform-like" organisms isolated from blood culture at The Prince of Wales Hospital.

Gentamicin	**S**
Ciprofloxacin	**S**
Ampicillin	**R ---** β-lactamase present
Imipenem	**R ---** zone size 6 mm but resistant mutants present
Cefotaxime	**R** --- induction of cephalosporinase demonstrated AND resistant mutants
Timentin	**R** --- although zone > 6 mm resistant mutants are present

• *Mechanism of resistance to β-lactams*

Resistant to ampicillin indicates that some type of β-lactamase is present. Also the phenomenon of induction of a cephalosporinase is present as indicated by flattening of the zone of inhibition around cefotaxime adjacent to the imipenem disc. Also, there are resistant mutants present within the zones of inhibition around cefotaxime and Timentin. Furthermore, although the annular radius of the zone of inhibition around imipenem is 6 mm there are resistant mutant colonies present within the zone. Again, the salient observation is that the annular radius of the zone of inhibition around imipenem is reduced in comparison with that around cefotaxime and resistant mutant colonies are present in the zone of inhibition around Timentin. These observations are similar to those demonstrated with *Aeromonas sobria* in the previous slide but in contrast, induction of a cephalosporinase is demonstrated and resistant mutants are present within the zone of inhibition around cefotaxime.
In all, the pattern of susceptibility to β-lactam antibiotics indicates that the isolate produces two types of β-lactamase:

1. A **carbapenemase/penicillinase Class 3 metalo-enzyme** type β-lactamase is present and this is indicated by the similar pattern of susceptibility to β-lactams which was demonstrated with *Aeromonas sobria.* Although not apparent on this photograph, this enzyme is INDUCIBLE and confers resistance to penicillin and carbapenem antibiotics (imipenem and meropenem) but not to cefotaxime or ceftriaxone.

2. **An inducible Class 1 cephalosporinase** which confers resistance to cephalosporins, cephamycins and aztreonam.

• Summary.

Two types of inducible β-lactamases are present: a **Class 3 metallo-enzyme type penicillinase/carbapenemase** and a **Class 1 cephalosporinase**. Both β-lactamases are found in *Aeromonas hydrophila* and *Aeromonas caviae*.

• *Most likely identification.*

Aeromonas hydrophila or *Aeromonas caviae*

Note : *Aeromonas caviae* colonies are usually NON β-HAEMOLYTIC on horse blood agar.

9. A heavy growth of *Streptococcus pneumoniae* was isolated from the sputum of an 18 year old female who presented to her General Practitioner with shortness of breath, a fever and a cough which had been present for 4 days.
The susceptibility to antibiotics which may be prescribed commonly in General Practice is shown on the slide. How would you report the susceptibility of the antibiotics shown ? Is there any thing else you would like to perform which at present, you are not capable of doing so ? If there is, what is it and why would you perform it ?

• *Reporting the susceptibilities*

The antibiotics tested are those which are used for pneumococci isolated from sputum specimens at The Prince of Wales Hospital.

Penicillin	**R** --- NO ZONE --- MIC ≥ 0.5 mg. Perhaps 1 mg/L
Erythromycin	**R**
Tetracycline	**R**
Chloramphenicol	**R**
Co-trimoxazole	**R**
Vancomycin	**S**

• *What else would you do.*

Perform a formal penicillin MIC or an E-test. However, facilities to perform these procedures may not be available to all laboratories.

• CDS Users will probably test cefotaxime 0.5 μg and ceftriaxone 0.5 μg. If so, the annular radius of the zones of inhibition around these antibiotic discs with this isolate was 3 mm and accordingly, when related to the CDS MIC breakpoint of ≤ 0.25 mg/L for susceptible strains the isolate will be considered resistant to these antibiotics.

B. THE QUALITY ASSURANCE PROGRAMME.

JohnTurnidge presented QAP data related to antibiotic susceptibility testing.

Overall, CDS Users performed well in the QAP when they followed the recommendations issued in CDS Users Group Newsletters. However, there was some confusion in relation to what antibiotics should be tested, the interpretation of the annular radius of the zone of inhibition for susceptible strains and the reporting of results when Users referred to the Surrogate Disc Testing Table.

The following suggestions should be noted.

a. Make sure that you have the most recent CDS Users Group Newsletter (Newsletter No 8) and examine "Table 1 Calibrations" paying particular attention to footnotes, flagged antibiotics and the annular radius of the zone of inhibition for susceptible strains which differ from the usual 6 mm.

b. Test only those antibiotics which have been calibrated (see Table 1 Calibrations). If the QAP asks for the result of an antibiotic which is not calibrated AND surrogate disc reporting is not possible, then answer "NOT TESTED". For example it is a mistake to test *Strep. pneumoniae* against ciprofloxacin because, although the QAP may have this antibiotic in their standard antimicrobial test list, this antibiotic is not calibrated for the CDS method with this species. When testing antibiotics for the QAP where a clinical scenario is supplied, consider the isolate as a "routine clinical specimen" and TEST ONLY those antibiotics which are calibrated. If required, report susceptibilities according to the Surrogate Disc Testing Table and state that the result is derived from the table.
No marks will be lost if an antibiotic is not tested because it is not calibrated. However, it is incorrect if CDS Users test an antibiotic which is not calibrated.

c. CDS Users are reminded to refer to the Surrogate Disc Testing Table (including its footnotes) and if appropriate, report additional antibiotics when required. For example, with *Staph. aureus,* report the susceptibly of cephalosporin antibiotics which might be used by your client doctors according to the type of specimen and the clinical situation ie. the susceptibility to cephalothin, cefazolin and cephalexin may be reported based on the result obtained with a 5 μg methicillin disc.

d. We advise that CDS Users approach QAP testing in four ways.

1. Identify the isolate.

2. Test the isolate against those antibiotics which you use routinely for such an organism from the site which was specified. For example, antibiotics which are used to test "coliform-like" organisms from the urine may be different from those used to test the same species if it was isolated from blood culture. If additional antibiotic susceptibility tests help you with the identification eg. you are suspicious that an ESBL might be present, use Augmentin or Timentin adjacent to a cefotaxime disc to demonstrate the typical patterns which might be found with ESBL production. **Note**: Augmentin and Timentin are not requested to be tested by the QAP.

3. We advise that you perform antibiotics susceptibility tests on all isolates even though this may not be requested. For example, the pattern of susceptibility observed with sulphafurazole adjacent to trimethoprim together with no zone around imipenem would confirm with greater probability that the isolate is a *Stenotrophomonas maltophilia*. Some kits may give an identification but susceptibility testing with appropriate antibiotics may indicate that the identification is incorrect, especially when the interaction of β-lactams with β-lactamase is observed.

4. Once you have identified the isolate and performed the relevant antibiotic susceptibility tests, look at the clinical scenario given and decide if you will report the susceptibilities. This a clinical decision. For, example the reporting of antibiotic susceptibilities for *Acinetobacter lwoffffi* from a catheter specimen of urine is not justified. Conversely, if an ESBL producing organism is isolated from a catheter specimen of urine, reporting susceptibilities may be justified for infection control purposes. Use common sense when reporting.

Three examples of how to report answers to QAP questions are given below. There is some degree of subjectivity in the reporting which may differ between laboratories. The examples are how we, might answer the questions. **PLEASE NOTE THAT THE REPORTING RECOMMENDATIONS** eg. **NOT TESTED**, **NOT REPORTED** etc. are subjective and may differ between laboratories. These reports are a guide only and no marks are deducted by the QAP if laboratories report differently.
The QAP lists antibiotics in a standard format for all organisms which might be tested against antibiotics.

ITEM 97:5:2A.
This specimen represents a swab from an infected wound following surgery to the neck of a 23 year old woman. A Gram stained smear reveals ++ pus cells and ++ Gram positive cocci.

The isolate was *Staphylococcus aureus* (MRSA).

The following style of reporting is that which we recommend to CDS Users. Once the isolate was identified as *Staphylococcus aureus* test those antibiotics which you would normally use against staphylococci isolated from a wound swab and if found resistant to methicillin, test additional, appropriate antibiotics.

Antibiotic	Zone diameter	Annual radius	MIC	Interpretation S or R
Ampicillin				R (Surrogate result from penicillin 0.5 u) Do not report
Amoxycillin				R (Surrogate result from penicillin 0.5 u) Do not report
Cefotaxime				Not Tested
Ceftriaxone				Not Tested
Ceftazidime				Not Tested
Cephalexin				R (Surrogate result from methicillin 5 μg) Do not report
Cephalothin				R (Surrogate result from methicillin 5 μg) Do not report
Ciprofloxacin		3.0 mm		R (Do not report)
Erythromycin		0 mm		R (Do not report)
Gentamicin		7 mm		S (Do not report)
Imipenem				Not tested
Methicillin		0 mm		R (Do not report)
Norfloxacin				Not tested
Penicillin		0 mm		R (Do not report)
Piperacillin				Not tested
Rifampicin		11.5 mm		S (Do not report)
Tetracycline		2 mm		Inappropriate to report
Trimethoprim				Inappropriate to test
Vancomycin		3.0 mm		S (Do not report)

Words to the effect "Methicillin-resistant *Staphylococcus aureus* isolated" might be warranted. However, the issuing of susceptibility test results may be withheld. MRSA frequently colonises wounds but may not be pathogenic. The decision to treat depends on the clinical circumstances.

ITEM 97:5:3B.
A 62 year old man in intensive care is catheterised. His urine has become cloudy.

The isolate was *Enterococcus faecium*.

Antibiotic	Zone diameter	Annual radius	MIC	Interpretation S or R
Ampicillin		0 mm		R (Do not report)
Amoxycillin				R (Surrogate result from ampicillin 5 µg) Do not report
Cefotaxime				Not Tested
Ceftriaxone				Not Tested
Ceftazidime				Not Tested
Cephalexin				Not Tested
Cephalothin				Not Tested
Ciprofloxacin				Not Tested
Erythromycin				Not Tested
Gentamicin				Inappropriate to test gentamicin 200 µg
Imipenem				Not tested
Methicillin				Not tested
Norfloxacin				Not tested
Penicillin				Not tested. Do not report from surrogate testing of ampicillin
Piperacillin				Not tested
Rifampicin				Not tested
Tetracycline				Not tested
Trimethoprim				Not tested
Vancomycin		4.0 mm		S (Do not report)

Words to the effect "Antibiotic susceptibilities will NOT be reported for catheter specimens of urine" seems reasonable in the clinical context here. **Note**: If the organism was found to be resistant to vancomycin ie: it was a VRE, report the susceptibility to vancomycin since there are implications for infection control policies.

ITEM Future 2000: 1: 1A
This organism was isolated from blood culture of a patient with signs of septic shock.

The isolate was *Providencia stuartii* (this is the organism from QAP 97:7:2B).

Antibiotic	Zone diameter	Annual radius	MIC	Interpretation S or R
Ampicillin		5 mm +RV*		R
Amoxycillin				Inappropriate to report
Cefotaxime		12 mm +RV*		R+
Ceftriaxone				R (Surrogate result from cefotaxime 5 µg)
Ceftazidime				R+
Cephalexin				Inappropriate to test & report
Cephalothin				R (Surrogate result from Ampicillin 25 µg)
Ciprofloxacin		8 mm		S
Erythromycin				Not tested
Gentamicin		1 mm		R
Imipenem		8 mm		S
Methicillin				Not tested
Norfloxacin				Inappropriate to test
Penicillin				Not tested
Piperacillin				Not tested
Rifampicin				Not tested
Tetracycline				Inappropriate to test
Trimethoprim				Inappropriate to test
Vancomycin				Not tested

*RV= resistant variant colonies.
+ Induction of cephalosporinase demonstrated.

C. THE ASIG WORKSHOP.

Nichalas Nuttall, Jan Bell & Narelle George convened the ASIG workshop which was not only entertaining but informative and ideas were exchanged in an open forum.

132 laboratories participated in antimicrobial susceptibility testing of 6 organisms sent out by ASIG.
The following is a synopsis of the results obtained not only by CDS Users but those who performed other methods of antimicrobial susceptibility tests.

There are some important hints which we would like CDS Users to take note of.

1. *Staphylococcus aureus* (from a wound swab). This was a MRSA and possessed the mec A gene.
The organism was resistant to penicillin (no zone) and methicillin. However, the annular radius of the zone of inhibition around methicillin 5 µg was approximately 5.0 mm and resistant colonies were present within the zone of inhibition. This rare strain of methicillin-resistant *Staph. aureus* was unusual in two respects: It was resistant to benzylpenicillin and methicillin but not erythromycin or tetracycline, ie. it was not "multiply resistant". Also, resistance to methicillin was not "well" expressed ie. the phenotypic expression of the mec A gene was incomplete. Twenty six CDS Users reported the organism S and 27 reported R. Other results were: NCCLS: 27 S, 15 R; Vitek: 4 S, 6 R; Microscan: 3 S, 10 R; Agar dilution: 7S, 2 R.
Note: The zone around a methicillin 5 µg disc recorded with a *Staph. aureus* resistant to penicillin is usually around 8 mm. Therefore, when the zone is reduced to approximately 6 mm, one should realise that the organism is not behaving in the usual way and this should alert the operator that methicillin-resistance may be present. Ensure that the inoculum is correct. If it is too light, resistant colonies may not be observed within the zone of inhibition. If in doubt about the presence of resistant colonies reincubate the plate for an additional 24 hrs and examine the zone around methicillin again.

2. *Enterococcus faecalis* (from a blood culture).
With this isolate, the MIC of vancomycin was 16 mg/L. When tested by the CDS method using a 5 µg vancomycin disc there was hazy growth up to the edge of the disc, a pattern described in Newsletter No 8. Amongst the 46 laboratories who used the CDS method, 20 reported S and 26 R. Laboratories from Queensland used a 60 µg vancomycin disc prior to the change to a potency of 5 µg. Some of these laboratories reported the isolate R because the annular radius of the zone of inhibition around the 60 µg potency disc was < 6 mm. They obtained the correct result for the wrong reason ! Other results were NCCLS: 11 S, 3 I, 25 R; Vitek: 3 S, 1 I; Microscan: 1 I, 8 R; Agar dilution: 2 S, 1 I, 4 R).
With a vancomycin 5 µg disc, CDS Users should not miss the typical pattern observed with low level resistance to vancomycin in enterococci.

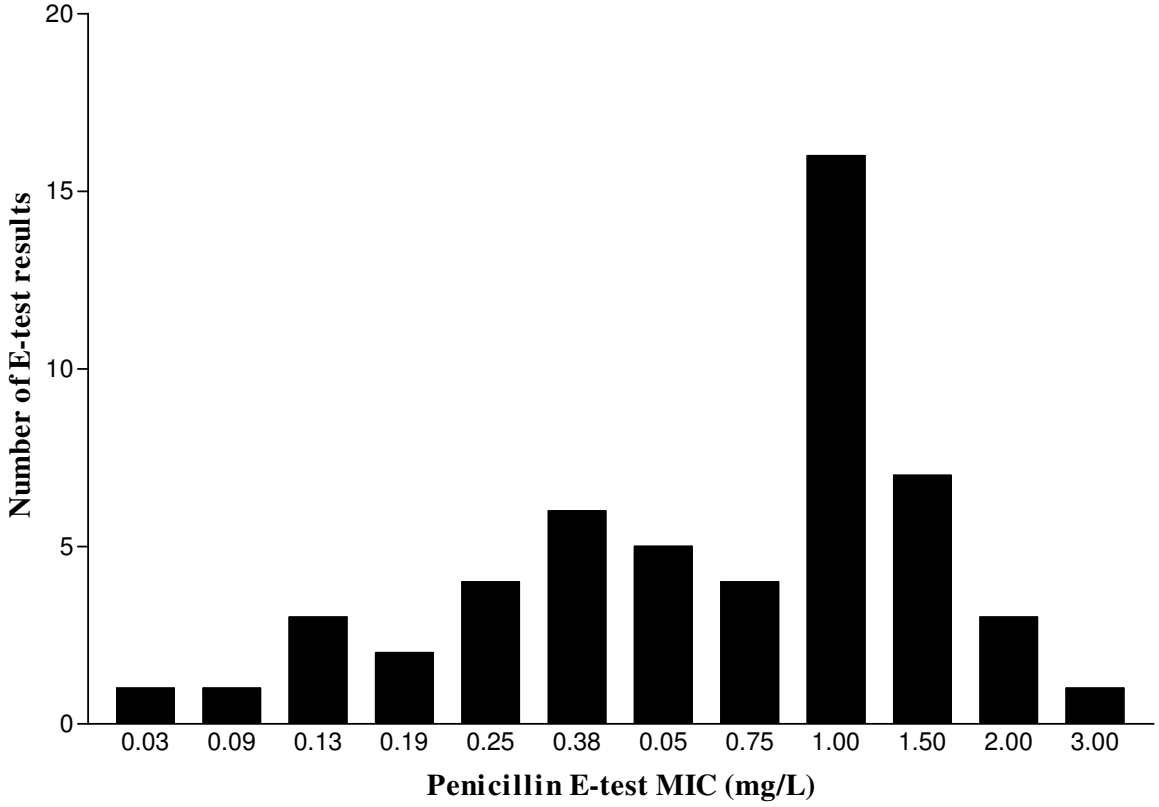
3. *Citrobacter koseri* producing an ESBL (from a blood culture).
CDS Users did not have any problem recognising the typical "key-hole" phenomenon exhibited by this member of the *Enterobacteriaceae* denoting the presence of an extended spectrum β-lactamase (ESBL). All CDS Users reported correctly that the organism was resistant to: cefotaxime, ceftriaxone, ceftazidime or aztreonam (which ever they chose to report). Results for testing aminoglycosides and quinolones were excellent and almost all CDS Users obtained the correct result.

4. *Citrobacter freundii* (from a catheter urine).
A small number of CDS Users reported incorrectly that the organism was susceptible to Augmentin, cephalexin and cefotaxime even though induction of a **Class 1 cephalosporinase** was demonstrated. There were no problems with the testing of other antibiotics.

5. *Stenotrophomonas maltophilia* (from sputum).
CDS Users are reminded that only sulphafurazole needs to be tested for this species. However, the resistance to imipenem (no zone) and a "comet tail effect" observed between sulphafurazole and trimethoprim could be used as an aid to the identification of this species. The organism was susceptible to sulphafurazole and could be reported susceptible to co-trimoxazole. In the rare situation where the organism is resistant to sulphafurazole then test Timentin, aztreonam and ciprofloxacin. Discuss the results with a clinical microbiologist regarding the implication of therapy with a combination of these agents.

6. *Streptococcus pneumoniae* (from a CSF).
There was no zone around a penicillin 0.5 u disc and the annular radius of the zone of inhibition was 3.5 to 4 mm around cefotaxime 0.5 µg and ceftriaxone 0.5 µg. Therefore, the organism was reported resistant to penicillin, cefotaxime and ceftriaxone (MIC > 0.25 mg/L according to CDS criteria. The agar dilution MIC of penicillin was 1.0 mg/L and 0.5 mg/L for both cefotaxime and ceftriaxone. In general, CDS Users obtained correct results: R to penicillin and cefotaxime and S to vancomycin. **Note**: The E-test (ASIG reference) results were on the low side: 0.38 mg/L for penicillin and 0.25 mg/L for cefotaxime. Also, there was a considerable variation in penicillin E-test results between laboratories (Figure 2).

Figure 2. Numbers of E-tests results issued by different laboratories related to the penicillin E-test MIC. The agar dilution MIC of penicillin was 1 mg/L. This result was obtained by a number of laboratories using an agar dilution method.



HANDLING OF REFERENCE STRAINS USED FOR QUALITY CONTROL OF ANTIBIOTIC SUSCEPTIBILITY TESTING BY THE CDS METHOD.

1. Upon receipt, subculture the strains onto blood agar *(H. influenzae* onto chocolate agar) immediately.
2. Prepare a heavy suspension of an overnight culture in sterile 20% glycerol in nutrient broth.
3. Store in cryogenic vials at -20^{0}C or preferably at -70^{0}C.

4. Recovery of cultures.
   * Remove the tube from freezer.
   * **DO NOT THAW.**
   * With the tip of a Pasteur pipette, aseptically remove a sample of the frozen suspension and inoculate a suitable medium.
   * Return the tube to freezer immediately.
5. Subculture the reference strains from cultures kept in the freezer when necessary.

Important notes:

1. Cultures of reference organisms grown overnight on blood agar plates or chocolate agar plates (for *H. influenzae)* **should be kept at** 4^{0}C to slow down autolysis. This is critical for *S. pneumoniae and H. influenzae.* Subculture reference strains **at least once a week** or as often as is required. Reference strains will keep their properties if maintained as described. Go back to stock cultures kept in the deep freezer when the reference strains fail to give zone sizes within the recommended range.

2. *H. influenzae* strains may not survive storage at -20^{0}C for more than 3 months. Therefore, it is advisable to renew deep-freeze cultures of these strains every 3 months. This has not been a problem with cultures stored at -70^{0}C.

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Revised Table 1a. Calibrations, 1998: Antibiotics, disc potencies, the MIC breakpoint for susceptible strains, the media and incubation conditions used.

Antibiotic	Disc potency (µg)		MIC for susceptible strains (mg/L)
Branhamella catarrhalis			
(Blood Sensitest, 5%CO_2, 35°C)			
Benzylpenicillin	0.5 u		≤ 0.25
Cefaclor	30		≤ 4.0
Cefpodoxime	10		≤ 2.0
Ciprofloxacin	2.5		≤ 1.0
Erythromycin	5		≤ 0.5
Tetracycline	30		≤ 4.0
Campylobacter spp.			
(Blood Sensitest, microaerophilic, 42°C)			
Ciprofloxacin	2.5		≤ 1.0
Erythromycin	5	4 mm *	≤ 0.5
Gentamicin	10		≤ 1.0
Tetracycline	30		≤ 4.0
Enterobacteriaceae*, *Vibrionaceae*, & *Acinetobacter spp.			
(Sensitest, air, 35°C)#			
Amikacin	30		≤ 4.0
Ampicillin	25		≤ 8.0
Augmentin •	60		≤16.0/8.0
Aztreonam	30		≤ 8.0
Cephazolin	30		≤16.0
Cefotaxime	5		≤ 1.0
Cefotetan	10		≤ 4.0
Cefoxitin	30		≤ 8.0
Cefpirome	10		≤ 2.0
Cefpodoxime	10		≤ 2.0
Ceftazidime	10		≤ 4.0
Ceftriaxone	5		≤ 1.0
Cephalexin+	100		≤ 16.0
Chloramphenicol	30		≤ 8.0
Ciprofloxacin	2.5		≤ 1.0
Enoxacin	10		≤ 4.0
Gentamicin	10		≤ 1.0
Imipenem	10		≤ 4.0
Kanamycin	50		≤ 8.0
Meropenem	5		≤ 2.0
Nalidixic acid +	30		≤ 4.0
Netilmicin	30		≤ 2.0
Nitrofurantoin +	200		≤ 32.0
Norfloxacin +	10		≤ 4.0
Sulphafurazole	300		≤ 64.0
Tazocin	55		≤ 16.0/2.0
Tetracycline	30		≤ 4.0
Timentin	85		≤ 32.0/2.0
Tobramycin	10		≤ 1.0
Trimethoprim	5		≤ 2.0
Enterococci			
(Blood Sensitest, air, 35°C)			
Ampicillin	5	4 mm *	≤ 2.0
Chloramphenicol	30	4 mm *	≤ 8.0
Gentamicin	200	4 mm *	≤ 512
Nitrofurantoin +	200	4 mm *	≤ 64.0
Teichoplanin	15	2 mm ©	≤ 8.0
Vancomycin	5	2 mm ©	≤ 4.0

* The annular radius of the zone of inhibition for susceptible strains is ≥ 4 mm. + For testing urine isolates only
© The annular radius of the zone of inhibition for susceptible strains is ≥2 mm. . # *Yersinia enterocoliticai* is incubated in air at 30˚C.
• If an ESBL is present, report Augmentin for isolates from URINE ONLY.

Revised Table 1b. Calibrations, 1998: Antibiotics, disc potencies, the MIC breakpoint for susceptible strains, the media and incubation conditions used.

Antibiotic	Disc potency (µg)		MIC for susceptible strains (mg/L)
***Haemophilus influenzae/Haemophilus* spp**			
(HTM[@] agar, 5%CO_2, 35°C)			
Ampicillin	5		≤ 1.0
Augmentin	3	4 mm [*]	≤ 2.0/1.0
Cefaclor	30		≤ 4.0
Cefotaxime	0.5		≤ 0.25
Cefpodoxime	10		≤ 2.0
Ceftriaxone	0.5		≤ 0.25
Chloramphenicol	10		≤ 2.0
Ciprofloxacin	2.5		≤ 1.0
Co-trimoxazole	25		≤ 1.0/19.0
Tetracycline	30		≤ 4.0
Listeria monocytogenes			
(Blood Sensitest, air, 35°C)			
Ampicillin	5		≤ 1.0
Gentamicin	10		≤ 1.0
Neisseria meningitidis			
(Blood Sensitest, 5% CO_2, 35°C)			
Benzylpenicillin	0.5 u	4 mm [*]	≤ 0.25
Cefotaxime	0.5		≤ 0.25
Ceftriaxone	0.5		≤ 0.25
Chloramphenicol	10		≤ 2.0
Ciprofloxacin	2.5		≤ 1.0
Rifampicin	1		≤ 0.5
Pasteurella multocida			
(Blood Sensitest, air, 35°C)			
Ampicillin	5		≤ 1.0
Ciprofloxacin	2.5		≤ 1.0
Tetracycline	30		≤ 4.0
***Pseudomonas* spp. & *Burkholderia* spp.**			
(Sensitest, air, 35°C)			
Amikacin	30	4 mm[*]	≤ 16.0
Aztreonam	30		≤ 8.0
Cefpirome	10		≤ 2.0
Ceftazidime	10		≤ 4.0
Ciprofloxacin	2.5		≤ 2.0
Gentamicin	10	4 mm[*]	≤ 4.0
Imipenem	10		≤ 4.0
Meropenem	5		≤ 2.0
Netilmicin	30	4 mm[*]	≤ 8.0
Norfloxacin [+]	10		≤ 4.0
Piperacillin	50		≤ 16.0
Polymyxin	300 u	4 mm[*]	≤ 1.0
Tazocin	55		≤ 16.0/2.0
Ticarcillin	75		≤ 32.0
Timentin	85		≤ 32.0/2.0
Tobramycin	10	4 mm[*]	≤ 4.0
Trimethoprim	5		≤ 2.0

[*] The annular radius of the zone of inhibition for susceptible strains is ≥ 4 mm [+] For testing urinary isolates only.
[@] Haemophilus Test Medium containing 15mg/L of freshly prepared haematin and NAD.

Revised Table 1c: Calibrations, 1998: Antibiotics, disc potencies, the MIC breakpoint for susceptible strains, the media and incubation conditions used.

Antibiotic	Disc potency (µg)		MIC for susceptible strains (mg/L)
Staphylococci			
(Sensitest, air, 35°C)			
Ampicillin •	5		≤ 0.5
Benzylpenicillin $	0.5 u		≤ 0.06
Cephalexin •	100		≤ 16.0
Chloramphenicol	30		≤ 8.0
Ciprofloxacin	2.5		≤ 1.0
Erythromycin	5		≤ 0.5
Fusidic acid	2.5		≤ 0.5
Gentamicin	10		≤ 1.0
Kanamycin	50		≤ 8.0
Methicillin $	5		≤ 4.0
Nitrofurantoin +	200		≤ 32.0
Rifampicin	1		≤ 0.5
Sulphafurazole	300		≤ 64.0
Teichoplanin	15	2 mm ©	≤ 8.0
Tetracycline	30		≤ 4.0
Trimethoprim	5		≤ 2.0
Vancomycin	5	2.mm ©	≤ 4.0
Streptococci			
(Blood Sensitest, air, 35°C) @			
Benzylpenicillin	0.5 u		≤ 0.25
Cefotaxime	0.5		≤ 0.25
Ceftriaxone	0.5		≤ 0.25
Chloramphenicol	30		≤ 8.0
Co-trimoxazole &	25		≤ 0.5/9.5
Erythromycin	5		≤ 0.5
Nitrofurantoin +	200		≤ 32.0
Rifampicin	1		≤ 0.5
Teichoplanin	15	2mm ©	≤ 8.0
Tetracycline	30		≤ 4.0
Vancomycin	5	2.mm ©	≤ 4.0
Stenotrophomonas maltophilia			
(Sensitest, air, 35°C)			
Sulphafurazole	300		≤ 64.0

* The annular radius of the zone of inhibition for susceptible strains is ≥ 4 mm. + For testing urine isolates only
© The annular radius of the zone of inhibition for susceptible strains is ≥2 mm. $ NOT for testing *Staph.saprophyticus*.
• ONLY for testing isolate of *Staph.*saprophyticus. @ *Strep.pneumoniae* & *Strep.anginosus* (milleri) are incubated in 5% CO_2.
& ONLY for testing *Strep.*pneumoniae and group B streptococci.